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Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains

Dongyou Liu *, Mark L. Lawrence, A. Jerald Ainsworth, Frank W. Austin

Department of Basic Sciences, College of Veterinary Medicine, Mississippi State University, P.O. Box 6100, Mississippi, MS 39762, USA

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Abstract

Listeria monocytogenes is an opportunistic bacterial pathogen of man and animals that has the capacity to survive under extreme environmental conditions. While our knowledge on *L. monocytogenes* and its ability to sustain within wide pH and temperature ranges and salt concentrations has been largely built on the virulent strains of this species, relatively little is known about avirulent strains in this regard. In this study, we extend our analysis on avirulent *L. monocytogenes* strains. By subjecting three virulent (EGD, 874 and ATCC 19196) and three avirulent (ATCC 19114, HCC23 and HCC25) strains to various pH and salt concentrations, it was found that *L. monocytogenes* recovered well after treatment with 100 mM Tris at pH 12.0, and to a lesser extent at pH 3.0. Interestingly, avirulent *L. monocytogenes* strains showed a somewhat higher tolerance to alkali than virulent strains. This unique feature of avirulent *L. monocytogenes* strains may potentially be exploited for the development of a rapid technique for differentiation between avirulent and virulent strains. Furthermore, all *L. monocytogenes* strains tested were resistant to saturated NaCl (about 7 M, or 40% w/v) for a long period of time (20 h and possibly longer). Together, these results highlight that acid, alkali, and/or salt treatments commonly used in food product processing may not be sufficient to eliminate *L. monocytogenes*, and therefore stringent quality control measures at the beginning and end of the food manufacturing process is essential to ensure that such food products are free of listerial contamination.

Keywords: *Listeria monocytogenes*; Acid; Alkali; Salt; Virulence

1. Introduction

Being a Gram-positive facultative intracellular bacterium, *Listeria monocytogenes* has increasingly emerged as an opportunistic human foodborne pathogen. The fact that this bacterium is ubiquitously present in all environments and tolerant to extreme environmental stresses may account for its prevalence. It is well known that *L. monocytogenes* bacteria can sustain wide ranges of pH (4.0–9.5) and temperature (<1–45 °C) and are able to survive in the presence of high salt concentrations (up to 10% NaCl) [1–5]. This renders many of the conventional food manufacturing processes ineffective in eliminating the bacterium. With manufactured, ready-to-eat food products being consumed in increasing quantities nowadays, it is no surprise that the incidence of listeriosis through contaminated foods has become more frequent.

While being pathogenic at the species level, *L. monocytogenes* is actually made up of multiple strains that display varied virulence and pathogenic potential. On the one hand, many *L. monocytogenes* strains are

* Corresponding author. Tel.: +1 662 325 3559; fax: +1 662 325 1031.

E-mail address: liu@cvm.msstate.edu (D. Liu).

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virulent and capable of causing a deadly disease, particularly in infants, the elderly, pregnant women and immuno-suppressed individuals. On the other, some strains are non-pathogenic, producing no apparent malaise and are rapidly cleared by hosts [6–10]. Through experimental studies on *L. monocytogenes* virulent strains, much is known about their capacity to survive at extreme pH, and salt concentrations and their ability to invade host tissues, with the identification of various virulence-associated, and stress response genes [11–13]. However, we know relatively little about avirulent strains in this regard. For example, in spite of possessing nearly identical virulence gene cluster (comprising *prfA*, *hlyA*, *plcA*, *mpl*, *actA* and *plcB*), and other virulence-associated genes (e.g., *inlA*, *inlB* and *iap*) in comparison with virulent strains, *L. monocytogenes* avirulent strains fail to pass through certain critical stage(s) of the infection cycle.

In this report, we attempt to address this imbalance by examining the ability of *L. monocytogenes* avirulent strains to recover after exposure to a range of pH and salt concentrations. A detailed understanding on *L. monocytogenes* avirulent strains and the molecular mechanisms behind their avirulence will help provide a more comprehensive picture on listerial virulence and pathogenicity. This in turn may lead to the development of novel, and more effective treatment and prevention strategies for listeriosis.

2. Materials and methods

2.1. Bacteria and culture conditions

Six *L. monocytogenes* strains of known virulence (i.e., virulent strains EGD, 874 and ATCC 19116; and avirulent strains ATCC 19114, HCC23 and HCC25) were examined in this study (Table 1). These strains came either from the American Type Culture Collection (ATCC) and the National Collection of Type Culture (NCTC), or from clinical or catfish specimens [6,8]. They were previously characterized by PCR targeting *L. monocytogenes* putative transcriptional regulator and internalin genes and by mouse virulence assay [6,8,9]. *L. monocytogenes* was initially cultivated on 5% sheep blood agar plates (TSA II, Becton Dickinson Microbiology Systems, Cockeysville, MD), from which several colonies of each strain were used to inoculate 50 ml of brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) in flasks and incubated at 37 °C for 18 h with aeration before undergoing acid, alkaline or salt treatments.

2.2. Acid and alkaline treatments

Cell densities of *L. monocytogenes* BHI broth cultures were measured at $OD_{540\ nm}$, *L. monocytogenes* bacteria were then centrifuged at 2500*g* for 10 min, washed once with 20 ml of sterile physiologic saline (0.9% NaCl) and resuspended in about 10 ml saline (the volume was adjusted to one fifth of that at an $OD_{540\ nm}$ value of 1.35 for each strain). In the first experiment, 100 mM Tris solutions with pH ranging from 2.0 to 5.0, and 9.0 to 12.0 were prepared by adding appropriate amounts of 1 M HCl or 1 M NaOH. In a 15 ml tube containing 5 ml of 100 mM Tris solution, 0.25 ml of each *L. monocytogenes* strain was added, mixed by inversion and left at room temperature for 1 h. Then 40 μl of each treated sample was transferred into an eppendorf tube containing 960 μl of saline and serially diluted to 10^{-6} and 10^{-8}, from which 25 μl was used to inoculate one half of a BHI agar (Difco Laboratories, Detroit, MI) plate (11 cm in diameter). After overnight incubation at 37 °C, the colony forming unit (CFU) of each treated sample was enumerated in accordance with a conventional plate count method (Bacteriological Analytical Manual, FDA). The experiment was performed in duplicate, so that the CFU for a test strain was averaged from the two separate plate counts each with 25 μl inoculon of treated *L. monocytogenes*. Therefore, the final CFU/ml values would be the products of these averaged CFU numbers and the dilution factors (40 and 10^8). As a control, individual tubes containing 5 ml of sterile saline were added with 0.25 ml of *L. monocytogenes* strains, incubated and emunerated as treated samples.

In the second experiment, 10 mM HCl and 100 mM NaOH were prepared in sterile saline. In a 15 ml tube

Table 1
L. monocytogenes strains of known virulence used in the study

Strain	Serovar	Source	*lmo2821* PCR[a]	Pathogenicity[b]
EGD (NCTC7973)	1/2a	Guinea pig	+	V
874	4c	Cow brain	+	V
ATCC 19116	4c	Chicken	+	V
ATCC 19114	4a	Human	–	A
HCC23	4a	Catfish brain	–	A
HCC25	4a	Catfish kidney	–	A

[a] *lmo2821* is a *L. monocytogenes* putative internalin gene that has proven valuable for determination of listerial virulence [8,10].
[b] The pathogenicity of these strains was previously determined by mouse virulence assay, with V being virulent and A being avirulent [6,8,9].

containing 5 ml of 10 mM HCl or 100 mM NaOH, 0.25 ml of each *L. monocytogenes* strain was added as above, mixed by inversion and incubated at room temperature. Forty microliters of each treated sample was taken out at 1, 5 and 15 min post incubation, transferred into an eppendorf tube containing 960 μl of saline, serially diluted, cultured and emunerated as above.

2.3. Salt treatment

NaCl at 1, 3, and 5 M as well as at saturated concentration (about 7 M, or 40% w/v) was used. In a 15 ml tube containing 5 ml of NaCl solution, 0.25 ml of each *L. monocytogenes* strain was added, mixed by inversion and inoculated at room temperature for 1 h. Then, 40 μl of each treated sample was transferred into an eppendorf tube containing 960 μl of saline, serially diluted, cultured and counted as above. In addition, 40 μl of samples treated with saturated NaCl was also taken out at 20 h post incubation, plated and counted. As a control, individual tubes containing 5 ml of sterile saline were added with *L. monocytogenes* strains, incubated and counted as treated samples.

3. Results

As shown in Fig. 1, both *L. monocytogenes* virulent (EGD, 874 and ATCC 19116) and avirulent (ATCC 19114, HCC23 and HCC25) strains showed remarkable tolerance to a wide range of pH. After 1 h treatment with 100 mM Tris at pH 5.0, and 9.0–12.0, these strains displayed no obvious reductions in their CFU, as calculated from the overnight BHI agar plates at the dilution of 10^{-8}, in comparison with the saline control. However, at pH 4.0, decreases in CFU values from both virulent and avirulent strains were noticeable. At pH 3.0, the CFU values of these strains equalled to only about 20% of the controls treated with saline. At pH 2.0, the CFU values from these strains became negligible at the dilution of 10^{-8} (Fig. 1), but showed a slight increase at the dilution of 10^{-6} (data not shown). The detrimental effect of low pH on these *L. monocytogenes* strains was further confirmed upon treatment with 10 mM HCl (Fig. 2). While both virulent and avirulent strains were able to sustain 10 mM HCl for 1 and 5 min, they essentially failed to recover after 15 min of such treatment.

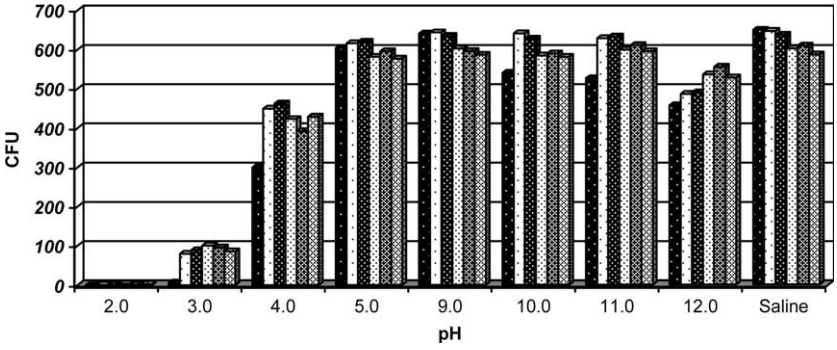

Fig. 1. The CFUs of *L. monocytogenes* strains treated with 100 mM Tris at various pH for 1 h, with saline treatment as a control. As in Table 2, the CFUs shown in this and other figures were averaged from two plate counts each with 25 μl inoculon of treated *L. monocytogenes* at a dilution of 10^{-8}. Therefore, the final CFU/ml values would be the products of the averaged CFU numbers and the dilution factors 40 and 10^8. The order of *L. monocytogenes* strains tested under each pH condition is as follows (from left): EGD, 874, ATCC 19116, ATCC 19114, HCC23 and HCC25.

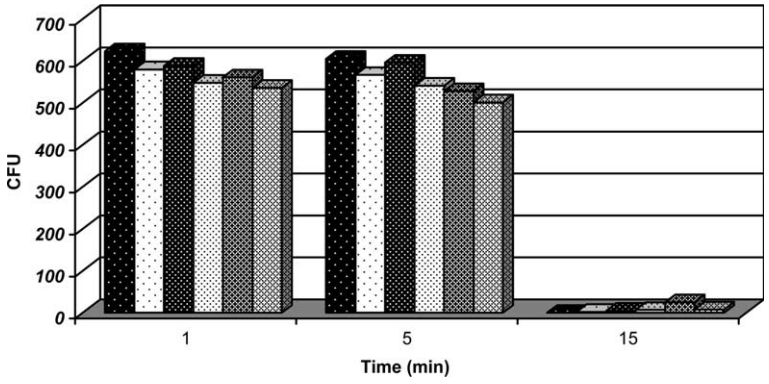

Fig. 2. The CFUs of *L. monocytogenes* strains after treatment with 10 mM HCl at various time points. The order of *L. monocytogenes* strains tested at each time point from left is: EGD, 874, ATCC 19116, ATCC 19114, HCC23 and HCC25.

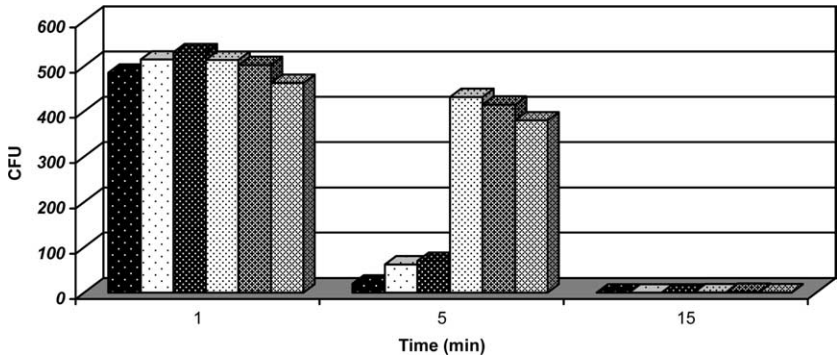

Fig. 3. The CFUs of *L. monocytogenes* strains after treatment with 100 mM NaOH at various time points. The order of *L. monocytogenes* strains tested at each time point from left is: EGD, 874, ATCC 19116, ATCC 19114, HCC23 and HCC25.

Table 2
The CFU values of *L. monocytogenes* strains treated with 100 mM NaOH at various time points

Strain	CFU[a] after 100 mM NaOH treatment for		
	1 min	5 min	15 min
EGD (NCTC7973)	484 (SD[b] ± 14)	20 (SD ± 3)	0
874	515 (SD ± 19)	62 (SD ± 4)	0
ATCC 19116	531 (SD ± 21)	70 (SD ± 7)	0
ATCC 19114	514 (SD ± 23)	432 (SD ± 18)	1 (SD ± 0)
HCC23	503 (SD ± 17)	415 (SD ± 15)	2 (SD ± 1)
HCC25	463 (SD ± 25)	380 (SD ± 22)	1 (SD ± 1)

[a] The CFU was averaged from two plate counts each originated with 25 μl inoculon of treated *L. monocytogenes* at a dilution of 10^{-8}. Thus, the final CFU/ml values for these strains would be the products of the averaged CFU numbers and the dilution factors 40 and 10^8.
[b] SD, standard deviation.

On the other hand, *L. monocytogenes* avirulent strains (ATCC 19114, HCC23 and HCC25) demonstrated a somewhat higher resistance to alkaline treatment than virulent strains (EGD, 874 and ATCC 19116). This was exemplified by the observations that after treatment with 100 mM NaOH for 5 min, the avirulent strains were able to recover, and produced CFU values that amounted to 80% of those from the same strains treated for 1 min. By contrast, the virulent strains only generated CFU values of less than 20% of those from the same strains treated for 1 min (Fig. 3; Table 2). Indeed, the difference of CFU values between virulent and avirulent strains treated with 100 mM NaOH for 1 and 5 min was statistically significant ($P < 0.001$) as assessed by χ^2 test. Furthermore, both virulent and avirulent strains of *L. monocytogenes* were highly tolerant to salt as their growth was not significantly affected after treatment with NaCl at 1, 3, 5 and 7 M for 1 h, or 7 M for 20 h (Fig. 4).

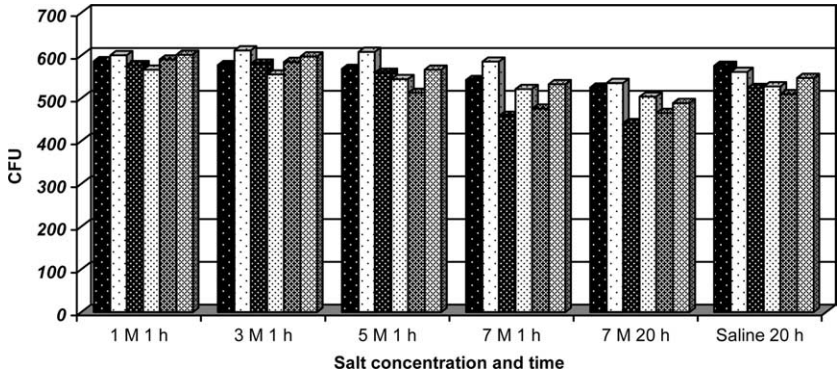

Fig. 4. The CFUs of *L. monocytogenes* strains after treatment with NaCl at various concentrations for 1 or 20 h. The order of *L. monocytogenes* strains tested under each condition is shown from left: EGD, 874, ATCC 19116, ATCC 19114, HCC23 and HCC25.

4. Discussion

The ability of *L. monocytogenes* virulent strains to tolerate and survive under harsh environmental conditions such as extreme pH (<4.0 or >9.0) and high concentration of salt (around 10%) has been well documented [1–5]. These external stresses often result in inhibition of *L. monocytogenes* growth, and once the stress factors are removed, this bacterium will resume its rapid multiplication. The genetic mechanisms underlining *L. monocytogenes* resistance to external influences such as acid, salt and low temperature have become clearer, following the identification of several stress response genes. In particular, an alternative sigma factor gene *sigB* and its dependent osmolyte transporter genes *betL*, *gbu*, and *opuC* have been shown to play vital roles in the general stress responses of *L. monocytogenes* to acid, low temperature and salt [12,13].

In this study, we extended the analysis of listerial acid, alkali and salt tolerance to *L. monocytogenes* avirulent strains. The results conformed that both *L. monocytogenes* virulent and avirulent strains are able to tolerate a much wider pH ranges and salt concentrations than previously thought. This was evidenced by the fact that *L. monocytogenes* bacteria grew well after the treatment with 100 mM Tris at pH 12.0 for 1 h, and also showed remarkable recovery from the treatment with 100 mM Tris at pH 3.0 for 1 h. With regard to salt tolerance, all six *L. monocytogenes* strains appeared to be unaffected by high concentrations of salt, generating reasonably high CFUs after incubation in saturated NaCl (about 7 M, or 40% w/v) for 20 h. This level of salt tolerance by *L. monocytogenes* bacteria is significantly higher than what has been generally recognized (up to 10%).

Moreover, *L. monocytogenes* avirulent strains seemed to be more tolerant to alkali than virulent strains, as the former produced much higher CFUs than the latter after treatment with 100 mM NaOH for 5 min (Fig. 3; Table 2). This observation was somewhat unexpected as conventional wisdom would suggest that virulent strains have higher tolerance to environmental extremes than avirulent strains, given that the former possess many more virulence-associated genes than the latter [8,10]. Perhaps, *L. monocytogenes* avirulent strains have evolved over long periods of time with accompanied genetic changes to survive under even harsher environmental conditions than virulent strains, which are adapted to propagate in host tissues. There is some tangible evidence supporting this hypothesis from another related non-pathogenic *Listeria* species, *L. grayi*, in which a species-specific gene region encoding an oxidoreductase has been isolated [14]. Interestingly, the translated protein of this *L. grayi*-specific oxidoreductase gene demonstrates a >50% amino acid similarity with an oxidoreductase from *Oceanobacillus iheyensis*, an alkaliphilic and extremely holotolerant *Bacillus*-related bacterium [15]. By the same token, *L. monocytogenes* virulent strains may have also acquired many unique virulence-associated genes to facilitate their survival in animal and human hosts [8,10]. The fact that *L. monocytogenes* avirulent strains display significantly higher tolerance to alkali than virulent strains may offer the potential for future development of a rapid and easy-to-perform technique to distinguish avirulent from virulent strains, much like the prior KOH treatment in the isolation of *Yersinia pseudotuberculosis* from other bacteria [16].

From a food safety point of view, an obvious implication from this and other studies [1–5] is that use of acid, alkali and/or salt in food product processing may not be sufficient to eliminate all *L. monocytogenes* bacteria, as a certain number of this species will survive at very extreme pH and salt conditions. This puts good manufacturing practice to the forefront to ensure that processed, ready-to-eat food products are free of *L. monocytogenes* contamination. In addition, the value of establishing a stringent monitoring/testing routine at the beginning and end of the food manufacturing process in the prevention of human listeriosis can never be underestimated.

In light of the findings that both *L. monocytogenes* virulent and avirulent strains are tolerant to pH 3.0 and lower, it is conceivable that like virulent strains, avirulent strains are capable of surviving the stomach's acidic environment once ingested by hosts via contaminated foods during a typical mode of infection. Therefore, the inability of *L. monocytogenes* avirulent strains to cause disease is most likely due to their failure to complete some other stage(s) of infection, including cell adhesion/internalizaton, escape from primary vacuoles, intracellular growth and cell-to-cell spread. Further studies by using in vitro cell assays involving epithelial, enterocyte, hepatic and macrophage cell lines and also by animal models will help pinpoint the critical stage(s) where *L. monocytogenes* avirulent strains are unable to get through. In addition, comparative RT-PCR analysis of mRNA levels of key virulence-associated genes (e.g., *prfA*, *plcA*, *hly*, *mpl*, *acta*, *plcB*, *inlA*, *inlB*, *iap* and *sigB*) in *L. monocytogenes* virulent and avirulent strains will also provide new insights on the molecular mechanisms of listerial avirulence.

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References

[1] Patchett, R.A., Kelly, A.F. and Kroll, R.G. (1992) Effect of sodium chloride on the intracellular solute pools of *Listeria monocytogenes*. Appl. Environ. Microbiol. 58, 3959–3963.

[2] Arizcun, C., Vasseur, C. and Labadie, J.C. (1998) Effect of several decontamination procedures on *Listeria monocytogenes* growing in biofilms. J. Food Prot. 61, 731–734.
[3] Vasseur, C., Rigaud, N., Hebraud, M. and Labadie, J. (2001) Combined effects of NaCl, NaOH, and biocides (monolaurin or lauric acid) on inactivation of *Listeria monocytogenes* and *Pseudomonas* spp. J. Food Prot. 64, 1442–1445.
[4] Dykes, G.A. and Moorhead, S.M. (2000) Survival of osmotic and acid stress by *Listeria monocytogenes* strains of clinical or meat origin. Int. J. Food Microbiol. 56, 161–166.
[5] Taormina, P.J. and Beuchat, L.R. (2002) Survival of *Listeria monocytogenes* in commercial food-processing equipment cleaning solutions and subsequent sensitivity to sanitizers and heat. J. Appl. Microbiol. 92, 71–80.
[6] Erdenlig, S., Ainsworth, A.J. and Austin, F.W. (2000) Pathogenicity and production of virulence factors by *Listeria monocytogenes* isolates from channel catfish. J. Food Prot. 63, 613–619.
[7] Gracieux, P., Roche, S.M., Pardon, P. and Velge, P. (2003) Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid' L. mono media. Int. J. Food Microbiol. 83, 133–145.
[8] Liu, D., Austin, F.W., Ainsworth, A.J. and Lawrence, M. (2003) Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. J. Med. Microbiol. 52, 1066–1070.
[9] Liu, D. (2004) *Listeria monocytogenes*: comparative interpretation of mouse virulence assay. FEMS Microbiol. Lett. 233, 159–164.
[10] Doumith, M., Cazalet, C., Simoes, N., Frangeul, L., Jacquet, C., Kunst, F., Martin, P., Cossart, P., Glaser, P. and Buchrieser, C. (2004) New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. Infect. Immun. 72, 1072–1083.
[11] Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J. and Kreft, J. (2001) *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14, 584–640.
[12] Sue, D., Boor, K.J. and Wiedmann, M. (2003) Sigma(B)-dependent expression patterns of compatible solute transporter genes opuCA and lmo1421 and the conjugated bile salt hydrolase gene bsh in *Listeria monocytogenes*. Microbiology 149, 3247–3256.
[13] Wemekamp-Kamphuis, H.H., Sleator, R.D., Wouters, J.A., Hill, C. and Abee, T. (2004) Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of *Listeria monocytogenes* at low temperatures. Appl. Environ. Microbiol. 70, 2912–2918.
[14] Liu, D., Lawrence, M., Ainsworth, A.J. and Austin, F.W. Isolation and PCR amplification of a species-specific, oxidoreductase-coding gene region in *Listeria grayi*. Can. J. Microbiol. (in press).
[15] Takami, H., Takaki, Y. and Uchiyama, I. (2002) Genome sequence of *Oceanobacillus iheyensis* isolated from the Iheya Ridge and its unexpected adaptive capabilities to extreme environments. Nucleic Acids Res. 30, 3927–3935.
[16] Fukushima, H. (1992) Direct isolation of *Yersinia pseudotuberculosis* from fresh water in Japan. Appl. Environ. Microbiol. 58, 2688–2690.