and an anaerobic count of 4.2 log CFU/g was detected in all three samples.

No bacterial growth was detected by aerobic cell counts in any of the samples, as expected.

Neurotoxin assay. Neurotoxin detection data are shown in Table 1. The higher the sample pH value, the earlier the start of neurotoxin production was observed. Neurotoxin was detected after 2 weeks of incubation in samples at pH 5.4 or above, while it took 4 weeks for the toxin to be detected in samples at pH 5.2 to 5.3, and 12 weeks in samples at pH 5.0 to 5.1. In samples at pH 4.8 or below, no toxin was detected during the experimental period (24 weeks).

Type A toxin was detected in samples of pH $\geq$ 5.0. Type B toxin was detected only in samples of pH $\geq$ 5.4.

Uninoculated samples were not included in the neurotoxin assay as negative control because they were confirmed to be free of bacteria in aerobic and anaerobic counts.

DISCUSSION

When *C. botulinum* spores alone were inoculated into rice samples at a concentration of 2 log CFU/g, counts of this pathogen increased, and neurotoxin was detected in samples at initial pH 5.4 or above after 2 weeks of storage at 30°C. This result indicates that MAP-steamed rice products, which are usually at pH 6.5, are at risk of *C. botulinum*–toxin formation when secondary contamination with this organism occur. On the other hand, Kazama et al. *(9)* reported that at least 1 month was needed before the toxin could be detected in the same type of sample stored at 30°C, with much higher inoculation load (4.2 log CFU/g). The only difference between their experimental design and ours was the control of atmospheric conditions in the packaging. They used a deoxidant pack to decrease the O_2 content but did not pack the samples under a modified atmosphere. Their method might have allowed high initial and subsequent O_2 content in the package, as our previous experiments showed that the use of deoxidant pack alone is not sufficient to produce a completely anaerobic atmosphere (data not shown). In fact, our data show that a long incubation time (2 to 3 months) is required for *C. botulinum*–toxin production and growth in rice products stored at oxygen concentration of 0.4% (data not shown). Since Kazama et al. *(9)* did not indicate the oxygen concentration in their report, it is not known what O_2 content prevents the growth of *C. botulinum*. In contrast to their experiments, our experiments were based on the worst-case scenario, using oxygen concentration of $\leq$0.3%, since we think that risk assessment of any foodborne pathogen must be carried out based on the worst-case scenario.

In samples at pH 4.9 or below, anaerobic counts did not increase from the initial counts, and neurotoxin was not detected during the entire experimental period used in this study. It is well reported that toxin production is not limited above pH 4.6 in pure cultures of *C. botulinum* isolated from food contamination cases *(21)*. This inconsistency of pH threshold may be attributed to the unique properties of aseptically steamed rice samples. Rice is composed almost entirely of starch, with little protein *(18)*, and proteolytic *C. botulinum* has limited ability to utilize starch as a carbon source *(15)*, although under optimized conditions, at least some strains could utilize starch *(20)*, and high-starch foods such as potatoes could be the source of an outbreak *(1)*. Moreover, when *C. botulinum* and *Bacillus subtilis* were grown together on rice, *B. subtilis* was reported to promote the growth and toxin production of *C. botulinum (8)*, as amylase produced by *B. subtilis* digests starch that then stimulates the growth of *C. botulinum*. Other researchers have also reported that the presence of other microbial species allows *C. botulinum* to grow and produce neurotoxins *(6, 14)*. For example, the presence of molds results in increased *C. botulinum* growth and allows neurotoxin production at pH 4.2 *(7)*. This is possible because molds not only produce amylase, but also locally increase the pH *(7)*. In the present study, we inoculated *C. botulinum* alone into rice samples. Thus, the effects of co-inoculation with other microorganisms should be studied further. Also, since botulinum toxin type A was produced at pH 4.75 and water activity of 0.97 in vacuum-packed potatoes acidified with organic acid, other factors contributing to inhibition remain to be identified *(4)*.

Unexpectedly low anaerobic count corresponding with no toxin production was observed for samples at pH 5.2 to 5.3 collected at week 12 (Table 1). All three samples had similar anaerobic counts, and the pH values and atmospheric compositions were sufficient for *C. botulinum* to multiply and produce toxins. Therefore, the reason for these results is not known.

In conclusion, we demonstrated the worst-case scenario of botulism in steamed rice products after inoculation of *C. botulinum* spores alone under an atmosphere of $\leq$0.3% oxygen. We conclude that aseptically steamed rice products must be adjusted to pH 4.9 or below to avoid the risk of botulism from consumption of these MAP foods.

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Research Note

Use of Single-Strand Conformation Polymorphism of Amplified 16S rDNA for Grouping of Bacteria Isolated from Foods

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ABSTRACT

The grouping method for isolated strains from foods using single-strand conformation polymorphism (SSCP) after PCR amplification of a portion of 16S rDNA was developed. This method was able to group the strains from various food samples based on 16S rDNA sequence. As 97.8% of the isolated strains from various foods were grouped correctly, use of the PCR-SSCP method enables the prompt and labor-saving analysis of microbial population of food-derived bacterial strains. Advantages in speed and accuracy of bacterial population identification by the PCR-SSCP method have practical application for food suppliers and testing laboratories.

For food manufacturers, aerobic plate count (APC) is widely used as a standard method for microbiological quality control. Although the importance of the APC remains unchanged, the demand for identification techniques of bacterial species is increasing to obtain the detailed information about APC result. An abnormal APC, a result that deviated from specification(s) formulated by public administration or the food industry, requires further analysis in order to identify isolates to analyze the microbial population.

Denaturing gradient gel electrophoresis (DGGE) *(16)*, one of the electrophoretic typing methods of DNA, is widely used to analyze microbial communities in combination with DNA sequencing of the separated DNA fragments *(6, 10, 21–23)*. DGGE is a useful method for rapid and comparative analysis of complex bacterial flora. However, as the DGGE method uses DNA extracted directly from the sample and amplified by PCR, comparative analysis with APC is difficult because the nonculturable bacteria on the APC plate are also detected by DGGE. In addition, the quantitative differences of bacterial species in a sample are not clearly identified by the DGGE method. The microbial population analysis by the food manufacturers is generally carried out by identifying 10 to 20 of the randomly isolated strains from the countable APC plates. Analysis of microbial populations by this conventional method enables the semiquantitative comparison of the constituents. However, identification of 10 to 20 of the bacterial isolates is both a time- and labor-intensive process.

The strategy of selecting representative strains from isolated strains following DNA typing is an easy and simple way to decrease the identification operation. The PCR–restriction fragment length polymorphism (RFLP) method is frequently used for this purpose. We consider that the PCR-RFLP targeting 16S rDNA is a simple and useful technique; however, it is limited in terms of analyzing bacterial populations promptly. The technique is time-consuming in terms of restriction enzyme processing, and the resolution is not sufficient. DGGE, temperature gradient gel electrophoresis, and single-strand conformation polymorphism (SSCP) are widely used for microbial community analysis. The SSCP method can recognize a single polymorphism in a DNA fragment *(19)*, and recently, this method has been applied to microbial community analysis *(9, 15, 20)*, including the identification of specific species *(25, 28)*. SSCP electrophoresis separates the single-stranded DNA not only according to its base length, but also according to the secondary structures of DNA. The secondary structure of DNA is affected by its sequence. We consider that SSCP is highly advantageous for grouping many strains in terms of rapidness and resolution. In this study, we evaluated a strategy based on the PCR-SSCP method for analyzing the microbial populations found in food products. We tested the accuracy of this grouping method in the samples of fresh fish products, meat, and vegetables.

MATERIALS AND METHODS

Bacterial isolation. Foods purchased from retail food shops in Tokyo and comprising three samples each of fish, meat, and vegetables were transported to the laboratory on ice. Twenty-five grams of each sample was homogenized with 225 ml of 0.85% NaCl saline, using a stomacher (Seward Co., Ltd., London, UK) for 1 min. Serial 10-fold dilutions (10^{-1} to 10^{-6}) were prepared, and 100 μl from each was plated in duplicate onto modified tryptic soy agar (Difco, Becton Dickinson, Sparks, Md.) supplemented with 1% NaCl and incubated at 30°C for 24 h. Colonies were counted, and 20 colonies from each sample type were randomly isolated from countable plates, without considering the appearance of colonies, and identified using the method below.

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TABLE 1. *The result of PCR-SSCP analysis and identification results of 16S rDNA sequencing of food isolates*

Sample	Bacterial counts (CFU/g)	No. of groups of isolated strains by PCR-SSCP	Confirmation of PCR-SSCP grouping by 16S rDNA sequencing[a]			
			SSCP group	Closest relatives of 16S rDNA sequence (accession no. of closest relative)[b]	% similarity	No. of strains
Horse mackerel	4.3×10^3	9	1A	*Pseudoalteromonas elyakovii* (AF082562)	98	7
			1B	*Psychrobacter glacincola* (U85877)	98	6
			1C	*Arthrobacter globiformis* (AB098573)	94	1
			1D	*Brochothrix thermosphacta* (AY543029)	98	1
			1E	*Flavobacterium frigidarium* (AY771722)	99	1
			1F	*Photobacterium angustum* (D25307)	96	1
			1G	*P. phosphoreum* (AY780009)	98	1
			1H	*Pseudomonas tolaasii* (EF111117)	97	1
			1I	*Vibrio rumoiensis* (DQ530292)	98	1
Tuna	1.0×10^4	9	2A	*Acinetobacter johnsonii* (X81663)	97	7
			2B[c]	*Chryseobacterium/Flavobacterium*	—	2
			2C	*Acinetobacter haemolyticus* (X81662)	98	3
			2D	*Psychrobacter glacincola* (U85878)	99	2
			2E	*Stenotrophomonas maltophilia* (AY367030)	97	2
			2F	*Arthrobacter bergeri* (AJ609633)	99	1
			2G	*Pseudomonas fluorescens* (AY512614)	97	1
			2H	*P. lurida* (AJ581999)	98	1
			2I	*Sphingobacterium spiritivorum* (EF090267)	92	1
Young yellowtail	1.2×10^3	6	3A	*Shewanella frigidimarina* (U85902)	99	7
			3B	*A. johnsonii* (DQ911549)	98	4
			3C	*Acinetobacter lwoffi* (U10875)	89	2
			3D	*P. fluorescens* (DQ084459)	99	3
			3E	*Chryseobacterium benhlgensis* (EF154516)	97	3
			3F	*Pseudomonas putida* (EF690402)	99	1
Pork	8.1×10^3	11	4A	*Carnobacterium maltaromaticum* (AF270798)	99	5
			4B	*A. johnsonii* (EF204268)	98	4
			4C	*P. putida* (AY456706)	99	2
			4D	*Staphylococcus* spp.	99	2
			4E	*Acinetobacter baumannii* (X81667)	96	1
			4F	*Aeromonas hydrophila* (AY264937)	100	1
			4G	*Carnobacterium maltaromaticum* (AF184247)	98	1
			4H	*Chryseobacterium soldanellicola* (AY883415)	96	1
			4I	*Enterobacter agglomerans* (AF157694)	97	1
			4J	*Pseudomonas migulae* (AF074383)	99	1
			4K	*Staphylococcus* spp.	99	1
Chicken	5.2×10^4	12	5A	*Pseudomonas libanensis* (DQ288882)	99	7
			5B	*A. hydrophila* (X87271)	98	3
			5C	*Acinetobacter junii* (EF429000)	99	1
			5D	*Brochothrix thermosphacta* (AY543017)	99	1
			5E	*Carnobacterium divergens* (AY543016)	99	1
			5F	*Chryseobacterium indoltheticum* (M58774)	97	1
			5G	*P. fluorescens* (AF228367)	98	1
			5H	*P. fluorescens* (DQ178230)	99	1
			5I	*Pseudomonas meridiana* (AJ537602)	99	1
			5J	*P. fragi* (D84014)	98	1
			5K	*P. migulae* (AY047218)	97	1
			5L	*P. fluorescens* (DQ178232)	99	1
Beef	8.8×10^5	3	6A	*Carnobacterium maltaromaticum* (AY543018)	99	18
			6B	*Moraxella osloensis* (Y15855)	99	1
			6C	*Staphylococcus* spp.	99	1
Daikon radish sprout	2.0×10^8	9	7A	*Stenotrophomonas maltophilia* (AJ293473)	99	8
			7B	*S. rhizophia* (AJ293463)	97	3
			7C	*S. maltophilia* (AB021406)	99	3
			7D	*Chryseobacterium piscium* (AM040439)	99	1
			7E	*Comamonas testosteroni* (AF519533)	99	1
			7F	*A. johnsonii* (AB099655)	99	1
			7G	*Pseudomonas fulgida* (AJ492830)	99	1
			7H	*P. putida* (AY647158)	98	1
			7I	*Sphingobacterium multivorum* (AB100738)	99	1

TABLE 1. *Continued*

Sample	Bacterial counts (CFU/g)	No. of groups of isolated strains by PCR-SSCP	Confirmation of PCR-SSCP grouping by 16S rDNA sequencing[a]			
			SSCP group	Closest relatives of 16S rDNA sequence (accession no. of closest relative)[b]	% similarity	No. of strains
Lettuce	5.6×10^7	7	8A	*Pseudomonas cichorii* (AB021398)	99	11
			8B[d]	*Enterobacter/Erwinia*	—	3
			8C	*P. putida* (AY456706)	99	2
			8D	*Chryseobacterium formosense* (AY315443)	98	1
			8E	*Pseudomonas veronii* (AB056120)	99	1
			8F	*P. marginalis* (Z76663)	99	1
			8G	*P. borealis* (AJ012712)	99	1
Bean sprout	2.2×10^7	7	9A[e]	*Enterobacter/Erwinia/Raoultella*	—	11
			9B	*Pseudomonas* spp.	98	4
			9C	*P. oryzihabitans* (AM262973)	98	1
			9D	*E. agglomerans* (AF130961)	96	1
			9E	*E. amnigenus* (EF426859)	98	1
			9F	*E. asburiae* (EF059885)	99	1
			9G	*P. oryzihabitans* (AY850170)	99	1

[a] Isolated strains from each food samples were grouped by PCR-SSCP, and all strains were identified by 16S rDNA sequencing.
[b] Identification by 16S rDNA sequencing was performed using an approximately 500-bp portion of 16S rDNA (*E. coli* position 50 to 500). After the database was searched, strains were identified as the closest relatives.
[c] Group 2B consisted of two species: two strains of *C. piscium* (DQ862541, 97%) and a strain of *Flavobacterium denitrificans* (AJ308927, 95%).
[d] Group 8B consisted of two species: two strains of *E. agglomerans* (Z96083, 99%) and *Erwinia persicina* (AM184098, 97%).
[e] Group 9A consisted of three species: nine strains of *E. agglomerans* (AF130939, 98%), *Erwinia rhapontici* (AJ233417, 100%), and *Raoultella planticola* (Y17663, 97%).

Identification of isolated strains by 16S rDNA sequencing. Samples of DNA for PCR templates were extracted based on chaotropic extraction, followed by absorption onto silica-coated magnetic beads using a commercially available DNA extraction kit (Mag Extractor-Genome, Toyobo Co., Ltd., Tokyo, Japan) according to manufacturer's instructions. Briefly, 1 ml of the overnight culture of each isolated strain in tryptic soy broth (Difco, Becton Dickinson) supplemented with 1% NaCl was centrifuged (15,000 × *g*, 5 min), resuspended in 850 μl of lysis buffer, applied to 40 μl of silica-coated magnetic beads, and vortexed vigorously for 10 min. The magnetic beads were then precipitated by tabletop centrifugation (2,000 × *g*, 15 s), washed twice in 900 μl of washing buffer and once in 900 μl of 70% ethanol, and finally resuspended in 100 μl of Tris-EDTA buffer. After the suspension was vortexed vigorously for 10 min, the magnetic beads were precipitated by tabletop centrifugation (2,000 × *g*, 15 s), and the supernatant was collected for use in PCR reactions.

All strains were identified by amplifying and sequencing an approximately 450- to 500-bp portion of 16S rDNA (*Escherichia coli* positions 50 to between 450 and 500) *(2)*. Amplification was performed using universal primers 27F and 1492R *(27)*, and products were purified by ultrafiltration (Montage PCR centrifugal filter devices, Millipore Corp., Bedford, Mass.). Purified products were then directly sequenced using Texas Red–labeled primers 27F and 536R *(27)* by the DNA sequencer SQ5500E (Hitachi, Ltd., Tokyo, Japan) with the Thermo Sequenase primer cycle sequencing kit (GE Healthcare UK, Ltd., Amersham Place, Little Chalfont, Buckinghamshire, UK). The BLAST 2.0 algorithm was used to compare the derived sequences with 16S rDNA sequences in the DDBJ database (http://www.ddbj.nig.ac.jp, Shizuoka, Japan).

SSCP analysis of 16S rDNA V3 region. In the PCR-SSCP analysis, we used precast polyacrylamide gel, followed by silver staining because of the high sensitivity of silver staining. This method visualizes even a small amount of nonspecific amplification product; therefore, several PCR primers and thermal profiles were tested for specificity and the difference of PCR efficiency. The primer set SRV3-1 (5′-CGG YCC AGA CTC CTA CGG G-3′) *(15)* for forward primer and V3R53 (5′-GTA TTA CCG CGG CTG CTG GC-3′), which was newly designed based on 536R *(27)* with minor modifications for reverse primer, gave acceptable results. PCR amplification was performed in 100-μl reaction mixtures composed of 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 four dNTPs, 2.5 U of TaKaRa *Taq* DNA polymerase (Takara Bio, Shiga, Japan), and 50 ng of template DNA. To minimize amplification of nonspecific products, touchdown PCR *(7)* was performed; the initial annealing temperature was set at 6°C above the target annealing temperature and decreased by 0.6°C every second cycle until the target annealing temperature, 61°C, was reached, and then 5 additional cycles were carried out with the target annealing temperature. Amplifications were carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, Calif.), using the following cycle: denaturation at 94°C for 30 s, annealing at the temperature regime described above for 30 s, and primer extension at 72°C for 10 s for touchdown cycles, and 72°C for 30 s for the last 5 additional cycles.

SSCP analysis of PCR products was performed as described previously *(23)*. Briefly, PCR products were mixed 1:2 with loading buffer (98% formamide–10 mM EDTA–0.5% bromophenol blue), denatured by heating for 10 min at 100°C, cooled on ice, loaded in a precast, ready-to-use gel (GeneGel Excel 12.5/24 kit, GE Healthcare), and electrophoresed on a GenePhor electrophoresis unit (GE Healthcare) at 650 V, 25 mA, and 15°C until the bromophenol blue front reached the anode buffer strip (about 90 min). The gel was stained with a PlusOne DNA silver staining kit (GE Healthcare). Scanned photographs of SSCP gels were stored as TIFF images.

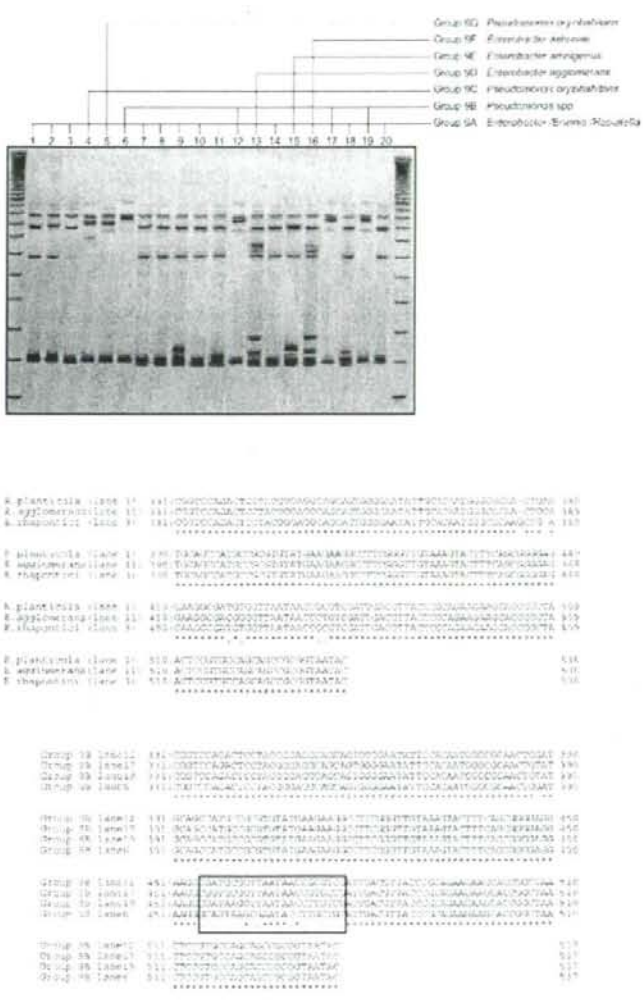

FIGURE 1. *Representative SSCP analysis results of the V3 region PCR product (bean sprout isolates). (A) Lanes: 1–3, 7–11, 14, 18, and 20 are group 9A; lanes 6, 12, 17 and 19 are group 9B; and lanes 4, 13, 15, 16, and 5 are group 9C to 9G, as shown in Table 1. The 100-bp DNA size marker was loaded on both sides, without heat denaturation. The bands that correspond to approximately 200 bp were double-stranded DNA. (B) Representative sequence alignment of the V3 region of group 9A strains. The fragments generated from these three genera were not separated by the PCR-SSCP method because of the high sequence similarity. (C) Sequence alignment of the V3 region of group 9B strains. The SSCP electrophoresis could not discriminate the polymorphisms indicated by the box, as described in the text.*

RESULTS AND DISCUSSION

Optimization of SSCP analysis. We selected the V3 region of 16S rDNA as the amplification target of the PCR-SSCP analysis because the V3 region of 16S rDNA is informative in the differentiation of bacterial strains. Nine of the V (hypervariable) regions, which contain many polymorphisms among species, are present in bacterial 16S rDNA *(18)*, and some are useful for bacterial classification alone or in combinations of two or more regions *(3)*. The V3 region is the third V region from the 5′ end of 16S rDNA and is positioned at 330 to 530 by *E. coli* numbering *(14)* and is widely used for typing or sequence identification of bacteria *(12, 29)*.

Amplification was performed using the touchdown PCR method *(7)*, in order to minimize nonspecific amplification products. In 80 strains (60 isolated strains from fresh fish products and 20 isolated strains from chicken; Table 1), the primer pair and the touchdown PCR conditions produced sufficient amounts of amplification products, with no undesired products (data not shown). The electrophoresis temperatures were also optimized for optimal separation of the 16S rDNA V3 fragment, and the optimal running temperature was determined to be 15°C. Theoretically, denaturation of a double-stranded DNA fragment provides two single-stranded DNA with different sequences. For this reason, two bands should be observed in PCR-SSCP analysis. However, some samples produced three or four bands by the PCR-SSCP method used here (Fig. 1; e.g., lanes 13 and 16). This occurred due to the high resolution of the PCR-SSCP method, which can detect substitutions of just a few bases. These results had no effect on the groupings since the banding was due to the heterogeneity among multiple copies of 16S rDNA *(4, 11, 17, 20)*.

Evaluation of the accuracy of SSCP analysis. We isolated 180 bacterial strains from nine food samples of fresh fish products, meat, and vegetables. All isolated strains were typed by the PCR-SSCP method (Fig. 1, representative result of bean sprout isolates), and then all strains were identified by 16S rDNA sequencing to confirm the grouping by PCR-SSCP. The 180 strains isolated in our study (20 strains each from nine food samples) were analyzed by PCR-SSCP, and 20 isolates from each food sample category were classified into 3 (beef) to 12 (chicken) groups (Table 1). Groupings by PCR-SSCP and 16S rDNA sequencing showed good correlation to the genus level (Table 1). For example, the groupings based on PCR-SSCP analysis of beef samples showed perfect correspondence to those of 16S rDNA sequencing analysis (Table 1). The perfect correspondences of grouping by PCR-SSCP analysis to 16S rDNA sequence were obtained for the other samples except tuna, lettuce, and bean sprouts as discussed below. These results indicate that PCR-SSCP is useful for grouping strains isolated from food samples. There is not a large influence in microbial population analysis results, although some of the isolates from tuna, lettuce, and bean sprout samples were not grouped perfectly.

Undesirable discrimination patterns of PCR-SSCP would be the grouping together in the same SSCP group of different strains belonging to different genera. Such imperfect grouping leads to the overlooking of the constituent bacteria in the sample. In this study, it was difficult to categorize some strains into the correct group. As shown in Table 1, two strains of *Chryseobacterium* and one strain of *Flavobacterium* from tuna fillets were grouped together. In isolates from lettuce, two strains of *Enterobacter* and a strain of *Erwinia* were grouped together (Table 1). In isolates from bean sprouts, nine strains of *Enterobacter*, one strain of *Raoultella*, and one strain of *Erwinia* were grouped together (Table 1 and Fig. 1A). When minority strains in a group for which two genera were observed, PCR-SSCP, as performed in this study, misidentified these 4 strains (2.2%) among a total of 180 strains. These inconsistencies were caused by the close genetic relationships among these strains. In fact, *Chryseobacterium* and *Flavobacterium* (isolates of both were found in tuna fillets) had been formally classified as the same genus *Flavobacterium* *(26)*. *Enterobacter* and *Raoultella* (formally classified in *Klebsiella*, *(8)*) found in lettuce and bean sprouts could not be discriminated by 16S rDNA sequencing because they have sequence differences of only a few bases in the V3 region of their 16S rDNA (Fig. 1B). Also, the 16S rDNA sequence of *Erwinia* spp. found in bean sprouts was very similar to that of *Raoultella* in a DNA database search (98.5%). The results in our study indicate that substitutions of at least five bases in the V3 region of the 16S rDNA (2.5% difference) were reflected in the banding patterns of PCR-SSCP, except the substitutions at a specific position (*E. coli* position 457 to 480; Fig. 1C). The region 457 to 480 contains stem-loop structure of the 16S rRNA, and some of the base changes occurring at the loop area would not influence the secondary structure of the 16S rRNA. Since the banding patterns of SSCP electrophoresis reflect the single-strand conformation of the DNA, mutations at the position 457 to 480 are not always reflected in the PCR-SSCP banding patterns. This insufficiency did not lead the isolated strains belonging to the different genus into the same SSCP group, and only observed in SSCP group 9B of bean sprout sample used in this study. In general, molecular identification using 16S rDNA is a powerful tool in bacterial identification. However, it is not always sufficient for identification to the species level among groups containing genetically diverged species (*Bacillus* group) *(1)*, genetically closed species (*Staphylococcus* group) *(24)*, or ambiguously defined species (*Clostridium* group) *(5, 13)*. These cases indicate the resolution limitations of the method using the V3 region of 16S rDNA. It is remarkable that our PCR-SSCP method did not fail to discriminate distant genera and clearly reflected the results of 16S rDNA sequencing.

In this study, nine food samples were tested and the 20 isolates from each food sample category were grouped into 3 to 12 (average of 8) groups by the PCR-SSCP method, and the same isolates were grouped into 3 to 8 (average of 6) genera by 16S rDNA sequencing (Table 1). Although the PCR-SSCP method occasionally grouped the same species into different groups (Table 1, SSCP group 4A and 4C, 5G and 5H, 7A and 7C, 9C and 9G), this did little to influence the accuracy of the analysis. Cost of analysis is increased because sequencing is carried out for the strains grouped together where it is not necessary. These problems occur due to the high resolution of this method, which can discriminate even a few bases of mutation.

We also analyzed the similarities of PCR-SSCP banding patterns and 16S rDNA sequences between the isolated strains using band imaging software (BioNumerics, Applied Maths BVBA, Kortrijk, Belgium), which identifies the strains by banding patterns. There were no correlations observed between the similarities of SSCP banding patterns and 16S rDNA sequences, because SSCP banding patterns are not directly affected by sequence substitutions, but rather by differences in secondary structure of the single-strand DNA caused by sequence substitution *(15)*. The SSCP banding pattern database may be provided in future experiments for the strain identification by only using this PCR-SSCP method.

The analyses carried out in this study required a total of 4 days. On the first day, we plated food samples onto medium; on the second day, colonies were counted and were picked. On the third day, chromosomal DNA of isolated strains were extracted using the commercially available kit, the V3 regions of 16S rDNA were amplified, and the DNA samples were applied to SSCP analysis and the representative strains selected from each SSCP group. The 16S rDNA sequences of the representatives were determined and strains were identified on the fourth day. Since precast gels and all reagents are commercially available as a ready-to-use package, the PCR-SSCP method has excellent reproducibility among experiments and technicians. Moreover, the multiprocessor (GE Healthcare) is now available and makes technician-free operation possible for the staining step. The total cost to analyze 20 isolates (one food

sample) was almost $70 for the PCR-SSCP analysis, followed by 16S rDNA sequencing. The cost of the PCR-SSCP method can be reduced because the high reproducibility of PCR-SSCP enables the grouping of strains between different samples.

In this study, we evaluated the PCR-SSCP process as a grouping method for isolated strains from plate count analysis and showed good correlation between the PCR-SSCP analysis and 16S rDNA sequencing. Among 180 strains from various foods, 2.2% were misgrouped due to their phylogenetic relationships. This is not a substantial problem of the PCR-SSCP method because the most important aspect of this grouping method for isolates used by food suppliers is the practical usefulness. The PCR-SSCP method meets these requirements in various aspects, such as sufficient accuracy, high throughput, high reproducibility, and ease of operation. This PCR-SSCP method can also be used as the grouping method of isolates followed by identification using the identification kits and classical identification by biochemical characterization.

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Multiple-locus variable-number of tandem-repeats analysis distinguishes *Vibrio parahaemolyticus* pandemic O3:K6 strains

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Abstract

A specific serotype of *Vibrio parahaemolyticus*, O3:K6, has recently been linked to epidemics of gastroenteritis in Southeast Asia, Japan, and North America. These pandemic O3:K6 strains appear to have recently spread across continents from a single origin to reach global coverage, based on profiling of strains by several molecular typing methods. In this study, variable-number tandem repeats (VNTR)-based fingerprinting was applied to clinical and environmental *V. parahaemolyticus* O3:K6 strains in an attempt to develop a molecular method with increased sensitivity for discriminating strains; the relative discriminatory powers were compared with ribotyping and pulsed-field gel electrophoresis (PFGE). All clinical strains tested were independent human isolates obtained from different outbreaks or from sporadic cases in Tokyo during the period from 1996 to 2003. Multiple-locus VNTR analysis (MLVA) was shown to have high resolution and reproducibility for typing of *V. parahaemolyticus* clones. MLVA analysis of 28 pandemic *V. parahaemolyticus* O3:K6 strains isolated from human cases produced 28 distinct VNTR patterns. The VNTR loci displayed between 2 and 15 alleles at each of eight loci with Nei's diversity index ranging from 0.35 and 0.91. These data demonstrated that MLVA is useful for individual strain typing of new O3:K6 strains, which appear to be closely related by other molecular methods.

Keywords: DNA typing; MLVA; *Vibrio parahaemolyticus*; VNTR

1. Introduction

Vibrio parahaemolyticus is a gram-negative marine bacterium that causes seafood-borne gastroenteritis; but not all strains have the same pathogenic potential. Infections are usually caused by *V. parahaemolyticus* of diverse serotypes, and until 1996, infections were characterized by sporadic cases caused by multiple, diverse serotypes. However, recent studies have shown the emergence of serotype O3:K6, a unique serotype that is characterized by the potential to spread and to be associated with infections more often than other serotypes. In February 1996, strains belonging to the O3:K6 serotype were first documented in Calcutta, India, and accounted for 50 to 80% of the strains of *V. parahaemolyticus* responsible for infections occurring annually since then (Okuda et al., 1997). Furthermore, strains belonging to the new O3:K6 serotype have been isolated from other Southeast Asian countries, from travelers at a quarantine station in Japan (Okuda et al., 1997), and from a food-borne outbreak in the United States (Centers for Disease Control and Prevention, 1999). These isolates possessed the *tdh* gene encoding thermostable direct hemolysin (TDH), lacked the *trh* gene encoding TDH-related hemolysin, and showed very similar profiles by several molecular methods (Matsumoto et al., 2000; Nasu et al., 2000; Wong et al., 2000), suggesting the presence of a common ancestor.

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A variety of molecular typing methods have been applied to *V. parahaemolyticus*; ribotyping (Bag et al., 1999; DePaola et al., 2003; Gendel et al., 2001), pulsed-field gel electrophoresis (PFGE) (Bag et al., 1999; Marshall et al., 1999), group-specific PCR (GS-PCR) (Matsumoto et al., 2000), arbitrarily primed PCR (AP-PCR) (Hara-Kudo et al., 2003; Matsumoto et al., 2000; Okuda et al., 1997), and multilocus sequence typing (MLST) (Chowdhury et al., 2004). However, newly identified O3:K6 strains are shown to be genetically homogeneous, and it makes difficult to distinguish them by above methods. Therefore, we decided to develop a method that targets the short tandem repeat sequences (TRs), which undergo rapid evolution in the bacterial genome. Increasingly, variable-number tandem repeats (VNTRs) have been used to discriminate among individual strains within several food- or waterborne pathogen with little genetic variation, including *Escherichia coli* O157:H7 (Lindstedt et al., 2004a; Noller et al., 2003), *Pseudomonas aeruginosa* (Onteniente et al., 2003), *Staphylococcus aureus* (Sabat et al., 2003), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Lindstedt et al., 2004b). Short sequence repeats, including VNTRs, consist of unique DNA elements that are repeated in tandem. Individual strains within a bacterial species often maintain the same sequence elements but with different copy numbers, variation introduced by slipped-strand mispairing during DNA replication (Metzgar et al., 2001). Since sequence homologies between strains exist in the flanking-sequences, specific primers can be used to determine the variation in copy numbers of repeat units, reflecting intraspecific genetic diversity.

The primary aim of this study was to develop a high-resolution typing system for *V. parahaemolyticus* serotype O3:K6 based on polymorphisms at genomic VNTR loci. We demonstrate the utility of this approach by comparing PFGE results for clinical strains of *V. parahaemolyticus* serotype O3:K6 from different outbreaks in Tokyo occurring from 1996 to 2003. This study shows for the first time that clonal pandemic O3:K6 strains are distinguishable by differing VNTR patterns.

2. Materials and methods

2.1. Bacterial strains

All *V. parahaemolyticus* strains ($n=34$) were collected by the Tokyo Metropolitan Institute of Public Health and provided to the Food Microbiology Laboratory at the Tokyo University of Marine Science and Technology (Table 1). Among them, 28 were clinical strains isolated from single patients associated with different outbreaks or sporadic cases in Tokyo during the period from 1996 to 2003 and the other 6 strains were environmental strains isolated from either food or seawater during the period from 1999 to 2003. All *V. parahaemolyticus* strains were grown in LB broth or on LB agar (1.5% agar) with 3% sodium chloride. All strains were serotyped by the slide agglutination test with O- and K- antigen using commercially available antisera (*V. parahaemolyticus* antisera Seiken set, Denka Seiken, Tokyo, Japan), and the presence of the *tdh* gene and *trh* gene in the isolates were determined by the primers described previously (Okura et al., 2003).

2.2. GS-PCR

GS-PCR for *toxRS* sequence of the newly emerging *V. parahaemolyticus* serotype O3:K6 clones (*toxRS/new*) and old O3:K6 strains (*toxRS/old*) was performed according to the reports by Okura et al. (2003), and ORF8 PCR for detection of the f237 filamentous phage which is unique to the newly emerged O3:K6 clones (Nasu et al., 2000) was also performed (Okura et al., 2003).

2.3. Automated ribotyping

Ribotyping was carried out using the Qualicon RiboPrinter Microbial Characterization System (Qualicon, Inc., Wilmington, Del.) according to the manufacturer's instructions. Riboprint patterns for each strain were compared to the patterns produced for all other strains using the same restriction enzyme using the software supplied with the Riboprinter system. Strain-to-strain comparisons were used to define ribogroups, each consisting of patterns that were >0.90 similar. The sample number of the first pattern in each group became the label used to identify that group. The analysis software derived a single average pattern for each ribogroup, as well as information on the similarity of each pattern within the group to the group average pattern.

2.4. Typing O3:K6 strains by PFGE

PFGE typing of strains was performed on genomic DNA digested with restriction enzyme *Not* I, as described elsewhere (Hara-Kudo et al., 2003; Yeung et al., 2002) with minor modifications. All strains were grown overnight at 30 °C in LB broth. Bacteria were harvested by centrifugation and resuspended in 100 μl resuspension buffer (Bio-Rad Laboratories Ltd., Richmond, Calif.). Agarose plugs were prepared by mixing equal volumes of bacterial suspensions. Suspensions were transferred to disposable plug molds and cooled to 4 °C. Bacterial cells in the agarose plugs were treated with lysozyme solution at 25 °C for 2 h, after which, the plugs were suspended in 300 μl of proteinase buffer containing 3 μl of proteinase K and incubated at 50 °C for overnight. Agarose plugs were washed once with wash buffer, once with 1 mM PMSF, twice with wash buffer, and once with 0.1 × wash buffer with wash time of 1 h each. Agarose plugs containing genomic DNA were digested with 10 U of *Not* I (Takara Bio Inc., Shiga, Japan) at 25 °C overnight. PFGE was performed with 1% agarose gel (Seakem Agarose Gold; FMC Bioproducts, Rockland, Me.) on a CHEF-DR II apparatus (Bio-Rad) in 0.5 × TBE buffer at 14 °C. Electrophoresis was performed at 6 V/cm for 18 h with a 2- to 40- s linear ramp time.

2.5. Searching potential VNTRs

All VNTR loci were selected using MICAS (http://www.cdfd.org.in/micas/) (Sreenu et al., 2003) and the Tandem Repeat Finder program (http://tandem.biomath.mssm.edu/trf/trf.html) (Benson, 1999) from the entire genomic sequence of *V. parahaemolyticus* RIMD2210633, Kanagawa-phenomenon positive, serotype O3:K6 (Makino et al., 2003), GenBank

Table 1
Vibrio parahaemolyticus strains used in this study

Source	Strains[a]	Serotype[b]	Year[b]	*tdh*[c]	*trh*[c]	GS-PCR *toxRS/new*[d]	GS-PCR ORF8[d]	Riboprinting DuPont ID	Riboprinting Ribogroup[e]
Clinical	V96-110	O3:K6	1996	+	−	+	+	<none>	172-48-s-3
	V96-178	O3:K6	1996	+	−	+	+	DUP-6626	172-48-s-3
	V96-223	O3:K6	1996	+	−	+	+	DUP-6626	172-48-s-3
	V97-19	O3:K6	1997	+	−	+	+	DUP-6626	172-54-s-4
	V97-49	O3:K6	1997	+	−	+	+	DUP-6626	172-48-s-3
	V97-204	O3:K6	1997	+	−	+	+	DUP-6626	172-48-s-3
	V98-10	O3:K6	1998	+	−	+	+	DUP-6626	172-48-s-1
	V98-290	O3:K6	1998	+	−	+	+	DUP-6626	172-48-s-3
	V98-324	O3:K6	1998	+	−	+	+	DUP-6626	172-48-s-3
	V99-38	O3:K6	1999	+	−	+	+	DUP-6626	172-48-s-3
	V99-107	O3:K6	1999	+	−	+	+	DUP-6626	172-48-s-3
	V99-205	O3:K6	1999	+	−	+	+	DUP-6626	172-48-s-3
	V00-76	O3:K6	2000	+	−	+	+	DUP-6626	172-48-s-3
	V00-145	O3:K6	2000	+	−	+	+	DUP-6626	172-48-s-3
	V00-161	O3:K6	2000	+	−	+	+	DUP-6626	172-48-s-3
	V01-38	O3:K6	2001	+	−	+	+	DUP-6626	172-48-s-3
	V01-141	O3:K6	2001	+	−	+	+	DUP-6626	172-48-s-3
	V01-151	O3:K6	2001	+	−	+	+	DUP-6626	172-48-s-3
	V02-21	O3:K6	2002	+	−	+	+	DUP-6626	172-48-s-1
	V02-36	O3:K6	2002	+	−	+	+	DUP-6626	172-48-s-1
	V02-64	O3:K6	2002	+	−	+	+	DUP-6626	172-48-s-3
	V02-106	O3:K6	2002	+	−	+	+	<none>	172-48-s-4
	V02-123	O3:K6	2002	+	−	+	+	DUP-6626	172-48-s-3
	V02-207	O3:K6	2002	+	−	+	+	DUP-6626	172-48-s-3
	V02-279	O3:K6	2002	+	−	+	+	DUP-6626	172-48-s-3
	V03-80	O3:K6	2003	+	−	+	+	DUP-6626	172-48-s-1
	V03-108	O3:K6	2003	+	−	+	+	DUP-6626	172-48-s-3
	V03-159	O3:K6	2003	+	−	+	+	DUP-6626	172-56-s-8
Environment	V19	O3:K6	1999	−	−	−	−	<none>	172-58-s-1
	V37	O3:K6	1999	−	−	−	−	<none>	172-58-s-2
	V71	O3:K6	1999	−	−	−	−	<none>	172-58-s-3
	V237	O3:K6	2000	−	−	−	−	<none>	172-58-s-4
	V238	O3:K6	2000	−	−	−	−	<none>	172-58-s-5
	V282	O3:K6	2003	−	−	−	−	<none>	172-58-s-5

[a] All strains were isolated in Tokyo, Japan.
[b] Year of isolation.
[c] The presence of these genes were determined by the PCR as described previously (Okura et al., 2003).
[d] Determined by the group-specific PCR for the newly emerged O3:K6 strains, performed as described previously (Okura et al., 2003).
[e] Ribogroups were designated such that identical riboprint patterns are grouped into the same ribogroup.

accession numbers BA000031 and BA000032 (Fig. 1). PCR primers were designed from sequences flanking the respective loci (Table 2).

2.6. MLVA typing

Chromosomal DNA was extracted and purified from overnight cultures by phenol-chloroform extraction and ethanol precipitation according to the method of Murray and Thompson (1980).

Primers were designed for the amplification and sequencing of the target repeat region (Table 2) to verify that the observed differences were due to variability in the tandem repeat region and not other genetic characteristics. Each 50 μl PCR mixture contained 5 μl of 10×PCR buffer, 4 μl each deoxyribonucleotide, 5 μM of each primer, 0.25 μl of *Taq* DNA polymerase (Takara Bio Inc.), and 1 μl of DNA template. The samples were placed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, Calif.) and the temperature was raised to 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. The final hold was for 7 min at 72 °C. All steps in the PCR thermal cycling program were identical for the 7 primer pairs, except for annealing temperatures (given in Table 2). The PCR products were then purified by polyethyleneglycol precipitation (Sambrook et al., 1989).

The forward and reverse strands of the PCR products were sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Contigs were created using the base calling and fragment assembling software programs, GENETYX/ATSQ (Software Development, Tokyo, Japan) and the numbers of repeats in aligned sequences were counted. The resulting data were imported back into BioNumerics software version 4.0 (Applied-Maths, Kortrijk, Belgium) for use clustering analysis with the categorical coefficient and Ward clustering parameter. Use of the categorical coefficient implies that the character states are considered unordered. The

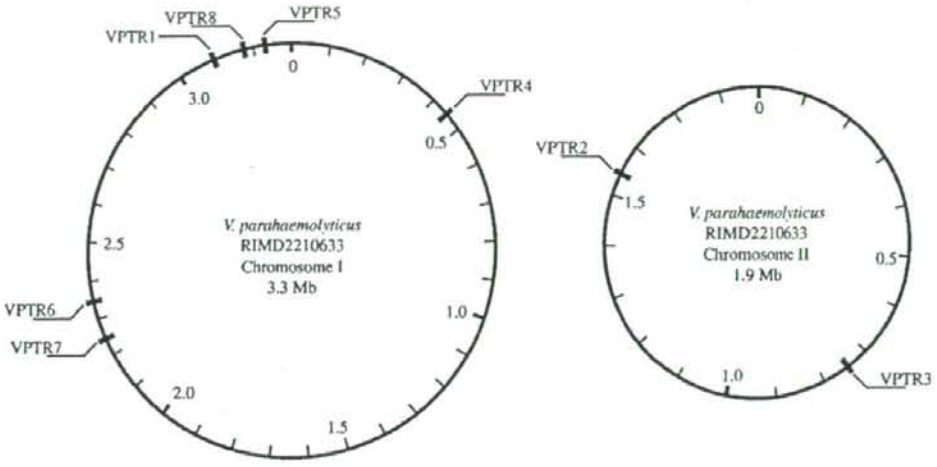


Fig. 1. VNTR maker locations within the physical map of the *V. parahaemolyticus* RIMD2210633 genome. Positions are given with reference to predicted origin of replication (set as position 0).

same weight is given to a large or a small number of differences in the number of repeats at any locus. The information index or Nei's diversity index (D) was calculated for each markers as $1-\Sigma(\text{allele frequency})^2$.

3. Results

The serotypes, virulence attributes, the results of GS-PCR for *toxRS* and ORF8, ribotyping and the sources of the 34 tested *V. parahaemolyticus* strains are shown in Table 1. PCR analysis confirmed the presence of *tdh* and the absence of *trh* in all 28 clinical strains of *V. parahaemolyticus* serotype O3:K6, while all 6 environmental strains of *V. parahaemolyticus* serotype O3:K6 lacked both *tdh* and *trh*. In addition, all 28 clinical strains were confirmed to be pandemic strains by the GS-PCR assay for *toxRS* and the ORF8 PCR assay which detects the presence of the f237 phage (Nasu et al., 2000). On the other hand, all 6 environmental strains were distinguished as nonpandemic strains by these PCR assays.

3.1. Automated ribotyping

Riboprint patterns generated for all 34 strains of *V. parahaemolyticus* O3:K6 using *Eco*RI (Table 1) showed that among the 28 clinical strains, 26 strains were identified as DuPont ID 6626 (DUP-6626; *V. parahaemolyticus*). Two

Table 2
Characteristics of the VNTR locus in *V. parahaemolyticus* and primers for MLVA

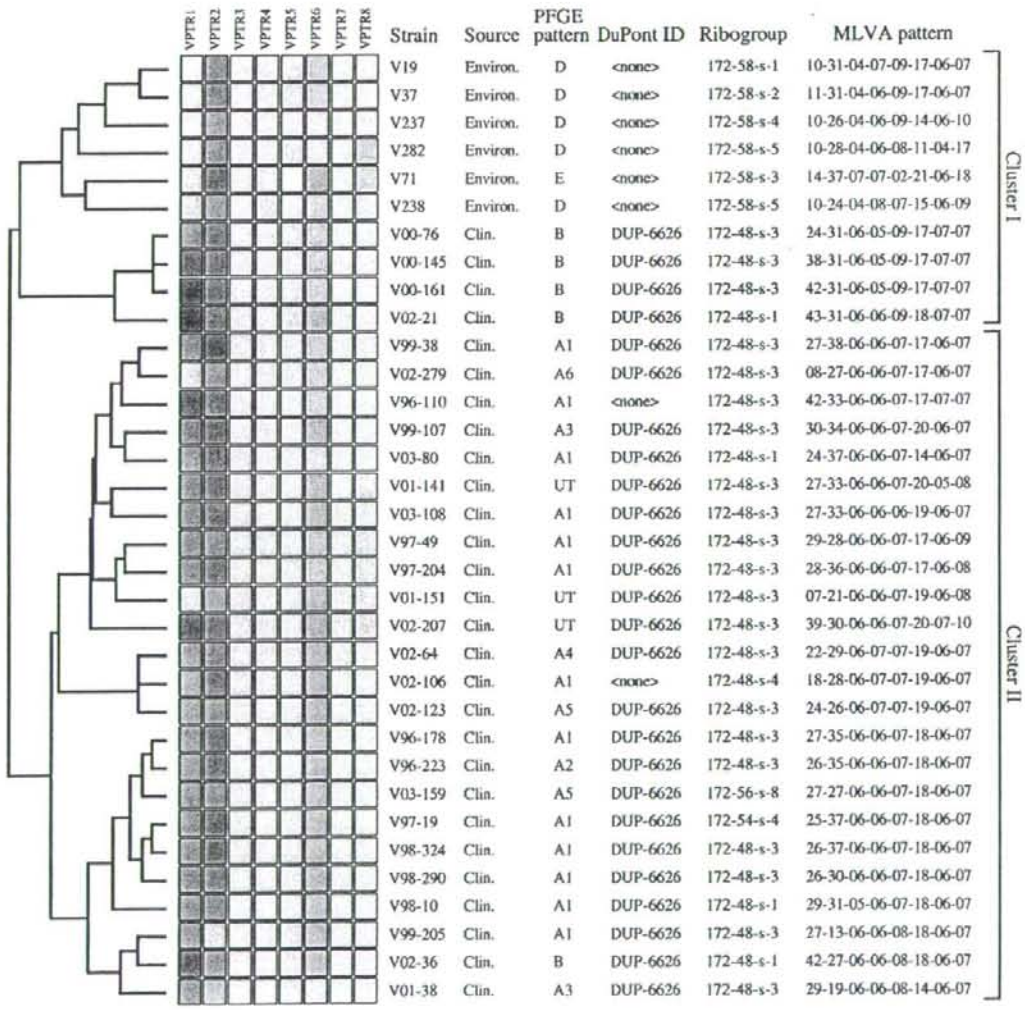
Locus	Repeat motif	Repeat times[a]	Function	Diversity[b]	Primer	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)
VPTR1	ATAGAG	28	hypothetical protein	0.91	VPTR1-F	TAACAACGCAAGCTTGCAACG	60	255
					VPTR1-R	TCATTCTCGCCACATAACTCAGC		
VPTR2	CAGCAA	28	hypothetical protein	0.90	VPTR2-F	GTTACCAAACTGGCGATTACGAAG	60	615
					VPTR2-R	CGGAATTCAGGATCATCCTGAT		
VPTR3	ATCTGT	6	putative collagenase	0.35	VPTR3-F	CGCCAGTAATTCGACTCATGC	60	333
					VPTR3-R	AAGACTGTTCCCGTCGCTGA		
VPTR4	TGTGTC	7	putative hemolysin	0.43	VPTR4-F	AAACGTCTCGACATCTGGATCA	61	229
					VPTR4-R	TGTTTGGCTATGTAACCGCTCA		
VPTR5	CTCAAA	7	Non-coding region	0.56	VPTR5-F	GCTGGATTGCTGCGAGTAAGA	60	202
					VPTR5-R	AACTCAAGGGCTGCTTCGG		
VPTR6	GCTCTG	17	hypothetical protein	0.79	VPTR6-F	TGTCGATGGTGTTCTGTTCCA	60	312
					VPTR6-R	CTTGACTTGCTCGCTCAGGAG		
VPTR7	CTGCTC	6	hypothetical protein	0.38	VPTR7-F	CAACAGTTCTGCTCTAATCTTCCG	56	221
					VPTR7-R	CAAAGGTGTTACTTGTTCCAGACG		
VPTR8	CTTCTG	7	Cell division protein	0.44	VPTR8-F	ACATCGGCAATGAGCAGTTG	60	306
					VPTR8-R	AAGAGGTTGCTGAGCAAGCG		

[a] Numbers of tandem repeats were counted using the genome sequence of *V. parahaemolyticus* RIMD2210633 (Makino et al., 2003).
[b] Diversity is based on Nei's marker diversity, which is $1-\Sigma(\text{allele frequency})^2$.

strains not identified as DUP-6626, V96-110 and V02-106, showed similarities with DUP-6626 of 84% and 74%, respectively (Table 1). The majority (22 strains) of the 28 clinical strains were grouped in ribogroup 172-48-s-3 (average internal similarity, 95.9%), and 4 strains in ribogroup 172-48-s-1 had an average internal similarity 97.5%. Ribogroup 172-48-s-3 has similarity of 93%, and 172-48-s-1, 91%, with DUP-6626. All the environmental strains had no DuPont ID (Table 1).

3.2. PFGE profiles

In this study, PFGE was carried out with *Not* I. Previous experiments indicated that pandemic O3:K6 clones show similar PFGE patterns (Arakawa et al., 1999; Chowdhury et al., 2000; Yeung et al., 2002). In this study, obvious distinction between clinical and environmental strains was noted (Fig. 2). Furthermore, clinical O3:K6 group strains had highly similar PFGE patterns; all pandemic strains tested in this study displayed PFGE pattern A, except for the isolates V00-76, V00-145, V00-161, V02-21 and V02-36, which were internally identical and showed small one-band differences from the pattern A PFGE profile (PFGE pattern B). Three strains, V01-141, V01-151, and V02-207, were untypeable producing only a smear of bands on the gels. This is in accordance with previous studies (Marshall et al., 1999; Yeung et al., 2002) and suggests that the utility of PFGE for differentiating *V. parahaemolyticus*



Strain	Source	PFGE pattern	DuPont ID	Ribogroup	MLVA pattern	
V19	Environ.	D	<none>	172-58-s-1	10-31-04-07-09-17-06-07	Cluster I
V37	Environ.	D	<none>	172-58-s-2	11-31-04-06-09-17-06-07	
V237	Environ.	D	<none>	172-58-s-4	10-26-04-06-09-14-06-10	
V282	Environ.	D	<none>	172-58-s-5	10-28-04-06-08-11-04-17	
V71	Environ.	E	<none>	172-58-s-3	14-37-07-07-02-21-06-18	
V238	Environ.	D	<none>	172-58-s-5	10-24-04-08-07-15-06-09	
V00-76	Clin.	B	DUP-6626	172-48-s-3	24-31-06-05-09-17-07-07	
V00-145	Clin.	B	DUP-6626	172-48-s-3	38-31-06-05-09-17-07-07	
V00-161	Clin.	B	DUP-6626	172-48-s-3	42-31-06-05-09-17-07-07	
V02-21	Clin.	B	DUP-6626	172-48-s-1	43-31-06-06-09-18-07-07	
V99-38	Clin.	A1	DUP-6626	172-48-s-3	27-38-06-06-07-17-06-07	Cluster II
V02-279	Clin.	A6	DUP-6626	172-48-s-3	08-27-06-06-07-17-06-07	
V96-110	Clin.	A1	<none>	172-48-s-3	42-33-06-06-07-17-07-07	
V99-107	Clin.	A3	DUP-6626	172-48-s-3	30-34-06-06-07-20-06-07	
V03-80	Clin.	A1	DUP-6626	172-48-s-1	24-37-06-06-07-14-06-07	
V01-141	Clin.	UT	DUP-6626	172-48-s-3	27-33-06-06-07-20-05-08	
V03-108	Clin.	A1	DUP-6626	172-48-s-3	27-33-06-06-06-19-06-07	
V97-49	Clin.	A1	DUP-6626	172-48-s-3	29-28-06-06-07-17-06-09	
V97-204	Clin.	A1	DUP-6626	172-48-s-3	28-36-06-06-07-17-06-08	
V01-151	Clin.	UT	DUP-6626	172-48-s-3	07-21-06-06-07-19-06-08	
V02-207	Clin.	UT	DUP-6626	172-48-s-3	39-30-06-06-07-20-07-10	
V02-64	Clin.	A4	DUP-6626	172-48-s-3	22-29-06-07-07-19-06-07	
V02-106	Clin.	A1	<none>	172-48-s-4	18-28-06-07-07-19-06-07	
V02-123	Clin.	A5	DUP-6626	172-48-s-3	24-26-06-07-07-19-06-07	
V96-178	Clin.	A1	DUP-6626	172-48-s-3	27-35-06-06-07-18-06-07	
V96-223	Clin.	A2	DUP-6626	172-48-s-3	26-35-06-06-07-18-06-07	
V03-159	Clin.	A5	DUP-6626	172-56-s-8	27-27-06-06-07-18-06-07	
V97-19	Clin.	A1	DUP-6626	172-54-s-4	25-37-06-06-07-18-06-07	
V98-324	Clin.	A1	DUP-6626	172-48-s-3	26-37-06-06-07-18-06-07	
V98-290	Clin.	A1	DUP-6626	172-48-s-3	26-30-06-06-07-18-06-07	
V98-10	Clin.	A1	DUP-6626	172-48-s-1	29-31-05-06-07-18-06-07	
V99-205	Clin.	A1	DUP-6626	172-48-s-3	27-13-06-06-08-18-06-07	
V02-36	Clin.	B	DUP-6626	172-48-s-1	42-27-06-06-08-18-06-07	
V01-38	Clin.	A3	DUP-6626	172-48-s-3	29-19-06-06-08-14-06-07	

Fig. 2. The dendrogram of all the MLVA-typed *V. parahaemolyticus* O3:K6 strains. The frequencies of tandem repeats of each locus were visualized by the grayscale matrices (the color density indicates the frequency of each tandem repeats). The PFGE profile and DuPont ID generated from ribotyping are also shown. Ribogroups were designated such that identical riboprint patterns were grouped into the same ribogroup. Abbreviations: <none>, No corresponded DuPont ID; UT, untypeable.

Table 3
The number of tandem repeats of the *V. parahaemolyticus* strains used in this study

		Repeat times (Repeat motif)							
		VPTR1	VPTR2	VPTR3	VPTR4	VPTR5	VPTR6	VPTR7	VPTR8
Source	Strains	(ATAGAG)	(CAGCAA)	(ATCTGT)	(TGTGTC)	(CTCAAA)	(GCTCTG)	(CTGCTC)	(CTTCTG)
Clinical	V96-110	42	33	6	6	7	17	7	7
	V96-178	27	35	6	6	7	18	6	7
	V96-223	26	35	6	6	7	18	6	7
	V97-19	25	37	6	6	7	18	6	7
	V97-49	29	28	6	6	7	17	6	9
	V97-204	28	36	6	6	7	17	6	8
	V98-10	29	31	5	6	7	18	6	7
	V98-290	26	30	6	6	7	18	6	7
	V98-324	26	37	6	6	7	18	6	7
	V99-38	27	38	6	6	7	17	6	7
	V99-107	30	34	6	6	7	20	6	7
	V99-205	27	13	6	6	8	18	6	7
	V00-76	24	31	6	5	9	17	7	7
	V00-145	38	31	6	5	9	17	7	7
	V00-161	42	31	6	5	9	17	7	7
	V01-38	29	19	6	6	8	14	6	7
	V01-141	27	33	6	6	7	20	5	8
	V01-151	7	21	6	6	7	19	6	8
	V02-21	43	31	6	6	9	18	7	7
	V02-36	42	27	6	6	8	18	6	7
	V02-64	18	29	6	7	7	19	6	7
	V02-106	18	28	6	7	7	19	6	7
	V02-123	24	26	6	7	7	19	6	7
	V02-207	39	30	6	6	7	20	7	10
	V02-279	8	27	6	6	7	17	6	7
	V03-80	24	37	6	6	7	14	6	7
	V03-108	27	33	6	6	6	19	6	7
	V03-159	27	27	6	6	7	18	6	7
Environment	V19	10	31	4	7	9	17	6	7
	V37	11	31	4	6	9	17	6	7
	V71	14	37	7	7	2	21	6	18
	V237	10	26	4	6	9	14	6	10
	V238	10	24	4	8	7	15	6	9
	V282	10	28	4	6	8	11	4	17

might be limited by some of isolates untypeable due to DNA degradation.

3.3. PCR amplification and sequence analysis of potential VNTRs

A total of eight VNTR loci were analyzed in the *V. parahaemolyticus* genome, which consists of two circular chromosomes; six VNTRs were localized on chromosome I, and two were localized on chromosome II (Fig. 1). All eight VNTR loci were successfully amplified, and sufficient variability was confirmed in the eight VNTR loci by sequencing. We found that all eight loci had multiple alleles with substantial variability. In all cases, the size variation observed among PCR products was attributable to the number of TRs.

The VNTRs loci displayed a wide range of polymorphisms in the O3:K6 strains, with the VPTR1 and VPTR2 being the most polymorphic (Table 3). Among 28 clinical strains, VPTR1 had 18 different alleles, and VPTR2 had 16, VPTR6 had 5, VPTR5 and VPTR8 each had 4, VPTR4 and VPTR7 each had 3, and VPTR3 had only 2. The Nei's diversity index (*D*) is based on the number of alleles and the allele frequency and provides a better measure of discriminatory power than allele number; *D* values for VNTR markers in this study ranged from 0.35 for VPTR3 to 0.91 for VPTR1. VNTR analysis showed a high degree of discrimination of the O3:K6 strains.

3.4. MLVA dendrogram

The extent of genetic diversity among the tested strains based on the MLVA dendrogram revealed that each strain has a distinct profile; that is, 34 strains produced 34 patterns (Fig. 2). Only minor discrepancies were noted between the cluster pattern profiles generated by MVLA and the PFGE (Fig. 2). In MLVA analysis, two main groups, denoted as groups I and II, each were comprised of smaller groups or individual isolates. Cluster I contained all environmental O3:K6 strains and 6 pandemic O3:K6 strains. Cluster II contained the remaining pandemic O3:K6 strains. Closer inspection of cluster I, however, revealed that the pandemic O3:K6 isolates in this

cluster (V00-76, V00-145, V00-161, V02-21) in MLVA had distinct,one-band differences from the major group of the pandemic O3:K6 strains identified by PFGE (data not shown). On the other hand, the majority of strains that clustered together in PFGE produced distinct VNTR profiles, suggesting that distinct populations of *V. parahaemolyticus* serotype O3:K6 strains may have circulated during sporadic cases or in outbreaks in Tokyo during the period from 1996 to 2003. The lack of multiple isolates from the same outbreak did, however, prevent a through analysis of isolate populations.

4. Discussion

The main finding of this study was the high discrimination power of MLVA for the pandemic serotype O3:K6 strains of *V. parahaemolyticus*. All 28 of pandemic serotype O3:K6 strains tested here could be discriminated as individual strains (28 different MLVA profiles, Fig. 2). This is important since no other available typing method provides high-resolution discrimination among pandemic serotype O3:K6 strains. Previous studies using molecular analysis of O3:K6 isolates collected recently from several countries had suggested the genetic homogeneity of O3:K6 (Arakawa et al., 1999; Chowdhury et al., 2000; Matsumoto et al., 2000). The genetic homogeneity of these newly isolated O3:K6 strains were also confirmed by GS-PCR, ribotyping, and PFGE in this study. Although the clinical strains used in this study were isolated from different outbreaks or from sporadic cases during period from 1996 to 2003 in Tokyo, almost all were shown to be identical by these methods (Table 1, Fig. 2), supporting the view of previous studies that pandemic strains might have originated from the same clone (Chowdhury et al., 2000; Okuda et al., 1997). However, our MLVA results showed a high resolution for these pandemic strains, indicating substantial genetic heterogeneity at the VNTR loci among pandemic *V. parahaemolyticus* O3:K6 strains. The finding of great diversity within the small set of *V. parahaemolyticus* O3:K6 strains studied here suggests that there is still a great deal of unsampled *V. parahaemolyticus* O3:K6 diversity to be discovered. One potential concern is that VNTRs evolve so rapidly that multiple MLVA types emerge during outbreak or cultural transfers. A number of studies, however, have revealed that the composition of the VNTR loci is relatively stable and does not change even after prolonged storage or subculture in laboratory settings (Adair et al., 2000; Keim et al., 2000; Sabat et al., 2003; Truman et al., 2004). In this study, we have not tested the stability and heterogeneity within the bacterial population after extensive subculturing of individual colonies of *V. parahaemolyticus*. Thus, further studies on cultural stability and larger collections from various origins including outbreak strains are necessary to validate the application of VNTRs for the characterization of *V. parahaemolyticus*.

The functions of VNTRs used for MLVA typing in this study remain unknown, and the relationships between VNTRs and potential mechanisms for metabolic regulation as well as antigenic variation and environmental adaptation should be further examined. The VNTR loci analyzed here are widely distributed across chromosome I and II of RIMD2210633 (Fig. 1). With the exception of VPTR5, which is located in a non-coding region, VNTRs analyzed here are all located in open reading frame regions. The repeat units in the VNTRs studied in this study were all 3-bp multiples, indicating that variation in the number of repeats in these genes results in altered amino acid sequence, but not in inactivation of genes due to frame shifting. VPTR1, VPTR2, VPTR6, and VPTR7 are located in open reading frames that hypothetically codes for proteins. Allele states of the VPTR1 and VPTR2 loci are highly variable, having 18 and 16 alleles respectively. VPTR3 is located in an open reading frame that codes for the putative collagenase gene. Collagenase digests collagen, affecting the basic structure of membranes in eucaryotic cells. Studies in *V. parahaemolyticus* and *V. cholerae* have shown that collagenase activity may play a role on the virulence and be a factor in host infection and pathogenesis. Apparently, variation in VPTR3 for this gene is limited, and clinical strains, except for V98-10, have 6 repeats, indicating the essential role of putative collagenase gene in the bacterial cell. VPTR4 is located in an open reading frame that codes for the putative hemolysin gene. Pathogenicity of *V. parahaemolyticus* has been correlated to well-characterized hemolysin, TDH and TRH (Honda and Iida, 1993; Naim et al., 2001). Thermolabile direct hemolysin (TLH) (Taniguchi et al., 1990) and lectin-dependent hemolysin (LDH) (Shinoda et al., 1991) have also been reported as the virulence factors of this bacterium. However, the putative hemolysin gene which includes VPTR4 is apparently different from the above hemolysin genes. Since both clinical and environmental strains of *V. parahaemolyticus* have this gene with variation in VPTR4, these genes do not to seem to be key to the virulence of this organism. Examination of the observed allelic differences of these genes among pandemic strains and the relationship to virulence or physiological differences will be interesting for future studies.

In this study, we have shown that MLVA is a valuable typing technique for characterizing recently emerged and highly homogeneous pandemic strains of *V. parahaemolyticus* serotype O3:K6. The data presented here demonstrate the utility of this approach for individual strain identification. Although MLVA loci in pandemic O3:K6 strains seem to mutate too rapidly to be useful in determining global phylogenetic relationships, they are useful for strain identification and may identify rapidly evolving polymorphisms that are useful for discriminating very closely related strains, such as *V. parahaemolyticus* serotype O3:K6 strains. In addition to high resolution power, MLVA is a simple and rapid method, which can be used to produce strain profiles that are easily exchanged electronically via the BioNumerics database as character strings. In this study, we sequenced VPTR1 to VPTR8 amplicons to verify PCR specificity and to confirm that any observed length polymorphisms were due solely to variation in VNTR copy number. However, for practical purposes, sequencing will not be necessary and this method can be further improved by using primers tagged with multiple fluorescent dyes, allowing accurate sizing of amplicons by automated DNA sequencer analysis. This method therefore gives fast, discriminative, and reproducible results for epidemiological surveillance of *V. parahaemolyticus* pandemic strains.

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ORIGINAL ARTICLE

Induction of the histidine decarboxylase genes of *Photobacterium damselae* subsp. *damselae* (formally *P. histaminum*) at low pH

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Aims: To elucidate the detailed mechanism of histamine production by *Photobacterium damselae* subsp. *damselae*.

Methods and Results: Histidine decarboxylase and related genes of *P. damselae* subsp. *damselae* were cloned, and three open reading frames named as *hdcT*, *hdcA* and *hisRS* were identified. The *hdcA* gene encodes a polypeptide of 377 amino acids and is considered to be the pyridoxal-P dependent histidine decarboxylase. The *hdcT* gene is assumed to be a histidine/histamine antiporter, and the *hisRS* gene is considered to be a histidyl-tRNA synthetase. Recombinant *Escherichia coli* strains harbouring plasmids carrying the *P. damselae hdc* genes were shown to over-excrete histamine extracellularly. Northern blot analysis and quantitative RT-PCR revealed high levels of mono- and bi-cistronic transcripts of *hdcA*, *hdcT* and *hisRS* genes under conditions of low pH and histidine excess.

Conclusions: The *hdcA* gene of *P. damselae* was constructed as an operon with putative histidine/histamine antiporter and histidyl-tRNA synthetase. Mono- and poly-cistronic transcripts and acid induction were detected.

Significance and Impact of the Study: This is the first report of cloning the histidine decarboxylase gene cluster in Gram-negative bacteria. Also, these genes were induced under acidic conditions and in the presence of excess histidine.

Introduction

Histidine decarboxylase catalyses the decarboxylation of histidine to histamine and CO_2. Histamine is well known for its vasoactive properties and, along with other biogenic amines produced by bacteria in several foods, is a consumer health threat. In general, bacterial amino acid decarboxylases, such as lysine decarboxylase (Park *et al.* 1996; Merrell and Camilli 1999, 2000), arginine decarboxylase (Castanie-Cornet *et al.* 1999) and glutamate decarboxylase (Lin *et al.* 1996) are greatly induced under acidic conditions and are hypothesized to play a role in controlling pH as a countermeasure to acidity resulting from anaerobic fermentation. One of these enzymes, lysine decarboxylase (Cad A) of *Escherichia coli*, has been well characterized (Meng and Bennett 1992a,b; Watson *et al.* 1992). CadA decarboxylates intracellular lysine to cadaverine, consumes a proton, and then, cadaverine is transported extracellularly via a lysine-cadaverine antiporter (CadB). These processes provide a clear survival advantage under acidic conditions. Similar mechanisms have been proposed for other decarboxylase systems, such as glutamate decarboxylase (Waterman and Small 1996; Sanders *et al.* 1998; Castanie-Cornet *et al.* 1999; Cotter *et al.* 2001) and arginine decarboxylase (Lin *et al.* 1996), and it is now clear that these amino acid decarboxylase systems play an important role in the survival of bacteria under acidic conditions (Bearson *et al.* 1997; Merrell and Camilli 2002).

The known bacterial histidine decarboxylases fall into two groups: those that contain pyridoxal-P as the essential coenzyme and those that contain a covalently bound pyruvoyl residue at the active site. While histidine decarboxylases from Gram-positive bacteria are

pyruvoyl-dependent enzymes, those from Gram-negative bacteria are pyridoxyal-P-dependent enzymes. The amino acid sequence of pyridoxal 5′-phosphate dependent histidine decarboxylase has been studied in four bacterial species: *Morganella morgani* (Vaaler *et al.* 1986), *Raoultella planticola* (formally named *Klebsiela*), *Enterobacter aerogenes* (Kamath *et al.* 1991) and *Photobacterium phosphoreum* (Morii *et al.* 2006). However, transcriptional analysis of these enzymes under acidic conditions has not yet been carried out.

In surveys of histamine-producing bacteria in marine fish and seawater, we isolated several strains of facultatively anaerobic, halophilic bacteria that produced histamine to as large an extent as *M. morganii*. This bacterium was renamed to a new species as *P. histaminum* (Okuzumi *et al.* 1994). Recently, this species has been proposed to be a subjective synonym of *P. damselae* subsp. *damselae*, an opportunistic pathogen of fish and mammals (Kimura *et al.* 2000; Yamane *et al.* 2004). A study on the effect of culture conditions of L-histidine decarboxylation activity of this bacterium showed that decarboxylation was highest in acidic conditions (Kurihara *et al.* 1993). These analyses suggest that expression of the gene encoding histidine decarboxylase in this bacterium is controlled by acidity in the environment.

In the study reported here, we cloned and performed transcriptional analysis of the *hdc* gene cluster, which encodes histidine decarboxylase and the related genes of *P. damselae* subsp. *damselae* ATCC51805. This is the first report of the full sequence of the *hdc* gene cluster and the first transcriptional analysis of the *hdc* gene cluster for Gram-negative bacteria.

Materials and methods

Strains and media

The strain *P. damselae* ATCC 51805 used in this study was originally isolated from fish and first named as *P. histaminum* (Okuzumi *et al.* 1994) and recently reclassified as *P. damselae* subsp. *damselae* (Kimura *et al.* 2000). Half-strength artificial seawater (ASW)-PMGY broth was described previously (Okuzumi *et al.* 1981). PY broth contained 10 g of bacto peptone, 3 g of bacto yeast extract in 50% ASW and histidine broth (HB) contained 10 g of bacto peptone, 3 g of bacto yeast extract and 5 g of L-histidine per liter in 50% ASW (Okuzumi *et al.* 1981). PY-7·5 and HB-7·5 media were prepared as PY or HB buffered at pH 7·5 with a final concentration of 100 mmol l^{-1} 3-(*N*-morpholino) propanesulfonic acid (MOPS), and PY-4·5 and HB-4·5 were PY or HB buffered at pH 4·5 with a final concentration of 100 mmol l^{-1} 2-(*N*-morpholino) ethane sulfonic acid (MES).

Escherichia coli JMI09 (Yanisch-Perron *et al.* 1985) was used as host cells for the cloning and sequencing procedures and *E. coli* BL21 (DE3) was used as host cells for expression of the *hdcA* and *hdcB* genes. Luria–Bertani medium (LB, 1% tryptone, 0·5% yeast extract, and 1% NaCl) was used as a complex medium for *E. coli*. Cultures of *E. coli* were incubated aerobically at 37°C and *P. damselae* was incubated at 30°C. The plasmid pUC18 was used for cloning, and plasmids pUC18 or pUC19 (Takara Bio, Shiga, Japan) were used for expression of the cloned *hdc* genes. When required, ampicillin (50 μg ml^{-1}) was added to the culture media.

Analysis of N-terminal amino acid sequence

Cells from a 5l culture of *P. damselae* ATCC 51805 in half-strength ASW-PMGY broth were harvested in early stationary phase by centrifugation (4000 *g*, 5 min), and resuspended in buffer A (50 mmol l^{-1} KH_2PO_4, 50 mmol l^{-1} succinic acid, 2 mmol l^{-1} EDTA, 1 mmol l^{-1} DTT, 10 μmol l^{-1} PLP, pH 6·0) (Vaaler *et al.* 1986). After cells were disrupted at 4°C by ultrasonication, the supernatant was purified through ammonium sulfate precipitation and several chromatographic separations; DEAE cellulofine A-550 (Seikagaku Corporation, Tokyo, Japan), Phenyl Sepharose CL-4B (Amersham Biosciences Corp., Piscataway, NJ, USA), and Amicon ultrafiltration cell (Millipore Corp., Bedford, MA, USA). The dialysed preparation of the semi-purified effluent was applied to an anion exchange column (DEAE), gel filtrated and applied to reversed phase chromatography using HPLC. The N-terminal amino acid sequence of purified protein was determined by automated Edman degradation using a Beckman LF3000 protein sequencer (Beckman Coulter, Fullerton, CA, USA).

Cloning of the *hdc* genes and sequence analysis

Chromosomal DNA of *P. damselae* was purified by standard methods (Murray and Thompson 1980). Southern blot hybridization analysis (Sambrook *et al.* 1989) was performed using DNA probes prepared using primers 5′-GAY GCN TTY TGG GCN CAY TGY G-3′ (designed from the N-terminal amino acid sequence, DAFWAHCV) and 5′-GAR CCR ATC ATT TTG TGK CCG C-3′ (designed from known *hdcA* sequences). Based on Southern blot analysis, the restriction enzyme digests including 4·5kb fragment of *SacI–SalI* was cloned into pUC18 plasmid using DNA ligation kit (Takara Bio) and transformed into *E. coli* JM109 competent cell (Toyobo, Tokyo, Japan). Then, based on the sequence data of the cloned 4·5-kb *SacI–SalI* fragment, the adjacent two fragments were cloned based on Southern blot hybridization: 4·5kb

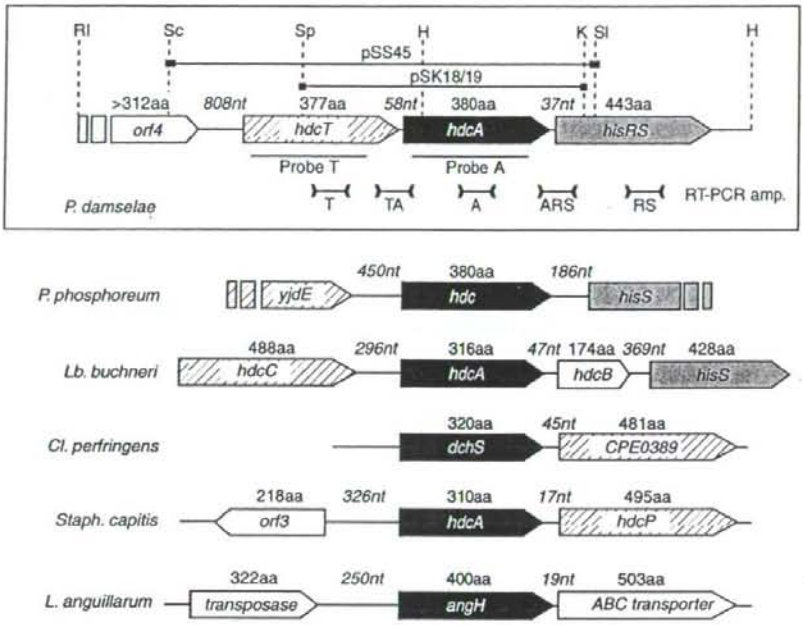

Figure 1 Schematic representation of the histidine decarboxylase gene clusters of *P. damselae* (in the box), *P. phosphoreum* (5' and 3' incomplete) (AY223843), *Lactobacillus buchneri* (AJ749838), *Clostridium perfringens* (BA000016), *Staphylococcus capitis* (AM283479) and *Listonella anguillarum* (AY312585). The restriction enzymes used in this report are indicated above of the *P. damselae* schematic with dashed lines. The regions targeted by DNA probes for Northern blot hybridization analysis are indicated by lines labelled with probe T or probe A, and amplification target sites for real-time PCR are indicated by the double-arrowed lines as follows: T, *hdcT* mRNA; TA, *hdcT-hdcA* bi-cistronic mRNA; A, *hdcA* mRNA; ARS, *hdcA-hisRS* bi-cistronic mRNA; and RS, *hisRS* mRNA. Restriction enzymes are abbreviated as follows: RI, *Eco*RI; Sc, *Sac*I; Sp, *Sph*I; H, *Hind*III; K, *Kpn*I; SI, *Sal*I. Genes with identical putative functions are depicted by identical shading. The gene names are as in previous reports.

fragment of *Hind*III–*Hind*III, including downstream of *hdcA*, and 2·3-kb fragment of *Eco*RI–*Sph*I, including upstream of *hdcT* (Fig. 1). Positive clones were selected by colony blot hybridization (Sambrook *et al.* 1989), and the obtained plasmids were designated as pSS45, pHH45 and pES23 (Table 1).

Plasmid DNA from *E. coli* strains was extracted by a commercially available kit and sequenced using a Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on a 373A DNA Sequencer (Applied Biosystems). Sequence data were compiled and analysed using Genetyx-Mac genetic information processing software (Software Development, Tokyo, Japan). Possible transcriptional terminator, ribosome binding site and promoter elements were predicted by Genetyx-Mac. The BLAST program was used to identify sequences similar to the deduced amino acid sequences in the DNA Data Bank of Japan (DDBJ) (Altschul *et al.* 1997). The hydrophobicity and the possible transmembrane domains of *hdcT* gene products were calculated and plotted using the TMpred program (Hofmann and Stoffel 1993) and by the SOSUI program (Hirokawa *et al.* 1998). Multiple sequence alignment and calculation of the phylogenetic relationships were performed using CLUSTALW (Thompson *et al.* 1994) with exclusion of alignment gaps for analysing phylogenetic relationships.

Expression of *hdc* gene in *E. coli*

The *Sac*I–*Kpn*I fragment containing the *hdcA* gene was subcloned into the pUC18 and pUC19 plasmids to obtain pSK18 and pSK19 and transformed into *E. coli* BL21 (DE3) cells (Novagen, Madison, WI, USA). Recombinant *E. coli* BL21 (DE3) cells harbouring plasmids pSS45 (containing both of *hdcT* and *hdcA*), pSK18, and pSK19 were cultured in 5 ml of modified LB medium (LB broth containing 10 g l^{-1} L-histidine). After centrifugation at 15 000 *g* for 10 min, the supernatant was filtered through

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
Photobacterium damselae		
ATCC 51805	Isolated strain from fish as a histamine producer	Okuzumi *et al.* (1981), Kimura *et al.* (2000)
Escherichia coli		
JM109	*recA1, endA1, gyrA96, thi, hsdR17(rK- mK+), Δ lac-proAB/F'[traD36,proAB+, lac I^q, lacZ Δ M15]*	Toyobo
BL21 (DE3)	F-, *lon*, *ompT*, *hsdS$_B$*($r_B^-m_B^-$), *dcm*, gal, (DE3)	Novagen
Plasmids		
pUC18	*lacZ*, Amp^r, Multiple cloning site	Takara bio
pUC19	*lacZ*, Amp^r, Multiple cloning site	Takara bio
pSS45	pUC18 plasmid vector containing 4.5 kb *Sac*I-*Sal*I fragment including *hdc A* and *hdc T*	This study
pES23	pUC18 plasmid vector containing 2.3 kb *Eco*RI-*Sph*I fragment from *P. damselae* genomic DNA covering the upstream region of *hdc T*	This study
pHH45	pUC18 plasmid vector containing 2.3 kb *Hin*dIII fragment from *P. damselae* genomic DNA covering the downstream region of *hdc A*	This study
pSK18*	pUC18 plasmid vector containing *hdc A* gene together with 0.8 kb of the upstream region and 0.3 kb of the downstream region	This study
pSK19†	pUC19 plasmid vector containing *hdc A* gene together with 0.8 kb of the upstream region and 0.3 kb of the downstream region	This study

*The inserted fragment was placed in the opposite orientation from *lac* promoter of pUC18 plasmid.
†The inserted fragment was placed under the control of *lac* promoter originated from pUC19 plasmid.

0·22 μm filtre (Advantec Toyo, Tokyo, Japan) and the histamine content of the supernatant was analysed by reversed phase HPLC (Yamanaka and Matsumoto 1989).

RNA isolation and Northern hybridization

Aliquots (100 μl) of overnight *P. damselae* cultures in PY-7·5 were inoculated into 50 ml of PY-7·5 in 100ml culture flasks and incubated aerobically at 30°C in a reciprocal shaker set at 200 rev min^{-1}. When the cultures reached an optical density of around 0·1 at 550 nm, 10 ml aliquots were removed and centrifuged, and cells were resuspended in 10 ml of PY-4·5, PY-7·5, HB-4·5, or HB-7·5 which were prepared as described above, and incubated in screw cap tubes (20 × 125 mm) in a stationary incubator at 30°C for 3 h. Tubes were shaken gently every 30 min to prevent cells from settling. Cells from these cultures were harvested by centrifugation at 10 000 *g* for 5 min and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) by the acid guanidium isothiocyanate–phenol–chloroform method. Samples containing 20 μg of total RNA were electrophoresed on 1% agarose gel containing 6·6% formaldehyde along with a 0·2- to 10-kb RNA ladder (Novagen) for size determination. RNA was transferred to a positively charged nylon membrane (Hybond-N$^+$; Amersham Biosciences) and hybridized to labelled DNA probes. Hybridization was performed with AlkPhos Direct Nucleic acid labeling and detection system using DNA probes specific for *hdcA* or *hdcT*, prepared by PCR using primers 5′-ATG CTT TTT GGG CTC ACT GC-3′ and 5′-GAT GGT GAG CAG ATA CCA CC-3′ for *hdcA*, and 5′-TTT ATT GGA TTA GCG CAT GG-3′ and 5′-ACA GAT TTA CCA CTG TGT GC-3′ for *hdcT*.

RT-PCR for mRNA quantification

Primer selection

All reverse transcription reactions were carried out with randomly synthesized hexanucleotide [random hexamer, d(N)$_6$, Takara Bio]. A total of five primer sets were designed to study the transcription of *hdcT*, *hdcA* and *hisRS* gene of *P. damselae* with the following transcript sizes: *hdcT* (408 bp), 5′-CGG AAT TGT CGC TTA TGC CAA-3′ and 5′-CGC CTA AGA AAC CCC ACA ATG-3′, *hdcA* (273 bp), 5′-AAG AGC CAG GTT GTC GAG TCA-3′ and 5′-CGG CAT CGG CAT GGA TAT AA-3′; and *hisRS* (107 bp), 5′-TGA GGT ATG GCC TTT TCC ACT A-3′ and 5′-CTA CCC ATT GCC AAA TAG GTG T-3 ′. The *hdcT-hdcA* bi-cistronic cDNA was detected by primers 5′-TTT TAA CTG CCA TTG GGA CGA-3′ and 5′-AGT GCG CCC AAA ATG CAT CTA-3′, which were designed