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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.

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

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

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				

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,

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

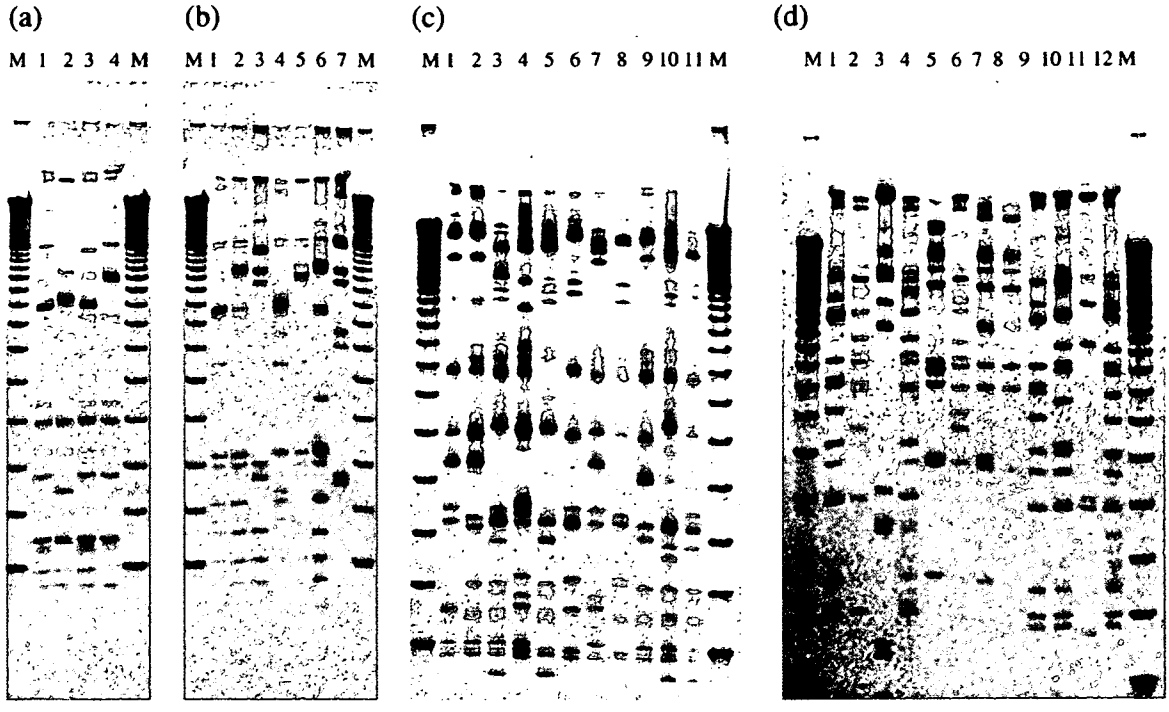

Fig. 2. SSCP gel images showing *hly* (a), *iap* (b), *actA* (c) and *inlB* (d) of the representative strains. The numbers assigned to each lane correspond to the groupings in Table 1.

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

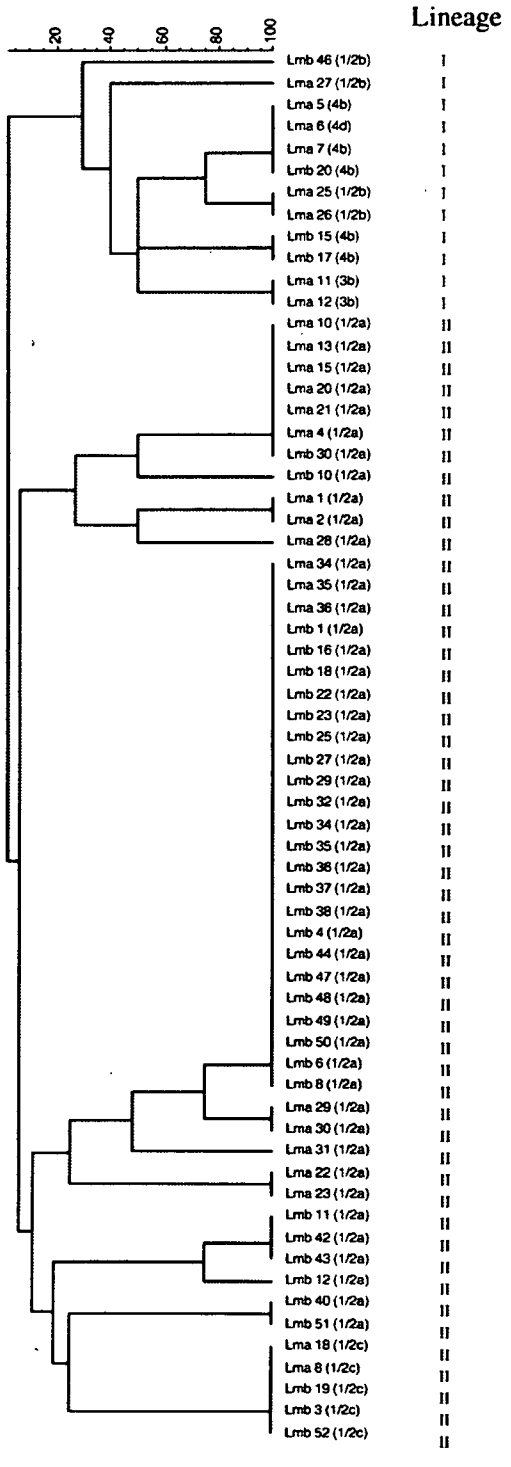

Fig. 3. Dendrogram constructed for MLSSCP analysis by the UPGMA method with BioNumerics v. 4.0 software showing the genetic relationships of 64 *L. monocytogenes* strains isolated from raw meat samples. Similarity percentages are shown above the dendrogram. Lineage classification was performed based 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

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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Keywords
acid tolerance, food, genes, lysine decarboxylase, regulation, stress response.

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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

1994). Many food- or water-borne diseases caused by *V. parahaemolyticus* (Sanyal and Sen 1974; Centers for Disease Control and Prevention 1998, 1999; Daniels *et al.* 2000) and *Vibrio cholerae* (Bennish 1994; Faruque *et al.* 1998) are important health problems worldwide. As these are gastrointestinal pathogens, it is implied that the pathogens successfully pass through the acidic stomach and colonize in the intestine. Waterman and Small (1998) reported that the surface-associated *V. cholerae* cells were protected from acidic environments. According to the report, *V. cholerae* cells cultured alone decreased over 5-log units in pH 5·0 Luria-Bertani (LB) medium, but in contrast, decreased only 3-log units even at pH 2·5 when inoculated with ground beef. As not all cases are transmitted by food, it is speculated that there is another factor participating in the acid resistance of Vibrionaceae. Merrell and Camilli (1999) reported that *V. cholerae* cells exposed to mildly acidic conditions (pH 5·7) survived lethal acid challenge (pH 4·5). Rhee *et al.* (2002) showed similar results in *Vibrio vulnificus*: their survival under acidic conditions was dependent on the lysine decarboxylase pathway. Although induced transcription of lysine decarboxylase genes under acidic pH is well described, the nature of transcription, particularly regarding the expression level of the genes, is not fully understood.

Wong *et al.* (1998) showed a similar result following mild acid exposure of *V. parahaemolyticus*. However, the detailed mechanism of acid resistance of *V. parahaemolticus* remained unknown. The phenomenon of mild acid exposure enhancing the ability of the pathogens to survive under lethal acidic conditions is now referred to as acid tolerance response (ATR). ATR was first recognized in *Salmonella typhimurium* by Foster and Hall (1990), and is now recognized by several researchers as the mechanism by which bacteria break through the gastric acid barrier (Foster 1999; Audia *et al.* 2001). ATR and its correlation with infectivity have also been reported elsewhere (Wilmes-Riesenberg *et al.* 1996; Merrell and Camilli 1999). *Vibrio parahaemolyticus*, an acid-sensitive gastric pathogen, may also utilize ATR in its mode of infection. In Japan, mildly acidic sushi (vinegar is usually added to the rice, pH 4·3–4·9) is frequently implicated in outbreaks of *V. parahaemolyticus* infection (Shimada and Arakawa 2000). This problem is suggestive of the importance of ATR in *V. parahaemolyticus* infection.

In this study, we focused on the ATR of *V. parahaemolyticus* using *in vitro* acid resistance tests. Molecular analysis for the ATR-associated gene, lysine decarboxylase, was also conducted, and the transcription patterns were precisely demonstrated by SYBR Green I real-time reverse transcriptional (RT) polymerase chain reaction (PCR) and Northern blot hybridization. Finally, a lysine decarboxylase gene-inactivated strain was constructed to clarify the role of this enzyme in ATR.

Materials and methods

Bacterial strains and growth conditions

A total of 11 *V. parahaemolyticus* strains, VpTK-(1, 3, 6, 8, 9, 10, 11, 12, 13, 17) and strain V02-64, representing the newly emerged clone of serotype O3:K6, were used. All *V. parahaemolyticus* strains (*tdh*+, Kanagawa phenomenon positive) isolated from food-poisoning patients were stored at −80°C using MicroBank microbial storage kits (Pro-Lab Diagnostics, Ontario, Canada) until use. All strains were serotyped by the slide agglutination test with O- and K-antigens using commercially available antisera (*V. parahaemolyticus* antisera Seiken set, Denka Seiken, Tokyo, Japan). Unless otherwise noted, all *V. parahaemolyticus* strains were cultured in LB broth or on LB agar (1·5% agar) with 3% sodium chloride. *Escherichia coli* strain JM109 used for genetic manipulation was grown in LB broth or on LB agar supplemented with 5 $\mu g\ ml^{-1}$ ampicillin unless otherwise specified.

Adaptive acid tolerance assays

Overnight cultures (300 μl) of each *V. parahaemolyticus* strain were inoculated into 30 ml fresh LB broth and incubated at 30°C with shaking until the OD_{600} reached 0·16–0·20 (log-phase culture). An aliquot of this culture (1 ml) was harvested by centrifugation at 15 000 *g* for 3 min at room temperature. Cells were resuspended in 1 ml of LB-MES (100 mmol l^{-1} 4-morpholinoethanesulfonic acid (MES), pH 5·5) or LB-HEPES (100 mmol l^{-1} [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7·5) and incubated at 37°C for 1 h. Cultures were centrifuged at 15 000 *g* for 3 min at room temperature, and the pellets were resuspended in 1 ml of LB broth (pH 4·0) and incubated at 37°C for 1 h. Immediately after resuspension in LB broth (pH 4·0), portions of samples were serially diluted in phosphate-buffered saline (PBS) (pH 7·0) and plated onto trypticase soy agar (TSA; Difco Laboratories, Detroit, MI, USA) with a spiral plater (IUL Instruments, Barcelona, Spain) (initial count). After 1 h of incubation in LB broth (pH 4·0), viable cells were enumerated in the same fashion (survival count), and the per cent surviving the acid challenge was calculated by dividing the viable counts at 1 h by the initial viable counts and multiplying by 100. The amount of some polyamines (tryptamine, putrescine, cadaverine, histamine, agmatine, tyramine, and spermidine) in the LB broth was measured by HPLC as previously described (Yamanaka and Matsumoto 1989).

Lysine decarboxylase-dependent acid-tolerance assays

Overnight cultures (500 μl) of each *V. parahaemolyticus* strain were inoculated into 50 ml fresh LB broth (1 : 100) and incubated at 30°C with shaking (170 rev min^{-1}) until OD_{600} reached 0·16–0·20 (log-phase culture). Cells were precipitated and resuspended in LB-MES, pH 5·5 (acid adaptation) or LB-HEPES, pH 7·5 (no adaptation). Chloramphenicol was added at 10 μg ml^{-1} to each treatment in order to assay the ATR in the absence of *de novo* protein synthesis during acid adaptation. After incubation for 1 h, cells were washed twice with no citrate E medium (NCE; Maloy and Roth 1983) at pH 5·5 or 7·5 (pH adjusted with HCl) and resuspended in equal volumes of NCE, pH 4·0 (acid challenge) with or without 1% L-lysine hydrochloride. Viable counts were performed immediately after resuspension in NCE and at subsequent 30-min intervals.

DNA manipulation and sequencing

Chromosomal DNA of *V. parahaemolyticus* was isolated according to the standard protocol (Sambrook *et al.* 1989) and plasmid DNA was purified using Quantum prep plasmid miniprep kits (Bio-Rad Laboratories, Richmond, CA, USA). A degenerate primer pair, 5′-GTN CTW TAY TAY CAC GCN AAC TGG A-3′ and 5′-GCY TSN CRN ACC ARC ATC ATC CA-3′, for lysine/cadaverine antiporter (*cadB*) was designed from other known CadB amino acid sequences: *E. coli* (sequence accession number: NC000913), *Salmonella typhimurium* (AE008816), *V. cholerae* (NC002505), and *V. vulnificus* (AF324470). After amplifying the homologous *V. parahaemolyticus cadB* fragment using this primer pair at an annealing temperature of 58°C, the PCR products were cloned into pT7-Blue T-vector (Novagen, Madison, WI, USA) and the sequences of several clones were determined.

Unidentified regions around *cadB* were amplified and cloned using partial sequence information and LA (long and accurate) PCR *in vitro* cloning kits (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. Briefly, chromosomal DNA digested with the appropriate restriction enzymes and adapters (double-stranded oligonucleotides) were ligated. Then, the fragments containing unknown regions were amplified using an adapter primer and a primer specific to the partial *cadB* sequence. The resulting products were cloned into pT7Blue T-vectors (all restriction enzymes and DNA ligase were provided by Takara Bio). Several clones were sequenced using BigDye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) and analysed on an ABI310 genetic analyser (Applied Biosystems). Sequences were assembled and analysed by GENETYX-MAC software (Software Development Co., Ltd, Tokyo, Japan).

RNA extraction and Northern blot hybridization

Log-phase cultures (45 ml) were pelleted and resuspended in 45 ml of LB-MES (pH 5·5) or LB-HEPES (pH 7·5). After 2 h of incubation at 30°C, total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by DNase I (RNase free, Wako Pure Chemical Industries, Japan) treatment for 30 min. Blotting of total RNA was performed as follows: 15 μg of total RNA was loaded onto 1% Seakem GTG (FMC BioProducts, Rockland, ME, USA) agarose gels, electrophoresed in MOPS buffer (0·4 mol l^{-1} 3-morpholinopropanesulfonic acid (MOPS), 0·1 mol l^{-1} sodium acetate, 15 mmol l^{-1} EDTA, pH 7·0) with size markers for RNA (Perfect RNA markers, 0·2–10 kb; Novagen), and blotted to Hybond-N+ membranes (Amersham Biosciences Corp., Piscataway, NJ, USA) with an electroblotter, NB-1513 (Nihon Eidoh, Tokyo, Japan). Hybridization was done with ECL (enhanced chemical luminescence) direct nucleic acid labelling kit (Amersham Biosciences) using DNA probes specific for *cadA* or *cadB*, prepared by PCR using primers 5′-TTA TCA CGC CAA CTG GAT TGG-3′ and 5′-GCG TGT AGC TTC ATG TAC TGA GC-3′ for *cadB*, and 5′-CCT CCA TTC AAC TAA AGC GCT A-3′ and 5′-CAA TGC CGT ACT GAG GTG AAG-3′ for *cadA*.

RT-PCR for mRNA quantification

In SYBR Green I real-time quantitative PCR, amplification of cDNA is measured by the increase in fluorescence resulting from the successive intercalation of SYBR Green I dye into the double-stranded DNA. The threshold cycle (*Ct*), the first PCR cycle in which the incremental increase in fluorescence can be detected, is used to calculate the initial amount of template DNA based on a standard curve plot of cDNA derived from the *Ct* values calculated from known concentrations of the standard DNA. Moreover, the specificity of all PCR reactions were verified based on the melting temperature (T_m) measured by the 'disassociation curve' analysis on an ABI 7900HT (Applied Biosystems) immediately after amplification. T_m is unique to the amplification products and is determined by the decrease in fluorescence from SYBR Green I dye during slow heating (from 60 to 95°C). Data collection and multicomponent analyses were performed with the Sequence Detection Software 2·0 supplied with ABI prism 7900HT (Applied Biosystems). An endogenous control of sample RNA, 16S rRNA, was also quantified and used for data analyses.

Primer selection

A total of four amplifications were designed to study the transcription of *cadBA* genes of *V. parahaemolyticus*. The *cadB* mRNA (519 bp) was amplified by primers 5′-ATT CGG TAG CTG GAC TGC AC-3′ and 5′-GCG TGT AGC TTC ATG TAC TGA GC-3′ and *cadA* mRNA (443 bp) was amplified by primers 5′-GTA TTC TTC CCT GTG CTT AAT GAC-3′ and 5′-GCA GTC ACA ATC GCA TGG CTA TCA-3′. The *cadBA* bi-cistronic mRNA (403 bp) was amplified by primers 5′-GCT TCG TAA TGC TGT TCT CTG G-3′ and 5′-CGT AGC CCG CTT TCT CAA GA-3′, which were designed to target the *C*-termini of *cadB* and the *N*-termini of *cadA*. The precise primer locations are shown in Fig. 2. Universal primers 510f and 920r were used to amplify 16S rRNA for endogenous reference. Reverse transcription was performed with randomly synthesized hexanucleotide [random hexamer, $d(N)_6$; Applied Biosystems].

RT of RNA

The RT reactions were carried out with the following recipe: 5·0 mmol l^{-1} of $MgCl_2$, 2·0 mmol l^{-1} of each dNTP, 2·5 μmol l^{-1} of $d(N)_6$, 0·4 U μl^{-1} of RNase Inhibitor, 1·25 U μl^{-1} of MultiScribe RTase, 200 ng of total RNA, and reacted in the supplied 10 × RT buffer at 1 × concentration. All reagents were purchased from Applied Biosystems. RNA samples extracted from each type of cells were purified by treatment with DNase I for the removal of residual DNA contamination. RT reactions were performed in a GeneAmp 9700 (Applied Biosystems) with a thermal profile of 25°C for 10 min, 37°C for 60 min followed by 95°C for 5 min to inactivate residual RTase, and a 4°C soak. Resultant cDNA were then applied to the SYBR Green I real-time quantitative PCR after purification by ethanol precipitation as described elsewhere.

Amplification

SYBR Green I real-time PCR was performed on an ABI prism 7900HT with Sequence Detector Software 2.0 (Applied Biosystems). Thermal cycling was performed with the following protocol: 50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 20 s, 58°C for 45 s, and 72°C for 45 s; and an extension phase for dissociation analysis of 95°C for 15 s followed by ramping from 60°C to 95°C in 30 min. During these cycles, fluorescent signals were measured at 521 nm every 7 s, and the data were analysed after all cycles and dissociation curve analyses had been finished. The 25-μl reaction mixture contained 1 × SYBR Green buffer, 3 mmol l^{-1} of $MgCl_2$, 1 mmol l^{-1} of d(ATP, CTP, GTP) and 2 mmol l^{-1} of dUTP, 0·2 μmol l^{-1} of each primer, 0·01 U μl^{-1} of uracil-*N*-glycosidase (AmpErase UNG), 0·025 U μl^{-1} of AmpliTaq Gold DNA polymerase, and 2 μl of template cDNA (or known concentration of genomic DNA) solution. All reagents except oligonucleotide primers were supplied from Applied Biosystems. All runs contained standard templates for calculating the standard curve, as well as controls with no reverse transcription and no templates. After checking the amplification specificity by disassociation curve analysis, results were subjected to data analysis as described next. All reaction mixtures were also electrophoresed on agarose gels followed by ethidium bromide staining for visualization.

Data analyses

The measured fluorescent signals were normalized against the reference dye (6-carboxy-X-rhodamine; ROX, included in the SYBR Green buffer) and were used to calculate the ΔRn using Rn^+ (normalized signal) – Rn^- (baseline *Rn* during cycles 3–15). Data were plotted as ΔRn against the PCR cycle number, with the threshold ΔRn being set at 10 times the SD of the mean baseline signal calculated for Rn^-. The obtained *Ct* values were plotted against the amount of DNA, and a standard curve was drawn using 10-fold serial dilutions of standard DNA. A unit of 1 × DNA standard was considered to contain 5 ng μl^{-1} (or 10^5 copies) of *V. parahaemolyticus* genomic DNA. The amount of mRNA was determined using this standard curve. In order to correct for the total cell number in the RNA extraction and extraction efficiency, the quantity of 16S rRNA in each sample was used for endogenous controls, assuming that the expression of 16S rRNA is constant for all cells used in this mRNA expression analysis.

Primer extension analysis

To determine the transcriptional start position of *cadB* and *cadA* genes, we performed primer extension analyses using 5′-Texas Red labelled primers 5′-GTA AAC AAA CGC AAG GCT CAG-3′ for *cadB* mRNA and 5′-TTA TCC CAG TCG AAC AGC AC-3′ for *cadA* mRNA. Aliquots of total RNA extracted from cells exposed to pH 5·0 were reverse transcribed with ReverTra Ace (Toyobo Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions. To map the transcriptional start site based on the fragment size, the extension products were separated on a 6% denaturing polyacrylamide gel on the DNA sequencer SQ5500E (Hitachi Ltd, Tokyo, Japan) along with a DNA sequence ladder prepared using the Thermo Sequenase primer cycle sequencing kit (Amersham Biosciences) to synthesize the target region with the same labelled primer.

Construction of the mutant strain

The *cadA* gene-inactivated strain, which was derived from *V. parahaemolyticus* V02-64 (serotype O3:K6) was con-

structed as previously described (Xu *et al.* 1994). Briefly, a partial fragment of the *cadA* gene of V02-64 was isolated by PCR using primers 5′-CAA TCT ACT TCC GTC CAA CTC G-3′ and 5′-CAA TGC CGT ACT GAG GTG AAG-3′. The amplified fragment was cloned into the pT7-Blue T-vector and then digested by *Bam*HI and *Pst*I. The digested fragment was subcloned into suicide vector, pKY719 (Xu *et al.* 1994), transformed into *E. coli* SM10*λpir*, and then re-introduced into *V. parahaemolyticus* cells through conjugation at 37°C overnight on LB agar. Cells were recovered by saline, plated onto thiosulfate citrate bile salts sucrose (TCBS; Eiken Chemicals Co. Ltd, Tokyo, Japan) supplemented with 10 $\mu g\ ml^{-1}$ of chloramphenicol, and incubated overnight at 30°C. Chloramphenicol-resistant *V. parahaemolyticus* cells with *cadA* genes inactivated by plasmid integration (single crossing-over), were screened by tests including southern blot hybridization and DNA sequencing. The lack of lysine decarboxylase activity was confirmed using an indicator broth (BBL Moeller decarboxylase broth base; Becton Dickinson, NJ, USA) and HPLC measurement as described before. Tests for biochemical characteristics were performed using ID32E API (BioMerieux, Marcy l'Etoile, France). The acid survival of the mutant and wild-type strains was measured by the same methods described before.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper is available in the DDBJ/EMBL/GenBank databases under accession number AB124819 (*V. parahaemolyticus* V02-64 *cadBA*).

Results

Vibrio parahaemolyticus possesses an adaptive ATR

The adaptive acid tolerance response in *V. parahaemolyticus* was examined in several strains (Table 1). In all strains, adapted cells (LB-MES, pH 5·5) showed better survival than nonadapted (LB-HEPES, pH 7·5) cells when exposed to acid (LB-MES, pH 4·0). As we have hypothesized that lysine decarboxylating activity plays a role in the ATR of *V. parahaemolyticus*, we analysed supernatants for the presence of polyamines. The HPLC findings show a large amount of cadaverine, which is generated from lysine through decarboxylation reactions, only in cells that survive lethal acidic conditions (adapted cells, data not shown). The recently emerged *V. parahaemolyticus* serotype O3:K6 was not more acid resistant than other *V. parahaemolyticus* strains used in this study.

Table 1 Effect of acid adaptation in the acid survival of *Vibrio parahaemolyticus* strains

Strain	Per cent survival* after 1 h at pH 4·0	
	Adapted†	Nonadapted
V02-64‡	$10{\cdot}1 \pm 6{\cdot}2_{AB}$§	$0{\cdot}23 \pm 0{\cdot}20_{x}$§
VpTK01	$32{\cdot}7 \pm 3{\cdot}4_{AB}$	$0{\cdot}21 \pm 0{\cdot}09_{x}$
VpTK03	$15{\cdot}9 \pm 3{\cdot}8_{AB}$	$0{\cdot}84 \pm 0{\cdot}14_{x}$
VpTK06	$7{\cdot}3 \pm 5{\cdot}6_{B}$	$0{\cdot}11 \pm 0{\cdot}05_{x}$
VpTK08	$36{\cdot}9 \pm 0{\cdot}6_{A}$	$0{\cdot}02 \pm 0{\cdot}01_{x}$
VpTK09	$8{\cdot}3 \pm 3{\cdot}0_{B}$	ND**
VpTK10	$18{\cdot}7 \pm 4{\cdot}5_{AB}$	$0{\cdot}03 \pm 0{\cdot}02_{x}$
VpTK11	$13{\cdot}0 \pm 0{\cdot}5_{AB}$	ND**
VpTK12	$30{\cdot}0 \pm 11{\cdot}8_{AB}$	$0{\cdot}03 \pm 0{\cdot}01_{x}$
VpTK13	$7{\cdot}0 \pm 5{\cdot}5_{B}$	$1{\cdot}00 \pm 0{\cdot}69_{x}$
VpTK17	$28{\cdot}4 \pm 6{\cdot}2_{AB}$	$0{\cdot}21 \pm 0{\cdot}07_{x}$

*Values are per cent survival after the acid challenge calculated by dividing the viable counts at 1 h by the initial viable counts and multiplying by 100. Values are the means (±SE) of three independent experiments.

†Acid adaptation was performed at pH 5·5 for 1 h at 30°C.

‡Serotype O3:K6.

§Means with the same letter within a column are not significantly different (Tukey-Kramer multiple comparison test, $P < 0{\cdot}05$).

**ND, not detected.

ATR of *Vibrio parahaemolyticus* requires external lysine

To demonstrate the dependence of ATR on lysine decarboxylase in *V. parahaemolyticus*, we determined the survival of the acid-adapted cells in NCE (pH 4·0) with or without 1% L-lysine. Tolerance of *V. parahaemolyticus* V02-64 (serotype O3:K6) to pH 4·0 NCE media was greater in the lysine supplied condition, as a threefold log reduction in survival was observed in the treatment without lysine after 60 min, while the reduction was only one order of magnitude in the treatment with lysine (Fig. 1). Nonadapted cells and adapted cells under the condition in which the protein synthesis was limited by the supplementation of the chloramphenicol in adaptation media were decreased to 10^{-3} at 30 min after resuspension in pH 4·0 NCE with lysine. This experiment was also performed for the other nine strains, and similar results were obtained. The mean per cent survival for the 10 strains at 30, 60, and 90 min at pH 4·0 was 17·8, 10·5, and 5·78, respectively, for the lysine-supplied cells, and 3·49, 0·94 and, <0·01, respectively, for the lysine-free culture conditions.

Molecular analyses of cadA and cadB genes of *Vibrio parahaemolyticus*

The high homology of bacterial lysine/cadaverine antiporter (*cadB*) genes enabled us to design degenerate

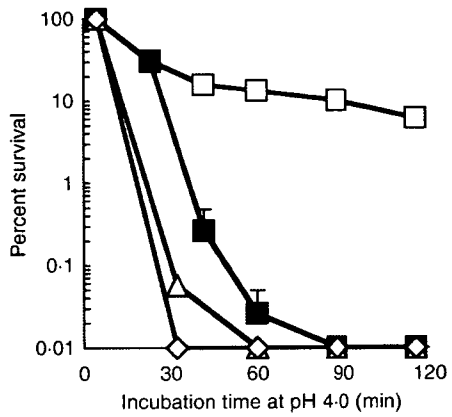

Figure 1 The fate of *Vibrio parahaemolyticus* V02-64 cells in lethally acidic environments in the presence or absence of lysine. Each cell treatment was acid adapted in Luria-Bertani (LB), pH 5·5 for 1 h and exposed to lethally acidic solution (no citrate E medium, pH 4·0) in the presence (open boxes) or absence (closed boxes) of lysine. It is notable that cells adapted with chloramphenicol (diamonds), and unadapted cells (triangles) both immediately decreased when lysine was present in the environment. All experiments were performed in triplicate, and some of the standard deviations (SD) were very small that the error bars do not extend beyond the data points.

primers based on alignments of *E. coli, S. typhimurium, V. cholerae,* and *V. vulnificus cadB* amino acid sequences. The resulting 631-bp amplification product from *V. parahaemolyticus* included six transmembrane domains as predicted by the SOSUI programme (Department of Biotechnology, Tokyo University of Agriculture and Technology; http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0E.html), and was considered to be *cadB* based on high homology of the determined DNA and the deduced amino acid sequences (82% and 92%, respectively) with *V. cholerae* sequences. The periphery sequences were completely determined using a commercial kit based on the cassette ligation-mediated PCR amplification technique. Two open reading frame (ORF) sequences coded on the same strand with lengths of 2136 and 1344 bp were matched to lysine decarboxylase and lysine/cadaverine antiporter of *V. cholerae* (91·7% and 88·3%, respectively) and other bacteria in the DNA Data Bank of Japan, (http://www.ddbj.nig.ac.jp). Therefore, considering the results from the mutational study (described next) into account, we designated the two ORF as *cadA* and *cadB* (Fig. 2a). The intergenic spacer region between *cadB* and *cadA* was 116 bp (Fig. 2a). This region has inverted repeat sequences and possibly forms a stem-loop structure in mRNA and may act as a terminator of the *cadB* sole transcript. RNA transcribed from the region has an eight-base stem and a four-base loop with no mismatches. Thus, taking into account the existence of the poly-A sequence, this region is the potential terminator (Fig. 2a). Recently, the entire genomic sequence of *V. parahaemolyticus* O3:K6 strain RIMD2210633 was reported (Makino *et al.* 2003); *cad* genes had sequence identity with those reported here of 99·7% (99·8% for the amino acid sequence) for *cadB* and 100% for *cadA*. In our study, *cadA* gene function was experimentally verified using the knockout mutant (described next).

Lysine decarboxylase genes are acid-induced operon

Northern blot hybridization revealed that *cad* genes are more highly expressed under acidic conditions than under neutral conditions (Fig. 3a, pH 5·5). However, hybridization data were not informative for the effect of lysine on transcription or for operon construction. Therefore, this RNA was subjected to the RT-PCR method using $d(N)_6$ and the primers shown in Fig. 2. Significant amounts of cDNA amplification products were produced using primers specific for *cadB*, *cadA* sole transcripts, and bi-cistronic transcripts of *cadBA* (Fig. 3b), showing that transcription of *cad* genes falls into the three patterns of *cadB*, *cadA*, and *cadBA*. Based on qualitative RT-PCR of RNA extracted from acid-stressed cells, significant amounts of amplification products (*cadB*, *cadA* and *cadBA*, Fig. 3b, lanes 1–4) were produced while only *cadA* amplification products were faintly observed in non-stressed cells (Fig. 3b, lanes 5 and 6). This shows that *cadBA* genes construct an operon, and the expression of *cadB*, *cadA*, and *cadBA* mRNA, especially *cadB* and *cadA*, are acid-inducible and low levels of transcription are also present at neutral pH.

RT-PCR gives more detailed information than the Northern method, although an accurate comparison of the expression ratio between these transcripts is not possible because of the 'plateau' effect of PCR end-point analysis and the variation in PCR efficiency between the experiments. Therefore, SYBR Green I real-time, quantitative RT-PCR was also performed to determine the relative expression of these mRNA species. Relative expression ratios showed that the expression of these genes was induced under the condition of low pH (Fig. 3c). For example, *cadA* mRNA exhibited about 40-fold greater expression at pH 5·0 (3·39 ± 1·5) than at pH 5·5 (0·082 ± 0·02). In cells exposed to pH 7·5, only the *cadA* transcript was detected, and this transcript showed the largest quantity under all conditions. In addition, the amount of *cadB* and *cadA* single transcripts was nearly 10- and 100-fold higher than the polycistronic *cadBA* transcript for each pH condition (Fig. 3c). For example, at pH 5·5 with the addition of lysine, the bi-cistronic *cadBA* transcript had a relative

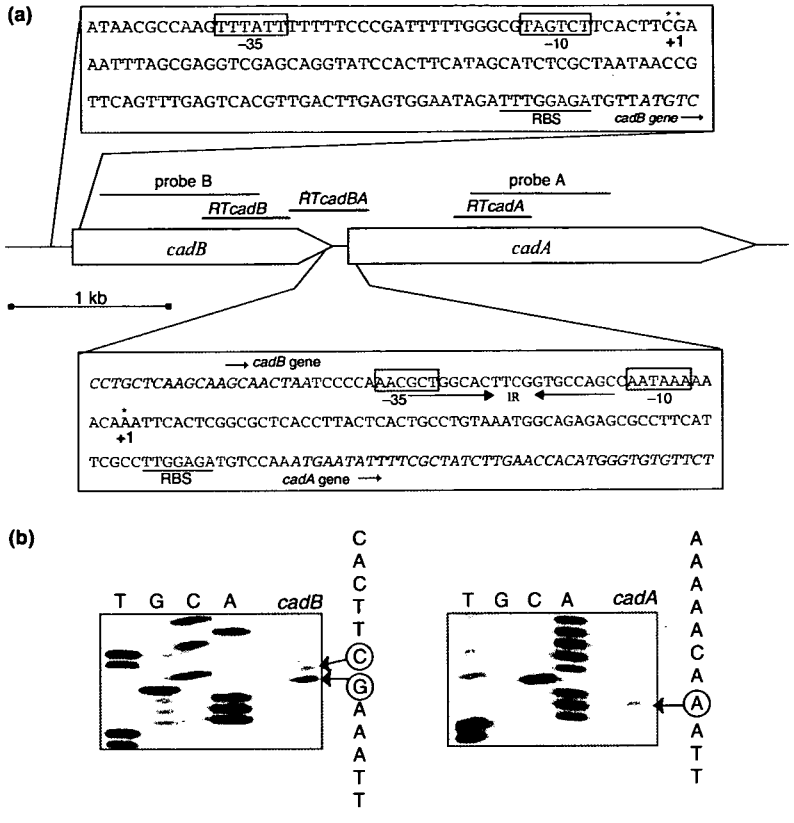

Figure 2 (a) Schematic representation of the *Vibrio parahaemolyticus* lysine decarboxylase gene cluster and intergenic region. Probes for the Northern blot hybridization of genes *cadB* and *cadA* and polymerase chain reaction (PCR) primers to measure mRNA species by quantitative reverse transcriptase (RT)-PCR are indicated by bars labelled with the probe name (probes B and A) and gene name (*RTcadB*, *RTcadBA*, and *RTcadA*), respectively. The *RTcadBA* fragment was targeted at the C-termini of *cadB* and N-termini of *cadA* to detect the bi-cistronic transcript. The periphery sequences of *cadB* and *cadA* are given in the inset. Protein coding regions are indicated by shading, the putative transcriptional terminator (inverted repeat, IR) is indicated by arrows, and the probable ribosome-binding site (RBS) is underlined. Transcriptional start sites (+1) of each gene is indicated by asterisks. Predicted promoter sites, −35 and −10, are indicated by boxes. The scale bar length of 1 kb of the sequence is indicated. (b) Primer extension analysis. The primer extension products with total RNA of *V. parahaemolyticus* were sized. Sequencing ladder obtained with the same primers were shown on the left.

expression of 0·198 ± 0·03, compared with 1·96 ± 0·89 for *cadB*, and 8·03 ± 0·08 for *cadA*. This suggests that the bi-cistronic transcript could not be visualized on Northern blot hybridization. The majority of the probe added to the total RNA might have been hybridized to the monocistronic transcripts because of their higher quantity (10- to 100-fold).

All data obtained from quantitative RT-PCR were normalized for cell numbers and extraction efficiency using the values for 16S rRNA endogenous control of 1 and 2·40 (with lysine) for pH 5·0, 0·738 and 0·643 (with lysine) for pH 5·5, and 0·487 and 0·683 (with lysine) for pH 7·5. No genomic DNA contamination was found in any of the RNA samples during 50 cycles of PCR with any of the primer pairs (data not shown).

Identification of the transcriptional start point of *cadA* and *cadB*

The transcriptional start point of *cad* genes of *V. parahaemolyticus* was determined, as shown in Fig. 2. The transcriptional start point of *cadB* mRNA was located 101 and 102 bases upstream from the start codon of *cadB*. The predicted promoter elements were TTTATT and TAGTCT located at −35 and −10, respectively. The *cadA* mRNA started from 75 bases upstream of the start codon, and the promoter elements were identified as follows: AACGCT at −35, AATAAA at −10. The promoter sequences of *cadA* and *cadB* have little resemblance to each other. Although these promoter elements have little similarity for the consensus sequences (TATAAT and TTGACA), except for the −10 box of *cadA*, they share significant homologies with the promoter elements of *V. chorelae cad* genes (Merrell and Camilli 2000).

Acid tolerance of *cadA* gene-inactivated strain

The acid tolerance of a mutant strain of the newly emergent *V. parahaemolyticus* clone, V02-64, serotype O3:K6 constructed with a disrupted *cadA* gene was also tested. The mutant strain has a plasmid integrated in its chromosome (single crossing over), with a corresponding decrease in lysine decarboxylating activity to 1/100 or less. The microscopic experiments, measurement of growth rate, and tests for biochemical properties using API ID32E (BioMerieux) revealed no changes to other characteristics by this manipulation. The 4·0-kb *Hin*cII fragment detected by southern blot hybridization using

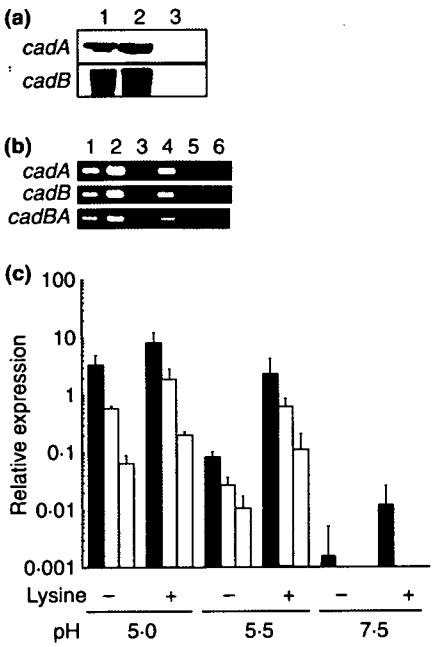

Figure 3 Transcriptional analysis of *cadBA* genes. (a) Northern blot hybridization analysis for *cadA* or *cadB* mRNA of *Vibrio parahaemolyticus* V02-64. Whole RNA (15 μg) was loaded, electrophoresed, blotted, and hybridized with probes specific for *cadA* or *cadB* under the following conditions: lane 1, pH 5·5; lane 2, pH 5·5 supplemented with 1% lysine; and lane 3, pH 7·5 with lysine. (b) Agarose gel images after 25 cycles of reverse transcriptase (RT)-polymerase chain reaction (PCR) under the following conditions: lane 1, pH 5·0; lane 2, pH 5·0 with lysine, lane 3, pH 5·5; lane 4, pH 5·5 with lysine; lane 5, pH 7·5; lane 6, pH 7·5 with lysine. (c) Relative expression analysis of *V. parahaemolyticus* lysine decarboxylase genes as detected by SYBR Green I real-time quantitative PCR under three pH conditions in the absence and presence of lysine. Total RNA extracted from the cells cultured in each condition was reverse transcribed by $d(N)_6$, and the quantity of cDNA consistent with that of the mRNA was quantified against a standard curve produced using genomic DNA. The relative amount of 1 is nearly equal to 10^4 copies of target DNA per 2 μl of the PCR template (=per reaction). Bars represent the averages of three to five experiments, and error bars indicate the SD (■, *cadA*; □, *cadB*; ■, *cadBA*).

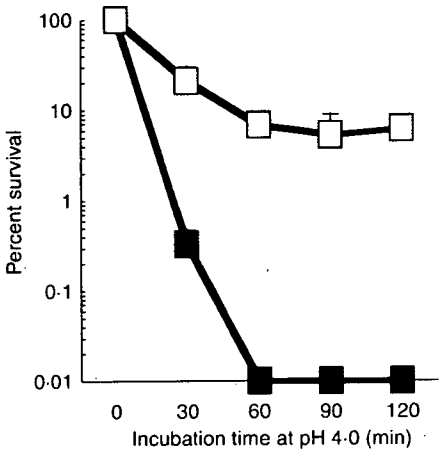

Figure 4 Acid resistance of the *Vibrio parahaemolyticus cadA* gene mutant and wild-type strains. All cell cultures were grown in Luria-Bertani (LB) media and collected at OD_{600} = 0·16–0·20 (log phase). Cells were all acid adapted in LB (pH 5·5) for 1 h to induce the acid-shock proteins. Acid-adapted cells were acid challenged in no citrate E medium (phosphate buffer, pH 4·0) with lysine at 37°C. Acid-challenged cells were then enumerated by plating onto trypticase soy agar medium at 30-min intervals. The survival of wild-type strain cells (open boxes) was reduced to one-tenths after 120 min, although survival of *cadA*-inactivated strain cells decreased immediately (closed boxes). Data represent the mean value of three measurements, and the error bars indicate the SD.

the *cadA* probe showed a band shift of 8·5 kb under the same conditions caused by plasmid integration in the mutant strain (data not shown). The acid resistance of this mutant strain at pH 4·0 in the phosphate buffer was weaker than in the parental strain; e.g. <0·01% of the mutant cells survived past 60 min of exposure, while 9·1% survival was observed for the wild-type strain (Fig. 4).

Discussion

The ATR is one of the strategies employed by the pathogens to infect and colonize mammalian hosts and has recently been reported in many enteropathogenic bacteria (Merrell and Camilli 2002). Among several proposed mechanisms of acid tolerance, amino acid decarboxylation has been confirmed in several bacteria. The molecular structure of lysine decarboxylase has been studied in some species, such as *E. coli* (Meng and Bennett 1992a,b), *S. typhimurium* (Foster and Hall 1991; Park *et al.* 1996), *V. cholerae* (Merrell and Camilli 1999, 2000), and *V. vulnificus* (Rhee *et al.* 2002). Among these species, lysine decarboxylase was confirmed to play a role in ATR in all but *E. coli*. Instead, *E. coli* utilizes glutamate decarboxylase as an alternative amino acid decarboxylase for ATR (Lin *et al.* 1995). Further, Samartzidou *et al.* (2003) have demonstrated that cadaverine, decarboxylated from lysine, plays a role in acid survival through porin inhibition in *E. coli*.

To achieve ATR via amino acid decarboxylation, two or more enzymes appear to be necessary (Bearson *et al.* 1997; Merrell and Camilli 2002); decarboxylation enzymes and their antiporters facilitate the coupled transport of amino acids and their decarboxylation products across the membrane. Each decarboxylation reaction consumes one intracellular proton to generate products including γ-aminobutyric acid (from glutamic acid) and cadaverine (from lysine), which are excreted to the extracellular space by antiporter protein and thereby decrease the

intracellular proton concentration and protect the cells from external acid stress. In our study, the molecular structure of the lysine decarboxylase gene cluster of *V. parahaemolyticus* was determined. Two ORF having sequences of 2136 and 1344 bp were identified as lysine decarboxylase (*cadA*) and lysine/cadaverine antiporter (*cadB*), respectively (Fig. 2a).

ATR of *V. parahaemolyticus* under low pH conditions is largely dependent on the presence of external lysine. Acid-adapted *V. parahaemolyticus* is resistant to low pH (4·0) only in the presence of external lysine (Fig. 1). Further, this adaptation effect is inhibited by chloramphenicol, an inhibitor of protein synthesis. Similar results have been obtained for all tested strains of *V. parahaemolyticus*, and these data suggest that *V. parahaemolyticus* gains resistance to conditions of low pH by using lysine and synthesizing proteins and that lysine-dependent pathways contribute significantly to the ATR of this pathogen. Thus, our findings corroborated previous reports that *V. parahaemolyticus* has the ability to mount a robust ATR, largely dependent on the presence of lysine.

In this study, transcriptional analyses by Northern blot hybridization and quantitative RT-PCR revealed that the *cad* genes of *V. parahaemolyticus* form an acid-inducible operon, while individual transcripts of *cadB* and *cadA* were also observed (Fig. 3a,b). The strength of induction of *cad* genes, which are related to acid adaptation, was shown to be inversely proportional to pH, suggesting that lysine decarboxylase genes are also responsible for the ATR of *V. parahaemolyticus*. The acid-inducible properties of the *cad* genes are the same in both *E. coli* and *V. cholerae*, the former has only a bi-cistronic transcript (Meng and Bennett 1992b), while the latter produces three transcripts comprising *cadBA*, *cadB*, and *cadA* (Merrell and Camilli 1999, 2000).

A small amount of *cadA* transcript is still produced under neutral pH conditions (Fig. 3c). This constitutive expression might be the result of substitution of another lysine decarboxylase (biosynthetic) found in *E. coli* (Kikuchi *et al.* 1997) as no other lysine decarboxylase was found in the entire genome of *V. parahaemolyticus* by southern hybridization (this study, data not shown) and whole genome sequencing (Makino *et al.* 2003). Cadaverine has been reported to play a role in the control of outer membrane permeability (Samartzidou *et al.* 2003) and cell division (Pruss *et al.* 1997) in *E. coli*. Therefore, decarboxylase protein CadA, the most important protein in the decarboxylation pathway, is expressed solely under neutral pH for the adjustment of the intracellular lysine or cadaverine concentration, although the expression level is low.

While the *cadB* and *cadA* genes of *V. parahaemolyticus* form an operon construct, the expression ratio between *cadB*, *cadA*, and *cadBA* was *c.* 10 : 100 : 1. The probable transcriptional terminator, the inverted repeat sequence of the *cadB* gene is indicated in Fig. 2a. Because this terminator-like sequence has sufficient length similar to a stem and a loop and includes several bases of the poly-A region, it is reasonable to assume that it acts as a transcription terminator. This likely explains the low production of *cadBA* mRNA, which is transcribed from the promoter upstream of *cadB* to the terminator of *cadA*. The *cadA* mRNA, the most abundant of the transcripts, is transcribed using its own upstream promoter. The predicted promoter regions of *cadB* and *cadA* have little resemblance. While both are acid-inducible promoters, differing transcriptional pattern were observed between *cadB* and *cadA* genes. The promoter sequence of *cadA* was more similar to the consensus sequence than that of *cadB* (Fig. 2a). Therefore, we consider that the *cadA* sole transcript is generated by its own promoter because the activity of promoter of *cadA* might be strong. When cells encounter low pH stress, *cadA* expression is increased and the *cadB* gene, which is indispensable in effecting acid tolerance, is also expressed. Some portion of the *cadB* transcripts do not terminate at the terminator located on the *cadB–cadA* intergenic region, and, thus, *cadBA* bi-cistronic transcripts are produced. In this study, the cadC-like ORF was observed in *V. parahaemolyticus* (data not shown). As *cadC* is reported to be a positive transcriptional regulator of the *cadBA* operon (Merrell and Camilli 2000; Rhee *et al.* 2005), the transcription of *cadBA* genes of *V. parahaemolyticus* might be regulated in the same way. The transcriptional regulation of the *V. parahaemolyticus cadBA* operon will be studied further.

To summarize, in the transcription of the *V. parahaemolyticus cad* operon, the *cadA* gene is constitutively expressed while *cadB* is not. Under low pH, the *cadA* gene is highly expressed, the *cadB* gene is also transcribed, and *cadB–cadA* bi-cistronic transcript is occasionally produced. This transcriptional regulation occurs because different amounts of CadA and CadB are required.

The mutant strain, with a disrupted *cadA* gene, was constructed to verify the role of *cadA* in ATR. The mutant strain was shown to have inactivated the *cadA* gene by an indicator broth, along with HPLC method, PCR, southern blot hybridization, and DNA sequencing (data not shown). All results showed the plasmid integration into the interior of the *cadA* gene, although a little cadaverine was detected from overnight cultures of the mutant strain. This remaining activity is speculated to be the result of imperfect mutation caused by the plasmid remaining in the chromosome. This mutant strain showed higher acid sensitivity than the wild-type strain, although other characteristics (growth rate, requirements for nutrition, biochemical characteristics, and colony