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Supplementary material

The following supplementary material is available for this article online:

Table S1. List of *V. cholerae* genes that were induced in the VBNC state.

Table S2. List of *V. cholerae* genes that were repressed in the VBNC state.

Table S3. List of *V. cholerae* genes that showed consistent ratio in the VBNC state compared with unstressed state.

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The sigma factor RpoN (σ54) is involved in osmotolerance in *Listeria monocytogenes*

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Listeria monocytogenes; RpoN, NaCl, carnitine.

Abstract

Listeria monocytogenes is able to grow under conditions of high osmolarity. We constructed a deletion mutant of *rpoN*, encoding the alternative sigma factor RpoN, and analyzed its response to osmotic stress. In a minimal medium with 4% NaCl and 1 mM betaine, the mutant showed a similar growth to that of the parental strain, EGD. In the same medium with 4% NaCl and 1 M carnitine, the growth rate of the mutant was greatly reduced, when the optical density at 600 nm (OD_{600}) at the starting point of growth, was 0.15. However, when growth of the culture was started at an OD_{600} of 0.025, the growth of the mutant was similar to that of EGD. The mutant's expression of two betaine transporter genes, *betL* and *gbuB*, and the carnitine transporter gene *opuCA*, was osmotically induced at a level similar to EGD, and its rate of carnitine uptake was similar to that of EGD. These results suggest that the growth defect from the *rpoN* mutant is caused not by the transcriptional regulation of *opuCA* or by a decrease in carnitine uptake, but possibly by larger amounts of carnitine being needed for growth of the mutant in minimal medium when NaCl is present.

Introduction

Listeria monocytogenes is known for its physiological characteristics of osmotolerance (Seeliger & Jones, 1986) and cryotolerance (Walker *et al.*, 1990), and these characteristics make it difficult to eliminate from many types of foods (Okutani *et al.*, 2004).

In general, bacterial survival under stress conditions requires rapid alterations in gene expression, controlled by the association of different alternative sigma factors with core RNA polymerase (Jishage *et al.*, 1996). Previous studies have reported the existence of four types of sigma factor in *L. monocytogenes*, including RpoD, σ^B, σ^H and RpoN. RpoD is recognized as the principal sigma factor (Metzger *et al.*, 1994), and σ^B has been recognized as a general stress-responsive sigma factor that contributes to survival under several adverse conditions, such as high osmolarity (Becker *et al.*, 1998), low temperature (Becker *et al.*, 2000) and acidity (Wiedmann *et al.*, 1998). In *L. monocytogenes*, σ^H, encoded by *sigH*, is induced by low pH (Phan-Thanh & Mahouin, 1999) and the growth of this mutant is impaired under alkaline conditions, as well as being affected by a minimal medium (Rea *et al.*, 2004). RpoN, encoded by *rpoN*, is involved in resistance to bacteriocin in *L. monocytogenes* (Robichon *et al.*, 1997; Dalet *et al.*, 2001, 2003). Arous *et al.* carried out whole-genome microarray and proteome analyses of an *rpoN* mutant, and showed that the expression of 77 genes and nine proteins were modulated by RpoN (Arous *et al.*, 2004). However, at present, the relationship between listerial sigma factors (excepting σ^B) and osmotolerance remains completely unknown. Our aim was to examine whether expression of the gene coding for RpoN can be osmotically induced, and to analyze the role of RpoN in growth under high osmotic conditions, utilizing a newly constructed *rpoN* mutant from *L. monocytogenes* EGD.

Materials and methods

Bacterial strains, PCR primers and media

Listeria monocytogenes EGD and *Escherichia coli* DH5α were used in this study. The sequences of the PCR primers used are shown in Table 1, all were designed using a database for the complete genome sequence of *L. monocytogenes* EGD (Glaser

Table 1. Primers used in this study

Name	Sequences (5′ → 3′)
Primer for mutant construction	
rpoNA1	AAAAGCGCTGGAAGTTCTG
rpoNB1	GCATCGTTTCGTAGTATGC
rpoNA2	GTAATTGATACCCACTGTGA
rpoNB2	CGATACGTCCAAAACCATTA
rpoNA3	ATTCAAGTTTCAAGAAGAGCT
rpoNB3	GTCTTCATTATAACATGCCAT
Primer for quantitative RT-PCR	
16SA1	CCGTCAAGGGACAAGCAGTT
16SB1	GCCGCGTGTATGAAGAAGGT
rpoDA1	CCGTATTCCGGTGCATATGG
rpoDB1	GAAGGATCGCGGCCTAAATC
sigBA1	GTAGAGTCCATCGCCCGAAA
sigBB1	ATTGTTGGCACAGCAAATGC
sigHA1	AGCGGGACGATTTAATCCAA
sigHB1	GCCGAAAGTAGCTGCCTGTT
rpoNA1	CCGGACGCAAAGAAACGTT
rpoNB1	TGAGCCGTGCGGTGAAT
ECFA1	TTCAACGATAAAATTTGGGACTATCTC
ECFB1	TCATTAAGCTGTTTGCTACATCTTTTG
betLA1	GCGCCTGATGCACGAAA
betLB1	AAGCCACCAAGCCCAATAAA
gbuBA1	CGGCCTTGAAATTCAAGGAA
gbuBB1	TCGCCGTAACCAGCTAAACC
opuCAA1	GCGGAAGATCCCGTCAAAC
opuCAB1	TCATATGTGGCATCAAGCCAAT

All primers were designed for this study.

et al., 2001). Brain Heart Infusion (BHI) broth and agar (Difco Laboratories) were used to culture *L. monocytogenes*, and LB broth and agar (Sambrook *et al.*, 1989) were used for *E. coli*. Where appropriate, antibiotics were used at the following concentrations: tetracycline (Tet), 5 μg mL^{-1} for *L. monocytogenes* and 10 μg mL^{-1} for *E. coli*; chloramphenicol (Cm), 5 μg mL^{-1} for *L. monocytogenes* and 10 μg mL^{-1} for *E. coli*; kanamycin (Km), 5 μg mL^{-1} for *L. monocytogenes* and 25 μg mL^{-1} for *E. coli*. To examine the efficacy of osmoprotectants in the *L. monocytogenes* strains, chemically defined minimal medium (Beumer *et al.*, 1994) supplemented with 20 mM glycine (Wako Pure Chemical Industries, Osaka, Japan), referred to hereafter as CDMMglycine, was used.

Analysis of listerial genome using database

Putative sigma factor-coding genes in the genome sequence of *L. monocytogenes* EGD were searched for using PEDANT software at the Munich Information Center of Protein Sequences (http://pedant.gsf.de/cgi-bin/wwwfly.pl?Set=Listeria_monocytogenes_EGD&Page=index).

Construction of the *rpoN* deletion mutant

To construct the *rpoN* deletion mutant, a thermo-sensitive plasmid, pHYTV32*ts*, was prepared from pHY300PLK (Ishiwa & Shibahara, 1985) partially digested with the *HaeIII* restriction enzyme and *SmaI*-digested pTV32*ts* (Youngman, 1987). The PCR fragment that included the complete *rpoN* gene and its flanking regions was cloned into pHYTV32*ts*, and then an inverted PCR was done to delete the *rpoN* gene from the resulting plasmid using rpoNA2/B2 primers (Fig. 2). This PCR fragment, which only includes the plasmid vector and *rpoN*-flanking regions, was ligated with the Km resistant gene cassette. The resulting plasmid was electroporated into an *L. monocytogenes* EGD strain, according to the methods described by Park and Stewart (1990). A clone selected by Cm and Km resistance was grown at temperatures of less than 30 °C to ensure retention of the thermo-sensitive plasmid. This clone was then incubated overnight at 42 °C to induce curing of the plasmid and homologous recombination between the plasmid and the *rpoN* coding region on the chromosome. The *rpoN* reconstructed clone was prepared from the *rpoN* deletion mutant and the rpoNA2/B2 PCR product cloned in pHYTV32*ts* using the same method as described above.

DNA and RNA isolation

To isolate chromosomal DNA from *L. monocytogenes*, bacterial cells from 3 mL of an overnight culture were washed once and then resuspended in 100 μL of 10 mM Tris (pH 8.0)/1 mM EDTA (pH 8.0)/50 mM NaCl. Aliquots of 100 μL of 10 mg mL^{-1} lysozyme and 10 μL of 10 mg mL^{-1} N-acetylmuramidase (Seikagaku Corporation, Chiyoda-ku, Tokyo) solutions were added to each bacterial suspension, and the mixture was incubated at 37 °C for 30 min, following incubation at 55 °C for 60 min with 100 μL of 20 mg mL^{-1} Proteinase K and 100 μL of 10% SDS. Two rounds of phenol extraction and two rounds of phenol/chloroform extraction were done to inactivate the enzymes and to extract the proteins from each sample. The DNA samples were purified by ethanol precipitation and their concentrations were determined by measuring the optical density at 260 nm. Plasmids were purified from overnight cultures with a Plasmid Mini Kit (Qiagen).

To isolate the total RNA, bacterial cells in BHI broth or in CDMMglycine were harvested by centrifugation at 4 °C and frozen at −20 °C for more than 18 h. The bacterial cells were suspended in 100 μL of lysozyme (30 mg mL^{-1} final concentration) in RNAsecure™ reagent (Ambion)/0.5 M sucrose (according to the methods of Dr Tomoko Hanawa, Kyorin University, Tokyo, Japan, personal communication) and 10 μL of 10 mg mL^{-1} N-acetylmuramidase, and incubated at 37 °C for 30 min. Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. All RNA samples were treated with DNaseI (Takara Shuzo) to remove contaminating DNA, and the preparations were confirmed to be DNA-free by RT-PCR

with and without MMLV reverse transcriptase (Promega) by the method described below.

RT-PCR

A 0.1-μg aliquot of total RNA was used for reverse transcription. After denaturation by heating at 70 °C for 10 min, 10 units of MMLV reverse transcriptase (Promega), 1 unit of RNase inhibitor (Toyobo) and 1.2 pM of downstream primer were added to each RNA preparation, and cDNA was synthesized by incubation at 37 °C for 60 min. One-tenth of the cDNA produced was used for PCR using a gene-specific primer pair (Table 1). Quantitative RT-PCR was done using an AB7000, TaqMan reverse transcription reagent (Applied Biosystems) with the primers shown in Table 1, according to the manufacturer's instructions. A 0.1-μg aliquot of total RNA was used for the reverse transcription. One-tenth of the cDNA was used for quantitative PCR. The amount of PCR product produced with primers designed for 16S ribosomal RNA was used as an internal control to normalize for the amount of total RNA in each sample. The primers were designed using Primer Express software (Applied Biosystems).

Growth in CDMMglycine supplemented with 4% NaCl and osmoprotectants

To examine the efficacy of osmoprotectants in the *rpoN* mutant of *L. monocytogenes* growing under conditions of high osmolarity, the OD_{660} values of the parental strain, the *rpoN* mutant and the *rpoN* re-constructed strain cultured in CDMMglycine were measured, as described previously (Okada *et al.*, 2002). In a typical experiment, bacterial cells grown in BHI broth overnight at 37 °C, were washed four times and then resuspended in CDMMglycine, CDMMglycine supplemented with 4% NaCl, or CDMMglycine with 4% NaCl that had been supplemented with either 1 mM glycine betaine (betaine) (Sigma) or 1 mM DL-carnitine (Sigma). The cultures were incubated at 37 °C with shaking, and growth was monitored by measurement of OD_{660} during the period shown in Fig. 3.

^{14}C-labelled carnitine uptake assay

The rates of uptake of ^{14}C-labelled carnitine by the *rpoN* mutant and its parental strain under osmotic stress were measured as previously described (Verheul *et al.*, 1997). The L-[methyl-^{14}C] carnitine hydrochloride was obtained from the Japan Isotope Association.

Results

Expression of sigma factor-coding genes in 3% NaCl

Previous studies have reported about four different types of sigma factor, including RpoD, σ^{B}, σ^{H} and RpoN, in *Listeria monocytogenes*. In addition to these sigma factors, the available evidence suggests that EGD contains at least one extra-cytoplasmic functional (ECF) type sigma factor, as we were able to demonstrate by an analysis using the PEDANT Database of the Munich Information Center of Protein Sequences (data not shown). We compared the transcriptional levels of five sigma-factor-coding genes in BHI, both with and without 3% NaCl, by quantitative RT-PCR, using RNA isolated from EGD at the early exponential growth phase (at an OD_{660} of 0.3). Expression of the *rpoN* gene was significantly induced ($P < 0.01$) under conditions of high osmotic strength (Fig. 1). In contrast, the expression of *lmo0423*, which encodes a putative ECF type sigma factor, was significantly reduced ($P < 0.01$) in BHI with 3% NaCl compared to BHI without additional NaCl. *rpoD* and *sigB* tended to have greater expression in BHI with NaCl than in BHI, but the effect was not significant under the conditions used in this study.

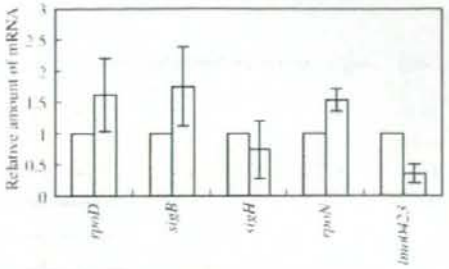

Fig. 1. Expression of sigma-coding genes under high-osmolarity conditions. Relative amounts of mRNA in the early exponential phase EGD grown in BHI (solid bars), and in BHI with 3% NaCl (open bars) are shown. Data are an average of three independent experiments. Standard deviation is shown by the error bar.

Construction and characterization of the *rpoN* mutant and the re-constructed strain

To analyze the role of RpoN in the osmotic responses, an *rpoN* deletion mutant, referred to as DN, was constructed by homologous recombination, induced by shifting the temperature up. Deletion of the *rpoN* gene in the mutant was confirmed by PCR using primers based on the *rpoN* DNA sequence, and by quantitative RT-PCR using an AB7000 and another pair of primers based on the *rpoN* sequence (Table 1, Fig. 2a). The *rpoN* re-constructed strain, RN, was also constructed by homologous recombination after electroporation of the thermo-sensitive plasmid carrying *rpoN* and its flanking regions. To restore an *rpoN* mutation, we constructed two types of *rpoN* complement clones using two different plasmids, pMK4 (Sullivan *et al.*, 1984)/*rpoN* and pHY300PLK/*rpoN*. Although transcription of the *rpoN* gene

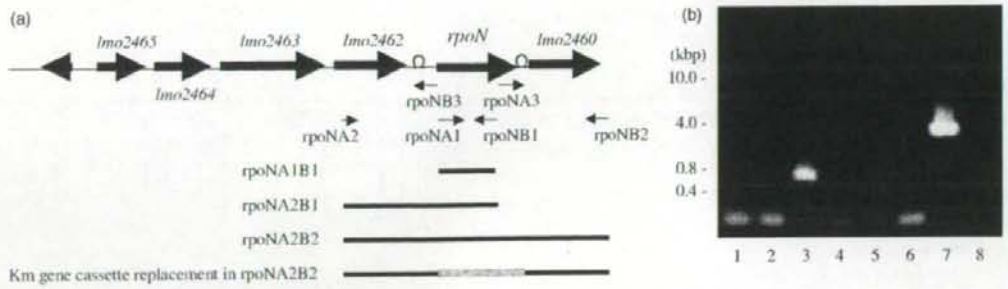

Fig. 2. Chromosomal organization of *rpoN* and its flanking regions and RT-PCR result from *rpoN* mRNA. (a) Chromosomal region around *rpoN*. The primers used to construct the *rpoN* mutant are shown by small arrowheads in the 5′ to 3′ direction. (b) RT-PCR was done with cDNA of *rpoN* mRNA synthesized using primer rpoNB1 plus primer rpoNA1 (lanes 1–4) or rpoNA2 (lanes 5–8) as upstream primers. Lanes 1 and 5: PCR products from *L. monocytogenes* EGD grown in BHI. Lanes 2 and 6: PCR products from EGD grown in BHI with 3% NaCl. Lanes 3 and 7: positive control. Lanes 4 and 8: negative control.

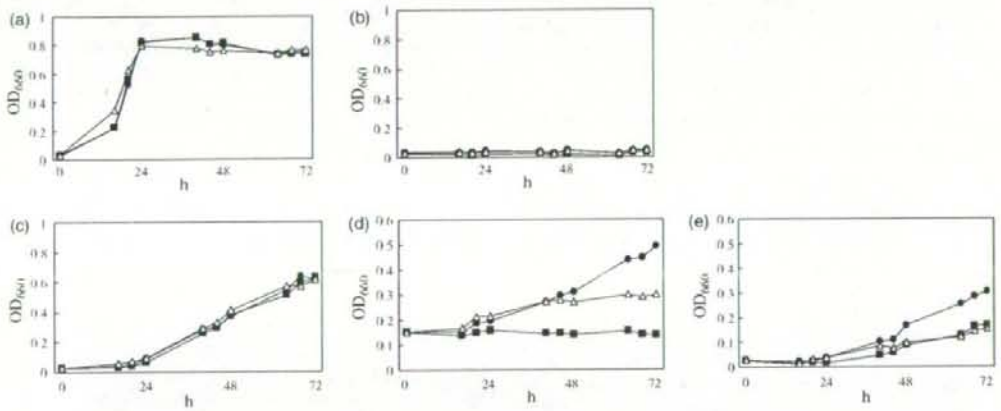

Fig. 3. Effect of osmoprotectants on the growth of the *rpoN* mutant in CDMMglycine. Growth of EGD (solid circle), *rpoN* mutant (solid square) and its re-constructed strain (open triangle) in (a) CDMMglycine, (b) CDMMglycine with 4% NaCl, (c) CDMMglycine with 4% NaCl and 1 mM betaine, (d, e) CDMMglycine with 4% NaCl and 1 mM carnitine. The OD_{660} at the starting point of growth was 0.025 (a, b, c, e) or 0.15 (d). Three independent experiments were performed and the results were similar.

was monocistronic (Fig. 2b), the transcriptional level of *rpoN* in these complement clones remained similar to that of the *rpoN* mutant, even though the plasmids had been retained (data not shown).

Growth of *rpoN* mutant in CDMMglycine with NaCl and osmoprotectant

We examined the *rpoN* mutant for a listerial well-characterized osmotolerance system, via osmoprotectant transportation (reviewed in Sleator *et al.*, 2003a). Since it has been reported that a mutation in *sigL*, a homologue of *rpoN* in *Bacillus subtilis*, causes a reduction in its ability to utilize arginine, leucine, isoleucine and valine (Debarbouille *et al.*, 1991), we tested the amino acid requirement of the *rpoN* mutant in *L. monocytogenes*. In contrast to from the *B. subtilis sigL* mutant, the *rpoN* mutant in *L. monocytogenes* showed only a reduced ability to utilize glutamine, and in the case of some amino acids such as glycine and serine, we confirmed that they could complement the reduction of growth (data not shown). Therefore, we supplied glycine to the original CDMM to prevent amino acid depletion (Fig. 3a). This phenotypic change in DN was recovered in the RN strain (data not shown). All three strains were unable to grow in CDMMglycine with 4% NaCl (Fig. 3b). The growth of DN in CDMMglycine with 4% NaCl and 1 mM betaine was similar to that of EGD and RN (Fig. 3c). The same result was obtained when the culture growth was

started at an OD_{660} of 0.15 (data not shown). In contrast, DN was unable to grow in CDMMglycine with 4% NaCl and 1 mM carnitine within the experimental period when the growth of the bacterial cells was started at an OD_{600} of 0.15, but the reduction of growth in CDMMglycine with NaCl and carnitine was partially recovered in the RN strain (Fig. 3d). On the other hand, the growth of this mutant in CDMMglycine with NaCl and carnitine was similar to that for EGD when growth was started at an OD_{600} of 0.025 (Fig. 3e).

Expression of osmoprotectant transporter genes in the *rpoN* mutant

The relative amounts of the mRNA of osmoprotectant transporter genes from listerial strains grown overnight in BHI and resuspended in CDMMglycine or CDMMglycine with 4% NaCl overnight were compared using a quantitative RT-PCR (Fig. 4). In all strains, the two betaine transporter genes, *betL* (Sleator *et al.*, 1999) and *gbuB* (Ko & Smith, 1999), and the single carnitine transporter gene, *opuCA* (Fraser *et al.*, 2000), were osmotically induced, although this effect only reached significance in some of the results (*rpoN* in EGD, $P < 0.01$; *gbuB* in EGD, $P < 0.01$; *opuCA* in EGD, $P < 0.05$, in RN, $P < 0.01$).

Uptake of ^{14}C-labeled carnitine by the *rpoN* mutant under osmotic stress

The *rpoN* mutant showed a salt-induced uptake of carnitine, and the level of carnitine uptake was similar to that for EGD (Fig. 5)

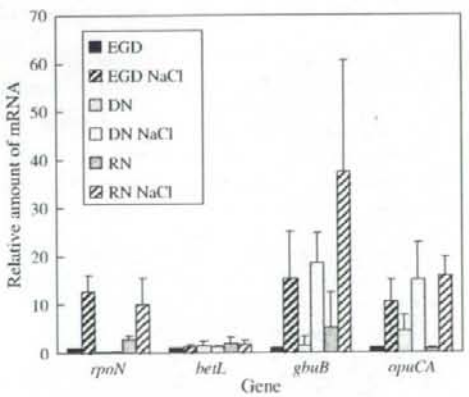

Fig. 4. Expression of osmoprotectant transporter genes. Relative amounts of mRNA from EGD grown in CDMMglycine with 4% NaCl (striped bars) or without additional NaCl (solid bars) after 24 h incubation. Data are the average of three independent experiments. Standard deviation is shown by the error bar

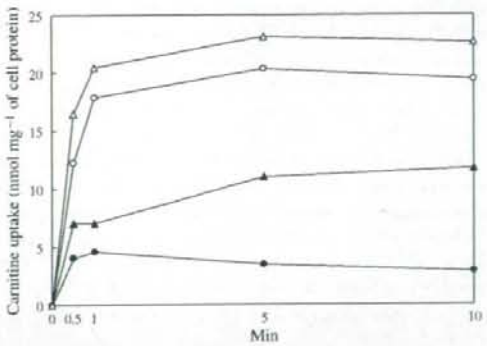

Fig. 5. Carnitine uptake under osmotic stress in EGD (circle) or in the *rpoN* mutant (triangle) with potassium phosphate buffer with (open symbols) or without (solid symbols) 0.8 M NaCl. Two independent experiments were performed and the results were similar.

Discussion

RpoN and its homologue, σ^L, are known to be metabolic regulators in many types of bacteria (Kohler *et al.*, 1989; Debarbouille *et al.*, 1991; Ali *et al.*, 2001). However, there have not been any reports on their association with osmotic stress. We constructed an *rpoN* mutant and analyzed the correspondence of RpoN with osmotolerance in *L. monocytogenes*. We examined the effects of the osmoprotectants, betaine and carnitine, on listerial growth under high-osmolarity conditions in a chemically defined minimal medium. The ability of the *rpoN* mutant to utilize extracellular betaine as an osmoprotectant was the same as in the parental and re-constructed strains (Fig. 3c). However, the ability of the *rpoN* mutant to utilize extracellular carnitine was considerably reduced compared with its parental and re-constructed strains when the growth of the culture was started at an OD_{600} of 0.15 (Fig. 3d). This observation indicates that RpoN is required for full osmotolerance in *L. monocytogenes*. Previous studies have shown that transcription of the *opuC* operon is regulated by σ^B (Fraser *et al.*, 2003; Cetin *et al.*, 2004), however, the level of expression of *opuCA* in the *rpoN* mutant appeared to be similar to that of the parental and re-constructed strains, as indicated by the results shown in Fig. 4. The *rpoN* mutant also showed no difference in rates of carnitine uptake under osmotic stress compared with EGD (Fig. 5). These results show that RpoN does not regulate the expression of *opuCA* transcriptionally or translationally, or the rate of carnitine uptake. Thus, the reduction in extracellular carnitine utilization in the *rpoN* mutant may not be caused by a change in carnitine transport. That the growth of the *rpoN* mutant was similar to EGD in CDMMglycine with 4% NaCl and 1 mM carnitine, when the growth of the bacteria was started at a low optical density (Fig. 3e), suggests that this mutant requires a larger amount of

carnitine in its cytoplasm to resist osmotic pressure and to grow under conditions of high osmolarity. It has been reported that RpoN in *L. monocytogenes* is associated with bacteriocin resistance (Robichon *et al.*, 1997; Dalet *et al.*, 2001, 2003), and that this resistance is involved in an alteration of the cell membrane (Vadyvaloo *et al.*, 2004). From these findings, it is possible that some changes in bacterial membrane composition caused by the absence of RpoN make the mutant more sensitive to extracellular osmopressure. A similar phenomenon was not observed when betaine was used as an osmoprotectant, but it is possible that betaine is more effective as an osmoprotectant than carnitine, as a previous study showed (Beumer *et al.*, 1994).

We also constructed two complement strains of the *rpoN* mutant after cloning the same chromosomal region as was used for preparing the RN strain into both Gram-negative and Gram-positive shuttle vectors, pMK4 and pHY300PLK, which are stable in *L. monocytogenes*. However, the level of *rpoN* gene expression in these strains was similar to that in the *rpoN* mutant strain (data not shown). Robichon *et al.* reported that their insertional *rpoN* mutant was complemented by the *rpoN*-harboring plasmid in *L. monocytogenes* LO28 (Robichon *et al.*, 1997). The DNA sequences of the intact *rpoN* gene in EGD and LO28 are completely identical (data not shown), and the *rpoN* genes are transcribed monocistronically in EGD (Fig. 2) and LO28 (Robichon *et al.*, 1997). In the present study, we were unable to clarify the basis for the discrepancy whereby *rpoN* is *cis*-acting in EGD, but *trans*-acting in LO28.

Here, we report that RpoN is necessary for the full osmotolerance of *L. monocytogenes*. This is the first study to demonstrate this, and we are now beginning to analyze osmotolerance by proteomic analysis further, in an attempt to elucidate the mechanisms of listerial stress response.

Acknowledgement

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Epidemiological data on food poisonings in Japan focused on *Salmonella*, 1998–2004

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In Japan, the numbers and cases of food poisonings must be reported as required by the Food Sanitation Law. This paper focuses on *Salmonella*, one of the leading food-borne pathogens in Japan, and it analyses the reported food poisoning data to assess the nature of *Salmonella*-associated food-borne disease. Obviously, these data do not exactly reflect the burden of food-borne illness associated with *Salmonella*; however, trends in *Salmonella* food poisoning and implicated foods could be identified for the purpose of setting priorities to mitigate the risk of food-borne salmonellosis. Summary information of *Salmonella* food poisoning investigation reports submitted by health departments of all prefectures and major cities between January 1998 and December 2004 was analysed. Both the number of reports and the cases of *Salmonella* food poisoning decreased drastically from 1999 (831 *Salmonella* food poisoning reports with 11,877 cases) to 2001 (265 reports with 7011 cases), increased in 2002, and then decreased again in 2003 and 2004 (231 reports with 3793 cases in 2004). About 80% of the *Salmonella* food poisoning reports and cases were associated with *Salmonella enteritidis* throughout the study period. Food vehicles were identified in 17–25% of the *Salmonella* food poisoning reports. Between 1998 and 2002, 45–60% of the *Salmonella* food poisoning cases were associated with eggs; however, the percentage dropped to 24.2% in 2003. The number of *Salmonella* food poisoning reports associated with beef, pork and poultry meat, and raw vegetables, which have been frequently reported in other countries, were very limited. Among the identified locations of disease break outs, 30–49% occurred in restaurant settings and the percentage of cases in restaurants increased during the study period. Thirteen to 41% of the *Salmonella* food poisoning cases occurred within the home, and the percentage declined. Phage types 1 and 4 were the predominant *S. enteritidis* isolated in 1998 and 1999; however, PT6, 14b, 36 and 47 were equally common afterward. In conclusion, even though both the number of *Salmonella* food poisoning reports and cases decreased during 1998–2004, the number of reported human salmonellosis cases remained significant. To improve the efficiency of control measures, the food poisoning investigation system should be strengthened the better to cover sporadic cases and to improve the identification of implicated foods. This information will contribute to establishing evidence-based priorities for *Salmonella* risk mitigation strategies.

Keywords: Salmonella; food poisoning; outbreak

Introduction

In Japan a food poisoning surveillance programme has been implemented under the Food Sanitation Law to contain food poisoning outbreaks by preventing their spread and recurrence (Government of Japan 2004). All information obtained from individual food poisoning investigations performed by health centres were described in food poisoning reports, which were sent to the Ministry of Health, Labour and Welfare (MHLW). However, the number of food poisoning cases reported under the Japanese food poisoning surveillance system is a vast underestimate of the true burden of food-borne diseases because most episodes never reach the reporting system for various reasons. Many patients do not seek medical care, medical practitioners may not order the stool sample tests that are needed to make a specific diagnosis, or the laboratories may not conduct the appropriate tests for isolating the causative pathogens. Sporadic cases are especially prone to be overlooked and not diagnosed as food poisoning. Even though there are limitations, the reported food poisoning data is, however, the only nationally available surveillance data regarding food-borne illness in Japan, and does provide considerable insight into food-borne disease trends. Food-borne *Salmonella* infections are a worldwide food safety problem. In the past decade, salmonellosis has been the first or second most common food poisoning in Japan in terms of both the number of food poisoning reports and patients (Kasuga et al. 2004). We therefore reviewed

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the summary of the Food Poisoning Investigation Report from 1998–2004 to identify the predominant serovars and significant food items associated with *Salmonella* food poisonings (SFP).

Material and methods

According to the Food Sanitation Law of Japan, any medical doctor who has examined a person poisoned or suspected to be poisoned by a food, or has examined the corpse of such a person, shall notify the director of the nearest health centre (HC) of such fact (Article of 58 of the Food Sanitation Law; MHLW 1947, first paragraph) within 24 h. On receiving this notification or otherwise knowing of the occurrence of a food poisoning incident, the director of the HC shall make an investigation and shall report the fact immediately to the governor of the prefecture (Article 58 of the Law, 2nd para.). The food safety departments of the prefecture's government shall then report in writing to the MHLW.

After the completion of the investigation, local health centre officials shall report the results of epidemiological investigations to the prefectural governors in the form of a 'Food Poisoning Investigation Report' (Abe et al. 2004). The report shall include the following information (Food Sanitation Law Enforcement regulations, Article 74; MHLW 1948):

- Name and address of the exposure location.
- Date of the first illness.
- Setting (type of business).
- Implicated food.
- Name and address of the location where the contamination happened.
- Aetiology.
- Total number of cases (total number of patients who suffered any adverse health symptoms).
- Total number of persons known to be exposed to the implicated food.
- Total number of cases involving hospitalization or death.

Prefecture governments reported the above information through an online information distribution system (Wide-area Information-exchange System for Health, Labour and Welfare (WISH) system) (Government of Japan 2004).

A food poisoning is defined as a food-borne illness that resulted from the consumption of a common food that contained food safety hazards and, accordingly, Food Poisoning Investigation Reports describe both single cases of food poisoning and outbreaks involving more than two patients. In this study, the Food Poisoning Investigation Reports (MHLW 2000, 2001, 2002, 2003, 2005, 2006, 2007) submitted by health departments of all prefectures and major cities between January 1998 and December 2004 were reviewed. Food vehicles, seasonal trends, predominant serovars, phage type (PT, if available) of the *Salmonella* isolates, and the places where contamination occurred were analysed in those reports indicating that *Salmonella* was an aetiological agent.

When information on serovars and PT were missing in the reports, any available information was obtained from bulletins of prefectural and municipal public health institutes (PHIs) where the microbiological examinations were performed. *Salmonella* phage typing is not required under the Food Sanitation Law and related regulations, however, some prefectural PHIs carried out phage typing to trace the sources of implicated food items, or to identify the linkage of diffuse outbreaks. Only PT data from 1998 to 2003 were analysed because the available data in 2004 were very limited.

Aetiologies were identified in 95–96% of all the food poisoning reports during the study period. Among these, the number of *Salmonella* isolates that were serotyped ranged from 34% to 74% according to the number of SFP reports and from 78% to 93% when based on the number of SFP cases.

Results

The total number of food poisoning reports was around 3000 in 1998, and thereafter decreased to around 1500 in 2003, and then slightly increased in 2004 (Figure 1). The percentage of SFP reports out of

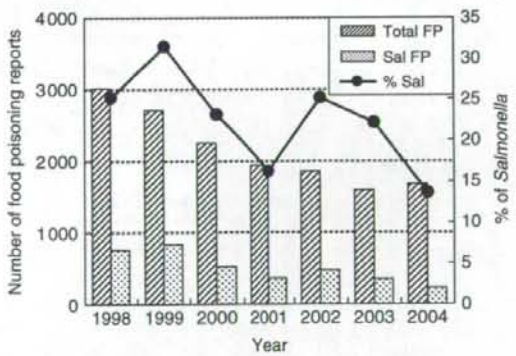

Figure 1. Numbers of total food poisoning reports, *Salmonella* (Sal) food poisoning reports, and the percentage of the total food poisoning reports associated with *Salmonella* during the period 1998–2004. Total FP, total number of food poisoning report; Sal FP, number of *Salmonella* food poisoning report; % Sal, percentage of *Salmonella* food poisoning reported out of total food poisoning reports.

the total food poisonings was at a peak (31%) in 1999, reduced to 16% in 2001, increased to 25% in 2002, and then reduced again to 14% in 2004. With regard to cases of total reported food poisonings, more than 46,000 cases were reported in 1998. The number of reported cases decreased in 1999, but increased again in 2000 due to a large, single outbreak that involved more than 13,000 cases and was associated with dairy products contaminated with *Staphylococcus aureus* toxin (Yamane 2006), Reported cases stayed around 25,000 to 29,000 between 2001 and 2004 (Figure 2). The percentage of SFP cases out of total food poisonings was also at a peak in 1999 (33%), reduced to 16% in 2000, increased again during 2001 and 2003 to 22%, and decreased below 14% in 2004 (Figure 2).

A majority of SFP reports were associated with a single case (Figure 3). More than 80% of the total single case SFP reports originated from one prefecture. Except for single case reports, the annual median number of cases per SFP report from 1998 to 2004 were nine, nine, seven, nine, nine, 15 and twelve, respectively. There were seven outbreaks of SFP involving more than 500 cases during this study period. The annual number of deaths due to SFP during this study period was one, three, one, zero, two, zero and two, respectively. The geographic distribution of the occurrence of the SFPs involving multiple cases in each prefecture is illustrated in Figure 4.

Salmonellosis has been the first or second most common food-borne disease in Japan in terms of both food poisoning reports and cases (Table 1). As shown in Figure 5, both the number of reports and cases of SFP decreased drastically from 1999 to 2001, increased in 2002, and then decreased toward 2004. The SFP report and cases were reduced in 2004 by 28% and 32%, respectively, from 1999 levels. Most SFP occurred in the warm weather months of May to October (Figure 6). Even though the annual percentage of serotypes identified among SFP were lower in terms of report number (35–74%), the percentage based on number of cases were still reasonably appropriate (90–98%) during the study period.

More than 68% of the serovars identified in SFP cases throughout the study period were associated with *Salmonella enteritidis* (SE), with a peak of 90% in 2002 (Figure 7). By year, *S. typhimurium*, ranked third, fourth, second, fourth, sixth, second, and third, respectively. Among the cases in which serovars were identified, the percentage of cases caused by *S. infantis* decreased from 4.8% in 1998 to 0.3% in 2003, and then increased to 13.5% in 2004. The increase in 2004 was due to a single outbreak involving 344 cases and linked to a catering establishment where a food handler continued to contaminate a variety of ready to eat foods for three days (MHLW 2007). The involvement of another serovar, *S. oranienburg*, in food poisoning cases increased suddenly in 1999 due to a diffuse outbreak of SFP caused by consumption of semi-dried squid (Niizuma et al. 2002).

The numbers of SE reports and the associated cases involving eggs decreased by 66% (25 from 74) and 85% (710 from 4824), respectively, from 1998 to 2004. However, the percentage of SE reports and associated cases linked to eggs out of the total SE reports and cases in which all implicated foods were identified through investigations were still relatively high (40–75%) when based on number of reports, and 56–81% when based on the number of cases (Table 2).

Food vehicles were identified in 17–25% of the annual SFP reports during the study period. The annual ratios ranged between 33% and 46% for SFP reports involving only multiple cases since the SFP investigations were not completed for SFPs involving single cases. Between 1998 and 2000, 45% (59 SFP involving eggs out of a total of 131 SFP with known

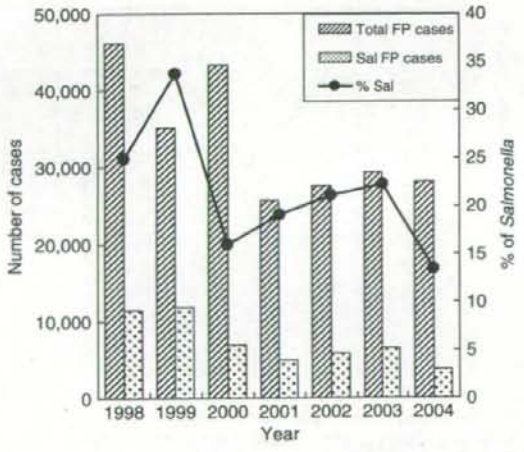

Figure 2. Numbers of total food poisoning cases (total FP cases), *Salmonella* food poisoning cases (Sal FP cases) and percentage of *Salmonella* cases out of total food poisoning cases (% Sal) from 1998 to 2004.

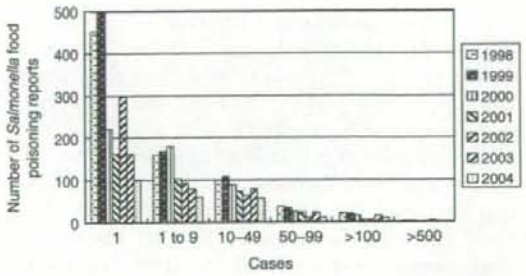

Figure 3. Number of cases involved in *Salmonella* food poisoning reports by year, 1998–2004.

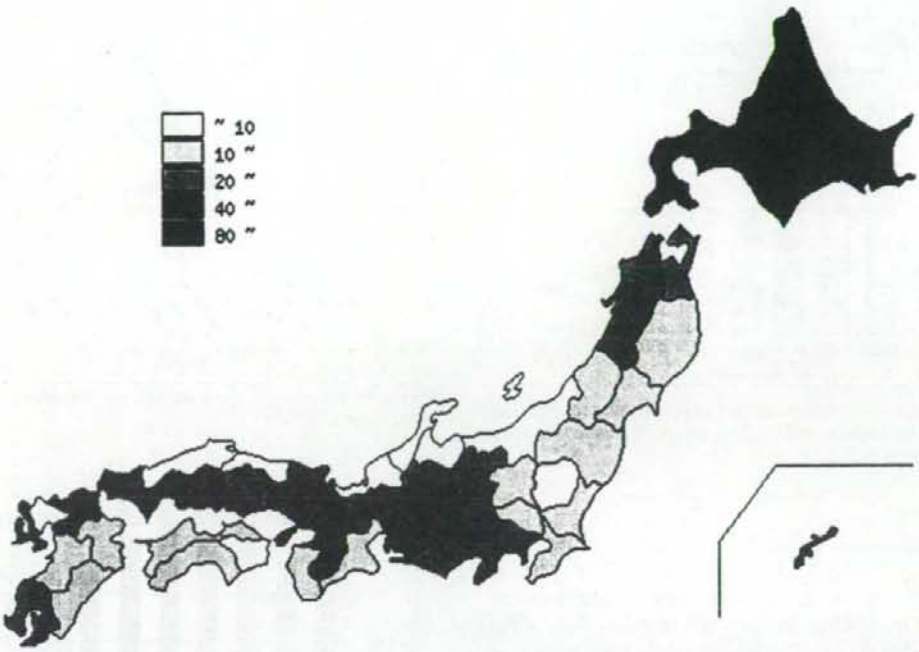

Figure 4. Occurrence of *Salmonella* food poisonings (SFP) by prefecture, 1998–2004. ~10, Less than ten SFP reports in 1998–2004; 10~, between ten and 19 SFP reports; 20~, between 20 and 39 SFP reports; 40~, between 40 and 79 SFP reports; and 80~, more than 80 SFP reports.

Table 1. Ranking of the top two aetiological agents in the Food Poisoning Report based on the number of reports and cases, 1998–2004.

Year	Rank	1998	1999	2000	2001	2002	2003	2004
Reports	1st	*Vibrio parahaemolyticus*	*Salmonella*	*Salmonella*	*Campylobacter*	*Salmonella*	*Campylobacter*	*Campylobacter*
	2nd	*Salmonella*	*Campylobacter*	*Campylobacter*	*Salmonella*	*Campylobacter*	*Salmonella*	Norovirus
Cases	1st	*Vibrio parahaemolyticus*	*Salmonella*	*Staphylococcus aureus*	SRSV	SRSV	SRSV	Norovirus
	2nd	*Salmonella*	*Campylobacter*	*Vibrio parahaemolyticus*	*Salmonella*	*Salmonella*	*Salmonella*	*Salmonella*

Note: SRSV, small, round structured virus, which was renamed as Norovirus in 2004.

vehicle) to 60% (63 SFP involving eggs out of a total of 139 SEP with known vehicle) of the SFP with known food vehicles were associated with eggs, however, after 2000, this percentage decreased to 24.2% (22 SFP involving eggs out of a total of 91 SEP with known vehicle) in 2003 (Figure 8). The number of cases involving eggs was at a peak (3804 cases) in 1999, declined to 358 in 2003, and increased slightly to 619 cases in 2004.

Of the cases where the food vehicle was identified, the percentage involving eggs increased from 39% (2733 out of 6959) in 1998 to 62% (2038 out or 3300) in 2000, and then decreased substantially to 8.5% (358 out of 4219) in 2003 (Figure 9). In 1999, the number of cases associated with fishery products was increased to 22% (1659 cases) due to a nationwide outbreak, involving 1634 cases, associated with the consumption of dried squid contaminated with *S. oranienburg* (Niizuma et al. 2002). The number of SFP that were associated with beef, pork, poultry, and raw vegetables, which were reported frequently in other countries (European Food Safety Authority (EFSA) 2006; Bowen et al. 2006; Sivapalasingam et al. 2004; FAO/WHO 2002, Kimura et al. 2004), were very limited (less than 10%) in Japan throughout the study period.

Among those SFP reports that contained information on the location of SFP outbreaks, 30–49% was linked to restaurant settings in 1998 to 2003. This percentage increased during the study period,

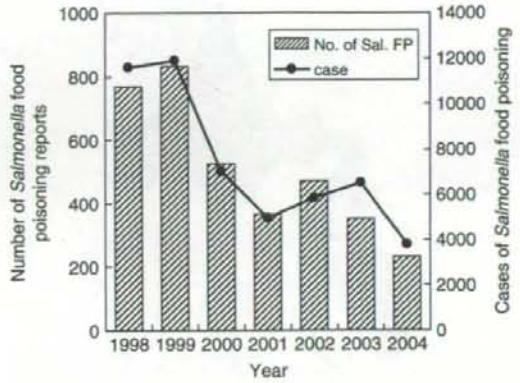

Figure 5. Number of *Salmonella* food poisoning reports and cases, 1998–2004. No. of Sal. FP, number of *Salmonella* food poisoning reports.

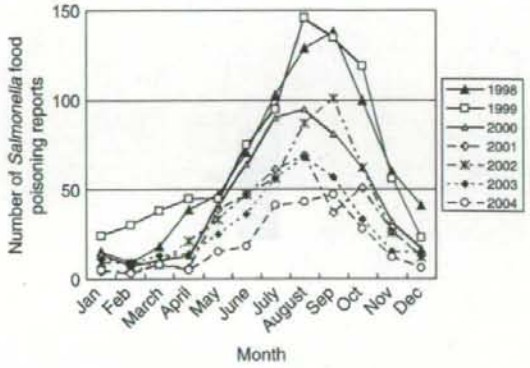

Figure 6. Number of *Salmonella* food poisoning reports by month, 1998–2004.

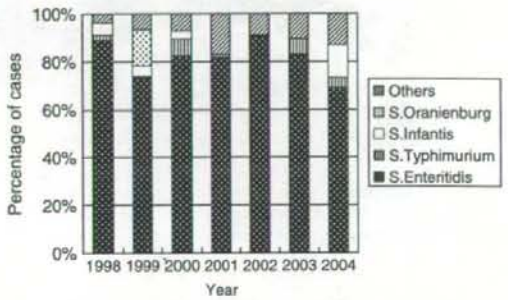

Figure 7. Percentage of *Salmonella* serotypes identified in food poisoning cases, 1998–2004.

with the exception of 2004, when the percentage was reduced to 20%. The percentage of SFP reports identifying the home as the location of involvement decreased from 42% to less than 10% in 2004, while the percentage that involved catering stayed at approximately the same level (4–8%) during the study period (Figure 11a). With regards to these cases, 30–40% of the SFP cases were associated with restaurant settings, and 10–30% of the SFP cases were associated with catering (Figure 11b).

Among the SE SFP reports that included phage type (PT) data, PT1 was the most common PT cause of SE SFP cases (884 cases out of 1232 phage typed SE cases (71%)) in 1998, PT4 became predominant (451 cases out of 575 phage typed SE cases (78%)) in 1999, PT14b became the most common (431 cases out of 730 phage typed SE cases (59%)) in 2000, and then PT47 became predominant (415 cases out of 956 phage typed SE cases (43%)) in 2001 onward. It should be noted that the percentage of reports in which the phage type was reported were very limited (between 2.2% and 11.8%) since it is not required by the regulations to be performed during SFP investigations. Therefore, while care should be taken when interpreting this information, the data at least indicates some trends on phage typing.

Discussion

Both the number of reports and the number of cases of SFP have been decreasing drastically since 1999. The data show that *Salmonella* is the first or second most prevalent cause of food poisoning in Japan and that serovar *enteritidis* accounts for up to 80% of all non-typhoid SFP, while only 1.8–4.0% of SFP reports in Japan involve serovar *typhimurium*. Similar distribution of *Salmonella* serovars in humans was reported in the Czech Republic, Greece, Slovakia and Slovenia in 2004 (EFSA 2006). However, a completely different serover distribution was observed in Australia in 2005, where *S. typhimurium* was the most commonly reported *Salmonella* serotype (The OzfoodNet Working Group 2006). The proportion of SFP caused by serovar *enteritidis* was still much higher than other serotypes. It should be considered that if SE was suspected, *Salmonella* isolates are more likely to be serotyped since SE is the only serotype required to be reported to the MHLW in Japan (MHLW 1948). Therefore, as far as is known, if during the course of the serotyping the results indicate that the involved isolates were not SE, some laboratories stopped the complicated serotype identification process due to limitations of financial and human resources.

The increased reports of SFP that were associated with serovar *enteritidis* infections are linked to the greater consumption of eggs and poultry that host the organism (Rodrigue et al. 1990). In the UK there has been a reduction in the number of cases of serovar *enteritidis* infection coincident with the introduction of vaccination of laying fowl against this serovar (Cogan and Humphrey 2003).

Table 2. Number of *Salmonella enteritidis* (SE) reports and associated cases involving eggs, 1998–2004.

Year	1998	1999	2000	2001	2002	2003	2004
Number of SE reports involving eggs	74	74	60	42	44	44	25
Percentage of SE with egg reports against implicated food identified	75.5	40.2	73.2	72.4	73.3	62.9	61.0
Cases of SE involving eggs	4824	3774	2266	1598	2342	2031	710
Percentage of SE with egg cases against implicated food identified	81.0	56.9	74.1	70.6	63.1	61.0	55.5

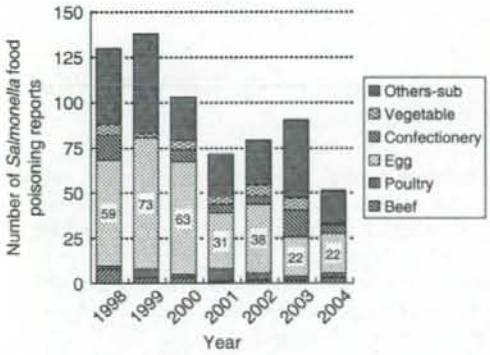

Figure 8. Number of *Salmonella* food poisoning reports by implicated food, 1998–2004.

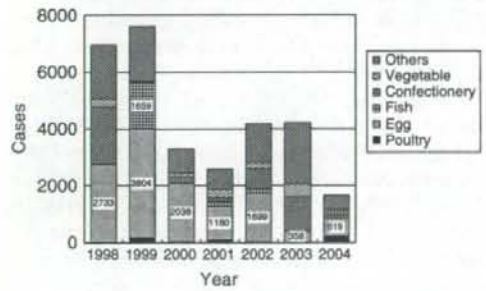

Figure 9. *Salmonella* food poisoning cases by implicated foods, 1998–2004.

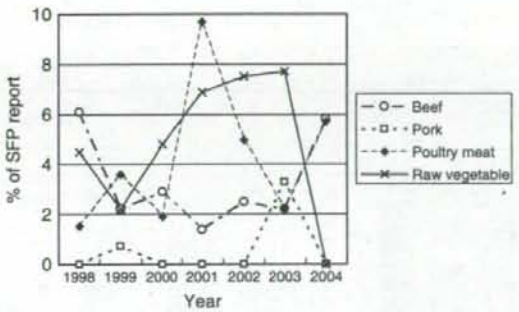

Figure 10. Percentage of *Salmonella* food poisoning (SFP) reports associated with beef, pork, poultry meat, and raw vegetables out of the total number of SFP reports with known food vehicles.

Data on serovar *enteritidis* phage typing obtained from SFP investigations were very limited in Japan. PT1 and PT4 were the major phagetypes (PT) in the early 1990s (Ito and Kusunoki 1996). However, in this study PT1 was the major PT in 1998; PT4 became the major PT in 1999; and PT14b became the major PT in 2000. Since 2001, PT47 has become the major PT although other PTs are still isolated. It has been suggested that the different PTs were predominant in Japanese egg flocks in different years (National Institute of Infectious Diseases (NIID) and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare, Japan 2003, 2006). PT1, 4, 6 and 14b were also commonly isolated from humans in Europe in 2004 (EFSA 2006), but PT21, which was also commonly isolated from humans in Europe, were not isolated in Japan.

In the present study, 24–60% of the *Salmonella*-caused outbreaks were egg-borne, and the majority of these outbreaks occurred in restaurant and catering settings. Patrick et al. (2004) reported that 80% of the outbreaks associated with SE were egg-borne in the USA, while Palmer et al. (2000) reported that the rate in the UK was 60%, and 48.3% of *Salmonella*-caused outbreaks were egg-borne in Catalonia, Spain (Dominguez et al. 2007).

In Japan the percentage of SE SFP cases was consistently 80% from 1998 to 2004. Both the percent of the reports and the number of cases of SFP associated with eggs have decreased since 2000. Among the cases in which the food vehicle was identified, the number of reports associated with eggs dropped from 79% in 2000 to 52% in 2004, while, with regard to the total cases, the percentage associated with eggs fell from 77% in 2000 to 46% in 2004.

In this study, SFP cases and reports associated with the consumption of raw vegetables were very limited. This could be due to efforts of the Ministry of Agriculture, Fisheries and Forestry (MAFF), which is the risk manager at a farm level, and industry to minimize microbiological contamination in the food chain, e.g. the development of the 'Fresh vegetable sanitary control guide' (MAFF 2005).

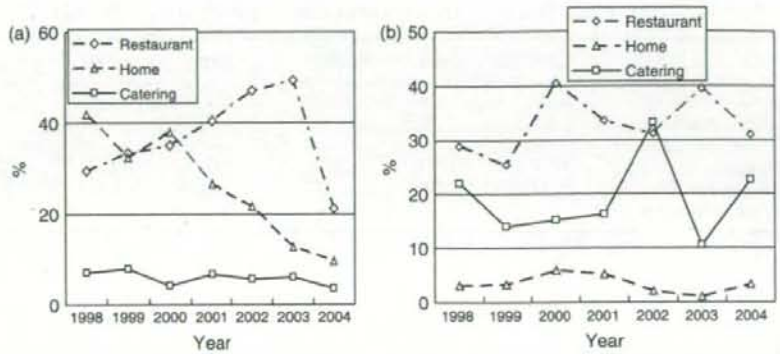

Figure 11. Percentage of *Salmonella* food poisoning reports (a) and cases (b) by settings (restaurants, home and caterings), 1998–2004.

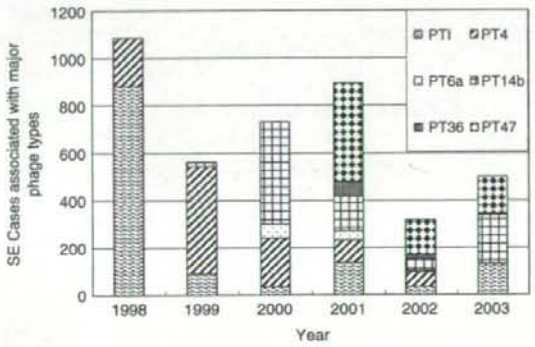

Figure 12. Cases of *Salmonella enteritidis* (SE) food poisonings associated with major phage types, 1998–2003, PT, phage type.

In addition, SFP associated with beef, poultry meat and pork were also less common than SFP associated with eggs in this study. This could be the effect of risk-management strategies put in place by MAFF (2002) to reduce the prevalence and concentration of *Salmonella* on farms and by MHLW to minimize cross-contamination and growth of *Salmonella* during the slaughter and processing steps at slaughter houses (MHLW 1996).

MHLW issued guidelines for *Salmonella* control measures at egg collection and grading centres in 1993 (MHLW 1993), and guidelines for hygienic practices in egg grading and packaging centres (MHLW 1998b). In November 1998 packages of control measures to reduce the burden of salmonellosis were announced (and were implemented in November 1999), including compulsory label requirements for: (1) the shelf life of shell eggs, (2) egg laying date, collecting date or packaging date for shell eggs for cooking, (3) the name and address of liquid egg processing plants, (4) the statement 'for raw consumption' for shell eggs intended for raw consumption, (5) the statements 'for cooked processing' and 'cooking required' for shell eggs for use in cooking or processing, (6) designating the 'time and temperature conditions for cooking' for pasteurized liquid eggs or (7) labelling liquid non-pasteurized eggs as 'not cooked' and 'require cooking'. In addition, microbial criteria for pasteurized and non-pasteurized liquid eggs as well as the temperature requirement for cold storage (less than 8°C) of liquid eggs were introduced (MHLW 1998a). These regulatory requirements were targeted towards a reduction of egg-borne salmonellosis and it has been suggested that these control measures, either individually or in combination, contributed to reducing the number and cases of SFP.

Furthermore, MAFF issued integrated control measures for *Salmonella* in eggs in 2005 (MAFF 2005) that also focused on *Salmonella* control at the farm level. According to the *Salmonella* in egg risk assessment (FAO/WHO 2002), reducing the prevalence of *Salmonella* in flocks is directly proportional to reduction of human risk of salmonellosis. Therefore, these control measures at farm level should also be implemented.

It is widely recognized that most statutory disease-notification systems suffer from substantial under-reporting of diagnosed cases and long delays in notification. Moreover, many people do not seek medical advice or are not diagnosed as suffering from a food-borne disease because of the non-specific nature of their symptoms (World Health Organization (WHO) 2007). Medical doctors should be well aware of the symptoms of food-borne diseases, and should report suspected cases of food-borne diseases. In addition, standardized serotyping and phage typing should be performed regularly to identify the linkage of potential outbreaks and implicated foods. Furthermore, to my knowledge, case control studies have never been performed to investigate food poisonings in Japan. Because no clearly defined 'cohort' of all exposed and non-exposed persons can be identified or

interviewed in many circumstances, case-control studies should be more frequently utilized to gather information systematically (WHO 2007). It should be noted that the identification of foods implicated in SFP through the food poisoning investigations was determined in 17–25% of the reports. This rate must be improved for preventing the recurrence of similar food poisonings and for identifying high-risk foods that need risk management strategies.

The Food Poisoning Statistics available under the Food Sanitation Law only include 'Food Poisonings' identified and reported by medical doctors. Therefore, the reported number of SFP and cases could be considered as only 'the tip of the iceberg' for the burden of food-borne diseases associated with *Salmonella* (New Zealand Food Safety Authority (NZFSA) 2008). Based on active food-borne studies conducted in other countries (Mead et al. 1999; Hall et al. 2005), it was assumed that a considerable number of patients, including sporadic cases, have been left unrecognized under the current surveillance system in Japan. It is important to establish risk management strategies based on both outbreak data and sporadic cases (Schlundt 1998).

Even though the number of both SFP reports and cases decreased during this study period, there is still a significant number of reported cases of human salmonellosis. To improve the efficiency of control measures, the food poisoning investigation system should be strengthened to cover sporadic cases, to improve the identification rate of implicated foods, and to set evidence-based priorities for *Salmonella* risk mitigation strategies.

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