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Nonsense-mutated *inlA* and *prfA* not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan

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Abstract

InlA is a surface protein participating in the entry of *Listeria monocytogenes* into mammalian non-phagocytic cells. PrfA is a positive regulatory factor that regulates the expression of a set of virulence genes. Recent studies revealed that some *L. monocytogenes* strains have a truncated form of these proteins because of nonsense mutations in their sequences, and these truncations contribute to the significant reduction in virulence of this pathogen. In this study, sequence analyses of *inlA* and *prfA* among *L. monocytogenes* isolated from ready-to-eat seafood revealed that only one out of 59 isolates had a nonsense-mutated *inlA* and all had non-mutated *prfA*. This indicated that these strains could be fully virulent based on the sizes of these proteins.
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Keywords: *Listeria monocytogenes*; Internalin A; Ready-to-eat; Seafood

1. Introduction

Listeria monocytogenes is an ubiquitous bacterium that can cause serious listeriosis infections in humans and animals. Both sporadic and epidemic cases of human listeriosis are mainly of food-borne in origin and have an associated mortality rate as high as 20–30% (Mead et al., 1999). Healthy adults are generally asymptomatic or develop only mild symptoms with simple gastroenteritis (Grif et al., 2001; Rocourt et al., 2000). However, infection in high-risk individuals, such as pregnant women, newborn infants, and immunocompromised people, can result in serious outcomes such as spontaneous abortion, septicemia, and meningoencephalitis. *L. monocytogenes* is therefore a public concern in terms of food safety and regulations to control this organism have been established in many countries. However, acceptable levels of this organism in ready-to-eat foods are defined differently from country to country. The United States adopted a zero-tolerance policy for all ready-to-eat foods whereas the EU allows 100 CFU/g of this pathogen at the best-before date for some classes of foods (European Commission, 2005). Establishing a definitive universal policy on acceptable levels of this organism is definitely required, and to this end, risk analysis is necessary to understand the actual dose response. However, it should be noted that these policies have been established based on the hypothesis that all *L. monocytogenes* strains are equally pathogenic, despite the heterogeneity of pathogenicity that has been reported to exist among isolates. This is indicated by most of the human listeriosis cases having been caused by strains of certain serotypes, such as 1/2a, 1/2b and 4b (Schuchat et al., 1991). Specifically, the strains of serotype 4b have been responsible for most food-borne epidemic listeriosis cases and the majority of sporadic cases (Farber and Peterkin, 1991; Schuchat et al., 1991). The varying levels of virulence were also demonstrated by virulence tests using chick embryo, various human cell lines, and mouse injection test (Bhunia et al., 1994; Nørrung and Andersen, 2000; Pine et al., 1991; Roberts et al., 2005; Roche et al., 2003; Roche et al., 2001; Stelma et al., 1987; Tabouret et al., 1991; Van Langendonck et al., 1998).

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The factors for this heterogeneity in virulence have been elucidated by various molecular methods. The observation that some *L. monocytogenes* isolates express a truncated non-functional form of internalin A (InlA) is of particular importance (Jonquières et al., 1998). InlA allows the pathogen to invade non-phagocytic cells, such as human intestinal epithelium cells (Gaillard et al., 1991), but strains expressing truncated InlA show a significant reduction in invasive ability into Caco-2 cells compared to ones lacking the nonsense mutation (Olier et al., 2005; Olier et al., 2002; Rousseaux et al., 2004). A truncated form of InlA was shown to be widely distributed in food isolates and less so in clinical isolates (Jacquet et al., 2004), indicating the critical role of InlA in the pathogenesis of human listeriosis. Reasons for strains of serotype 4b posing high risks for humans is still unknown, but having the intact form of *inlA* gene, rather than a nonsense-mutated form found in other strains, may play a partial role (Jacquet et al., 2004). In another recent study, truncation of this protein in a number of clinical and food isolates was confirmed using isolates from the United States (Nightingale et al., 2005a). Moreover, nonsense mutations were also found in the *prfA* gene (Roche et al., 2005), which regulates expression of a set of virulence factors. Although only three isolates were found to have nonsense-mutated *prfA*, all of them failed to enter human adenocarcinoma cells and were either avirulent or hypovirulent to mice because of their truncated PrfA proteins (Roche et al., 2005).

This virulence attenuation mechanism sheds light on questions about the rate of listeriosis cases. Although *L. monocytogenes* is widely present in ready-to-eat foods (Gombas et al., 2003), the number of cases of human infection is relatively low. This is also true in Japan where almost no foodborne listeriosis cases have been reported to date, although *L. monocytogenes* is known to be prevalent in many kinds of foods (Okutani et al., 2004). Despite significant consumption of these foods in Japan, it should be particularly noted that raw fish and ready-to-eat raw fish products have never been implicated in listeriosis in humans. This may be due to the low cell number of *L. monocytogenes* in these foods. Or, the presence of *L. innocua*, which is commonly found in foods (Karunasagar and Karunasagar, 2000), enhances host protective immunity against this pathogen (Vázquez-Boland et al., 2001). Alternatively, previous outbreaks simply have escaped recognition since *Listeria* detection from patients with diarrhea has not been routinely performed (Makino et al., 2005). However, the possibility that non-virulent or virulence-attenuated strains are prevalent in these foods cannot be ignored. Therefore, it is of extreme importance to determine whether truncation of virulence or virulence-associated genes could be a new tool for assessing risk of consuming food products contaminated with *L. monocytogenes*. Thus, we investigated *L. monocytogenes* isolates in ready-to-eat seafoods in this study to determine whether virulence-related genes *inlA* and *prfA* have nonsense mutations that leads to the truncated form of their respective proteins, InlA and PrfA. As sample foods, we specifically selected fish roe and minced tuna, since these have high levels of *L. monocytogenes* contamination (Handa et al., 2005) and risk assessment of these foods is urgently required.

2. Materials and methods

2.1. Bacterial isolates

The 59 seafood isolates used in this study are summarized in Table 1. A total of 10 isolates were from a previous study (Handa et al., 2005) and an additional 49 were selected from 64 isolates obtained from 531 ready-to-eat raw seafood retail products obtained in 61 different grocery stores in and around Tokyo between October 2004 and July 2005. The remaining15 isolates were excluded from further analyses because isolation sources (sampling date and store number), EcoRI ribotyping (Bruce, 1996) or MLST (Maiden et al., 1998; Zhang et al., 2004) data suggested that they were clonal isolates of other isolates already included in our list. Strains of the same serotype, ribotype and MLST profile were included in this study when the food samples were obtained on different dates or different stores.

2.2. Serotyping

Serotyping was carried out with commercial *Listeria* antiserum (Denka Seiken, Tokyo, Japan). O-antigen determination strains were grown on brain heart infusion agar (Becton Dickinson, Sparks, MD, USA) for 24 h at 35 °C. Cells were suspended in 0.2% sodium chloride and heated at 121 °C for 30 min followed by centrifugation at 3000 rpm for 20 min and resuspended in 0.5 ml of 0.2% sodium chloride. Slide agglutination tests using polyvalent type O-antiserum were performed first, followed by typing with individual O-antiserum. H-antigen strains were determined using the tube agglutination test. Briefly, sample cultures were incubated in semiliquid BHI medium (0.2% wt/vol agar) at room temperature (20–25 °C) for 24 h, repeated four times. The samples were incubated in a semiliquid BHI medium in Craigie tubes for 24 h followed by removal to BHI medium for an additional 24-h incubation. H-antigen type was determined after mixing two drops of antiserum with 0.5 ml of cell suspension with 1% formalin and incubating at 50 °C for 1 h.

2.3. Lineage designation

L. monocytogenes has been grouped into 3 distinct phylogenetic lineages based on genotypings such as sequencing analysis, ribotyping, and PCR-restriction fragment length polymorphisms (Rasmussen et al., 1995; Wiedmann et al., 1997). Each of the 59 strains used in this study was categorized into one of these 3 lineages using a method described previously (Ward et al., 2004). This method used multiplex PCR to produce a lineage-specific sized band on electrophoresis gels.

2.4. MLST (multilocus sequence typing)

Partial regions of 6 different virulence and virulence-associated genes were selected for MLST analysis according to Zhang et al. (2004) since they have reported a high discriminatory power of this method. DNA sequencing for each locus was performed

Table 1
L. monocytogenes strains isolated from ready-to-eat raw seafood

Strain	Serotype	Lineage	MLST type	Ribotype	Sampling date	Store no.	Sample type	Nonsense mutation		Reference
								inlA	*prfA*	
2-9	1/2a	II	18	1056	19-Nov-02	2	Salmon roe "sujiko"	–	–	Handa et al. (2005)
5-2	1/2a	II	14	1023	10-Dec-02	2	Cod roe "tarako"	–	–	Handa et al. (2005)
5-4	1/2a	II	26	1046	10-Dec-02	2	Salmon roe "sujiko"	–	–	Handa et al. (2005)
6-9	1/2a	II	12	1023	15-Dec-02	3	Minced tuna	–	–	Handa et al. (2005)
11-4	1/2a	II	20	1030	15-Jun-03	5	Cod roe "tarako"	–	–	Handa et al. (2005)
12-17	1/2a	II	12	1030	22-Jun-03	6	Minced tuna	–	–	Handa et al. (2005)
12-18	1/2a	II	9	1027	22-Jun-03	7	Cod roe "tarako"	–	–	Handa et al. (2005)
13-20	1/2a	II	11	1039	5-Nov-03	8	Minced tuna	–	–	Handa et al. (2005)
20-7-1	1/2a	II	15	16619	28-Oct-04	9	Cod roe "mentaiko"	–	–	This study
22-13-3	1/2a	II	13	1023	16-Nov-04	10	Minced tuna	–	–	This study
22-18-5	1/2a	II	20	1035	16-Nov-04	11	Minced tuna	–	–	This study
22-29-1	1/2a	II	24	1053	16-Nov-04	12	Cod roe "tarako"	–	–	This study
23-4-4	1/2a	II	12	16619	25-Nov-04	13	Salmon roe "sujiko"	–	–	This study
23-29-1	1/2a	II	9	1030	25-Nov-04	14	Salmon roe "sujiko"	–	–	This study
25-4-1	1/2a	II	12	16619	9-Dec-04	15	Salmon roe "sujiko"	–	–	This study
25-8-1	1/2a	II	22	1035	9-Dec-04	11	Minced tuna	–	–	This study
25-15-1	1/2a	II	25	1045	9-Dec-04	10	Cod roe "mentaiko"	–	–	This study
26-1-2	1/2a	II	12	1030	13-Jan-05	10	Minced tuna	–	–	This study
26-26-2	1/2a	II	8	1030	13-Jan-05	14	Salmon roe "sujiko"	–	–	This study
28-9-1	1/2a	II	16	1039	3-Feb-05	16	Salmon roe "ikura"	–	–	This study
29-13-2	1/2a	II	12	16619	17-Feb-05	18	Cod roe "mentaiko"	–	–	This study
30-8-1	1/2a	II	10	1030	17-Mar-05	15	Salmon roe "sujiko"	–	–	This study
30-11-1	1/2a	II	12	1039	17-Mar-05	2	Minced tuna	–	–	This study
30-29-1	1/2a	II	20	1035	17-Mar-05	11	Minced tuna	–	–	This study
32-27-1	1/2a	II	24	1045	14-Apr-05	10	Cod roe "mentaiko"	–	–	This study
36-6-1	1/2a	II	11	1039	2-Jun-05	2	Minced tuna	–	–	This study
36-17-1	1/2a	II	16	1039	2-Jun-05	24	Cod roe "tarako"	–	–	This study
36-25-1	1/2a	II	21	1039	2-Jun-05	22	Cod roe "tarako"	+	–	This study
36-25-2	1/2a	II	12	1053	2-Jun-05	22	Cod roe "tarako"	–	–	This study
37-1-1	1/2a	II	12	16619	9-Jun-05	25	Minced tuna	–	–	This study
37-3-1	1/2a	II	12	1053	9-Jun-05	25	Salmon roe "sujiko"	–	–	This study
38-16-1	1/2a	II	12	1039	16-Jun-05	19	Minced tuna	–	–	This study
38-16-3	1/2a	II	19	1030	16-Jun-05	19	Minced tuna	–	–	This study
39-2-1	1/2a	II	16	1039	21-Jul-05	24	Cod roe "tarako"	–	–	This study
40-4-1	1/2a	II	9	1030	26-Jul-05	25	Cod roe "tarako"	–	–	This study
40-6-1	1/2a	II	12	16619	26-Jul-05	2	Minced tuna	–	–	This study
22-19-2	3a	II	20	1035	16-Nov-04	11	Cod roe "mentaiko"	–	–	This study
22-28-5	3a	II	12	16619	16-Nov-04	12	Cod roe "mentaiko"	–	–	This study
26-2-3B	3a	II	12	1039	13-Jan-05	10	Cod roe "mentaiko"	–	–	This study
26-23-2	3a	II	20	1035	13-Jan-05	15	Cod roe "mentaiko"	–	–	This study
26-29-2	3a	II	12	1053	13-Jan-05	14	Cod roe "mentaiko"	–	–	This study
30-25-1	3a	II	23	1035	17-Mar-05	11	Cod roe "tarako"	–	–	This study
34-9-1	3a	II	17	1045	28-Apr-05	20	Cod roe "tarako"	–	–	This study
34-26-1	3a	II	20	1035	28-Apr-05	22	Cod roe "mentaiko"	–	–	This study
34-29-1	3a	II	9	1030	28-Apr-05	22	Cod roe "mentaiko"	–	–	This study
39-9-1	3a	II	12	16619	21-Jul-05	25	Cod roe "tarako"	–	–	This study
39-9-2	3a	II	9	1030	21-Jul-05	25	Cod roe "tarako"	–	–	This study
39-23-1	3a	II	20	1035	21-Jul-05	18	Cod roe "mentaiko"	–	–	This study
40-4-4	3a	II	12	1053	26-Jul-05	25	Cod roe "tarako"	–	–	This study
13-19	1/2b	I	2	1027	5-Nov-03	8	Cod roe "tarako"	–	–	Handa et al. (2005)
23-4-1	1/2b	I	1	1042	25-Nov-04	13	Salmon roe "sujiko"	–	–	This study
25-4-3	1/2b	I	1	1042	9-Dec-04	15	Salmon roe "sujiko"	–	–	This study
29-10-1	1/2b	I	4	1051	17-Feb-05	17	Minced tuna	–	–	This study
29-13-1	1/2b	I	5	1051	17-Feb-05	18	Cod roe "mentaiko"	–	–	This study
40-5-1	1/2b	I	2	1052	26-Jul-05	25	Salmon roe "sujiko"	–	–	This study
9-17	3b	I	3	1042	2-Feb-03	4	Salmon roe "sujiko"	–	–	Handa et al. (2005)
39-8-1	3b	I	2	1052	21-Jul-05	25	Salmon roe "sujiko"	–	–	This study
20-5-1	4b	I	6	1042	28-Oct-04	1	Cod roe "tarako"	–	–	This study
34-18-2	4b	I	7	1042	28-Apr-05	26	Cod roe "tarako"	–	–	This study

A total of 49 out of 59 *L. monocytogenes* isolates were collected from 531 ready-to-eat raw seafood retail products obtained at 61 different grocery stores in and around Tokyo between October 2004 and July 2005.

with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers as were used for PCR amplification (Zhang et al., 2004). For each locus, alleles differentiated by at least one nucleotide were arbitrarily assigned different allele numbers (Maiden et al., 1998). Obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Shizuoka, Japan) under accession numbers AB276438 through AB276791. Full sequences of *prfA* were deposited, but only positions 61–529 were used for MLST analysis.

2.5. Ribotyping

Automated ribotyping was carried out using a RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, DE, USA) according to manufacturer's instructions. Briefly, each isolate was streaked onto BHI agar plates, and following the overnight incubation at 30 °C, the appropriate amount of colonies were added to tubes containing sample buffer for cell lysis. Then the tubes were inserted into the RiboPrinter. This automated typing instrument employed *Eco*RI digestion of *L. monocytogenes* chromosomal DNA followed by Southern hybridization with an rRNA gene probe. Images were analyzed using RiboPrinter analysis software that normalized fragment pattern data for band intensity and band size relative to molecular weight markers, and were compared to database images for characterization. When an obtained ribotype pattern matched any one stored in the database with similarity of 0.85 or above, Dupont ID (e.g., DUP-1056) was automatically assigned.

2.6. Sequencing of complete inlA and prfA

The regions that contained complete sequences of *inlA* and *prfA*, respectively, and their flanking regions were amplified

Table 2
Amplification and sequencing primers used for *inlA* and *prfA* analysis

Gene	Primer name	Sequence (5'-3')	Product size (bp)
Amplification [a, b]			
inlA	inlA-F1	AATCCTATACAACGAAACCTGA	2490–2499
	inlA-R1	ATATAGTCCGAAAACCACATCT	
prfA	prfA-F1	TGTTGTTACTGCCTAATGTTTT	951
	prfA-R1	ACTCCATCGCTCTTCCAGAA	
Sequencing			
inlA	inlA-F2	TTTAAATCGGCTAGAACTATC	
	inlA-F3	AAGATATTAGCCCAATTTCT	
	inlA-F4	ATGCCTGCTAAAAACATCACC	
	inlA-R2	GGTGATGTTTTTAGCAGGCAT	
	inlA-R3	ATTTTTCACCGTGTTTGGA	
	inlA-R4	GATAGTTCTAGCCGATTTAAA	
prfA	prfA-F2	TTTAATGATTTTTCGATTA	
	prfA-R2	TAATCGAAAAATCATTAAA	

[a] For both *inlA* and *prfA* amplification, Mg^{2+} concentration was 1.5 mM, primer concentration was 1 mM, annealing temperature was 55 °C, and number of PCR cycles was 30.

[b] Amplification primers were also used as sequencing primers.

Table 3
Invasion efficiency of *L. monocytogenes* strains

Group	Isolates	Invasion %±SD [a]	Reference
1 Non-mutated *inlA*	EGDe	0.26±0.05	Nightingale et al. (2005a); Rousseaux et al. (2004)
	Scott A	0.64±0.17	Rousseaux et al. (2004)
	20-7-1	0.37±0.04	This study
	25-4-3	0.14±0.06	This study
	38-16-3	0.19±0.01	This study
	40-5-1	0.29±0.04	This study
2 Nonsense-mutated *inlA*	F2-563	0.05±0.004	Nightingale et al. (2005a)
	36-25-1	0.08±0.02	This study
3 *inlA* with 9 nucleotide deletions	20-5-1	0.18±0.04	This study
	34-18-2	0.30±0.07	This study

[a] The invasion rate was calculated as the number of bacteria recovered divided by the number of bacteria inoculated × 100.

in all 59 *L. monocytogenes* seafood isolates so that direct sequence analysis could be performed. DNA sequencing was performed with an ABI Prism 3100 (Applied Biosystems) and the obtained sequences were aligned with GENETYX-WIN software (Genetyx, Tokyo, Japan). The amplification and sequence primers are shown in Table 2. The obtained sequences have been deposited in the DDBJ under accession numbers AB276379 to AB276437 for *inlA* and AB276438 to AB276496 for *prfA*.

2.7. Caco-2 cell invasion assay

Early confluent cell monolayers of Caco-2 cells (ECACC No. 86010202) were prepared using the Biocoat HTS Caco-2 assay system (Beckton Dickinson) following the manufacturer's instruction. The cells were seeded onto fibrillar collagen-coated wells at a density of 2×10^5 cells/well and incubated for 24 h in DMEM-based Basal Seeding Medium supplemented with MITO-Serum Extender (DMEM-MITO). After aspirating the medium, 500 µl of Entero-STIM Medium supplemented with MITO-Serum Extender was added to each well and incubated for 48 h. *L. monocytogenes* strains were selected for this invasion assay from 3 different groups based on the mutation type of *inlA* gene (Table 3). As control strains, the group of non-mutated *inlA* included EGDe and ScottA and the group of nonsense-mutated *inlA* included F2-563 since their high (EGDe and ScottA) or low (F2-563) invasion abilities were previously reported (Nightingale et al., 2005a; Rousseaux et al., 2004). Other strains included in the group of full length of InlA were selected randomly from strains listed in Table 1. After growing at 30 °C in brain heart infusion broth, *L. monocytogenes* cells resuspended in DMEM-MITO were added to infect Caco-2 cells. Following 2 h of incubation at 37 °C, bacterial cells that did not adhere to Caco-2 cells were washed away with PBS. The cells were incubated at 37 °C for 1 h in 500 µl of DMEM-MITO including gentamicin (50 µg/ml) to kill extracellular adherent bacteria. The cells were washed 3 times with PBS and lysed by maintaining them for 10 min in cold PBS containing 1% tritonX 100. The number of viable bacteria released from the cells was counted on TSAYE

plates by appropriate dilutions. Each bacterial strain was tested in triplicate.

3. Results and discussion

In a previous study (Handa et al., 2005), enrichment of this organism in food samples followed by isolation on selective agars and identification with a RiboPrinter microbial characterization system (Bruce, 1996) showed that the contamination rates among these samples were 10.0–17.1%, which are relatively high compared to those of most ready-to-eat foods, including cheese, meat products, and vegetables sampled in other countries (Farber, 2000; Gombas et al., 2003; Hitchins, 1996; Soriano et al., 2001). This survey indicates frequent exposure of Japanese people to this pathogen and shows that urgent risk assessment of ready-to-eat seafood products is necessary. Therefore, we specifically selected these types of foods for our study samples.

In our study, *inlA* and *prfA* were successfully amplified in all 59 of the *L. monocytogenes* seafood isolates, and the sequence analysis revealed that 58 out of 59 isolates and all 59 isolates lacked the nonsense codon producing truncated forms of InlA and PrfA, respectively. Our results are consistent with those of Jacquet et al. (2004) in that all strains of serotypes 1/2b and 4b did not have any nonsense codon in the sequence of *inlA*. On the other hand, only one out of 36 isolates of serotype 1/2a had a nonsense mutation in *inlA*, while 37% of food isolates of this serotype had truncated InlA according to Jacquet et al. (2004). Only strain 36-25-1 had a nonsense mutation at position 526, where adenine was converted into thiamine, resulting in a codon change from lysine into a nonsense codon TAA. This is the first report of a nonsense mutation at this position, while nine other different nonsense mutation positions have been previously reported (Jonquières et al., 1998; Nightingale et al., 2005a,b; Olier et al., 2002; Rousseaux et al., 2004). This suggests that there could be other positions with nonsense mutation resulting in the truncation of InlA, making it difficult to establish an easy method to detect strains with truncated forms of InlA, although this kind of technique would be a particularly useful tool for the risk assessment of foods. The invasion efficiency of this strain with the nonsense mutation was tested by performing a Caco-2 cell invasion assay. The greatly reduced invasion efficiency of this strain compared to those of strains with non-mutated *inlA* was confirmed as expected (Table 3).

In addition to the identification of a strain with a nonsense mutation in the *inlA* sequence, we identified two other isolates, 20-5-1 and 34-18-2, having 9-nucleotide deletions in the membrane anchor region (nucleotides 2212 to 2220 of *inlA*). This type of deletion has never been reported before for *L. monocytogenes* strains of any origin to the best of our knowledge. This type of deletion may have no effect on anchoring of the protein to the bacterial surface since the LPXTG motif, which allows a covalent linkage of the protein to the cell wall peptidoglycan, is retained (Navarre and Schneewind, 1994; Schubert et al., 2002). However, statistical analysis (*t* test) showed no significant difference between these two isolates and isolates with nonsense-mutated *inlA* in terms of invasion efficiency (P value = 0.104) (group 2 and 3 in Table 3). Furthermore, the invasion efficiencies of these two isolates were not significantly different from those of the isolates of non-mutated *inlA*, either (P value = 0.234) (group 1 and 3 in Table 3), whereas there was a significant difference between the invasion efficiencies of strains of groups 1 and 2 (P value = 0.0093). Even though these two isolates having 3 amino acid residue deletions seemed to be closer to the isolates with non-mutated *inlA* than to the isolates with nonsense-mutated *inlA* based on the statistics, this needs to be confirmed by testing larger numbers of strains.

No case of seafood-borne listeriosis has been detected in Japan until now (Makino et al., 2005). According to Jacquet et al. (2004), 35% of food isolates of no less than four different serotypes analyzed, including seafood isolates, had truncated InlA. Other reports have also shown that truncation of this protein is not a rare event among food isolates (Jonquières et al., 1998; Nightingale et al., 2005a). Therefore, it was unexpected that just one out of 59 isolates collected from 531 ready-to-eat raw seafood retail products distributed in and around Tokyo was mutated to have a truncated form of InlA in our study. The role of InlA was previously evaluated in virulence toward chick embryos by Olier et al. (2005). They compared the virulence between wild type strains and mutants constructed by allelic exchange of the *inlA* region, providing evidence of the necessary, but not sufficient, role of InlA in in vivo infection. In fact, strains having a full-length InlA have been isolated from a healthy child (Olier et al., 2002) and strains having a truncated form of InlA have been isolated from clinical patients (Jacquet et al., 2004), indicating that other factors contribute to virulence attenuation or induction. Therefore, even though almost all of the investigated seafood isolates had non-mutated *inlA*, this does not directly equate with full-virulence of these strains. We need to conduct further research to determine whether these isolates are fully virulent, and if not, which gene(s) contributes to virulence attenuation.

Nonsense-mutated *prfA* was not detected in all 59 isolates in this study (Table 1). To the best of our knowledge, there is only one report of PrfA truncation, and in it, all three isolates with truncated PrfA were found to have nonsense mutations at the same position (Roche et al., 2005). The truncated form of this protein may be more prevalent, and there may be other positions with nonsense mutation resulting in the truncation of PrfA, as in the case of the *inlA* gene. Since *prfA* is important in *L. monocytogenes* virulence because of its regulatory function over several virulence determinants, further investigation of nonsense-mutated *prfA* prevalence is needed.

Among the seafood isolates we analyzed, their distribution among the serotypes was as follows: serotype 1/2a, 61.0%; 3a, 22.0%; 1/2b, 10.2%; 3b, 3.4%; 4b, 3.4% (Table 1). Isolates of serotypes 1/2a and 3a comprised 83% of all isolates, and all of them belonged to lineage II, which is more associated with food isolates than human or animal isolates. Most of the ribotypes we obtained from these isolates were widely prevalent among many kinds of ready-to-eat foods (Gray et al., 2004) and no ribotypes from those common to outbreak isolates, such as 1038 and 1042 (Jeffers et al., 2001), were found. Moreover, no specific patterns

for these seafood isolates were detected in MLST when compared to other food isolates (data not shown). These subtypings reveal that the seafood isolates we analyzed were not especially virulent ones, making our results of *inlA* sequences more surprising.

Seafood isolates of serotype 1/2c were not isolated from 531 seafood samples investigated in this study, and thus, were not included in this analysis. This is consistent with previous reports showing that strains of this serotype were frequently isolated from meat products (Fantelli and Stephan, 2001; Farber and Peterkin, 1991; Johnson et al., 1990; Thévenot et al., 2005), and from seafood products with much less frequency (Dauphin et al., 2001; Handa et al., 2005; Johansson et al., 1999; Nakamura et al., 2004). However, the reason for these observations remains unknown since the primary source of food contamination is most likely the processing environment, rather than the raw material itself (Kathariou, 2002), and the difference in biofilm formation ability among different serotypes is still under discussion (Borucki et al., 2003; Djordjevic et al., 2002; Lunden et al., 2000; Norwood and Gilmour, 1999). In a survey of the prevalence of strains with nonsense-mutated *inlA*, we found that all 4 meat isolates of serotype 1/2c that we analyzed in parallel with the fish isolates had a nonsense-mutated *inlA*. This is consistent with results previously reported (Jacquet et al., 2004). As an epidemiological study of strains of serotype 1/2c, a larger group of isolates of this serotype may be needed to ascertain this finding. Also, elucidation of the low incidence of *L. monocytogenes* of serotype 1/2c in seafood isolates is needed to contribute to progress in food safety.

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Development of multilocus single strand conformation polymorphism (MLSSCP) analysis of virulence genes of *Listeria monocytogenes* and comparison with existing DNA typing methods

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Abstract

Development of rapid and simple typing methods is required for analyzing the distribution and contamination routes of food-borne pathogens. We established a simple typing method for *Listeria monocytogenes* using MLSSCP (Multilocus Single Strand Conformation Polymorphism) analysis. Four virulence genes, *hlyA*, *iap*, *actA* and *inlB* were amplified by PCR, digested with endonucleases and applied to gels for SSCP. As banding patterns have been shown to reflect even a single nucleotide difference, this method has a potential discriminatory power comparable to that of sequencing analysis. The 64 strains isolated from five meat processing plants were divided into 18 groups by this MLSSCP. Additionally, clustering obtained with this method showed strong correspondence with phylogenetic lineages I and II, and was achieved with much less expenditure in time and cost than is required for other methods, such as MLST. The validity of the MLSSCP lineage classification was confirmed by PFGE, AFLP and ribotyping results. This newly developed MLSSCP method is suitable when obtaining accurate results quickly and simply is crucial.
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Keywords: *Listeria monocytogenes*; Typing; SSCP; Meat products

Listeria monocytogenes is an ubiquitous facultative intracellular pathogen, which can cause listeriosis in humans and animals. In the United States, this pathogen is responsible for approximately 2500 cases of human illness and an estimated 500 deaths per year, with 99% of listeriosis cases being food-borne (Mead et al., 1999). Accordingly, a zero-tolerance policy was introduced for this organism in ready-to-eat food in the United States and some other countries. On the other hand, effective January 1, 2006, the EU adopted a tolerance level of less than 100 cfu/g at the best-before date. This includes a condition that this pathogen be absent in 25 g prior to dispatch for *L. monocytogenes* supportive ready-to-eat foods other than those intended for infants and for special medical purposes. This more realistic standard has been set because many ready-to-eat foods have been reported to contain *L. monocytogenes* at considerable rates. This pathogen mainly causes listeriosis in pregnant women, neonates, infants, and immunocompromised patients (Farber and Peterkin, 1991; Schuchat et al., 1991). On the other hand, 1–6% of the entire population is estimated to carry this bacterium without showing any symptoms (Macgowan et al., 1991; Schuchat et al., 1993). These studies indicated that *L. monocytogenes* virulence may differ from strain to strain, as was also suggested by some previous studies (Barbour et al., 2001; Jacquet et al., 2002; Olier et al., 2002; Roche et al., 2001; Tabouret et al., 1992).

Food processing plants have been suspected as being the primary place of contamination with this organism for some types of foods (Autio et al., 1999, 2004; Nesbakken et al., 1996; Rørvik et al., 1995). Therefore, local epidemiological study for this

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organism in food processing plants is urgently needed in order to clarify the contamination route and to trace the sources of these pathogenic bacteria. Virulence potential also needs to be identified so that a definitive universal policy on acceptable levels of this organism in foods can be established. In order to trace the contamination route and to identify type-specific virulence potential using phylogenetic classification systems, various fragment-based typing methods have been developed to replace serotyping. Serotyping alone is not sufficient to distinguish these strains (Heir et al., 2004; Wagner et al., 2000), and it is laborious and limited in use due to the limited availability of antisera (Palumbo et al., 2003). Examples of fragment-based methods are pulsed-field gel electrophoresis (PFGE) (Autio et al., 1999; Brosch et al., 1996; Giovannacci et al., 1999; Kerouanton et al., 1998; Larsen et al., 2002; Louie et al., 1996), amplified fragment length polymorphism (AFLP) (Guerra et al., 2002; Keto-Timonen et al., 2003), and random amplified polymorphic DNA (RAPD) (Giovannacci et al., 1999; Kerouanton et al., 1998; Lawrence and Gilmour, 1995; Wagner et al., 1996). These methods have been extensively studied and demonstrated to be accurate and useful for epidemiological typing of *L. monocytogenes* for contamination route determination and virulent strain detection. Moreover, high discriminatory power of these methods has been reported as well. However, they are time-consuming and the sample preparation and analysis requires high-level skills. RAPD is relatively easy to handle and is used in many food-testing laboratories for a wide range of microorganisms; however, its lack of reproducibility due to variation in primer and DNA concentration, DNA template quality, gel electrophoresis conditions, and the type of DNA polymerase has been reported (Tyler et al., 1997; Valsangiacomo et al., 1995). Ribotyping is also known to be an easy-to-use fully automated method, but it has a rather low discriminatory power for strains and is too costly for routine operation in food testing laboratories. In addition, results obtained by these fragment-based typing methods are limited with respect to interlab comparability (Wiedmann, 2002).

Recently, DNA sequencing-based methods have been developed to compensate for drawbacks of these fragment-based methods. Especially, the relationship between sequences of some protein-coding genes and evolutionary lines has been investigated by multilocus sequence typing (MLST) (Cai et al., 2002; Rasmussen et al., 1995; Revazishvili et al., 2004; Salcedo et al., 2003). In particular, MLST using virulence genes for *L. monocytogenes* has been reported to have discriminatory power comparable to that of PFGE (Zhang et al., 2004). This method established a strong presence in a phylogenetic classification system, which categorizes this organism into three distinct lineages in order to understand its ecology and population dynamics. The classification done by this system correlates with flagellar antigen types. That is, lineage I strains mainly include serotypes 1/2b, 3b, 4b, 4d and 4e whereas lineage II includes serotypes 1/2a, 1/2c, 3a, and 3c (Brosch et al., 1994; Graves et al., 1994), lineage III includes serotypes 4a and 4c (Rasmussen et al., 1995; Wiedmann et al., 1997). The presence of lineage-specific virulence potential has also been suggested; for example, lineage I strains are more common among human listeriosis cases than are the strains belonging to other lineages and lineage II strains more strongly associated with foods. Lineage III strains, a less populous group, are more commonly associated with animal listeriosis cases (Jeffers et al., 2001) and they are rarely isolated from human cases (Rasmussen et al., 1995). The MLST method is not only a reliable tool for phylogenetic classification analysis, but it is advantageous in that the allelic profiles of isolates can easily be compared to those obtained by other laboratories via the Internet. This is of particular value in situations where the profiles of a wide variety of isolates need to be compared to each other. However, it is doubtful that this comparability is required in every situation. For example, in food processing plants, having simple and quick approaches to local epidemiology is more critical than having interlab comparability of data because practicable routine monitoring methods need to be established for clarifying the contamination route and tracing the movement of pathogenic bacteria within these plants. Moreover, MLST cannot be characterized as a simple and low cost method which allows anyone to easily and routinely run analyses for quick risk assessment.

In the present study, we developed a simple and quick method using multilocus single strand conformation polymorphism (MLSSCP) analysis. MLSSCP is similar to MLST in that multilocus gene diversity is analyzed for genomic typing, but it uses longer sequences of the genes. We demonstrated that this method has a discriminatory power potentially comparable to that of sequencing analysis being equivalent to that of MLST but with a much lower time and cost requirement.

1. Materials and methods

1.1. Bacterial strains in this study

A total of 64 *L. monocytogenes* strains used in this study were isolated from various raw meats during the routine monitorings in five different meat processing plants, each of which handles meats originating from more than one country (Table 1). Isolation procedures were basically conducted according to the method of Handa, Kimura, Takahashi, Koda, Hisa, and Fujii (2005) with some modifications. Briefly, 25 g of meat sample was homogenized in 225 ml of UVM-*Listeria* selective enrichment broth modified (Merck, Darmstadt, Germany) and incubated at 30 °C for 48 h. Then, a portion of the enrichment culture was streaked onto Palcam agar (Oxoid, Basingstoke, United Kingdom) plates and incubated at 35 °C for 48 h. Typical colonies were picked and inoculated on sheep blood agar plates (Nissui Pharmaceutical, Tokyo, Japan), and colonies showing β-hemolysis activity were streaked onto tryptic soy agar (TSA; Becton Dickinson, Sparks, MD, USA) plates and incubated at 35 °C for 24 h. Colonies on TSA plates were identified using a RiboPrinter microbial characterization system (Qualicon Inc., Wilmington, DE, USA) following the manufacturer's instructions (shown below).

1.2. Serotyping of isolates

Serotyping was carried out with commercial *Listeria* antiserum (Denka Seiken, Tokyo, Japan). O-antigen determination

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Table 1
Comparison of typing methods by serotype, SSCP, ribotyping, PFGE and AFLP

Strain no.	Source		Serotype	SSCP type					Ribotyping	PFGE	AFLP
	Sample	Country		*hlyA*	*iap*	*actA*	*inlB*	Combination of 4 genes			
Lma 5	Pork	United States	4b	2	3	3	3	1	7	8	5
Lma 7	Pork	United States	4b	2	3	3	3	1	7	5	5
Lmb 15	Pork	Canada	4b	2	3	10	11	2	2	7	6
Lmb 17	Pork	Denmark	4b	2	3	10	11	2	2	6	6
Lmb 20	Pork	Denmark	4b	2	3	3	3	1	7	4	6
Lma 25	Pork	Japan	1/2b	2	3	3	7	3	2	3	1
Lma 26	Pork	Japan	1/2b	2	3	3	7	3	2	3	1
Lma 27	Chicken	Japan	1/2b	2	4	3	8	4	2	1	2
Lmb 46	Pork	Denmark	1/2b	2	7	11	7	5	2	2	3
Lma 6	Pork	United States	4d	2	3	3	3	1	7	8	5
Lma 11	Pork	United States	3b	2	3	5	5	6	5	9	4
Lma 12	Pork	United States	3b	2	3	5	5	6	5	9	4
Lma 8	Pork	United States	1/2c	3	2	4	4	8	3	34	30
Lma 18	Pork	United States	1/2c	3	2	4	4	8	8	33	30
Lmb 3	Pork	Denmark	1/2c	3	2	4	4	8	3	33	32
Lmb 19	Pork	Denmark	1/2c	3	2	4	4	8	3	31	32
Lmb 52	Pork	Canada	1/2c	3	2	4	4	8	3	32	31
Lma 1	Pork	Korea	1/2a	1	1	1	1	9	6	13	28
Lma 2	Pork	Korea	1/2a	1	1	1	1	9	6	13	29
Lma 4	Pork	United States	1/2a	1	2	2	2	10	1	12	16
Lma 10	Chicken	Japan	1/2a	1	2	2	2	10	1	10	16
Lma 13	Pork	United States	1/2a	1	2	2	2	10	1	11	16
Lma 15	Pork	Canada	1/2a	1	2	2	2	10	1	10	16
Lma 20	Other	China	1/2a	1	2	2	2	10	1	10	17
Lma 21	Other	China	1/2a	1	2	2	2	10	1	10	17
Lma 22	Pork	Denmark	1/2a	3	4	6	6	11	8	26	19
Lma 23	Pork	Denmark	1/2a	3	4	6	6	11	8	26	19
Lma 28	Chicken	Japan	1/2a	1	1	7	9	12	1	27	7
Lma 29	Chicken	Unknown	1/2a	1	4	7	4	13	4	19	13
Lma 30	Chicken	Unknown	1/2a	1	4	7	4	13	4	23	13
Lma 31	Chicken	Unknown	1/2a	4	4	1	1	14	1	30	8
Lma 34	Pork	Unknown	1/2a	4	4	7	4	7	4	23	13
Lma 35	Pork	Unknown	1/2a	4	4	7	4	7	4	19	13
Lma 36	Pork	Unknown	1/2a	4	4	7	4	7	4	23	13
Lmb 1	Pork	Denmark	1/2a	4	4	7	4	7	9	17	10
Lmb 4	Pork	Denmark	1/2a	4	4	7	4	7	4	21	14
Lmb 6	Pork	Denmark	1/2a	4	4	7	4	7	4	19	14
Lmb 8	Pork	Denmark	1/2a	4	4	7	4	7	4	22	14
Lmb 10	Pork	Denmark	1/2a	1	2	8	1	15	4	28	9
Lmb 11	Pork	Denmark	1/2a	3	5	9	10	16	10	25	27
Lmb 12	Pork	Denmark	1/2a	2	5	9	10	17	10	25	21
Lmb 16	Pork	Denmark	1/2a	4	4	7	4	7	4	14	14
Lmb 18	Pork	Denmark	1/2a	4	4	7	4	7	9	19	14
Lmb 22	Pork	Denmark	1/2a	4	4	7	4	7	4	19	14
Lmb 23	Pork	Denmark	1/2a	4	4	7	4	7	4	21	18
Lmb 25	Pork	Denmark	1/2a	4	4	7	4	7	9	23	14
Lmb 27	Pork	Denmark	1/2a	4	4	7	4	7	4	22	18
Lmb 29	Pork	Denmark	1/2a	4	4	7	4	7	4	20	15
Lmb 30	Pork	Denmark	1/2a	1	2	2	2	10	1	12	11
Lmb 32	Pork	Denmark	1/2a	4	4	7	4	7	9	19	10
Lmb 34	Pork	Denmark	1/2a	4	4	7	4	7	4	15	10
Lmb 35	Pork	Denmark	1/2a	4	4	7	4	7	4	14	24
Lmb 36	Pork	Denmark	1/2a	4	4	7	4	7	4	14	10
Lmb 37	Pork	Denmark	1/2a	4	4	7	4	7	4	16	10
Lmb 38	Pork	Denmark	1/2a	4	4	7	4	7	4	18	10
Lmb 40	Pork	Denmark	1/2a	3	6	2	12	18	3	29	22
Lmb 42	Pork	Denmark	1/2a	3	5	9	10	16	10	24	20
Lmb 43	Pork	Denmark	1/2a	3	5	9	10	16	10	24	20
Lmb 44	Pork	Denmark	1/2a	4	4	7	4	7	4	22	10
Lmb 47	Pork	Denmark	1/2a	4	4	7	4	7	4	14	12
Lmb 48	Pork	Denmark	1/2a	4	4	7	4	7	4	22	24
Lmb 49	Pork	Denmark	1/2a	4	4	7	4	7	9	23	26

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Table 1 (*continued*)

Strain no.	Source		Serotype	SSCP type					Ribotyping	PFGE	AFLP
	Sample	Country		*hlyA*	*iap*	*actA*	*inlB*	Combination of 4 genes			
Lmb 50	Pork	Denmark	1/2a	4	4	7	4	7	4	14	25
Lmb 51	Pork	Denmark	1/2a	3	6	2	12	18	3	29	23
Total			5	4	7	11	12	18	10	34	32

strains were grown on brain heart infusion (BHI) agar (Becton Dickinson) for 24 h at 35 °C. Cells were suspended in 0.2% sodium chloride and heated at 121 °C for 30 min followed by centrifugation at 3000 rpm for 20 min and resuspended in 0.5 ml of 0.2% sodium chloride. Slide agglutination tests using polyvalent type O-antiserum were performed first, followed by typing with individual O-antiserum. H-antigen strains were determined using the tube agglutination test. Briefly, sample cultures were incubated in semiliquid BHI medium (0.2% wt/vol agar) at room temperature (20–25 °C) for 24 h, repeated four times. The samples were incubated in semiliquid BHI medium in Craigie tubes for 24 h followed by removal to BHI medium for an additional 24-h incubation. H-antigen type was determined after mixing two drops of antiserum with 0.5 ml of cell suspension with 1% formalin and incubating at 50 °C for 1 h.

1.3. MLSSCP analysis using four virulence-associated genes

Primer sets and PCR conditions designed for amplification of *hlyA*, *iap*, *actA*, and *inlB* are shown in Table 2. Primers were designed based on alignments of sequences of each gene from DNA Databank of Japan using GENETYX-WIN software version7 (Genetyx, Tokyo, Japan) and tested for their suitability using Primer Express software (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed in mixtures (100 μl) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmol each of primer, 0.2 mM each of 4 dNTPs, 50 ng of template DNA and 2.5 U of TaKaRa Taq DNA polymerase (Takara Bio, Otsu, Japan) using a GeneAmp 9700 thermal cycler (Applied Biosystems). Aliquots (5 μl) of the PCR products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels to confirm that amplification products of the expected size were produced. The amplified fragments were treated with polyethylene glycol, cooled on ice for 1 h, and pelleted by centrifugation at 15,000 ×*g* for 20 min. The pellets were washed with 70% ethanol, dried, and dissolved in TE buffer. As the purified fragments used in this study had approximate lengths of 1000–1700 bp, they were considered to be too long for detecting mutations by SSCP analysis and were subsequently digested with endonucleases at 37 °C for 3 h according to manufacturer's instructions to produce a number of shorter fragments: *Hha*I (Takara) for *iap* and, *Alu*I (Takara) for *hlyA*, *actA*, and *inlB*. The digested products were electrophoresed on 1.5% (wt/vol) agarose gels to confirm the correct digestion, mixed 1:2 with loading buffer (98% formamide-10 EDTA-0.5% bromophenol blue), denatured by heating for 10 min at 100 °C, cooled on ice, and loaded in precast ready-to-use gels (GeneGel Excel 12.5/24 kit; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). SSCP electrophoresis was performed at 5 °C for 90 min (until the bromophenol blue reached the anode buffer strip) using a GenePhor (GE Healthcare Bio-Sciences) electrophoresis unit at 750 V, 25 mA. The gels were stained with PlusOne DNA silver staining kit (GE Healthcare Bio-Sciences).

1.4. Confirmation of sequence polymorphism using representative gene, iap

To confirm polymorphism, representative gene, *iap*, was sequenced for all 64 strains using an ABI310 genetic analyzer (Applied Biosystems) with the Big Dye terminator v.3.1 cycle sequencing kit (Applied Biosystems).

1.5. PFGE analysis

Enzymes and electrophoretic conditions were according to the method of Graves and Swaminathan (2001). DNA plugs for PFGE were made using CHEF bacterial plug kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Briefly, *L. monocytogenes* strains were grown overnight in BHI

Table 2
PCR primers and conditions of 4 genes used in this study

Gene	Primer	Sequence (5′–3′)	Product size (bp)	PCR conditions		Reference
				No. of cycles	Cycle steps	
hylA	*hylA – f*	GAGAGGAGGGGCTAAACAGTAT	1702	35	94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min	Mengaud et al. (1988)
	hylA – r	TTTCGTGTGTGTTAAGCGGT				This study
iap	*iap – f*	CAACTATCGCGGCTACAGCT	1428	35	94 °C for 1 min, 60 °C for 1 min 72 °C for 1 min	This study
	iap – r	ATACGCGACCGAAGCCAACT				
actA	*actA – f*	CACAGATGAATGGGAAGAAGAA	963	30	94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min	Revazishvili et al. (2004)
	actA – f	CTTGTAAAACTAGAATCTAGCGA				
inlB	inlB – f	GCCAACGCCAATCAAGCAA	1749	30	94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min	This study
	inlB – r	CTTTCGTCCAACCAATGAAAG				

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medium. After adding chloramphenicol to yield a final concentration of 180 μg/ml in order to allow further DNA replication but inhibit protein synthesis, the cultures were incubated for up to 1 h at 30 °C. The cells were pelleted by centrifugation at 10,000 × g for 5 min, resuspended in 100 μl of cell suspension buffer, and equilibrated to 50 °C. Bacterial suspensions were mixed with equal volumes of CleanCut agarose (2%, 50 °C) and poured into a mold to form agarose plugs (15 min, 4 °C). Plugs with immobilized bacteria were incubated for 2 h at 37 °C with lysozyme solution, rinsed with sterile water and incubated overnight at 50 °C with Proteinase K reaction buffer. Plugs were washed four times with 1 ml of 1× wash buffer with gentle mixing at room temperature for 1 h each time. After the buffer was aspirated, the plugs were incubated overnight at room temperature with 30 U of *Apa*I (New England Biolabs, Beverly, MA, USA) in 300 μl of 1× enzyme buffer. Following overnight digestion, the plugs were incubated in 1 ml of 1× wash buffer for 30 min with gentle agitation. One-third of each plug was placed in 1.2% agarose (Seakem Agarose Gold; FMC Bioproducts, Rockland, ME, USA) gel in 0.5× TBE buffer. PFGE was performed at 14 °C with the CHEF-DR II system (Bio-Rad) at 6 V/cm with switch times ranging between 4 and 40 s for 22 h. Gels were stained with ethidium bromide, and DNA banding patterns were photographed after transillumination with UV light. To facilitate intergel comparison, one *L. monocytogenes* strain (Lma 12) was used as a marker in all gels.

1.6. AFLP analysis

AFLP analysis was performed using an AFLP microbial fingerprinting kit (Applied Biosystems). Briefly, 10 ng of bacterial DNA extracted by the method of Sambrook, Fritsch, and Maniatis (1989) was digested with 2.5 U of *Mse*I and 5 U of *Eco*RI restriction enzymes and was subsequently ligated to *Mse*I and *Eco*RI restriction site-specific adapters overnight at room temperature. Preselective PCR was carried out in 20 μl reaction mixtures containing 4 μl of the restriction ligation mixture, 0.5 μl of preselective *Mse*I primer, 0.5 μl of preselective *Eco*RI primer, and 15 μl of AFLP amplification core mix. After amplification, the PCR samples were diluted 20:1 with $TE_{0.1}$ buffer (20 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). For selective PCR, 1.5 μl of the diluted PCR samples were amplified in 10 μl (final volume) mixtures under the reaction conditions described above with the substitution of *Mse*I and fluorescently labeled *Eco*RI selective primers containing adjacent nucleotides A, C, G or T.

PCR was performed according to the AFLP microbial fingerprinting protocol using a GeneAmp 9600 thermal cycler (Applied Biosystems). Aliquots (0.5 μl) of the selective amplification products were mixed with 25 μl of deionized formamide and 1 μl of GeneScan-500 [ROX] size standard (Applied Biosystems). Samples were denatured at 95 °C for 3 min and immediately cooled on ice. AFLP capillary electrophoresis was performed on an ABI Prism 310 genetic analyzer (Applied Biosystems). The AFLP patterns were automatically analyzed using GeneScan Analysis software (Applied Biosystems).

1.7. Ribotyping

Automated ribotyping was carried out using the RiboPrinter (Qualicon Inc., Wilmington, DE, USA) with normalized data according to manufacturer's instructions. This automated typing method involves *Eco*RI digestion of *L. monocytogenes* chromosomal DNA followed by Southern hybridization with an rRNA gene probe. Images are analyzed using RiboPrinter analysis software that normalizes fragment pattern data for band intensity and band size relative to a molecular weight marker.

1.8. Data analysis

The obtained banding patterns of SSCP, PFGE and AFLP were analyzed with BioNumerics v.4.0 software (Applied Maths, Sint–Martens–Latem, Belgium). For MLSSCP, scanned photographs of gels were analyzed with this software to identify lanes with identical banding patterns. Different type numbers were assigned to lanes of different banding patterns for each locus, the numbers were entered into the software, and clustering analysis for MLSSCP was done with the numerical profiles for each isolate with the categorical coefficient and unweighted pair group method with arithmetic mean (UPGMA) clustering parameter. Likewise, scanned photographs of PFGE gels and banding patterns of AFLP analyses obtained after conversion of the peak patterns generated on an ABI 310 Genetic analyzer were stored as TIFF format files and processed with BioNumerics software. Entire PFGE gels were used for analysis. For AFLP analysis, only fragments in the range of 40 bp to 600 bp were considered. Cluster analysis was performed by UPGMA analysis using Dice coefficient with 1.0% band position tolerance and 0.4% optimization value for PFGE, and Pearson coefficient with 0% optimization value for AFLP.

1.9. Discriminatory index (D. I.)

Discriminatory power of the methods was calculated with Simpson's index of diversity (Hunter and Gaston, 1988).

1.10. Lineage classification

Each of the 64 *L. monocytogenes* strains was designated into one of three evolutionary lineages using a method described previously (Ward et al., 2004). Briefly, multiplex PCR was carried out using primers designed for the identification of lineage-specific regions in *actA* or *plcB* to produce a lineage-specific sized band on electrophoresis.

2. Results

2.1. Fragment-based whole-genome typing

First, a total of 64 strains of *L. monocytogenes* isolates were serotyped and classified into lineages: 12 isolates were assigned into lineage I and the remaining 52 isolates into lineage II. Strains belonging to lineage III were not detected. Strains of serotypes 1/2b, 3b, 4b, and 4d were classified into lineage I,

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Fig. 1. (a), (b). Dendrograms constructed by the UPGMA method using PFGE and AFLP analyses. Similarity percentages shown above the dendrogram were calculated using the Dice coefficient (a) or the Pearson product–moment correlation (b).

whereas those of serotypes 1/2a and 1/2c were classified into lineage II.

Then, these strains were characterized by whole-genome typing methods PFGE, AFLP and ribotyping. In PFGE analysis, a total of 34 different banding patterns obtained for the 64 *L. monocytogenes* strains were largely divided into 2 main clusters (Table 1, Fig. 1a): lineage I strains (serotypes 1/2b, 3b, 4b, and 4d) and lineage II strains (serotypes 1/2a and 1/2c). For AFLP

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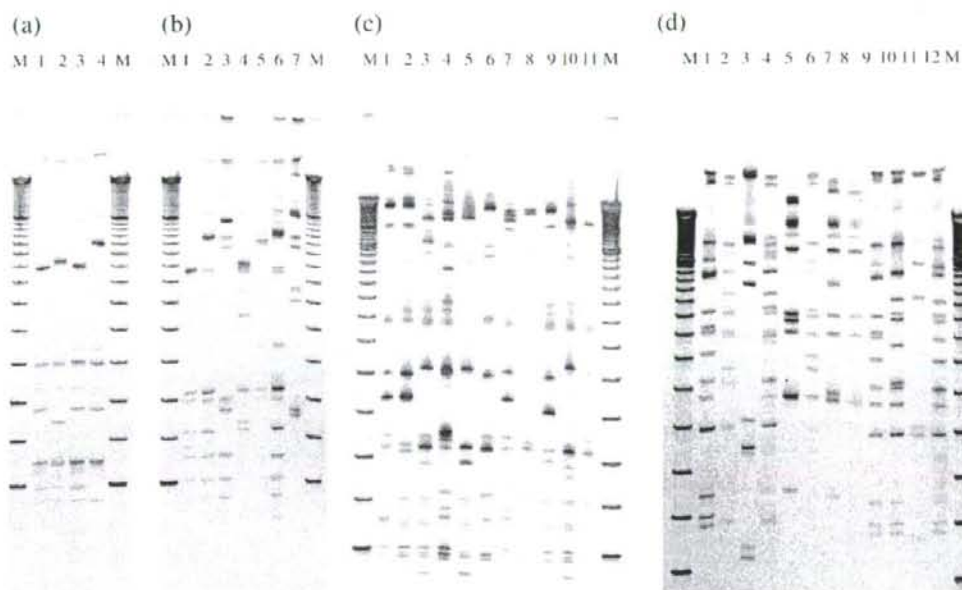

Fig. 2. SSCP gel images showing *hly* (a), *iap* (b), *actA* (c) and *inlB* (d) of the representative strains. The numbers assigned to each lane correspond to the groupings in Table 1.

analysis, the selected primer set (*Mse*I+G, *Eco*RI+G) distinguished the 64 strains into 32 groups when the cut-off value was set at 95% (Table 1, Fig. 1b). The two main clusters of *L. monocytogenes* strains matched those identified by PFGE. Of the two clusters, lineage I included strains of serotypes 1/2b, 3b, 4b, and 4d and lineage II included strains of serotypes 1/2a and 1/2c. By contrast, ribotyping analysis separated the 64 strains into only 10 groups, and the list of ribotypes in lineages I and II differed (Table 1). Discriminatory ability (D. I.; Discriminatory index) of PFGE, AFLP and ribotyping was 0.971, 0.964, and 0.821, respectively.

2.2. MLSSCP analysis

The MLSSCP analysis developed in this study used four virulence genes, *hlyA*, *iap*, *actA*, and *inlB*, with distinct banding patterns obtained for each (Fig. 2 and Table 1): *hlyA*, 4 distinct types; *iap*, 7; *actA*, 11; and *inlB*, 12. Combination of SSCP banding patterns of all 4 genes produced 18 distinct banding patterns for the 64 *L. monocytogenes* strains (Table 1).

The dendrogram produced based on MLSSCP analysis corresponded to the lineage classification produced by previous analyses (Wiedmann et al., 1997; Salcedo et al., 2003; Zhang et al., 2004). That is, two main clusters were observed, with one consisting of only lineage I strains and the other consisting of only lineage II strains (Fig. 3). SSCP types for each gene also reflected these lineage classifications, but each had a few exceptions (Table 1): for example, *iap*, lineage I strains were basically included in SSCP type Nos. 3 and 7, and lineage II strains were included in type Nos. 1, 2, 4, 5, and 6, and only one 1/2b strain (Lma 27) was included in type No. 4. In contrast, combination of SSCP typing for all 4 genes, MLSSCP, completely reflected the lineages. For lineage II strains, serotype 1/2a strains were included in SSCP types 7 and 10–19, serotype 1/2c in types 8 and 9, and for lineage I strains, serotype 1/2b strains were included in types 3–5, serotype 3b in type 6, serotype 4b in 2, and serotypes 4b and 4d were included together in SSCP type 1. The D. I. value of MLSSCP was 0.830.

The variation on SSCP banding patterns was compared to that for sequence alleles using *iap*, one of the genes we used. This comparison confirmed that our SSCP analysis reflected even minor sequence variations. Seven different *iap* sequence allele types corresponded with 7 different SSCP types. The similarity for *iap* sequences ranged from 93.3 to 99.8%.

3. Discussion

MLST, which recently came to the forefront as a useful typing system in population dynamics and evolutionary biology (Maiden et al., 1998), has high discriminatory power when highly polymorphic regions are targeted. This method is advantageous in that databases built-up in other laboratories are easily available via Internet, and in this regard, MLST is superior to fragment-based typing methods such as PFGE, AFLP and RAPD. However, a time- and cost-intensive method of this kind is not practicable in food processing plants where exchanging data is not as important as being able to conduct a quick local epidemiological study. Furthermore, relatively short

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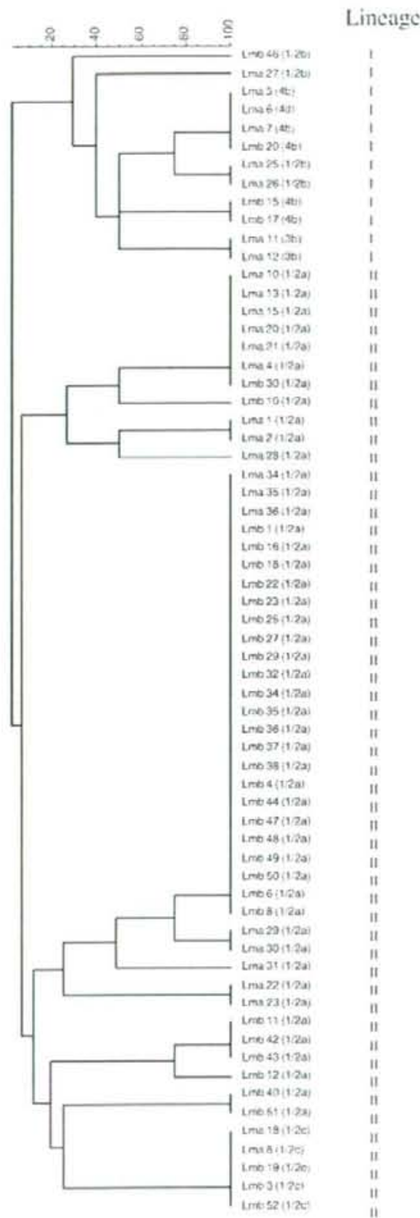

Fig. 3. Dendrogram constructed for MLSSCP analysis by the UPGMA method with BioNumerics v. 4.0 software showing the genetic relationships of 64 *L. monocytogenes* strains isolated from raw meat samples. Similarity percentages are shown above the dendrogram. Lineage classification was performed based on the multiplex PCR method described previously (Ward et al., 2004).

sequences with lengths around 400–600 bp are appropriate for this method, as they can be sequenced accurately with a single primer (Cai et al., 2002; Maiden et al., 1998; Zhang et al., 2004).

We developed an SSCP method using restriction enzyme digestion of multiple genes as a rapid, simple and inexpensive method for use in place of MLST. This MLSSCP method, which analyzes many, rather than only one, genetic regions and reflects every single nucleotide polymorphism, has the potential to have a high discriminatory power equivalent to that of MLST. Compared to whole genome typing methods such as PFGE and AFLP, our MLSSCP method has lower discriminatory power, hence our method cannot replace PFGE or AFLP. However MLSSCP is more practical when time and costs are limited, and moreover, this method is easier to handle than whole genome typing methods. Differentiation methods for *L. monocytogenes* using SSCP analysis have been reported previously (Lehner et al., 1999; Manzano et al., 1997; Wagner et al., 2000). However, these reports have analyzed only a single gene having a length of up to 500 bp, and consequently, either the discriminatory power was low or amplification of many short regions was needed in order to obtain an appropriate degree of discriminatory power. Moreover, the produced banding patterns were too similar to be visually distinguished. We, in fact, performed conventional SSCP with PCR fragments of around 400 bp for the *inlB* gene following the published reports. As expected, however, it was difficult to visually distinguish banding patterns in cases where 2–4 bands with similar electrophoretic mobilities were detected in a single lane (data not shown). In this regard, we determined that conventional SSCP analysis was not sufficient for typing *L. monocytogenes* because of its low discriminatory power for vague banding pattern differences. We then amplified longer sequences of the genes and digested them with restriction enzymes prior to SSCP analysis and consequently achieved success with more easily discriminated banding patterns (Fig. 2). Discriminating power for our method was higher than that of conventional SSCP analysis; for example, 12 different banding patterns were obtained for the *inlB* gene compared to only 9 for the conventional method using shorter sequences. Direct DNA sequencing of *iap* confirmed that the detected variations reflected even minor differences and that misdigestion had not occurred. Our enzyme-used SSCP method was demonstrated to be highly valid since every single nucleotide polymorphism was reflected in the SSCP banding pattern. In this regard, MLSSCP is superior to restriction fragment length polymorphism (RFLP) and PCR-RFLP. Although all three methods are similar in terms of labor required to electrophorese enzyme-digested fragments, single or several nucleotide differences in double-stranded DNA among fragments of the same length would not be detected in RFLP or PCR-RFLP since gel migration speed of double stranded fragments of the same length is almost identical. However, in our MLSSCP method, double-stranded DNA is denatured and the resulting 2 single strands form different conformation structures based on DNA sequence that affect migration speed of fragments through gels, even for fragments of identical length.

Unlike the previous researches on SSCP analysis described above, Lee, Lo, and Choo (1992) used restriction enzyme in their SSCP method so that they could analyze longer sequences (>1 kb). However, they analyzed only single DNA region since

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their objective was detecting mutations in this region. Combining multiple loci, on the other hand, we could obtain higher discriminatory power than when analyzing only a single locus.

A total of 64 *L. monocytogenes* strains were analyzed by four virulence genes: *hlyA* encoding listeriolysin O (Mengaud et al., 1988), *iap* encoding p60, an invasion-associated protein (Köhler et al., 1990), *actA* encoding ActA, an actin recruitment and polymerization protein (Kocks et al., 1992), and *inlB* encoding InlB, an invasion protein (Dramsi et al., 1995). These four genes were selected as target genes since all of them are virulence genes. Virulence genes were reported to be more polymorphic than housekeeping genes since they have evolved more rapidly due to frequent changes of the environment (Cai et al., 2002; Zhang et al., 2004). Specifically, these genes have been reported to be highly or moderately polymorphic (Bubert et al., 1992; Cai et al., 2002; Ericsson et al., 2000; Jacquet et al., 2002; Jeffers et al., 2001; Lehner et al., 1999; Rasmussen et al., 1995; Revazishvili et al., 2004; Wiedmann et al., 1997; Zhang et al., 2004). We selected the PCR primers to amplify the variable region for *actA* from a previous study (Revazishvili et al., 2004) and newly designed the primers to amplify nearly the entire sequences for *hlyA*, *iap* and *inlB*. As for *hlyA*, however, we obtained only 4 distinct alleles for the 64 strains included in our study, whereas Revazishvili et al. (2004) obtained 15 alleles in analysis of 157 strains using a shorter fragment of *hlyA*. This difference in number of different alleles could not be due to the amplified region-difference since our target region included the region they analyzed, but rather was due to strain differences, since we used only food isolates from meat processing plants while they used both clinical and food isolates. With respect to localization in the genome, these four genes are located in different *L. monocytogenes* virulence gene islands, except for *hlyA* and *actA*, which are located in the same virulence locus (Glaser et al., 2001). Another important consideration is that these four genes have been reported to reflect phylogenetic lineage classification (Ericsson et al., 2000; Jacquet et al., 2002; Rasmussen et al., 1995). Our findings supported these classifications, with a few noted exceptions. These exceptions in individual gene findings, however, were corrected by using MLSSCP analysis of the combination of all four genes (Fig. 3).

The dendrogram constructed in our MLSSCP analysis showed that the 64 strains were distinguished into two lineages with a large dissimilarity distance (Fig. 3). This lineage classification was supported by other methods using multiplex PCR, as described previously (Ward et al., 2004). This strain grouping showed a certain level of agreement among AFLP, PFGE and ribotyping (Table 1), and moreover, the validity of this dendrogram was supported by other dendrograms based on PFGE and AFLP analyses (Fig. 1). The similar branch topologies produced by multiple clustering methods indicate that the established phylogeny was accurate (Kim, 1993). The phylogenetic classification methodologies of *L. monocytogenes* have been discussed previously (Jinneman and Hill, 2001; Meinersmann et al., 2004; Mereghetti et al., 2002; Rasmussen et al., 1995; Ward et al., 2004; Wiedmann et al., 1997; Zhang et al., 2003). Our SSCP method compares very favorably with results obtained by all of these methods.

Overall, our present study showed that MLSSCP analysis has a higher discriminatory power, which could be comparable with that of MLST. SSCP using pre-cast gels, as in this study, is simple, rapid, and inexpensive compared to other methods and is effective for inferring contamination routes at plants. The resolution achieved by this method may be further improved by using additional genes.

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ORIGINAL ARTICLE

Lysine decarboxylase of *Vibrio parahaemolyticus*: kinetics of transcription and role in acid resistance

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Abstract

Aim: The aim of this study was to investigate the detailed mechanisms of acid resistance in *Vibrio parahaemolyticus*.

Methods and Results: All 11 strains of *V. parahaemolyticus* survived lethal acidic conditions following acid adaptation, and accumulation of cadaverine was detected. The addition of lysine improved survival, suggesting that lysine decarboxylase plays a role in the adaptive acid tolerance response. Two open reading frames (ORF) in *V. parahaemolyticus*, which are separated by a non-coding region, were found to be highly homologous to bacterial lysine decarboxylase (*cadA*) and lysine/cadaverine antiporter (*cadB*) genes. Transcriptional analyses of this operon revealed acid induction and enhanced induction by external lysine. The relative expression ratio of each transcript was found to follow the trend of *cadA* mRNA > *cadB* mRNA > *cadBA* bi-cistronic mRNA. A mutated strain, with a disrupted *cadA* gene, showed attenuated acid survival.

Conclusions: We identified the lysine decarboxylase gene operon of *V. parahaemolyticus*. Expression of this operon was induced under acidic conditions. The *cadA*-mutated strain constructed in this study showed weaker tolerance to acidic conditions than the wild-type strain.

Significance and Impact of the Study: *Vibrio parahaemolyticus* utilizes the lysine decarboxylation pathway for survival in acidic conditions.

Introduction

Vibrio parahaemolyticus, a ubiquitous marine pathogen found in seafood, causes human diarrhoea, especially through the consumption of raw fish and shellfish. *Vibrio parahaemolyticus* is a gram-negative halophilic bacterium, which is distributed worldwide in estuarine environments (Joseph *et al.* 1982). The virulent factors of this pathogen, mainly thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH), show haemolytic, cytotoxic, enterotoxic, and cardiotoxic activities against mammalian hosts (Honda and Iida 1993; Raimondi *et al.* 2000; Shimada and Arakawa 2000; Naim *et al.* 2001). While it is well established that fresh seafood generally contains *V. parahaemolyticus*, only a small fraction of bacteria carries these virulent genes (Wagatsuma 1974; DePaola *et al.* 1990; Kaysner *et al.* 1990). Over 400 cases of food poisonings caused by *V. parahaemolyticus* have been reported in Japan in 2000, illustrating the extent of this public health problem. Furthermore, the pandemic spread of *V. parahaemolyticus* serotype O3:K6, which has emerged since 1996, is a new topic in the control of this pathogen (Matsumoto *et al.* 2000). Bacteriophage f237, which is unique to the newly isolated O3:K6 clones, has been reported (Nasu *et al.* 2000), and some virulence-associated characteristics seem to be enhanced in O3:K6 clones. This newly emerging clone has also been implicated in a number of outbreaks in Japan and other countries.

Vibrio parahaemolyticus and other Vibrionaceae are generally thought to be more sensitive to low pH than other bacteria (Nishikawa *et al.* 1993; Waterman and Small 1998). Therefore, the infective dose (ID) of these pathogens is not low (Sanyal and Sen 1974; Bennish

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