resentative strains from the outbreak were tested for their resistance to lactic acid (pH 4.0). Although most strains died within 48 h of incubation in PBS-LA, one food strain, 668, showed prolonged survival for up to 72 h (Table 1). Although the human clinical strain 690 shared identical PFGE profiles to the food strain 668 (Fig. 1A), it did not show prolonged survival, similar to the other strains (Table 1). Taking these data into account, we reasoned that food strain 668 and clinical strain 690 might have originated from the same source; if so, the acid sensitivity of the food strain potentially increased during the infection in human.

3.2. Animal passage alters acid resistance of L. monocytogenes

To investigate the above idea, BALB/c mice were orally infected with *L. monocytogenes* strains 668 and 690. Bacteria were recovered from their intestines 3 days post infection. This period of infection is sufficient for the dissemination of this pathogen in mice (Asakura et al., 2006). Of total 150 colonies recovered from the mice infected with strain 668, 19 (12.7%) exhibited no survival after a 48-h incubation in PBS-LA (Table 2). While, the animal passage did not cause the restoration of acid resistance in strain 690 (Table 2). Both strains 668 and 690 did not show altered acid resistance after incubated in PBS or cultivated in BHI broth for 3 days (Table 2). Neither clinical strain 690 nor five representative mouse-passaged variants of strain 668 with increased acid sensitivity (which were separately isolated from different animals, designated strains mp1–5) exhibited any alterations in their PFGE or ribotyping profiles (Fig. 1A and B). Together, these results demonstrated that the *L. monocytogenes* food strain 668 increased in acid sensitivity by passage through an animal host without undergoing changes in its macrogenetic profile.

Table 2
In vivo passage increases acid sensitivity in *L. monocytogenes* strain 668 and 690.

Passage	Acid resistance (c.f.u. ml^{-1})	No. Isolates*					
		668			690		
		Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
In vivo mouse							
	>200	11	20	18	0	0	0
	20–200	14	19	15	0	0	0
	1–20	17	5	12	47	50	48
	0	8	6	5	3	0	2
PBS							
	>200	50	47	49	0	0	0
	20–200	0	3	1	0	0	0
	1–20	0	0	0	48	49	46
	0	0	0	0	2	1	4
BHI-broth							
	>200	50	49	48	0	0	0
	20–200	0	1	2	0	0	0
	1–20	0	0	0	46	48	47
	0	0	0	0	4	2	3

BALB/c mice were orally infected with *L. monocytogenes* cells grown overnight at 37 °C. At 3 days post inoculation, bacteria were recovered from the mouse intestines, and 50 randomly selected colonies on PALCUM agars were subjected to the acid resistance test, to determine the viable numbers after 48 h-incubation in PBS-LA (pH 4.0). Separately, bacteria grown overnight at 37 °C, were incubated in PBS (pH 7.4) or in BHI-broth for 3 days at 37 °C. Bacteria were then recovered and 50 randomly selected colonies were subjected to the acid resistance test in same manner. Each test was performed in three independent sets (Exp1–3).

* The numbers of isolates exhibiting >200, 20–200, 1–20, 0 c.f.u. ml^{-1} after a 48 h-incubation in PBS-LA (pH 4.0) were shown. Parental strains 668 and 690 exhibited this mean at 251 ± 54 or 2.4 ± 0.5 c.f.u. ml^{-1}, respectively. Five representative colonies of strain 668 recovered from mice, exhibiting no viability in the acid resistance test in Exp 1 were used as mouse-passaged variants of strain 668 (mp1–5) for subsequent studies.

3.3. The SigB response is linked to bacterial viability in an acidic environment

To elucidate the molecular basis of the altered acid resistance, we conducted semi-quantitative RT-PCR analyses to determine the expression levels of RNA polymerase sigma subunit genes (*sigB*, *rpoN*, *rpoD* and *sigH*) because of their pivotal role in regulating global gene expression in response to external stresses (Chaturongakul et al., 2008; Marles-Wright and Lewis, 2007). Strains 668 and 690 showed clear differences in *sigB* mRNA levels: the *sigB* gene was transcribed in strain 668 but not in strain 690, regardless of exposure to acidic pH (Fig. 2A). In agreement with this result, the gene *ctsR*, which controls class III heat shock gene expression in a SigB-dependent manner (Nair et al., 2000), was transcribed only in strain 668 (Fig. 2A). The mp1–5 variants of strain 668 also exhibited undetectable *sigB* transcript levels (Fig. 2B). Western blot analysis detected the SigB protein in strain 668 but not in strains 690 or mp1–5 (Fig. 2C). The level of SigB expression in strain 668 was increased by exposure to acidic pH (Fig. 2D), suggesting a pivotal role for SigB in the acid resistance of this strain. Comparative proteomic analyses revealed that three genes regulated by SigB, *lmo0211* (putative general stress protein, Ctc), *lmo2391* (hypothetical protein, similar to the *Bacillus subtilis* YhfK protein), *lmo0794* (hypothetical protein, similar to the *B. subtilis* YwnB protein) (Oliver et al., 2009), were more abundant in strain 668 than strain 690 under acidic conditions (Fig. S1 and Table S2). Together, these findings indicated that the host environment led to the selection of bacteria containing mutations that alter the transcriptional regulation of *sigB*.

3.4. Mutations in rsbW correspond to impairment of SigB-dependent acid resistance in L. monocytogenes

Having observed altered SigB activity in *L. monocytogenes* strains 668 and 690, we reasoned that the host environment might have triggered a mutation leading to *sigB* downregulation in the genome of strain 668. To investigate this possibility, we performed comparative genome sequencing analyses of *L. monocytogenes* strains 668 and 690. The SNP analysis showed potent mutation in several genes during host passage (Table S3). By focusing in approximately 4.3 kb portion of the *sigB* operon (*rsbR–rsbS–rsbT–rsbU–rsbV–rsbW–sigB*) (Wiedmann et al., 1998), we found a point mutation in the *rsbW* ORF in strain 690, the product of which acts in a partner-switching module for the control of SigB (Lee et al., 2004) (Fig. 3A). Four of 5 mouse-passaged variants of strain 668 (mp1, 2, 3, 5) also exhibited a mutation in this locus similarly, all of which generated stop codon in the RsbW sequence (Fig. 3). Thus, these data indicated that mutation in the *rsbW* gene was induced during the intrahost period, coincident with the onset of altered SigB-dependent acid resistance.

3.5. Intrahost mutation modestly attenuates growth/invasion properties of L. monocytogenes in tissue culture cells

This species is capable of surviving and replicating inside phagocytes, which is a prerequisite for it to spread to surrounding areas at an early stage of infection (Myers et al., 2003; Lee and Swanson, 2007). There is a mounting body of evidence indicating that SigB transcriptionally regulates a set of virulence genes in *L. monocytogenes* (Chaturongakul et al., 2008; Ollinger et al., 2009; Sue et al., 2004). We thus examined each strain's ability to survive in macrophages to investigate the effect of intrahost mutation in the intracellular behaviours. Our gentamicin protection assay showed that strain 668 exhibited better survival inside the J774 macrophage cells than strains 690 and mp1 at 2–4 h post infection (Fig. 4A). Likewise, strain 668 also exhibited a greater ability to in-

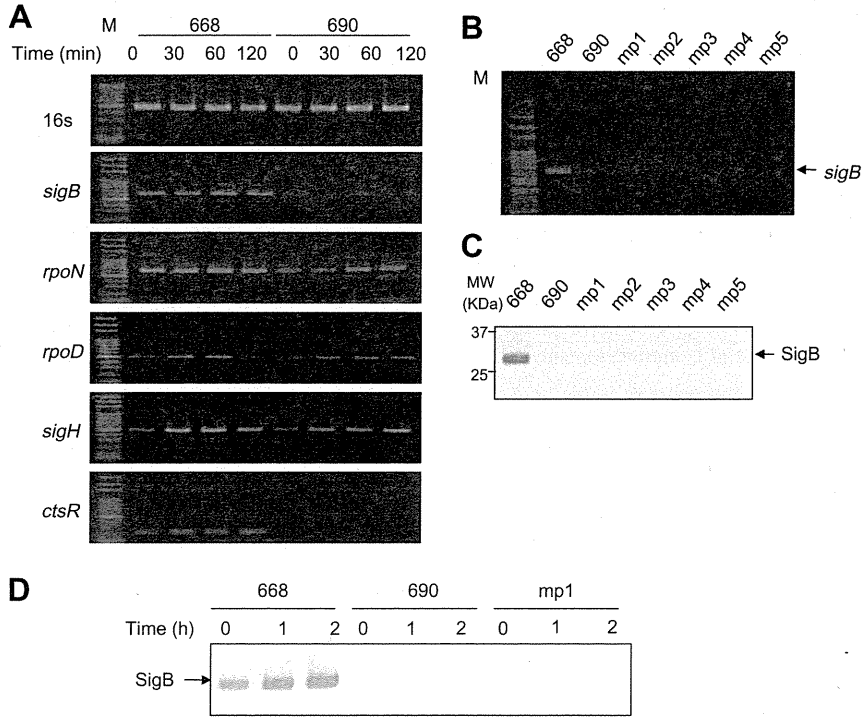


Fig. 2. Acid stress response of *L. monocytogenes*. (A) Strains 668 and 690 were incubated in PBS-lactic acid (PBS-LA; pH 4.0) for 0, 30, 60, or 120 min at 37 °C, and transcript levels of sigma factors (*sigB*, *rpoN*, *rpoD*, and *sigH*), the sigB-controlled gene *ctsR*, and 16S rRNA (control) were measured by semi-quantitative RT-PCR. M indicates the molecular marker (100-bp ladder). (B–C) Levels of *sigB* transcripts (B) and SigmaB protein (C) in *L. monocytogenes* strains 668, 690, and mouse-passaged variants mp1–5 in the absence of acidic stress. (D) Reactivity of the SigB protein to acidic pH stress in *L. monocytogenes* strains 668, 690, and mp1. Bacterial cells incubated for 0, 60 or 120 min in PBS-LA were subjected to Western blotting to detect SigB.

vade Caco-2 cells than strains 690 or mp1, in an m.o.i-dependent manner (Fig. 4B). Mice infection assay, which is capable of assessing the property of listeriolysin (LLO), ActA, and PlcAB (Lecuit, 2005), however showed that the strains 668, 690, and mp1 exhibited mice mortality with no significant differences (Fig. 4C). Taken together, we demonstrated that the acid sensitive strains were modestly attenuated for the growth and invasion in tissue culture cells, not affecting the systemic infection in mice.

4. Discussion

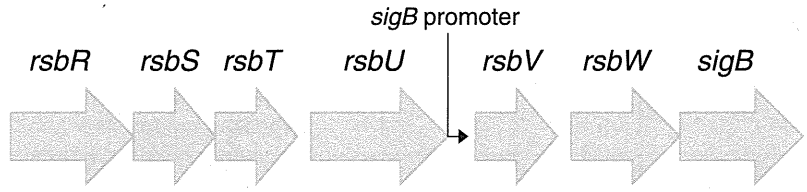
There is a growing body of evidence supporting the widespread presence of *L. monocytogenes* in a variety of habitats, including many foods (Ivanek et al., 2006; Swaminathan and Gerner-Smidt, 2007). We found that *L. monocytogenes* strains of a certain epidemiological background showed altered acid resistance after *in vivo* passage. Comparative genetic/proteomic studies in combination with *in vitro* cell-based assays revealed that an intrahost passage negatively altered the SigB-mediated gene regulation, thereby promoting the changes in bacterial acid resistance and its growth/invasion in tissue culture cells.

The SigB-dependent general stress response in Gram-positive bacteria can be elicited by a variety of stress factors, including starvation, acidic, osmotic, and oxidative stress, via a complex upstream signalling cascade (Ferreira et al., 2001). At least 11 proteins in *L. monocytogenes* have been shown to respond to acid and osmotic stress in a SigB-dependent manner (Abram et al., 2008). Our data linking acid resistance with *sigB* transcript levels suggest that the intrahost environment stimulated certain alterations in the genome of this pathogen that resulted in the downregulation of the *sigB* gene transcript.

Most prokaryotes develop a variety of protective mechanisms in response to foreign stressors, leading to alterations in their morphology and metabolism (Galhardo et al., 2007). To adapt to such stressful conditions, bacterial genomes evolve via many mechanisms (Smith et al., 1993; Feil et al., 1999, 2000; Lawrence and Hendrickson, 2003). However, compared with other model microorganisms such as *E. coli*, it seems likely that *L. monocytogenes* is only capable of limited diversification beyond plasmid gain/loss and phage conversion (Orsi et al., 2008), and homologous recombination rarely occurs (Ragon et al., 2008). Indeed, the macrogenotypes of the *L. monocytogenes* strains examined in this study did not alter upon host infection (Fig. 1). The observed phenotypic shift that did occur in the host environment thus led us to perform a comparative genomic analysis to unravel the molecular shift relating to this phenomenon.

Our comparative genetic analyses revealed point mutations in the *rsbW* locus during host infection. In the related Gram-positive pathogen *B. subtilis*, RsbW and RsbV are central players for regulating the SigB activity; RsbW directly binds to SigB, sequestering it from associating with RNA polymerase core enzyme (Benson and Haldenwang, 1993; Boylan et al., 1993). RsbW also acts as a kinase on RsbV (van Schaik et al., 2005), while RsbV conversely dephosphorylates RsbW, leaving SigB free to bind to the RNA polymerase core enzyme (Benson and Haldenwang, 1993; Dufour and Haldenwang, 1994). SigB-activating signalling pathway is however not completely understood in *L. monocytogenes*, and functional studies for the *rsbW* gene in this pathogen would be thus required to dissect the potent role of RsbW in SigB activity. Nevertheless, a previous study demonstrating that a mutation in the *rsbW*-homologue *rsbA* gene inhibited the *sigB* transcription and reduced salt-resistance in *Streptomyces coelicolor* (Lee et al., 2004), suggests that the mutation in *rsbW* gene occurred during host infection might promote the reduced SigB activity in *L. monocytogenes*.

Among the *sigB* operon genes in several gram-positive bacteria, RsbST and SigB proteins are highly conserved while other Rsb

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668         ATGGCAACAATGCATGACAAAATTACATTACAACTTCCTGCCAAGCCTGAATATGTTAGT
690         ATGGCAACAATGCATGACAAAATTACATTACAACTTCCTGCCAAGCCTGAATATGTTAGT
mp1-3       ATGGCAACAATGCATGACAAAATTACATTACAACTTCCTGCCAAGCCTGAATATGTTAGT
mp5         ATGGCAACAATGCATGACAAAATTACATTACAACTTCCTGCCAAGCCTGAATATGTTAGT
EGDe        ATGGCAACAATGCATGACAAAATTACATTACAACTTCCTGCCAAGCCTGAATATGTAAGT
            ************************************************************ ***

668         TTAGGTAGACTTTCATTATCAGGAATTGCAAGTCGCGCAGGATTTTCTTATGAAGCAATT
690         TTAGGTAGACTTTCATTATCAGGAATTGCAAGTCGCGCAGGATTTTCTTATGAAGCAATT
mp1-3       TTAGGTAGACTTTCATTATCAGGAATTGCAAGTCGCGCAGGATTTTCTTATGAAGCAATT
mp5         TTAGGTAGACTTTCATTATCAGGAATTGCAAGTCGCGCAGGATTTTCTTATGAAGCAATT
EGDe        TTAGGTAGACTTTCATTATCAGGAATTGCAAGTCGCGCAGGATTTTCTTATGAAGCAATT
            ************************************************************

668         GAAGATTTGAAAATAGCCGTAAGTGAAGCCATCACTAATTCTGTAAAGCACGCATTTAAA
690         GAAGATTTGAAAATAGCCGTAAGTGAAGCCATCACTAATTCTGTAAAGCACGCATTTAAA
mp1-3       GAAGATTTGAAAATAGCCGTAAGTGAAGCCATCACTAATTCTGTAAAGCACGCATTTAAA
mp5         GAAGATTTGAAAATAGCCGTAAGTGAAGCCATCACTAATTCTGTAAAGCACGCATTTAAA
EGDe        GAAGATTTGAAAATAGCTGTAAGTGAAGCCATCACTAATTCCGTAAAGCATGCATTTAAA
            ****************** ********************** ******** *********

668         GGAGAAGATGATGGTGAGATTACAGTAGAATATCTTATTTATGAAGACAAATTAGAAGTT
690         GGAGAAGATGATGGT-AGATTACAGTAGAATATCTTATTTATGAAGACAAATTAGAAGTT
mp1-3       TGAGAAGATGATGGTGAGATTACAGTAGAATATCTTATTTATGAAGACAAATTAGAAGTT
mp5         GGTGAAGATGATGGTGAGATTACAGTAGAATAACTTATTTATGAAGACAAATTAGAAGTT
EGDe        GGGGAAGAAGATGGCGAAATCACAGTCGAATATCTTATTTATGAAGATAAGCTAGAAGTT
            ** ***** ***** ** ** ***** ***** ************** **  ********

668         CGTGTTTCTGATAATGGAACAAGCTTCGACTTAGAAACCCGTAAACAAGAAATTGGCCCA
690         CGTGTTTCTGATAATGGAACAAGCTTCGACTTAGAAACCCGTAAACAAGAAATTGGCCCA
mp1-3       CGTGTTTCTGATAATGGAACAAGCTTCGACTTAGAAACCCGTAAACAAGAAATTGGCCCA
mp5         CGTGTTTCTGATAATGGAACAAGCTTCGACTTAGAAACCCGTAAACAAGAAATTGGCCCA
EGDe        CGTGTTTCCGATAACGGCACAAGCTTCGACTTAGAAACCCGTAAACAAGAAATTGGCCCA
            ******** ***** ********************************************

668         TATGAGGTAGGGGAAGATGCGGAGATGATGCGTATCGGAGGTCTAGGTTTATTTTTAATT
690         TATGAGGTAGGGGAAGATGCGGAGATGATGCGTATCGGAGGTCTAGGTTTATTTTTAATT
mp1-3       TATGAGGTAGGGGAAGATGCGGAGATGATGCGTATCGGAGGTCTAGGTTTATTTTTAATT
mp5         TATGAGGTAGGGGAAGATGCGGAGATGATGCGTATCGGAGGTCTAGGTTTATTTTTAATT
EGDe        TATGATGTGGGCGAGGATGCGGAGATGATGCGTATCGGTGGGCTAGGTTTATTTTTAATT
            ***** ** ***************************** ** ******************

668         GAAACATTAATGGATGACGTGAAACTTTATTATGATGAAGGGGTTTCTGTCGTAATGACC
690         GAAACATTAATGGATGACGTGAAACTTTATTATGATGAAGGGGTTTCTGTCGTAATGACC
mp1-3       GAAACATTAATGGATGACGTGAAACTTTATTATGATGAAGGGGTTTCTGTCGTAATGACC
mp5         GAAACATTAATGGATGACGTGAAACTTTATTATGATGAAGGGGTTTCTGTCGTAATGACC
EGDe        GAAACATTAATGGATGACGTGAAACTTTATTATGATGAAGGGGTTTCTGTCGTAATGACC
            ************************************************************

668         AAATATATTAATGAAAAGCAGGTGGAGGAGAATGCCAAAAGTATCTCAACCTGA
690         AAATATATTAATGAAAAGCAGGTGGAGGAGAATGCCAAAAGTATCTCAACCTGA
mp1-3       AAATATATTAATGAAAAGCAGGTGGAGGAGAATGCCAAAAGTATCTCAACCTGA
mp5         AAATATATTAATGAAAAGCAGGTGGAGGAGAATGCCAAAAGTATCTCAACCTGA
EGDe        AAATATATTAATGAAAAGCAGGTGGAGGAGAATGCCAAAAGTATCTCAACCTGA
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Fig. 3. Mutations in *rsbW* locus on *L. monocytogenes* genome during host passage. Upper panel represents the *sigB* operon constituted in *L. monocytogenes* strain 668. Bottom panel shows the multiple alignment of *rsbW* nucleotide sequences in strains 668, 690, mp1–3, mp5 and EGDe. Boxed bases represent difference in comparison with the base in the strain 668. Asterisks indicate identical bases among the strains aligned. The generated stop codon was underlined.

proteins including RsbW exhibited low conservation (Ferreira et al., 2004), suggesting that these less conservative regions might be one of the hotspots for mutation throughout the bacterial lifecycle. Although we still do not know how the mp4 strain obtained the reduced *sigB* expression through animal passage, genome-wide transcriptomic analyses in our future study would clarify the

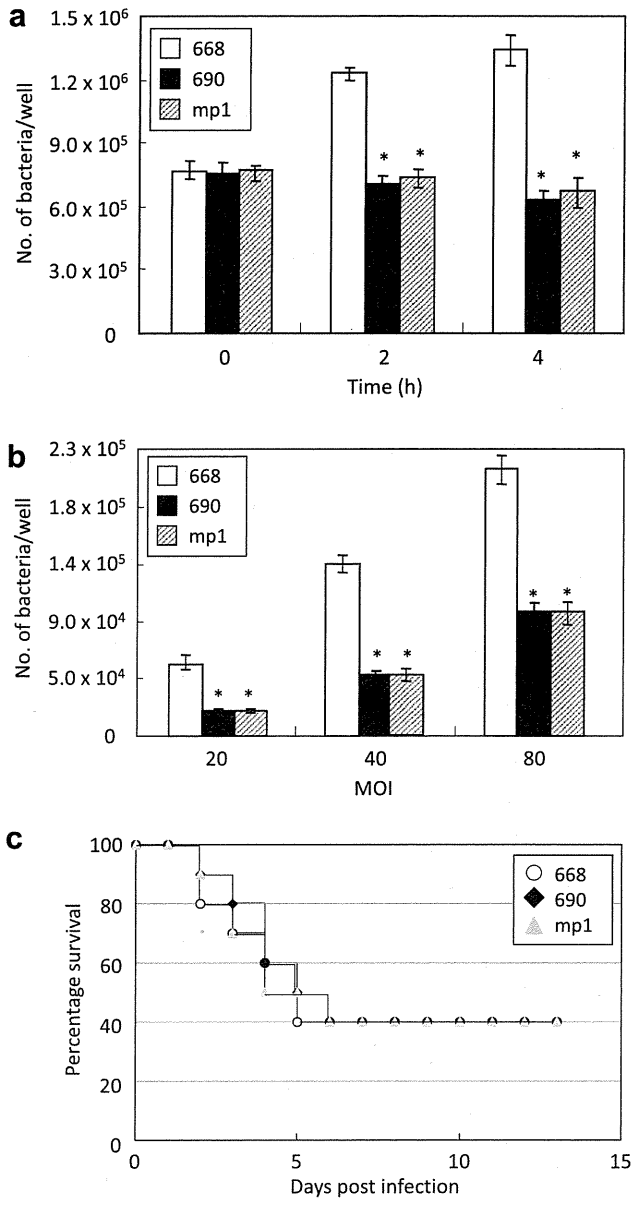

Fig. 4. Intrahost mutation leads to multiple phenotypic conversions. (A) Intracellular survival of *L. monocytogenes* strains 668, 690, and mp1 in the J774 mouse macrophage cell line was assessed by plate counts after gentamicin treatment at the indicated time points. (B) Intracellular viable counts of *L. monocytogenes* strains 668, 690, and mp1 in Caco-2 cells were generally determined as described in panel A, except that the infection process lasted only 1 h and involved different m.o.i (20, 40 or 80). The data in panels A & B are shown as means ± standard deviation from three independent experiments. Asterisks represent statistically significant differences from the means of strain 668. (C) Mice were intravenously inoculated with *L. monocytogenes* strains 668, 690, and mp1, and mortality (shown as percentage survival) was scored daily for 14 days. Differences in survival was calculated by Student's *t*-test ($p < 0.05$).

molecular basis behind this issue, with improving our knowledge on the stability and shift of *L. monocytogenes* genomic feature during host infection.

The SNPs found between strains 668 and 690 also supports that the host passage promotes mutation in the *L. monocytogenes* genome. However, we could not rule out the possibility that genome of the two strains might have been affected by any selective pressures differently in addition to the effect of host passage, associating with this SNP data.

SigB, downregulated by the intrahost passage, also plays a functional role in the host-pathogen interaction because it regulates PrfA-, and/or internalin A (InlA)-mediated gene expression, which is required for cell invasion and intracellular replication (Ollinger et al., 2009; Sue et al., 2004). This regulation could explain why strains 690 and mp1 showed reduced growth/survival within macrophages and invasion into Caco-2 cells. *L. monocytogenes* is likely to require SigB for adaptation to gastric intestinal (GI) environments including reduced pH, elevated osmolarity and bile salts, but not for systemic infection (Begley et al., 2005; Garner et al., 2006; Nadon et al., 2002). Consistently, our intravenous infection assay provided evidence that the reduced SigB activity occurred during host passage did not affect mortality. Future work using oral infection model (Disson et al., 2009; Wollert et al., 2007) would be advantageous to robustly assess the impact of the intrahost mutation on the bacterial virulence and fitness during infection processes.

In summary, we report the first example of an intrahost alteration of the SigB-dependent acid resistance and interaction to *in vitro* cell culture in the *L. monocytogenes*. As was found that *L. monocytogenes* strains from different outbreaks vary significantly in host cell invasion efficiency (Roberts et al., 2009), inactivation of SigB function may frequently occur in the host environment. Our study thus corroborates the prominent role of the host environment in genetic shift affecting bacterial fitness in multiple aspects.

Acknowledgements

This work was financially supported in part, by a Grant-in-Aid from Japan Food Safety Commission (No. 1101), a Grant-in-Aid of Scientific Research from Japan Society for Promotion of Science (JSPS, 22780275), and a Grant from the Ministry of Health, Labor and Welfare, Japan (Research on Food Safety).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.10.014.

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Epidemiol. Infect. (2012), **140**, 655–664. © Cambridge University Press 2011
doi:10.1017/S0950268811001105

Prevalence and growth kinetics of Shiga toxin-producing *Escherichia coli* (STEC) in bovine offal products in Japan

H. ASAKURA[1]*, E. SAITO[2], Y. MOMOSE[1], T. EKAWA[1], M. SAWADA[3], A. YAMAMOTO[2], A. HASEGAWA[4], J. IWAHORI[5], T. TSUTSUI[6], K. OSAKA[7], T. MATSUSHITA[4], M. KAKINUMA[4], K. MOTOYAMA[3], Y. HAYAMA[6], H. KITAMOTO[2], S. IGIMI[1] AND F. KASUGA[1]

[1] *Division of Biomedical Food Research, National Institute of Health Sciences, Tokyo, Japan*
[2] *Hyogo Prefectural Institute of Public Health and Consumer Sciences, Kobe, Japan*
[3] *Hitachi East Japan Solutions, Kawasaki, Japan*
[4] *Mitsubishi Research Institute, Tokyo, Japan*
[5] *Kochi Medical School, Nangoku, Japan*
[6] *National Institute of Animal Health, Tsukuba, Japan*
[7] *Tohoku University, Sendai, Japan*

(*Accepted 17 May 2011; first published online 21 June 2011*)

SUMMARY

Recent epidemiological data suggest a link between the consumption of bovine offal products and Shiga toxin-producing *Escherichia coli* (STEC) infection in Japan. This study thus examined the prevalence of STEC in various types of these foods. PCR screened 229 bovine offal products for the presence of Shiga toxin (*stx*) gene. Thirty-eight (16·6%) samples were *stx* positive, of which eight were positive for $rfbE_{O157}$ and three were positive for wzy_{O26}. Four O157 and one O26 STEC isolates were finally obtained from small-intestine and omasum products. Notably, homogenates of bovine intestinal products significantly reduced the extent of growth of O157 in the enrichment process compared to homogenates of beef carcass. As co-incubation of O157 with background microbiota complex from bovine intestinal products in buffered peptone water, in the absence of meat samples, tended to reduce the extent of growth of O157, we reasoned that certain microbiota present in offal products played a role. In support of this, inoculation of generic *E. coli* from bovine intestinal products into the homogenates significantly reduced the extent of growth of O157 in the homogenates of bovine intestinal and loin-beef products, and this effect was markedly increased when these homogenates were heat-treated prior to inoculation. Together, this report provides first evidence of the prevalence of STEC in a variety of bovine offal products in Japan. The prevalence data herein may be useful for risk assessment of those products as a potential source of human STEC infection beyond the epidemiological background. The growth characteristic of STEC O157 in offal products also indicates the importance of being aware when to test these food products.

Key words: Background microbiota, bovine offal products, growth kinetics, Shiga toxin-producing *Escherichia coli* (STEC).

* Author for correspondence: Dr H. Asakura, Division of Biomedical Food Research, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan.
(Email: hasakura@nihs.go.jp)

INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is a foodborne enteric pathogen that causes human illness ranging from mild diarrhoea to life-threatening haemolytic uraemic syndrome (HUS) [1]. Epidemiological studies have shown that STEC can potentially enter the human food chain from a number of animal sources; the most common sources are unpasteurized dairy products and meats cross-contaminated with faeces or intestinal contents after slaughter [2].

Because most causes of STEC outbreaks in the USA have been traced to beef containing the STEC serotype O157:H7 [3], epidemiological studies have focused on the prevalence of this serotype in beef and beef cattle. However, additional STEC serotypes, including serotypes O26, O91, O103, O111, O118, O145, and O166, have been isolated from beef and have caused human illnesses worldwide [4]. In Japan, the dominant serotype associated with human STEC infection is O157:H7, followed by O26:H11 and O111:H$^-$, with these three accounting for more than 95% of human STEC infections [5]. Of the 373 STEC serotypes isolated from cattle faeces or hides, 65 were identical to those detected in HUS patients, and 62 are known to cause other human illnesses [6]; these data highlight the fact that cattle and bovine food products are important reservoirs for human STEC infection.

Notably, in some Asian countries, it is customary to eat bovine offal products including liver, heart, and intestines; some are consumed raw, as so-called *sashimi*. Recent epidemiological data have indicated that of the 28 foodborne STEC O157 outbreaks in which their causative foods were identified in Japan during 2007–2010, 11 (39%) outbreaks were caused by the consumption of these bovine offal products [7]. These cases suggest that such food products might be one of the major sources of foodborne STEC infection in Japan. Despite this understanding, contamination rates and doses of STEC in those food products along their supply chain remain unclear, although a small-scale detection study has been reported [8].

In the present study, we examined the prevalence of STEC (particularly serotypes O157, O26, and O111) in various types of bovine offal products that are widely distributed in Japan. Throughout the comparative assessment of the growth kinetics of STEC, we found that these food products significantly affected the growth of STEC during enrichment.

Table 1. *Prevalence of* stx *and O157, O26, O111 serotype-specific genes in bovine internal organ foods*

Name	No. tested	No. *stx*-positive (%)	*No. positive*		
			$rfbE_{O157}$	wzy_{O26}	rfb_{O111}
Heart	6	2 (33·3)	—	—	—
Liver	36	5 (13·9)	1	—	—
Rumen	21	5 (23·8)	1	—	—
Reticulum	22	6 (27·3)	—	—	—
Omasum	38	4 (10·5)	2	1	—
Abomasum	24	2 (8·3)	—	—	—
Small intestine	54	10 (18·5)	3	1	—
Large intestine	22	4 (18·2)	1	1	—
Others*	6	0 (0·0)	—	—	—
Total	229	38 (16·6)	8	3	0

* Others represent kidney ($n=2$) and mixed intestinal products ($n=4$).

Therefore, we investigated background microbiota from the offal products and their effects on the growth of STEC.

MATERIALS AND METHODS

Food samples

A total of 229 raw bovine offal products that are commercially distributed throughout Japan via the internet were purchased from July to November 2010 (Table 1). These samples were delivered to the laboratory by rapid, cold road transport and were processed on arrival. These samples included liver ($n=36$), rumen ($n=21$), reticulum ($n=22$), omasum ($n=38$), abomasum ($n=24$), small intestine ($n=54$), large intestine ($n=22$), heart ($n=6$), and others (two kidney and four mixed intestinal products).

Culture enrichment and DNA extraction

A 25-g portion of each raw sample was diluted with 225 ml buffered peptone water (BPW) (pH 7·4) (Oxoid, UK) in a Stomacher bag (Eiken Kagaku, Japan), followed by homogenization for 1 min at 5·0 strokes/s using a Lab blender (IUL Instruments, Spain). After incubation at 42 °C for 20 h, a 1 ml aliquot of the enrichment broth was centrifuged at 21 500 *g* for 5 min, and the cell pellets were re-suspended in 100 μl PrepMan Ultra solution (Life Science Technology Inc., USA) to extract DNA according to the manufacturer's instructions.

PCR detection for *stx* and serotype-specific genes

The DNA extract prepared as described above was subjected to PCR to detect the Shiga toxin (*stx*) gene as described previously [9]. The *stx*-positive samples were further subjected to PCR to detect the following genes: $rfbE_{O157}$ [10], wzy_{O26} [11], and rfb_{O111} [12], which can specifically detect serotypes O157, O26, and O111, respectively.

Isolation and characterization of STEC from bovine offal products

Bacterial cultures that were PCR positive for any of the three serotype-specific genes were subjected to immunomagnetic separation using Dynabeads specific for O157 or O26 (Dynal, Norway) (according to the manufacturer's instructions) to isolate the target serotype bacteria. The following selective agar media were used: KBM STEC Chrom agar (Kojin Bio, Japan) and cefixime tellurite-sorbitol MacConkey (CT-SMAC) agar (Eiken Kagaku) for O157 and KBM STEC Chrom agar (Kojin Bio) and cefixime tellurite-rhamnose MacConkey (CT-RMAC) agar (Eiken Kagaku) for O26. The STEC isolates that were finally obtained were serotyped and genetically characterized for *stx*, *eaeA*, *EhlyA* and *uidA* as previously described [13]. Stx production was assayed with VTEC-RPLA (Denka Seiken, Japan).

Construction of kanamycin (Km)-resistant STEC O157

The plasmid pWM1007, which contains *kan* (Km-resistant) loci [14], was introduced into STEC O157 strains 204, 466, and 470 by electroporation. Successful transformants (designated 204-Km, 466-Km, 470-Km, respectively) (Table 2) were selected on CT-SMAC agar containing 50 μg/ml Km (CT-SMAC-Km).

Viable cell count of STEC O157 in bovine food homogenates

The STEC O157 strain 204-Km was grown in LB medium supplemented with 50 μg/ml Km (LB-Km) at 37 °C for 20 h. After being washed with sterile PBS twice, 100 μl each of the serially diluted suspension were separately used to inoculate 5-g portions of short-loin beef, liver, and small-intestine products in a Stomacher bag (Eiken Kagaku). Following suspension in 45 ml BPW, homogenization was performed in a Lab blender (IUL Instruments) at 5·0 strokes/s for 1 min. The homogenates were then incubated at 42 °C for 20 h. The numbers of viable O157 cells were determined by plating the homogenates onto CT-SMAC-Km at 0 h and 20 h post-incubation. To exclude strain-specific growth characteristics, we separately inoculated three O157 strains (204-Km, 466-Km, 470-Km) in bovine small-intestine products and their growth through BPW enrichment was also measured by plate count.

Measurement, isolation, and characterization of background microbiota in bovine offal

A 5-g portion of small-intestine product and short-loin beef was homogenized (1 min, 5·0 strokes/s) in 45 ml BPW as described above, and the serial dilutions were immediately plated on Nutrient agar (NA) plates (Eiken Kagaku) to determine aerobic plate counts (APCs) by incubation at 37 °C for 22 h, or MRS agar (BD Biosciences, USA) to measure the number of lactic acid bacteria (LAB) by anaerobic incubation using GasPak (BD Biosciences) at 37 °C for 48 h as described previously [15]. The remainder of the homogenates were thereafter incubated at 42 °C for 20 h, and also subjected to the same measurement of bacterial counts. To rule out bacteria that predominantly grew in the homogenates after incubation, six representative colonies grown on the NA and MRS plates from the 20-h culture were separately chosen, and their 16S rRNA sequences were determined by cycle sequencing using the primers 5′-CAGGCCTAACACATGCAAGTC-3′ and 5′-GGGGGGTGTACAAGGC-3′, in an ABI3730xl DNA analyser (Life Science Technology) according to the manufacturer's instructions. In the case of complete sequence similarity in the isolates from NA plates, one representative isolate was biochemically characterized with the Api-20E kit (bioMérieux, France) and used in the following studies.

Competitive growth assay

Co-incubation with background microbiota complex

A 5-g portion of short-loin beef or small-intestine samples was suspended in 45 ml BPW and vortexed for 60 s. Subsequently, the suspensions were filtered through a 20-μm-diameter filter (Millipore, USA) to remove the majority of the microbes from the food matrices. The filtrates were then centrifuged for

Table 2. *Bacterial strains used in the study*

Strain name	Taxonomy*	Source	*E. coli* serotype	Genotype				
				stx1	*stx2*	*eaeA*	*uidA*	*EhxA*
STEC isolates								
022-1	*E. coli*	Small intestine	O157:H7	−	+	+	+	+
066-1	*E. coli*	Small intestine	O157:H7	−	+	+	+	+
068-1	*E. coli*	Omasum	O26:H11	+	−	+	−	+
HG16	*E. coli*	Omasum	O157:H7	+	−	+	+	+
HG45	*E. coli*	Small intestine	O157:H21	−	−	+	+	+
Background microbiota								
SI-1	*E. coli* (AB609040)	Small intestine	OUT:H21	−	−	−	−	−
SI-2	*Aeromonas* sp. (AB609041)	Small intestine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
SI-3	*E. coli* (AB609042)	Small intestine	OUT:HUT	−	−	−	−	−
SI-4	*Enterococcus* sp. (AB609043)	Small intestine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
SI-7	*E. coli* (AB609044)	Small intestine	OUT:H16	−	−	−	−	−
LAB-1	*Lactobacillus* sp. (AB609049)	Small intestine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
LAB-2	*Lactobacillus sakei* (AB609050)	Small intestine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
LAB-3	*Lactobacillus* sp. (AB609051)	Small intestine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
B-1	*Hafnia alvei* (AB609045)	Short-loin beef	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
B-4	*Enterobacter* sp. (AB609046)	Short-loin beef	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
B-6	*Yersinia enterocolitica* (AB609047)	Short-loin beef	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
B-9	*Hafnia alvei* (AB609048)	Short-loin beef	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
STEC O157 strains for spike experiments (harbouring plasmid pWM1007)								
206-Km	*E. coli*	Human	O157:H7	−	+	+	+	+
466-Km	*E. coli*	Liver	O157:H7	+	+	+	+	+
470-Km	*E. coli*	Small intestine	O157:H7	−	+	+	+	+

n.t., Not tested.
All *E. coli* were genotyped for the presence (+/−) of *stx*, *eaeA*, *uidA*, and *EhxA* genes, and were O- and H-serotyped.
* Taxonomy of the background microbiota were based on the 16S rRNA sequences (GenBank accession numbers given in parentheses). Km-resistant STEC O157 strains 204-Km, 466-Km, 470-Km were constructed by introducing the plasmid pWM1007.

15 min at 2150 ***g***, and the bacterial pellets were carefully washed and resuspended in 45 ml fresh BPW. About $1{\cdot}5 \times 10^3$ cells of STEC O157 strain 204-Km were then used to inoculate background microbiota-containing BPW suspensions in a Stomacher bag and these cultures were then incubated at 42 °C for 20 h. After incubation, the growth of STEC O157 was determined by plate counts on CT-SMAC-Km.

Co-incubation with representative microbiota in food homogenates

A 5-g portion of short-loin beef or small-intestine products was added to 45 ml BPW, homogenized as described above in a Stomacher bag, followed by incubation for 30 min at 63 °C (heat+) to kill most bacteria, or kept at room temperature (heat–) not to kill bacteria (we confirmed that following the above

heating, both types of food homogenates contained <10 c.f.u./ml bacteria, enumerated by colony counts on NA agar). After cooling down to room temperature, the food homogenates were inoculated with $8{\cdot}6 \times 10^2$ cells of O157 strain 204-Km and co-cultured with approximately 10^2 c.f.u. orders of representative background microbiota from small-intestine products (SI-1, -2, -3, -4, and LAB-2) at 42 °C for 20 h. The growth of O157 cells was determined by plate count on CT-SMAC-Km. The food homogenates spiked only with O157 culture (without microbiota) were used as controls.

Nucleotide sequence data

The 16S rRNA sequences from the representative background microbiota were deposited in GenBank under accession numbers AB609039–AB609051.

Statistical analysis

Data for the plate counts represent means ± standard deviation from three sets in two independent experiments unless otherwise indicated. Statistical significance was calculated by Student's t test ($P<0{\cdot}05$).

RESULTS

Prevalence of STEC-related genes in bovine offal products in Japan

From July to November 2010, 229 bovine offal products that were commercially distributed throughout Japan were subjected to testing for the prevalence of STEC. PCR screening detected the *stx* gene in 38 (16·6%) samples. These samples included heart (33·3%), liver (11·1%), rumen (23·8%), reticulum (27·3%), omasum (10·5%), abomasum (8·3%), small intestine (18·5%), large intestine (18·2%), and others (0%) (Table 1). Of these samples, eight were positive for $rfbE_{O157}$, and three were positive for wzy_{O26}. One omasum sample was positive for both $rfbE_{O157}$ and wzy_{O26} (Table 1). The rfb_{O111} gene was not detected in any of the samples tested.

Isolation and characterization of STEC O157 and O26

Of the 10 samples that were positive for $rfbE_{O157}$ or/and wzy_{O26}, immunomagnetic separation in combination with the selective media enabled us to isolate three O157:H7, one O157:H21, and one O26:H11 STEC from small-intestine and omasum products (Table 2). All STEC strains except the strain HG45 (serotype O157:H21) produced cytotoxic Shiga toxins, and their *stx* genotypes consisted of *stx1* alone ($n=2$), and *stx2* alone ($n=2$) (Table 2). They all possessed the *EhxA* gene, and the *uidA* gene was detected in all O157 isolates (Table 2).

Type of food matrix that affects the growth kinetics of STEC O157

Having little information on the enrichment efficiency of the bovine offal products for STEC bacteria, we experimentally used the Km-resistant STEC O157 strain 204-Km to inoculate samples of short-loin beef, liver, and small intestine, followed by homogenization. After incubation of the homogenates, the growth of O157 cells was determined. The short-loin beef homogenates allowed the STEC bacteria to grow to a concentration ranging between $1{\cdot}1 \times 10^7$ and $2{\cdot}8 \times 10^7$ c.f.u./ml when inoculated with $2{\cdot}5 \times 10^3$ c.f.u. of STEC O157 strains (Fig. 1*a*). However, the inoculums were not significantly increased in the homogenates of small-intestine products, exhibiting only a 3·2- to 4·8-fold increase ($8{\cdot}1 \times 10^3$ to $1{\cdot}2 \times 10^4$ c.f.u./g) (Fig. 1*a*). The growth of O157 cells in the liver sample homogenates was intermediate; $5{\cdot}9 \times 10^4$ to $3{\cdot}2 \times 10^5$ c.f.u./g of STEC O157 were recovered under the same conditions (Fig. 1*a*). The reduced extent of STEC O157's growth in the homogenates of bovine small-intestine products was confirmed not to be a strain-dependent characteristic because three different O157 strains exhibited similar trends towards O157 growth (Fig. 1*b*). Taken together, the data demonstrated that homogenates of bovine digestive-tract products significantly reduced the extent of STEC O157 growth in the enrichment process compared to homogenates of loin beef and bovine liver products.

Background microbiota in bovine intestinal products plays a role in affecting STEC O157 growth in BPW

Due to the significant effect of the homogenates of small-intestine products on STEC O157 growth, we assumed that background microbiota in the homogenates might play a role in reducing the extent of growth of STEC bacteria in BPW. To examine this issue, we first isolated the background microbiota complexes from short-loin beef or bovine small-intestine products, using filtration-based isolation of the background microbiota; this enabled us to collect

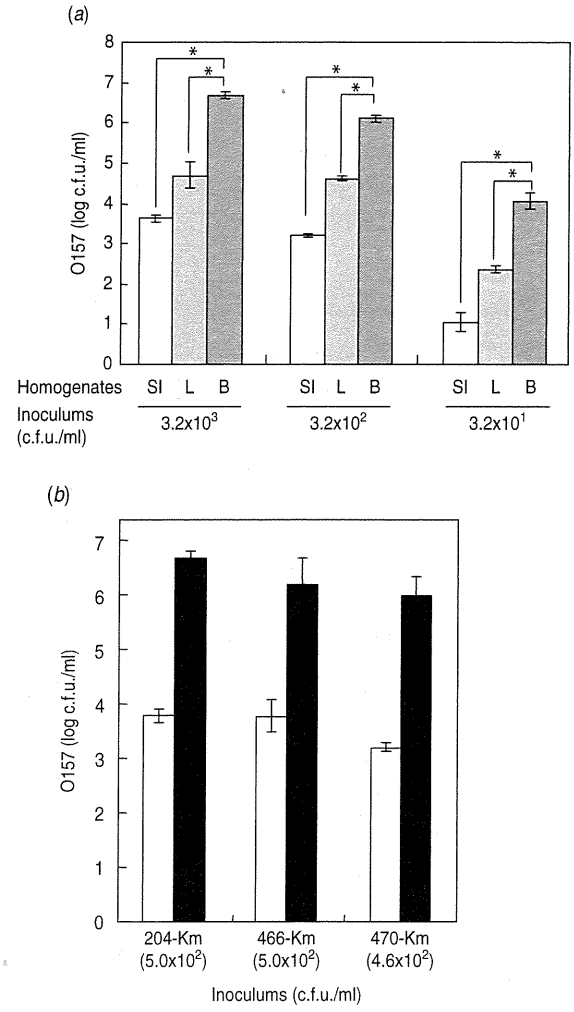

Fig. 1. Food matrixes of bovine digestive-tract products significantly affect the growth of STEC O157 during the enrichment process. (*a*) Different numbers of STEC O157 strain 204-Km ($3{\cdot}2\times10^3$, $3{\cdot}2\times10^2$, $3{\cdot}2\times10^1$ c.f.u.) were inoculated into 5-g portions of bovine products (SI, small intestine; L, liver; B, short-loin beef) and homogenized in BPW. After incubation, the STEC O157 cells were counted on CT-SMAC-Km. (*b*) Three STEC O157 strains (204-Km, 466-Km, 470-Km) were used to inoculate bovine small-intestine (□) and short-loin beef (■) products and homogenized in BPW. In panels (*a*) and (*b*), means represent viable numbers of O157 (c.f.u./ml), and error bars represent standard deviation. Asterisks indicate statistical significance ($P<0{\cdot}05$).

approximately $4{\cdot}8\times10^3$ or $5{\cdot}8\times10^3$ cells of the microbiota from 5-g short-loin beef and small-intestine products, respectively, which corresponded to 40% and 44·6%, respectively, of the APCs that were counted by direct plating of the homogenates ($1{\cdot}2\times10^4$ or $1{\cdot}3\times10^4$ cells, respectively) (Fig. 2*a*). Through the co-incubation with loin beef-originated background microbiota complex in the absence of food homogenates, the O157 strain 204-Km grew to a concentration ranging from $2{\cdot}8\times10^7$ to $4{\cdot}5\times10^7$ c.f.u./ml BPW medium (Fig. 2*b*). In comparison, co-incubation with background microbiota from small-intestine products allowed STEC cells to grow only to $7{\cdot}7\times10^5$ c.f.u./ml, which was about 30-fold less than from co-incubation with short-loin beef-derived background microbiota (Fig. 2*b*). These data thus suggested that the background microbiota complex in small-intestine products played a role in affecting the growth of STEC O157 in BPW.

Bovine intestinal products contain different composition of background microbiota than short-loin beef products

Given the different effects of background microbiota from the loin-beef and bovine small-intestine products on the growth of O157 under the experimental co-culture conditions, we next examined the population and composition of APCs, and LAB in the homogenates of bovine short-loin beef and small-intestine products before and after incubation. Between the two types of the homogenates, APCs were not significantly different throughout the enrichment ($1{\cdot}2\times10^3$ and $1{\cdot}3\times10^3$ c.f.u./ml at pre-incubation, and $7{\cdot}8\times10^7$ and $7{\cdot}1\times10^7$ c.f.u./ml at 20 h post-incubation, respectively) (Fig. 2*b*). Moreover, the homogenates of small-intestine products contained more LAB compared to the short-loin beef sample homogenates at pre-incubation ($6{\cdot}8\times10^3$ and $4{\cdot}2\times10^1$ c.f.u./ml, $P<0{\cdot}05$), whereas both were increased to almost equal levels after incubation (Fig. 2*b*). Through genetic and biochemical characterization, we subsequently identified representative bacterial colonies predominantly grown after incubation; the microbiota in small-intestine products consisted of *E. coli*, *Aeromonas* spp., and *Enterococcus faecium* (Table 2). In comparison, no *E. coli* were isolated from the short-loin beef homogenate sample, which instead contained *Hafnia alvei*, *Enterobacter* spp., and *Yersinia enterocolitica* (Table 2). In addition, representative LAB isolates from the homogenates of small-intestine products were genetically identified as *Lactobacillus* spp. and *L. sakei* (Table 2). These findings indicated that microbiota that grew predominantly in homogenates of bovine small-intestine products were compositionally different from those in the homogenates of short-loin beef.

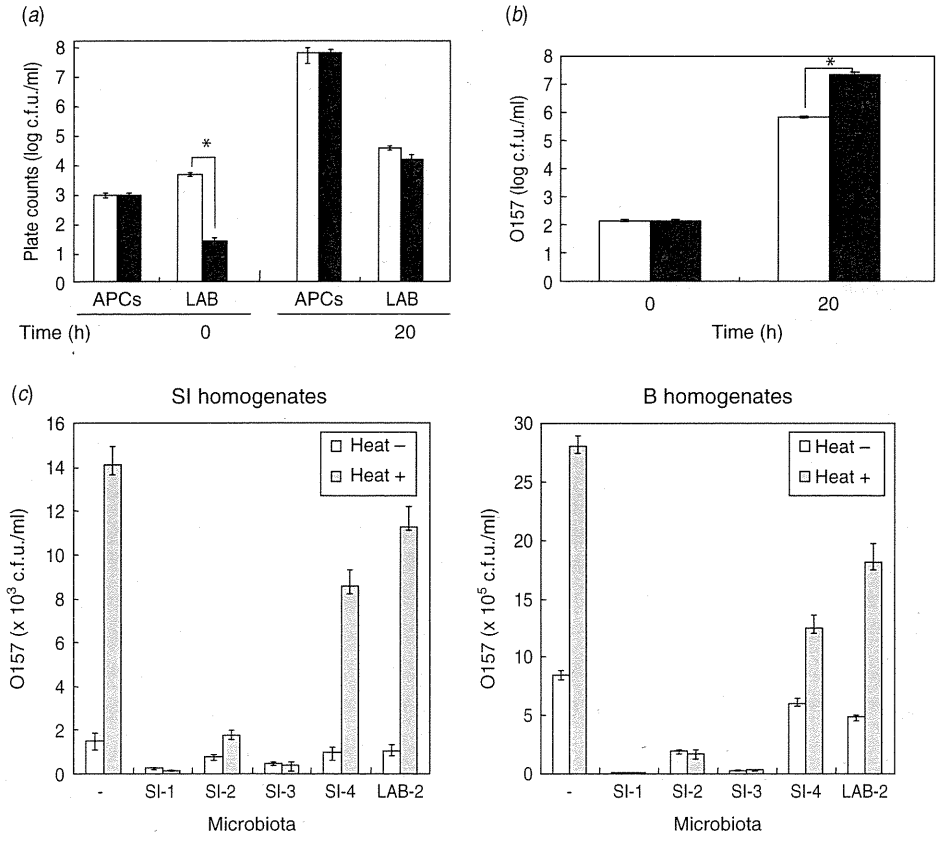

Fig. 2. Background microbiota affects the growth of STEC O157 during enrichment. (*a*) Aerobic plate counts (APCs) and lactic acid bacteria (LAB) counts in homogenates of small-intestine (□) and short-loin beef (■) products were counted before (0 h) and after (20 h) incubation. (*b*) Background microbiota complex was isolated from small-intestine (□) or short-loin beef products (■), then co-incubated with O157 strain 204-Km in BPW in the absence of food homogenates. At 0 h and 20 h post-incubation, numbers of STEC O157 were counted. Asterisks in section panels (*a*) and (*b*) indicate statistical significance ($P<0{\cdot}05$). (*c*) Homogenates of small-intestine products (left panel) or short-loin beef (right panel) were treated with heat (heat+) for 30 min or untreated (heat–). These food sample homogenates were then inoculated with O157 strain 204-Km and representative microbiota from small-intestine products (SI-1, -2, -3, -4, and LAB-2). After incubation, the growth of O157 was determined. Food sample homogenates spiked only with O157 (with no spiked microbiota) were used as control (–). Error bars in panels (*a*)–(*c*) indicate standard deviation.

Generic *E. coli* reduces the extent of STEC O157 growth in BPW homogenates

Having established that the background microbiota complex from bovine small-intestine products significantly reduced the extent of growth of O157 in BPW in the absence of food sample homogenates, and in which different composition of microbiota were predominantly grown compared to the homogenates of short-loin beef, we next examined the effect of representative microbiota isolates on STEC O157 growth by co-incubation with the food homogenates. Prior to incubation, the homogenates of small-intestine and short-loin beef products were treated with or without heating (63 °C, 30 min). These homogenates were then spiked with STEC O157 and equal orders of microbiota isolates from small-intestine products, following by incubation for 20 h. In all homogenates, the heat treatment increased the growth of O157 compared to unheated homogenates (Fig. 2*c*); in the absence of spiked microbiota, the heat treatment increased the number of O157 by 9·2-fold in small-intestine homogenates and by 3·3-fold in loin-beef homogenates, respectively (Fig. 2*c*). Of homogenates spiked with microbiota, those with generic *E. coli* microbiota (SI-1, SI-3) allowed less growth of O157 cells in small-intestine and loin-beef homogenates (Fig. 2*c*). Spiking with LAB (LAB-2) did not produce any marked influence on the growth of O157 in either type of homogenate, compared to generic *E. coli* (Fig. 2*c*). Taken together, we were able to demonstrate that generic *E. coli* was one of the predominant microbiota in reducing the extent of STEC O157 growth in the homogenates during the enrichment process.

DISCUSSION

The types of food associated with STEC outbreaks and the geographical distribution of cases differ between countries. These differences have originated from local food preferences, culinary customs, and patterns of food distribution. Recent epidemiological data in Japan have revealed that consuming bovine offal products seems to be linked to the occurrence of STEC infection [7]. Therefore, for the first time, this study examined the prevalence of STEC in various types of bovine offal products.

The efficacy of a detection method depends on the choice of the base medium, selective agents, and their concentrations. The interactions between these factors are also expected to affect the sensitivity of the detection method, especially when the test sample contains a small number of STEC cells. After slaughter, bovine offal products are often treated with chlorine, and it is likely that such sanitizing damages or kills many of the STEC bacteria [16]. Thus, it is conceivable that a portion of STEC in the test samples might have been damaged by chlorination. A comparative evaluation study on the recovery medium for STEC O157, O26, and O111 showed that BPW could efficiently recover damaged O157 bacteria culture, while tryptone soya and EC broths, and supplementation with vancomycin and novobiocin prevented bacterial growth [17, 18]. Further, BPW without additives was superior to BPW with additives for the isolation of STEC O157 by immunomagnetic separation from bovine faecal samples [19]. Therefore, we selected BPW without supplements as the enrichment media.

Many studies have focused the prevalence of STEC O157 in cattle faeces, hides, trim, or ground beef because of their epidemiological importance to public health [6]. In this study, the *stx* gene was detected from a variety of bovine offal products. However, it is difficult to conclude that these rates reflect the contamination rates of STEC in these products because of the different numbers tested. Conversely, successful isolation of STEC O157 and/or O26 only from digestive-tract products including small-intestine and omasum (Table 2) suggested that these products might be contaminated with high populations of STEC pathogens, compared to the others. Most O157 STEC strains relating to human infection possess H7 flagellin or no flagellin (H^-), while the serotype O157:H21 isolated from bovine small-intestine products in this study appears to be rare and the association with human infection is unknown, because no report exists for the isolation of this serotype from beef cattle and their products [6].

We repeatedly subcultured the samples that were positive for *stx* and either $rfbE_{O157}$ or wzy_{O26}, in different enrichment media for the isolation of STEC. Nevertheless, some of the STEC were not isolated. Possible explanations for this result include the following: the STEC had already died, the STEC were minimally damaged during storage and transport of these products, or the growth of STEC was competitively inhibited by certain microbes present in the food samples during the processes of storage and enrichment.

Our focus on the growth kinetics of STEC in the food matrix during the process of enrichment thereby revealed that bovine small-intestine homogenates greatly reduced the extent of STEC O157 growth compared to short-loin beef homogenates. Consequently, we are able to demonstrate that in a variety of background microbiota, generic *E. coli* predominantly played a role in reducing the extent of STEC growth in the homogenates of bovine offal products. Although we also found higher levels of LAB in small-intestine products compared to that in short-loin beef, co-incubation with LAB weakly affected STEC O157 growth relative to generic *E. coli* and others in the food sample homogenates (Fig. 2*c*). In addition, the numbers of LAB after incubation were not significantly different between the two types of food products and both reached only around 10^4 c.f.u./ml (Fig. 2*b*), further supporting our notion that LAB might not be the predominant microbiota to affect the growth of STEC in the enrichment process. Even with heat pretreatment to kill most microbiota, O157 still grew more in homogenates of short-loin beef than in homogenates of bovine intestinal products (Fig. 2*c*). This suggests the additional factors in the food matrices also influence the growth of O157. Although the bovine intestinal products contained greater amounts of fat content than the loin beef products (data not shown), a recent work by Bosilevac *et al.* reported that increased fat content did not interfere with recovery of STEC O157 from beef carcasses [20]. Further exploration for such factors would be valuable to improve the isolation method of STEC from bovine offal products.

The population of STEC may also be affected by the processing and storage conditions of the food products. A previous study reported that feedlot cattle are infected with O157 at a level of about log

1·17 MPN/g, and some cattle exhibit a ~1000-fold increase at that stage of slaughter (log 5·66 MPN/g) [21]. The samples tested in this study were further multi-processed with washing, cutting, packaging, storage and transportation at 4 °C before being subjected to examination. The data provided here are only for the final products, and therefore the compositional changes of STEC and microbiota in these foods during each process needs to be clarified in our future work. A global metabolomic approach will be valuable to gain comprehensive information on the composition and functional roles of background microbiota in food products.

In conclusion, we demonstrated the prevalence of STEC in various types of bovine offal products for the first time; the prevalence data (i.e. contamination rates with the isolate's virulence characteristics) herein may be useful for the risk assessment of those products as a potential source of human infection. Because a low dose of this pathogen can cause a relatively high attack rate, even survival without growth in bovine digestive-tract products may pose a public health risk if the food is insufficiently heat-treated or consumed raw. Other data such as 'annual consumption volumes/rates' for these foods, would further improve our understanding of the epidemiological importance. The characteristic growth kinetics of STEC O157 shown in this study also indicates the importance of being aware when to test these foods.

ACKNOWLEDGEMENTS

We thank Shin-ichiro Yoshida (Japan Food Research Laboratories) and Taku Yamaguchi (Japan Frozen Food Inspection Cooperation) for technical support in bacterial detection. This work was financially supported in part by a Grant-in-Aid from the Food Safety Commission, Japan (No. 0805) and a Grant from the Ministry of Health, Labor and Welfare, Japan.

DECLARATION OF INTEREST

None.

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Biochemical and Biophysical Research Communications 426 (2012) 654–658

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Membrane topology of *Salmonella* invasion protein SipB confers osmotolerance

Hiroshi Asakura [a,*], Tomoya Ekawa [a], Naoki Sugimoto [b], Yoshika Momose [a], Keiko Kawamoto [c], Sou-ichi Makino [d], Shizunobu Igimi [a], Shigeki Yamamoto [a]

[a] Division of Biomedical Food Research, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan
[b] Division of Food Additives, National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan
[c] Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, Inada 2-11, Obihiro, Hokkaido 080-8555, Japan
[d] Department of Life Science, Kyoto Seibo College, Fukakusatanicho 1, Fushimi-ku, Kyoto, Kyoto 612-0878, Japan

ARTICLE INFO

Article history:
Received 29 August 2012
Available online 10 September 2012

Keywords:
Salmonella enterica serovar Typhimurium
SipB
Osmotolerance

ABSTRACT

Salmonella enterica serovar Typhimurium is a major cause of human gastrointestinal illness worldwide. This pathogen can persist in a wide range of environments, making it of great concern to public health. Here, we report that the *salmonella* pathogenicity island (SPI)-1 effector protein SipB exhibits a membrane topology that confers bacterial osmotolerance. Disruption of the *sipB* gene or the *invG* gene (SPI-1 component) significantly reduced the osmotolerance of *S.* Typhimurium LT2. Biochemical assays showed that NaCl osmolarity increased the membrane topology of SipB, and a neutralising antibody against SipB reduced osmotolerance in the WT strain. The WT strain, but not the *sipB* mutant, exhibited elevated cyclopropane fatty acid C19:0 during conditions of osmotic stress, correlating with the observed levels of survival and membrane integrity. This result suggests a link between SipB and the altered fatty acid composition induced upon exposure to osmotic stress. Overall, our findings provide the first evidence that the *Salmonella* virulence translocon SipB affects membrane fluidity and alters bacterial osmotolerance.

1. Introduction

Salmonella enterica subsp. *enterica* serovar Typhimurium is one of the major causes of foodborne diseases worldwide. Central to *S.* Typhimurium pathogenesis are two Type 3 Secretion Systems (TTSSs) encoded within *salmonella* pathogenicity islands (SPI)-1 and -2. TTSSs are responsible for the secretion and translocation of a set of bacterial proteins known as effectors into host cells. This process facilitates host cell invasion and inflammation (by SPI-1) or survival and replication within phagocytes (for SPI-2) to establish a systemic infection [1]. The expression of SPI apparatus genes is known to be regulated by multiple environmental signals, including osmolarity, oxygen tension, pH, and the growth rate of bacteria [2–5].

This pathogen is known to be widespread in host and hostile environments and can survive for long periods of time in aquatic environments, making it a significant public health concern [6]. There is mounting evidence that SipB, a SPI-1-mediated effector protein that promotes host cell apoptosis and necrosis via caspase-1-mediated activation of IL-1β and IL-18 [7,8], exhibits bacterial outer membrane topology [9,10]. More recent studies have shown that this surface-association is mediated through SPI-1 assembly when bacteria are cultivated with NaCl osmolarity [11]. The osmo-responsible SipB translocation was shown to be regulated through the upregulation of *hilAD* systems [12] under the control of the ATP-dependent Lon protease [13].

Considering that bacterial membranes first sense environmental changes and then trigger a variety of response mechanisms to adapt [14], we hypothesised that the membrane localisation of SipB might affect bacterial osmotic stress tolerance. To examine this issue, we investigated the role of SipB membrane fusion in the osmotolerance of *S.* Typhimurium. Our studies revealed that the disruption of the *sipB* gene or the *invG* gene (encoding the SPI-1 multiring base required for SPI-1 mediated protein secretion) [15] clearly impaired the osmotolerance of this pathogen. Biochemical assays then provided evidence that the disruption of SipB weakened bacterial osmotolerance upon exposure to osmotic stimuli, coinciding with reduced membrane integrity and no clear upshift in the level of cyclopropane fatty acid C19:0.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The *Salmonella enterica* serovar Typhimurium LT2 strain was used as the wild type (WT) strain and for constructing the knockout mutants. *Escherichia coli* DH5α was used for genetic cloning.

* Corresponding author. Fax: +81 3 3700 9246.
E-mail address: hasakura@nihs.go.jp (H. Asakura).

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http://dx.doi.org/10.1016/j.bbrc.2012.09.012

Except where indicated otherwise, bacteria were routinely grown at 37 °C in Luria–Bertani (LB) broth or on LB agar (LA) plates with the appropriate antibiotics.

2.2. Disruption and complementation of the sipB/invG genes

The *S.* Typhimurium LT2-derived *invG* mutant and *sipB* mutant were constructed by the λRed recombinase method as described [16]. The primer sequences used for the replacement of the target genes are listed in Table S1. The antibiotic cassette was removed by FRT-mediated FLP recombination after transformation with pCP20 [16], and unmarked mutants were selected by sensitive antibiotics and verified by DNA sequencing. For complementation, the *invG* and *sipB* genes were PCR-amplified with primer sets (Table S1) and cloned into the *Bam*HI/*Sal*I site of the plasmid pACYC184 in *E. coli* DH5α. The plasmids were then used to transform the *invG* mutant or *sipB* mutants by electroporation. Successful transformants were screened on chloramphenicol (30 μg ml^{-1})-agar plates at 37 °C.

2.3. NaCl sensitivity test

Approximately 1.0×10^9 cells grown in TY medium (1% (w/v) tryptone and 0.5% (w/v) yeast extract) for 18 h at 37 °C were washed with phosphate-buffered saline (PBS) (pH 7.2) twice, followed by incubation in 50 ml of 1.36 M (equivalent to 8% (w/v)) NaCl in PBS (pH 7.2) at 37 °C for up to 9 days. At 24 h intervals, 100 μl of the suspension and serial dilutions was spread onto Tryptic soy agar (TSA) plates to count the number of viable cells. Simultaneously, the suspensions were fluorescently stained with Bac Light Bacterial viability kit (Invitrogen-Molecular Probes, Eugene, OR, USA) to measure the membrane integrity as described [17].

2.4. Real-time PCR

Total RNA was prepared from the bacterial cells with a RiboPure bacterial RNA isolation kit (Life Science Technologies, Carlsbad, CA) and subjected to cDNA synthesis using a first strand cDNA synthesis kit (Roche Diagnostics, Burgdorf, Switzerland). Real-time PCR assays were performed with oligonucleotide primers for *hilA*, *hilD*, *sipB*, and 16S rRNA (Table S1) using a Light Cycler 480 DNA analyser (Roche Diagnostics, Burgdorf, Switzerland). The transcript levels for each gene were determined according to the manufacturer's instructions by calculating the relative quantification and using 16s rRNA as a standard.

2.5. Expression and purification of SipB recombinant protein

The *sipB* coding sequence derived from *S.* Typhimurium LT2 was amplified by PCR with primers (Table S1), cloned into pBAD202-D-Topo (Life Science Technologies, Carlsbad, CA), and transformed into *E. coli* KRX competent cells (Promega, Fitchburg, WI, USA). Successful transformants were grown in 500 ml of LB broth at 37 °C to an A_{600} of 1.0 and induced with 0.1% L-arabinose (Sigma Aldrich, St. Louis, MO, USA) for 6 h. The recombinant SipB was purified with Ni–NTA agarose (QIAGEN, Hilden, Germany). The resultant rSipB protein was then used to produce rabbit anti-SipB antisera by in-house immunisation of white rabbits.

2.6. Subcellular fractionation, SDS–PAGE, and Western blot

Subfractions of *Salmonella* cells were obtained essentially as described [11]. Approximately 30 μg of the protein samples was loaded onto 10% acrylamide gels. The proteins on the gels were visualised with CBB (Coomassie Brilliant Blue) stain. For the western blot protocol, the proteins were simultaneously transferred onto PVDF membrane (Millipore). Rabbit anti-SipB or anti-OmpW antisera [17] were used as the primary antibodies. HRP-conjugated anti-rabbit IgG antibody (GE HealthCare, Little Chalfont, UK) was used as the secondary antibody. The protein signals were detected with the ECL detection system (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

2.7. Antibody neutralisation assay

Approximately 10^9 CFU of WT strain grown in TY medium for 18 h at 37 °C were washed twice with PBS (pH 7.2), followed by incubation at 37 °C in 50 ml of 1.36 M NaCl/PBS (pH 7.2) supplemented with 0, 1, 5, 10, and 20 μl of anti-SipB antiserum. At days 3, 5, and 7 post incubation, 100 μl of the suspension and serial dilutions was spread onto TSA plates to count the number of viable cells.

2.8. Fatty acid methyl ester (FAME) assay

Bacterial cells were harvested, washed three times with sterile PBS, and freeze-dried. The pellets were then used to extract and measure fatty acid methyl ester (FAME) using the Sherlock Microbial ID system (MIDI Inc., Newark, DE, USA) according to the manufacturer's instructions. Each sample was tested twice, and the mean values were used for a comparative analysis.

2.9. Statistics

Data for plate counts, membrane integrity, and real-time PCR represented means ± standard deviations (SD) from three independent experimental sets. Fatty acid profiling data represent a means from two testing.

3. Results

3.1. Disruption of the sipB and invG genes impaired osmotolerance of S. Typhimurium

To investigate the potential role of SipB in the osmotolerance of *S.* Typhimurium, we constructed a *sipB* mutant and an *invG* mutant (lacking functional SPI-1) with the backbones of the *S.* Typhimurium LT2 strain. Each strain's survival in 8% NaCl solution was examined by conducting a plate count each day for 9 days. As shown in Fig. 1A, the two mutants showed impaired survival under the high osmotic condition when compared with the wild-type (WT) strain (i.e., after 5 days of incubation, the WT strain exhibited a 1.3 log-reduction, and the *sipB* mutant exhibited a 3.1 log-reduction). Complementation of each gene restored the osmotolerance, while the introduction of the *sipB* gene into the *invG* mutant did not alter its phenotype (Fig. 1). A parallel measurement for membrane integrity using BacLight staining showed similar trends; 14.1% of the WT strain, 1.7% of the *sipB* mutant strain, and 1.5% of the *invG* mutant strain retained integrity after 5 days of incubation in the 8% NaCl solution (Fig. 1B). Thus, we demonstrated that both the *sipB* gene and the SPI-1 system are required for the osmotolerance of *S.* Typhimurium.

3.2. NaCl osmolarity enhances the translocation of SipB to the membrane

Given that the addition of NaCl to a broth culture enhances the secretion of SipB *in vitro* [12], we examined whether SipB translocates to the bacterial surface in response to stringent NaCl osmolarity (8% NaCl). After a 20 h incubation of the WT strain in 8% NaCl solution, protein subfractionation and western blot assays

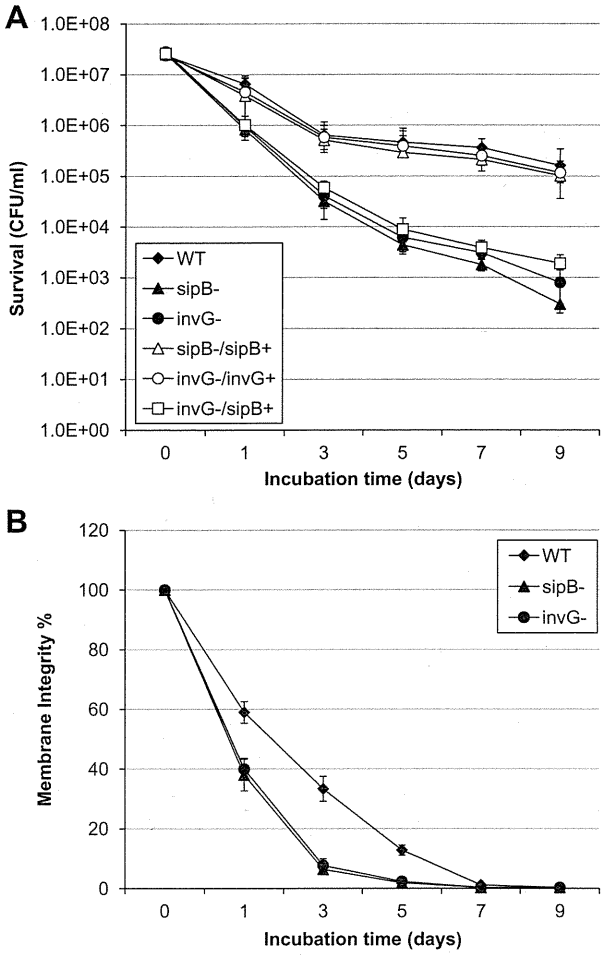

Fig. 1. The effect of *sipB* and *invG* gene disruption on the viability of *S.* Typhimurium under osmotic stress is shown. Bacteria were grown in TY medium for 20 h and were then incubated in 8% NaCl solution at 37 °C. At the indicated time point after incubation, 100 μl aliquots of the suspensions were serially diluted and plated on TSA agar plates to measure viable cell counts (section A, left *y*-axis). In parallel, the suspensions of the WT, sipB−, and invG− strains were subjected to LIVE/DEAD Bac Light viability stain to determine the percentage of cells with intact membranes (section B, right *y*-axis). The data in sections A and B represent the standard mean ± standard deviation from three independent tests. WT, wild-type LT2 strain; sipB−, *sipB* mutant; invG−, *invG* mutant; sipB−/sipB+, *sipB* mutant complemented with *sipB* gene; invG−/invG+, *invG* mutant complemented with *invG* gene; invG−/sipB+, *invG* mutant complemented with *sipB* gene.

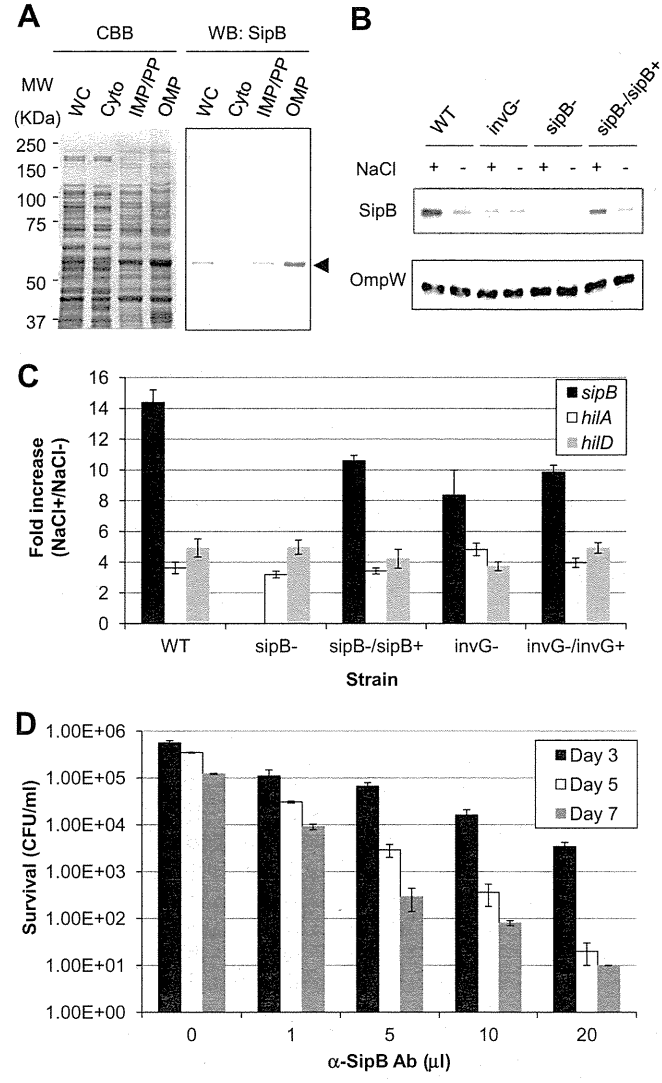

Fig. 2. NaCl osmolarity facilitates the membrane topology of SipB, affecting osmotolerance. (A) The SDS–PAGE and Western blot results of the *S.* Typhimurium LT2 strain incubated in 8% NaCl for 20 h are shown. Bacterial lysates (WC) were subfractionated into cytoplasmic proteins (Cyto), inner membrane/periplasmic proteins (IMP/PP), and outer membrane proteins (OMP), followed by separation on 10% acrylamide gels. Total proteins were visualised by CBB stain (left panel). The Western blot for the detection of SipB is shown in the right panel. (B) The membrane topology of SipB in *S.* Typhimurium in response to osmotic stimuli is shown. WT, *invG* mutant (invG−), *sipB* mutant (sipB−), and its complement (sipB−/sipB+) strains were incubated in 8% NaCl solution for 1 day. OMP fractions were separated before (NaCl−) and after (NaCl+) the incubation, and 30 μg proteins were subjected to Western blot analysis to detect SipB and OmpW. (C) Altered transcripts of *sipB*, *hilA*, and *hilD* genes in WT, invG−, sipB−, and sipB−/sipB+ strains after incubation in 8% NaCl solution for 2 h are shown. The data represent the fold increase compared with the transcript levels at the 0 h incubation. (D) The SipB-neutralisation antibody decreased survival of the WT strain under osmotic stimuli in a dose-dependent manner. The data represent plate counts (CFU/ml) on days 3, 5, and 7 post-incubation in 8% NaCl solution. The data in sections C and D represent the mean ± standard deviations (SD) from three independent tests.

showed that SipB was largely present in the sarcosyl-insoluble fraction (mainly consisting of outer membranes) (Fig. 2A). Similarly, greater amounts of SipB were detected in the OMP fraction of the WT strain and the *sipB* mutant/*sipB*-complemented strain (sipB−/sipB+) after 20 h incubation in 8% NaCl solution. In contrast, the *invG* mutant did not exhibit an NaCl-induced increase in the level of membrane-associated SipB (Fig. 2B), indicating that SPI-1 mediated the osmo-inducible translocation of SipB onto the bacterial surface. The qRT-PCR assays showed that the inactivation of the *sipB* or *invG* genes did not alter the transcription of the *hilAD* genes (Fig. 2C), which are central regulators of the *sipB* gene transcript [12]. This indicated that SPI-1-mediated SipB translocation is essential to conferring the osmotolerance. This result was supported by the observation that blocking the surface-localised SipB with neutralising antisera significantly reduced bacterial osmotolerance in a dose-dependent manner (Fig. 2D). Together, our data indicated that the SPI-1-mediated translocation of SipB to the bacterial surface plays a prominent role in the osmotolerance of this pathogen.

3.3. SipB induces bacterial fatty acid compositional shift in response to osmotic stress

Bacterial membranes initially sense harsh environmental stresses and then trigger a variety of response mechanisms to adapt to the changing conditions [14]. Because fatty acids constitute the main body of bacterial membranes, and the disruption of the *sipB* gene accelerated the reduction of membrane integrity upon exposure to osmotic stimuli (Fig. 1B), we hypothesised that

Table 1
The osmotic stimuli-induced modulation in the fatty acid compositions in *S.* Typhimurium LT2 WT and *sipB* mutant strains.

Fatty acid	Percentage					
	WT			*sipB* mutant		
	0 h	6 h	20 h	0 h	6 h	20 h
10:0	0.12	0.04	–	0.09	0.03	–
12:0 Alde	1.08	0.74	0.56	0.93	0.77	–
12:0	3.86	2.74	1.88	3.94	2.78	1.68
13:0	0.15	0.12	–	0.17	0.12	–
12:0 2OH	0.15	0.08	–	0.15	0.08	–
14:0	5.04	4.09	3.22	5.05	4.16	3.19
15:0	0.87	0.77	0.53	0.82	0.73	0.52
14:0 2OH	1.95	1.34	0.84	1.95	1.29	0.26
Sum in Feature 2 (C14:0 3OH/C16:1)	9.12	5.34	3.91	9.36	5.09	1.77
Sum in Feature 3 (C16:1 ω7c/15 iso)	7.66	6.59	5.39	7.89	7.87	4.12
16:1 ω5c	0.19	0.18	0.19	0.20	0.17	0.19
16:0	35.60	37.36	37.01	35.40	36.71	28.47
17:0 CYCLO	10.74	14.66	17.25	10.48	13.63	10.90
17:0	0.94	1.05	0.32	0.91	0.95	0.39
18:1 ω7c	14.96	15.75	17.18	15.20	17.54	17.07
18:0	0.87	1.05	2.11	0.95	1.02	0.70
11 methyl 18:1 ω7c	0.29	–	–	0.32	–	0.19
19:0 CYCLO ω8c	3.86	6.27	8.44	3.63	4.10	2.39
20:2 ω6,9c	0.28	0.08	–	0.28	–	–
CFA ratio (%)	14.60	20.93	25.69	14.11	17.73	13.29

Fatty acid methyl esters were detected in *S.* Typhimurium WT and the *sipB* mutant strains incubated in 8% NaCl solutions for 0, 6, and 20 h.

membrane topology of SipB may alter the fatty acid composition of *S.* Typhimurium under conditions of osmotic stress. The fatty acid analyses were able to detect a total of 19 fatty acids, including C10:0, C12:0 Alde, C12:0, C12:0 2OH, C13:0, C14:0, C14:0 2OH, C15:0, Sum in Feature 2 (C14:0 3OH/C16:1 ISO I), Sum in Feature 3 (C16:1 ω7c/15 iso 2OH_C15:0 ISO 2OH/C16:1 ω7c), C16:1ω5c, C16:0, C17:0 cyclo, C17:0, C18:1ω7c, C18:0, 11-methyl C18:1 ω7c, C19:0 cyclo ω8c, and C20:2 ω6, 9c (Table 1). No apparent differences in fatty acid composition were found between the WT and *sipB* mutant strains before the introduction of osmotic stimuli; however, this profile was significantly altered upon incubation in 8% NaCl solution. The most notable change we observed was an elevation in the percentage of cyclopropane fatty acid (CFA) C19:0 in the WT strain. This is in contrast to the *sipB* mutant, which showed no alteration in this CFA (Table 1). Together, these data showed that stringent osmotic stress increases CFA in *S.* Typhimurium, which correlates with the presence of the *sipB* gene.

4. Discussion

Here we showed that the disruption of the *sipB* gene or the *invG* gene reduced osmotolerance in *S.* Typhimurium. The osmotic stress increased the levels of membrane-associated SipB protein in the WT strain, and antibody neutralisation assays provided further evidence of the important function of surface-associated SipB in conferring bacterial osmotolerance. This osmotic stress modulated the composition of fatty acids constituting the main body of the membrane, and SipB was shown to associate with the elevated levels of CFA C19:0. Our data thus indicated that surface-localised SipB plays a role in this pathogen's stabilised membrane integrity and osmotolerance.

To date, genetic and transcriptomic studies have implicated a substantial number of genes and environmental conditions in the regulation of SPI-1 genes [18]. Upon exposure to osmotic stimuli, Lon protease is involved in the upregulation of the *hilA*/*hilD* genes, which directly regulate transcription of the SPI-1 apparatus genes in *S.* Typhimurium [12]. We observed no altered expression of *hilAD* genes in the WT, *sipB* mutant, and *invG* mutant strains during incubation in stringent osmolarity, suggesting that the surface-localised SipB was sufficient for retaining osmotolerance.

Next, we examined whether the surface-localised SipB might affect fatty acid composition. In *E. coli*, the cyclisation of fatty acid acyl chains is generally regarded as a means of controlling the penetration of undesirable molecules to adapt the cells to adverse conditions [19,20]. A previous study additionally reported that fatty acid membrane composition was drastically modulated in response to osmotic stimuli in *Lactococcus lactis* [21]. The authors demonstrated that NaCl osmolarity increased cyclopropane fatty acid (CFA) C19:0. CFAs are known to be present in many bacteria, but the physiological consequences of their contribution to membrane properties are not yet understood, especially concerning the modifications of membrane fluidity in response to environmental stress. In *S.* Typhimurium, CFA formation is known to affect acid tolerance [22] and heat resistance [23]; in both conditions, *Salmonella* cells showed increased C19:0. Our data demonstrated that while the SipB-positive WT strain had an increased level of CFA C19:0 during incubation in the 8% NaCl solution, the *sipB* mutant's fatty acid composition remained unchanged. This novel finding indicated that *S.* Typhimurium increased CFA levels in response to osmotic stress, which correlated with SipB-related osmotolerance. The implications for the potential interaction between the altered fatty acid composition and SipB's polarity remain unclear; nevertheless, our data suggest that the increased CFA levels might be involved in the global stress response mechanism(s) of this pathogen.

The SPI-1 cluster is widely distributed among different *S. enterica* strains [24]. Nevertheless, the recent availability of genome sequences has revealed variation in SPI-1 homology among the different serovars of *S. enterica* [25]. The virulence factor database VFDB [26] showed high conservation of SipB, whereas conservation of one SPI-1 component, InvF protein, seemed to vary among different *S. enterica* serovars (data not shown). This suggested that the SPI-1-mediated membrane topology of SipB might be strain-dependent, and if so, it would determine the strain-to-strain variation in osmotolerance phenotypes. Considering that a variety of *Salmonella* serovars have been found in aquatic environments [27], our data provide new insight into the ecological role of the virulence determinant, potentially linking it to the public health threat.

Overall, our data presented herein demonstrate that *Salmonella* transports SipB to the bacterial surface, affecting bacterial osmotolerance. The altered CFA levels observed upon exposure to osmotic stimuli correlated with the reduced membrane integrity observed under stress conditions. Because we found that anti-SipB antisera significantly reduced bacterial survival, this protein may also become a potential target for the control of this pathogen in the food supply and the environment, providing an attractive strategy for preventing human infection.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for Promotion of Science (22780275) and a grant from the Ministry of Health, Labor, and Welfare, Japan (H22-shokuhin-ippan-009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.012.

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