Cloning and Sequencing of the Histidine Decarboxylase Genes of Gram-Negative, Histamine-Producing Bacteria and Their Application in Detection and Identification of These Organisms in Fish

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The use of molecular tools for early and rapid detection of gram-negative histamine-producing bacteria is important for preventing the accumulation of histamine in fish products. To date, no molecular detection or identification system for gram-negative histamine-producing bacteria has been developed. A molecular method that allows the rapid detection of gram-negative histamine producers by PCR and simultaneous differentiation by single-strand conformation polymorphism (SSCP) analysis using the amplification product of the histidine decarboxylase genes (*hdc*) was developed. A collection of 37 strains of histamine-producing bacteria (8 reference strains from culture collections and 29 isolates from fish) and 470 strains of non-histamine-producing bacteria isolated from fish were tested. Histamine production of bacteria was determined by paper chromatography and confirmed by high-performance liquid chromatography. Among 37 strains of histamine-producing bacteria, all histidine-decarboxylating gram-negative bacteria produced a PCR product, except for a strain of *Citrobacter braakii*. In contrast, none of the non-histamine-producing strains (470 strains) produced an amplification product. Specificity of the amplification was further confirmed by sequencing the 0.7-kbp amplification product. A phylogenetic tree of the isolates constructed using newly determined sequences of partial *hdc* was similar to the phylogenetic tree generated from 16S ribosomal DNA sequences. Histamine accumulation occurred when PCR amplification of *hdc* was positive in all of fish samples tested and the presence of powerful histamine producers was confirmed by subsequent SSCP identification. The potential application of the PCR-SSCP method as a rapid monitoring tool is discussed.

Scombroid poisoning is a type of food poisoning that results from eating mishandled tuna and related scombroid fish containing elevated levels of histamine (34). Histamine intoxication is probably the best known sanitary problem of food-borne disease associated with eating fish (34). It has been suggested that the lack of relationship between histamine content and sensory attributes explains the high incidence of scombroid toxicity (50). Histamine forms in a variety of foods, including raw fish (25, 27, 28, 38), wine (8), cheese (60), fermented meat (53), and fish (31) products. While histamine in fermented products, such as wine (36), cheese (35, 59), and fish sauce (31, 57), is produced by gram-positive lactic acid bacteria, histamine in raw fish products is caused mostly by gram-negative enteric bacteria such as *Morganella morganii*, *Klebsiella* spp., and *Enterobacter* spp. (16, 28, 37, 39). During the decomposition of fish such as tuna and mackerel, histamine forms in significant amounts due to bacterial decarboxylation of histidine present in the muscle tissue (69).

Histamine-producing bacterial species are likely introduced into the fish and raw materials during handling after capture (37) and following temperature abuse. Therefore, it is important to develop rapid methods to detect the presence of histamine producers before dangerous levels of histamine are reached. Efforts to isolate histamine-producing bacteria have been hampered by the lack of a reliable and reproducible

method. Niven's medium (48) has been most widely used, although high rates of false positives and false negatives are observed, presumably due to the presence of competing bacteria (12, 25, 39). Several improved screening methods have been described (4, 22, 41, 45). However, most screening procedures generally involve the use of a differential medium containing a pH indicator. The histamine-forming capability of positive isolates was not confirmed in all reports, and some reports (52, 54) described false-positive reactions in some media due to the formation of alkaline compounds or false-negative responses due to fermentation by some bacteria. Moreover, conventional cultural assays can require 2 to 3 days to complete, making these methods inefficient for the rapid detection of histamine-producing bacteria.

Molecular methods for detection and identification of food-borne pathogens are becoming more widely accepted as an alternative to traditional culture methods. Rapid detection of gram-negative histamine producers is important for detecting and preventing microbial contamination and high histamine levels during processing of fish products. Since histamine is the decarboxylation product of histidine catalyzed specifically by the enzyme histidine decarboxylase (HDC), it is possible to develop a molecular detection method that detects the gene responsible for production of this enzyme. There are two distinct classes of HDCs: homometric pyridoxal 5'-phosphate (PLP)-dependent enzymes in animals and gram-negative bacteria (23) and heterometric enzymes that contain an essential pyruvoyl group but no PLP in gram-positive bacteria (33, 65). Although PCR detection of genes encoding the pyruvoyl-dependent HDC of gram-positive bacteria has been developed

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(21), to our knowledge, methods of detecting genes that encode PLP-dependent enzymes of gram-negative bacteria have not yet been developed. Rapid detection and identification of histamine-producing bacteria would be beneficial as a management tool for use in hazard analysis and critical control point systems.

In this study, we developed universal PCR primers to amplify the HDC gene of gram-negative histamine-producing bacteria in fish samples and other sources and provide a detailed analysis of HDC gene sequences. We also describe a rapid identification system for gram-negative histamine producers in fish products using PCR-driven single-strand conformation polymorphism (PCR-SSCP) (49).

MATERIALS AND METHODS

Bacterial strains and determination of histamine production. The bacterial strains used in this study were obtained from the American Type Culture Collection, Japan Collection of Microorganisms (Saitama, Japan), the Institute of Applied Microbiology (Tokyo, Japan), and various raw fish samples during routine screenings of histamine-producing bacteria (Table 1). Histamine-producing bacteria isolated in this study were collected from different types of fish during 2000 and 2001. A variety of fresh fish samples, including horse mackerel, yellowtail, tuna, skipjack tuna, bigeye tuna, autumn albacore, and young yellowtail were obtained as skinned fillets from various supermarkets in Tokyo, Japan. In all cases, market samples were kept on ice during transport to the laboratory and were processed within 1 h. Muscle tissue (5 g) was aseptically removed from these samples and placed in 45 ml of histidine broth (pH 6.5) containing (per liter) 10 g of Bacto Peptone (Difco Laboratories, Detroit, Mich.), 3 g of yeast extract (Difco), 15 g of NaCl, and 5 g of histidine (Wako Pure Chemical Ind. Ltd., Osaka, Japan). The mixture was homogenized for 1 min using a stomacher (model 400; Seward Co. Ltd., London, United Kingdom). Then, 1 ml of the homogenate was brought to a volume of 9 ml with fresh histidine broth and incubated at 25°C. After 24 h, 5 µl of culture was assayed for the presence of histamine using paper chromatography as described previously (46). Briefly, 5 µl of the culture was applied to Advantec filter paper (no. 51B; 40cm by 40cm; Toyo Roshi, Tokyo, Japan). A solvent consisting of 100% butanol and 10% NH₄OH (1:1) was applied for 90 min to develop the sample. After drying the filter papers, the histamine spots were visualized by applying Pauly's diazo reagent. Those samples testing positive for histamine were plated onto Trypticase soy agar (TSA) (BBL, Becton Dickinson, Cockeysville, Md.) containing 1.5% NaCl, and incubated at 25°C for 24 h on TSA plates. Colonies were randomly selected and used in PCR analysis and HDC activity tests. Histamine-forming activity of isolates was screened by paper chromatography using 24-h incubation cultures (25°C, in histidine broth). Since we could efficiently test multiple strains using paper chromatography, we used the method to identify histamine-producing bacteria. Isolates identified as having histamine-forming activity were further analyzed by high-performance liquid chromatography (HPLC) as described previously (68). Briefly, 1 ml of 24-h incubation cultures (25°C, in histidine broth) was filtered through a 0.2-µm-pore-size syringe filter (Toyo Roshi) and analyzed on an LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan), using ion-pair chromatographic partitioning on a C₁₈ reversed-phase column and a postcolumn reaction with *o*-phthalaldehyde to form fluorescent derivatives of amines.

Identification of isolates. All histamine-producing bacteria isolated from fish, except for *Photobacterium phosphoreum* and *Raoultella planticola*, were identified using the Vitek instrument, following manufacturer instructions (bioMérieux Vitek Systems Inc., Hazelwood, Mo.). Following isolation on TSA plates, colonies were transferred to TSA slants and incubated at 30°C for 18 h. The colonies from the slants were suspended in saline water (0.45% NaCl) at concentrations determined to be suitable by the Vitek colorimeter. The suspensions were inoculated automatically on gram-negative identification cards (GNI+) containing media for individual biochemical tests in each well. During incubation at 35°C, digital analog optical reading and identification were automatically carried out by the Vitek system (every 8 to 18 h depending on the strain). The identification of *R. planticola* was performed using API ID32E strips inoculated and incubated as described by the manufacturer (bioMérieux Vitek Systems). Briefly, following incubation on TSA slants at 30°C for 18 h, colonies were suspended in 0.9% NaCl solution and the turbidity was adjusted to McFarland standard number 4 by colorimetry. The API ID32E strips were inoculated and incubated at 30°C as

described by the manufacturer. Examination of the strips was conducted after 24 and 48 h, and the results from the 48-h analysis were used. The results were read and analyzed using a mini-API instrument (bioMérieux Vitek Systems). *P. phosphoreum* was identified according to the method of Fujii et al. (14).

The identity of all isolates was further confirmed by amplifying and sequencing approximately 400 to 450 bp of 16S ribosomal DNA (rDNA) (position 50-450 to 50-500 [*Escherichia coli*] numbering) (5). Amplification was performed using universal primers 27F and 1492R (66), and products were purified and sequenced directly using primer 27F. The BLAST 2.0 algorithm was used to compare the sequence derived to 16S rDNA sequences in the DDBJ database (Shizuoka, Japan) (<http://www.ddbj.nig.ac.jp>).

DNA extraction. The PCR templates were prepared using a DNA extraction kit (Mag Extractor-Genome; Toyobo Co. Ltd., Tokyo, Japan), based on chaotropic extraction followed by absorption onto silica-coated magnetic beads according to manufacturer instructions. Briefly, 1 ml of culture or fish homogenate 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 PCRs.

Strategies for primer design. Universal primers amplifying a 709-bp region were selected based on the sequences of the HDC genes of *M. morgani* (ATCC 35200), *Enterobacter aerogenes* (ATCC 43175), and *R. planticola* (formerly *Klebsiella planticola*) (ATCC 43176). To find the conserved region, we aligned *hdc* sequences of GenBank accession numbers M62745 (23), M62746 (23), and J02577 (64) in Genetyx-Mac (Software Development Co., Tokyo, Japan). Two highly conserved regions were identified, and a unique and specific primer pair, *hdc-f* (TCH ATY ARY AAC TGY GGT GAC TGG RG) and *hdc-r* (CCC ACA KCA TBA RWG GDG TRT GRC C), corresponding to the base pair positions 132 to 158 and 817 to 841 of the HDC gene, respectively, was selected. The primers were synthesized by Funakoshi (Tokyo, Japan).

PCR assay. PCR amplification was performed in 20-µl reaction mixtures: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 20 pmol of each primer, a 0.2 mM concentration each of the four deoxynucleoside triphosphates, 0.5 U of *Taq* DNA polymerase (Takara, Shiga, Japan), and template DNA (10 ng). Amplifications were carried out for 35 cycles (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min) in a GeneAmp PCR 2400 thermal cycler (Applied Biosystems, Foster City, Calif.) with an initial denaturation (94°C for 4 min) and a final extension (72°C for 4 min).

PCR products (10 µl) along with suitable molecular markers (100-bp ladder; Amersham Biosciences Corp., Piscataway, N.J.) separated by 1% agarose gel electrophoresis at 100 V in 1× TAE buffer (pH 8.3; 40 mM Tris, 20 mM acetate, 1 mM EDTA). Following electrophoresis, gels were stained by ethidium bromide and visualized under a UV (245 nm) trans-illuminator.

Southern blot hybridization. Genomic DNA of *Citrobacter braakii* was extracted and purified by standard procedures (56), digested with three restriction enzymes (*Eco*RI, *Hind*III, and *Kpn*I), and analyzed by Southern blot hybridization (56). The probe used for detection of HDC genes was the amplification product of HDC genes of *M. morgani*.

DNA sequencing and phylogenetic analysis. The 709-bp amplification fragments of the partial HDC genes from histidine-decarboxylating strains *M. morgani* (JCM1672^T, 87411, AP28, and JU27), *R. planticola* (8433 and 4131), *Proteus vulgaris* (AU34), *Erwinia* sp. (MB31), *Photobacterium damsela* (ATCC 33539^T and JCM8968), and *P. phosphoreum* (MB36) were cloned and sequenced. The amplified *hdc* fragments from these bacteria were treated with polyethylene glycol (PEG), cooled on the ice for 1 h, and pelleted by centrifugation at 15,000 × *g* for 20 min. The pellet was washed with 70% ethanol, dried, and dissolved in Tris-EDTA buffer. Purified DNA fragments were ligated into pT7 blue vectors (Novagen, Darmstadt, Germany) by DNA ligation kit (Takara), and transformed by *E. coli* JM109 (56). Transformed colonies were identified on Luria-Bertani agar plates containing ampicillin (50 µg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside) (400 µg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 µg/ml), and the inserted DNA sequences were determined using a SQ5500E DNA sequencer (Hitachi High-Technologies Corp., Tokyo, Japan) with the ThermoSequenase cycle sequencing kits (Amersham Biosciences). The newly determined sequences of *hdc* were aligned for phylogenetic analysis in CLUSTAL W (63). A phylogenetic tree was constructed using the neighbor-joining method (55) with genetic distances computed by Kimura's two-parameter model (32). Primer regions for PCR amplification were not taken into consid-

TABLE 1. Characteristics of bacterial strains used in this study

Strain ^a	PCR result	Histamine secretion ^f (mg/liter)	Source ^h
<i>Morganella morganii</i>			
ATCC 35200	+	1,350	ATCC
JCM1672 ^T	+	2,300	JCM
87411	+	1,770	Fish (horse mackerel)
AP28	+	1,990	Fish (yellowtail)
JU27	+	1,560	Fish (yellowtail)
4b19	+	2,320	Fish (tuna)
5a05	+	2,080	Fish (tuna)
5c15	+	2,160	Fish (skipjack tuna)
24a01	+	2,480	Fish (tuna)
25a32	+	2,440	Fish (tuna)
25b03	+	2,200	Fish (yellowtail)
27a19	+	2,300	Fish (tuna)
30a04	+	2,260	Fish (horse mackerel)
<i>Raoultella planticola</i> ^b			
ATCC 43176	+	2,230	ATCC
8433	+	2,370	Fish (horse mackerel)
4131	+	2,910	Fish (tuna)
5b09	+	2,710	Fish (bigeye tuna)
18a14	+	2,070	Fish (tuna)
20b06	+	2,210	Fish (bigeye tuna)
21b20	+	2,370	Fish (horse mackerel)
29a03	+	2,200	Fish (bigeye tuna)
31a10	+	2,010	Fish (autum albacore)
<i>Enterobacter aerogenes</i>			
ATCC 43175	+	2,110	ATCC
21a08	+	2,380	Fish (tuna)
<i>Enterobacter amnigenus</i> 23a06	+	2,330	Fish (horse mackerel)
<i>Photobacterium damsela</i>			
ATCC 33539 ^T	+	910	ATCC
JCM8968	+	790	JCM
6a20	+	1,860	Fish (horse mackerel)
<i>Photobacterium phosphoreum</i> ^c			
IAM12085	+	46	IAM
92436	+	481	Fish (horse mackerel)
N14	+	286	Fish (horse mackerel)
MB36	+	199	Fish (horse mackerel)
<i>Hafnia alvei</i> JCM1666	+	253	JCM
<i>Erwinia</i> sp. strain MB31 ^d	+	665	Fish (horse mackerel)
<i>Proteus vulgaris</i>			
AU34 ^e	+	2,400	Fish (young yellowtail)
19a28 ^e	+	2,160	Fish (horse mackerel)
<i>Citrobacter braakii</i> 8435	—	311	Fish (horse mackerel)
Other (470 strains)	—	ND ^g	Fish (various samples)

^a Unless otherwise indicated, strains isolated from fish were identified by the VITEK system. The identity of all isolates was further confirmed by partial sequences of 16S rDNA.

^b Identified by the API ID32E system.

^c Identified according to the method of Fujii et al. (14).

^d Unidentified by either the VITEK or the API ID32E system. The partial sequence of 16S rDNA demonstrated 98% similarity with those of *Erwinia* sp.

^e Unidentified by either the VITEK system or the API ID32E. The partial sequences of 16S rDNA demonstrated 98% similarity with those of *Proteus vulgaris*.

^f Incubation was carried out at 25°C for 24 h in histidine broth. The amount of histamine in the culture broth was measured by HPLC.

^g ND, not detected.

^h Bacteria were obtained from following sources: ATCC, American Type Culture Collection, Manassas, Va.; JCM, Japan Collection of Microorganisms, Saitama, Japan; IAM, Institute of Applied Microbiology, Tokyo, Japan; Fish, bacteria were unrelated strains from different types of fish.

eration for the calculation. The robustness of the phylogenetic tree topologies was evaluated by bootstrap analysis with 1,000 replications.

To investigate the phylogenetic relationships of the representative species based on the 16S rDNA sequences, the 1,411-bp nucleotide sequences of the 16S rDNA (covering base positions 30 to 1441 [*E. coli* numbering(5)] was determined for six species (12 strains) of gram-negative histamine-producing bacteria as previously described (57). For two strains of *P. damsela*, ATCC 33539^T and JCM8968, the 16S rDNA sequences already deposited in the DDBJ database were used (accession no. AB032015 and AB032014, respectively).

PCR detection of histamine producers. For detecting *M. morganii* by PCR in pure culture, an overnight culture of *M. morganii* (JCM1672^T) was diluted, suspended in fresh histidine broth (10^1 CFU/ml), and incubated at 30°C for 24 h. Culture growth and HDC activity of *M. morganii* were determined every 2 h. Viable cell counts were determined by pour plating 1-ml aliquots of the cultures serially diluted as necessary in duplicate on TSA plates and incubating for 48 h at 30°C. The amount of histamine in the culture broth was measured by HPLC as described above.

For detecting *M. morganii* by PCR from spiked tuna homogenates, a block of freshly caught tuna fish was purchased from a commercial processor. The block was aseptically processed on a clean bench in our laboratory. Specifically, the outer surfaces of the block were cut away to remove any potential cross-contamination from the surface to the inner muscle, which were presumed to reflect the previous handling of the fish. A 30-g portion of internal muscle tissue was placed in a sterile stomacher bag containing 270 ml of 1.5% NaCl solution, homogenized with a stomacher (model 400; Seward) for 2 min, and transferred to sterile containers. These homogenates were artificially contaminated with *M. morganii* strain JCM1672^T (8.8×10^1 CFU/g) and then incubated at 30°C. Histamine formation in the tuna homogenate was monitored by the HPLC method as described previously (68). To monitor proliferation of spiked *M. morganii*, 1-ml samples were taken immediately at 0 h and after 2, 4, 6, 9, 12, 15, 18, 21, 24, and 48 h. Tuna homogenates were serially diluted (10-fold), and 1 ml of each dilution was inoculated into three replicate tubes of 9 ml of fresh histidine broth and incubated at 30°C. After 48 h, 5- μ l aliquots of culture were analyzed for the presence of histamine using the paper chromatography method as described above. Cultures testing positive for histamine production were streaked onto TSA, and randomly selected isolates were confirmed as *M. morganii* using the Vitek instrument as described above. Numbers of histamine-producing bacteria in the tuna homogenates were calculated by the three-tube most-probable-number (MPN) method (6). Aliquots (0.1 ml) of the serially diluted homogenates were inoculated into tubes containing histidine broth at pH 6.5 and incubated at 30°C for 24 h. Histamine formation was determined in each tube by the paper chromatographic method as described above. Unspiked tuna homogenate samples (negative controls) were processed by the same methods to determine background histamine production and numbers of histamine producers. DNA was extracted from 1 ml of the spiked and the unspiked homogenates using the DNA extraction kit (Mag Extractor-Genome; Toyobo) as described above and analyzed by *hdc*-specific PCR.

For detecting histamine-producing bacteria in fish samples, we used histamine and PCR assays to examine 13 samples of fish purchased in supermarkets. Commercial fresh fish were purchased in the local supermarkets, transported on ice to the laboratory, and processed immediately. Muscle tissue (10 g) was aseptically cut out and placed in sterile stomacher bags containing 90 ml of 1.5% NaCl solution, homogenized with a stomacher (model 400; Seward) for 1 min, and incubated at 30°C for 24 h. Homogenates were sampled every 4 h. DNA was extracted from 1 ml of homogenate using a kit as described above and assayed by PCR. Simultaneously, another 1 ml of homogenate was analyzed for histamine accumulation by HPLC.

SSCP analysis of histamine producers. Since the 709-bp fragment used in this study was considered to be too long to detect mutations by SSCP analysis (49), the amplification products were digested with endonuclease *HinfI* (Toyobo) before SSCP analysis. *hdc*-specific PCR products (709 bp, as amplified by the protocol described above) from fish samples were purified using PEG as described above and digested overnight at 37°C with *HinfI* according to manufacturer instructions. The digested 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; and loaded in a precast, ready-to-use gel (GeneGel Excel 12.5/24 kit; Amersham Biosciences). SSCP electrophoresis was run on a GenePhor electrophoresis unit at 650 V, 25 mA, and 10°C until the bromophenol blue front reached the anode buffer strip (about 90 min). The gel was stained with PlusOne DNA silver staining kit (Amersham Biosciences). Pure-culture strains were amplified under the same conditions described above and used for identifying the SSCP profiles of fish samples. The identification of gram-negative histamine-producing bacteria from fish samples was also con-

firmed by direct sequencing of the original PCR products before digestion with endonuclease *HinfI*. After the PCR products were treated by PEG, the PCR products were directly sequenced as described above.

Nucleotide sequence accession numbers. Partial *hdc* and 16S rDNA sequences obtained in this study were deposited in the DDBJ nucleotide sequence databases. *hdc* sequences from the following strains were deposited under the indicated numbers: *M. morganii* strain JCM1672^T, AB083200; *M. morganii* strain 87411, AB083201; *M. morganii* strain AP28, AB083202; *M. morganii* strain JU27, AB083203; *R. planticola* strain 8433, AB083205; *R. planticola* strain 4131, AB083206; *P. vulgaris* strain AU34, AB083204; *Erwinia* sp. strain MB31, AB083208; *P. phosphoreum* strain MB36, AB084250; *P. damsela* strain ATCC 33539^T, AB084251; *P. damsela* strain JCM8968, AB084252. 16S rDNA sequences from the following strains were deposited under the indicated numbers: *M. morganii* strain JCM1672^T, AB089243; *M. morganii* strain ATCC 35200, AB089244; *M. morganii* strain 87411, AB089245; *M. morganii* strain AP28, AB089246; *M. morganii* strain JU27, AB099406; *R. planticola* strain ATCC 43176, AB099403; *R. planticola* strain 8433, AB099407; *R. planticola* strain 4131, AB099400; *P. vulgaris* strain AU34, AB099401; *E. aerogenes* strain ATCC 43175, AB099402; *Erwinia* sp. strain MB31, AB099404; *P. phosphoreum* strain MB36, AB099405.

RESULTS

Designing specific primers and PCR amplification. As expected, the *hdc*-specific primer set designed in this study generated a single, typical PCR product of 709 bp for the three reference strains (the strains used to design the primers): *M. morganii* (ATCC 35200), *E. aerogenes* (ATCC 43175), and *R. planticola* (ATCC 43176). Out of 507 bacterial strains including strains received from various culture collections and wild-type isolates randomly isolated from fish samples, 37 strains were positive for histamine production based on HPLC analysis (Table 1). Among these 37 histamine producers tested, 36 strains had a unique and clear PCR band of the expected size (709 bp). One strain identified as *C. braakii* by the Vitek instrument and determination of the partial 16S rDNA sequence had histidine-decarboxylating activity but was negative in the PCR assay. A positive PCR was not attained even when the annealing temperature was decreased to 48°C. This strain also failed to hybridize to a DNA probe made from the amplification product of *M. morganii* (Southern blot data not shown). Using PCR, there was no amplification for the 470 strains of nonhistamine producers.

DNA sequencing and phylogenetic analysis. The deduced amino acid sequences based on these nucleotide sequences of *hdc* from selected bacterial species were the same length in all strains (Fig. 1) and contained the conserved sequence Ser-X-His-Lys in the reduced active site peptides from PLP-dependent HDC (64). The similarities for the nucleotide sequences of 709-bp fragments in these species ranged from 73.8 to 99.2%. The similarities for the amino acid sequence ranged from 82.6 to 100%.

Phylogenetic trees of the isolates based on newly determined sequences of partial HDC genes and 16S rDNA sequences were compared (Fig. 2). The bootstrap scores observed for all the nodes are indicated. Overall, the phylogenetic tree determined by partial HDC gene was similar to that determined by the 16S rDNA. In both trees, the clusters of *M. morganii* and *P. vulgaris* were supported by high boot strap values (100% and 76% on 16S rDNA and partial HDC gene trees, respectively) putting the other enteric species in a separate cluster. Likewise, clusters of *R. planticola*, *E. aerogenes*, and *Erwinia* sp. were differentiated from the cluster containing all other spe-

<i>M. morgani</i> ATCC35200	1	EYCNLLNSFDFFEKEVMEYFADLFKIPFEQSWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>M. morgani</i> JCM1672	1	EYCNLLNSFDFFEKEVMEYFADLFKIPFEQSWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>M. morgani</i> 87411	1	EYCNLLNSFDFFEKEVMEYFADLFKIPFEQSWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>M. morgani</i> AP28	1	EYCNLLNSFDFFEKEVMEYFADLFKIPFEQSWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>M. morgani</i> JU27	1	EYCNLLNSFDFFEKEVMEYFADLFKIPFEKSWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>Pr. vulgaris</i> AU34	1	EYCNLLNSFDFFEKEVMAYFAQLFKIPFEKSWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>Erwinia</i> sp. MB31	1	EYCNLLNSFDFFEKEVMEYFASIFKIPFEESWGYVTNGGTEGNMFGCYLGRELFPDGTLY	60
<i>R. planticola</i> ATCC43176	1	EYCNLLNSFDFFEKEVMEYFAQLFKIPFEESWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>R. planticola</i> 4131	1	EYCNLLNSFDFFEKEVMEYFAQLFKIPFEESWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>R. planticola</i> 8433	1	EYCNLLNSFDFFEKEVMEYFAQLFKIPFEESWGYVTNGGTEGNMFGCYLGREIFPDGTLG	60
<i>E. aerogenes</i> ATCC43175	1	EYCNLLNSFDFFEKEVMEYFSGIFKIPFAESWGYVTNGGTEGNMFGCYLGRELFPDGTLY	60
<i>P. damsela</i> ATCC33539	1	EYCNLLNSFDFFEKEVIEYFAHTFKIPFDKCSWGYVTNGGTEGNMFGCYLGRELFPDGTLY	60
<i>P. damsela</i> JCM8968	1	EYCNLLNSFDFFEKEVIEYFAHTFKIPFDKCSWGYVTNGGTEGNMFGCYLGRELFPDGTLY	60
<i>P. phosphoreum</i> MB36	1	EYCNLLNSFDFFEKEVMEYFADLFKIPFEDSWGYVTNGGTEGNMFGCYLGRELFPDGTLY	60
<i>M. morgani</i> ATCC35200	61	YSKDTHTSVAKIVKLLRIKSOVVESSQPNGEIDYDDLKKIADDKKAHPPIIFANIGTTVRG	120
<i>M. morgani</i> JCM1672	61	YSKDTHTSVAKIVKLLRIKSOVVESSQPNGEIDYDDLKKIADDKKAHPPIIFANIGTTVRG	120
<i>M. morgani</i> 87411	61	YSKDTHTSVAKIVKLLRIKSOVVESSQPNGEIDYDDLKKIADDKKAHPPIIFANIGTTVRG	120
<i>M. morgani</i> AP28	61	YSKDTHTSVAKIVKLLRIKSOVVESSQPNGEIDYDDLKKIADDKKAHPPIIFANIGTTVRG	120
<i>M. morgani</i> JU27	61	YSKDTHTSVAKIVKLLRIKSOVVESSQPNGEIDYDDLKKIADDKKAHPPIIFANIGTTVRG	120
<i>Pr. vulgaris</i> AU34	61	YSKDTHTSVAKIVKLLRIKSRVVESSQPNGEVDYDDLKKITMDKKAHPPIIFANIGTTVRG	120
<i>Erwinia</i> sp. MB31	61	YSKDTHTSVAKIVKLLRIKSSVVESSQPNGEMDYDDLIRKIQRDNEHPPIIFANIGTTVRG	120
<i>R. planticola</i> ATCC43176	61	YSKDTHTSVAKIVKLLRIKSTLVESQPNGEMDYADLKKIKADNEKHPPIIFANIGTTVRG	120
<i>R. planticola</i> 4131	61	YSKDTHTSVAKIVKLLRIKSTLVESQPNGEMDYADLKKIKRDNEKHPPIIFANIGTTVRG	120
<i>R. planticola</i> 8433	61	YSKDTHTSVAKIVKLLRIKSTLVESQPNGEMDYADLKKIKRDNEKHPPIIFANIGTTVRG	120
<i>E. aerogenes</i> ATCC43175	61	YSKDTHTSVAKIVKLLRIKSLVVESSQPDGEMDYDDLIRKISGRHPPIIFANIGTTVRG	120
<i>P. damsela</i> ATCC33539	61	YSKDTHTSVAKIVKLLRIKSOVVESSQPNGEVNYDDLKKIEQDGEKHPPIIFANIGTTVRG	120
<i>P. damsela</i> JCM8968	61	YSKDTHTSVAKIVKLLRIKSOVVESSQPNGEVNYDDLKKIEQDGEKHPPIIFANIGTTVRG	120
<i>P. phosphoreum</i> MB36	61	YSKDTHTSVAKIVKLLRIKSLVVESSQPNGEIDYDDLKKIKQDDEKHPPIIFANIGTTVRG	120
<i>M. morgani</i> ATCC35200	121	ATDDIAETOKRLKAAIGIKREDYYLHADAALSGMILPFVDDAOPFTFADGIDSIGVSGHKM	180
<i>M. morgani</i> JCM1672	121	ATDDIAETOKRLKAAIGIKREDYYLHADAALSGMILPFVDDAOPFTFADGIDSIGVSGHKM	180
<i>M. morgani</i> 87411	121	ATDDIAETOKRLKAAIGIKREDYYLHADAALSGMILPFVDDAOPFTFADGIDSIGVSGHKM	180
<i>M. morgani</i> AP28	121	ATDDIAETOKRLKAAIGIKREDYYLHADAALSGMILPFVDDAOPFTFADGIDSIGVSGHKM	180
<i>M. morgani</i> JU27	121	ATDDITEITOKRMKAAGIKREDYYLHADAALSGMILPFVDDAOPFTFADGIDSIGVSGHKM	180
<i>Pr. vulgaris</i> AU34	121	ATDDIALIQORLKDAAGFKREDYYLHADAALSGMILPFVDDPOGFTFADGIDSIGVSGHKM	180
<i>Erwinia</i> sp. MB31	121	ATDNIAETIQORIGQLGIKRDYYLHADAALSGMILPFVNDPOPFNFADGIDSIGVSGHKM	180
<i>R. planticola</i> ATCC43176	121	ATDNIAETIQOSISELGIKRDYYLHADAALSGMILPFVNDPOPFNFADGIDSIGVSGHKM	180
<i>R. planticola</i> 4131	121	ATDNIAETIQOSISELGIKRDYYLHADAALSGMILPFVNDPOPFNFADGIDSIGVSGHKM	180
<i>R. planticola</i> 8433	121	ATDNIAETIQOSISELGIKRDYYLHADAALSGMILPFVNDPOPFNFADGIDSIGVSGHKM	180
<i>E. aerogenes</i> ATCC43175	121	AVDNIAETIQKRIAAIGIPREDYYLHADAALSGMILPFVEDPOPFNFADGIDSIGVSGHKM	180
<i>P. damsela</i> ATCC33539	121	ATDNIFEITODRIAKLGIAREDYYLHADAALSGMILPFVDEPOGFNFADGIDSIGVSGHKM	180
<i>P. damsela</i> JCM8968	121	ATDNIFEITODRIAKLGIVREDYYLHADAALSGMILPFVDEPOGFNFADGIDSIGVSGHKM	180
<i>P. phosphoreum</i> MB36	121	ATDDISKIQAMIGELGIKREDYYLHADAALSGMILPFVDEPOGFNFADGIDSIGVSGHKM	180
<i>M. morgani</i> ATCC35200	181	IGSPIPCGIVVAKKENVDRIISVEIDYISAHDKTITGSR	218
<i>M. morgani</i> JCM1672	181	IGSPIPCGIVVAKKENVDRIISVEIDYISAHDKTITGSR	218
<i>M. morgani</i> 87411	181	IGSPIPCGIVVAKKENVDRIISVEIDYISAHDKTITGSR	218
<i>M. morgani</i> AP28	181	IGSPIPCGIVVAKKENVDRIISVEIDYISAHDKTITGSR	218
<i>M. morgani</i> JU27	181	IGSPIPCGIVVAKKENVDRIISVEIDYISAHDKTITGSR	218
<i>Pr. vulgaris</i> AU34	181	IGSPIPCGIVVAKKANVDRIISVEIDYISGHDKTITGSR	218
<i>Erwinia</i> sp. MB31	181	IGSPIPCGIVVAKRKNVDRIISVEIDYISAHDKTITGSR	218
<i>R. planticola</i> ATCC43176	181	IGSPIPCGIVVAKKKNVDRIISVEIDYISAHDKTITGSR	218
<i>R. planticola</i> 4131	181	IGSPIPCGIVVAKKKNVDRIISVEIDYISAHDKTITGSR	218
<i>R. planticola</i> 8433	181	IGSPIPCGIVVAKKKNVDRIISVEIDYISAHDKTITGSR	218
<i>E. aerogenes</i> ATCC43175	181	IGSPIPCGIVVAKKANVDRIISVEIDYISAHDKTITGSR	218
<i>P. damsela</i> ATCC33539	181	IGSPIPCGIVIAKKNVDQITVEIDYIAAHDKTITGSR	218
<i>P. damsela</i> JCM8968	181	IGSPIPCGIVIAKKNVDQITVEIDYIAAHDKTITGSR	218
<i>P. phosphoreum</i> MB36	181	IGSPIPCGIVVAKRNVDAISVEIDYISAHDKTITGSR	218

FIG. 1. Alignment of partial HDC amino acid sequences of gram-negative histamine producers. Residues conserved in all sequences are shaded in black, and residues conserved in more than 50% of sequences are shaded in gray. The asterisk marks the lysine residue that binds PLP in the *M. morgani* holoenzyme (64).

cies (boot strap values of 89 and 47% on the 16S rDNA and partial HDC gene sequences, respectively). Also, consistent with previous literature (29), *Photobacterium* strains belonging to the marine bacterium appeared to be the most distant from

the other enteric species, regardless of the genes used for construction of phylogenetic trees (boot strap values of 100 and 99% on the 16S rDNA and partial HDC gene trees, respectively).

FIG. 2. Phylogenetic trees of 14 strains of gram-negative histamine producers based on 16S rRNA gene sequences (A) and partial HDC gene sequences (B). The trees were constructed by using the neighbor-joining method. The genetic distances were calculated using the Kimura's two-parameter method. The numbers on the nodes indicate the number of times (percentage) the species (shown on the right) grouped together in 1,000 bootstrap samples. Only values greater than 40% are shown.

Comparisons of sequence similarities within and between pairs of closely related species and subspecies showed that greater resolution of evolutionary branching was achieved using the partial *hdc* sequences compared to 16S rDNA sequences, with the exception of one pair of *R. planticola* (4131 and 8433), in which sequence similarity with partial HDC gene (99.2%) was almost the same with that of the 16S rDNA (99.6%). The average pairwise sequence similarity of the 16S rDNA within four species of *M. morganii* was 99.0%, while that of partial HDC gene was 97.0%; *M. morganii* JU27 was excluded from calculation because of its distant relationship with other strains of this species. Likewise, sequence similarity of the 16S rDNA between two subspecies of *P. damsela* (ATCC 33539^T and JCM8968) was 100%, while that of partial HDC gene was 98.6%.

PCR detection of histamine producers. The HDC gene was detected in *M. morganii* strain JCM1672^T by *hdc*-specific PCR at 6 h incubation when the culture concentration was 10⁴ CFU/ml (Fig. 3A). Histamine was detected by HPLC after 15 h when the strain had reached the stationary phase.

Similar results were obtained in spiked tuna (Fig. 3B), where initial levels of histamine producers (about 10¹ CFU/g) rose to 10⁷ MPN/g after 15 h of incubation followed by a rapid increase in histamine (from 3 to 43 mg/100 g of tuna) between 15 and 24 h while bacterial counts rose slightly during this period. In PCR assay, *M. morganii* recovered from inoculated tuna yielded positive amplification at 9 h of incubation (10⁵ MPN/g of histamine producers). MPN of histamine producers in spiked tuna was most likely the number of *M. morganii* because the sterile uninoculated tuna remained negative for both PCR

FIG. 3. Growth, histamine production, and detection of *hdc* by PCR for *M. morganii* (JCM1672^T) in histidine broth (A) and tuna homogenates inoculated with *M. morganii* at 30°C (B). Arrows indicate sampling times at which PCRs became positive for the first time. Symbols: ○, histamine; ●, *M. morganii*; ▲, histamine producers.

detection and histamine production determined by HPLC (data not shown).

The *hdc* was successfully amplified from 10 out of 13 samples of fish, and was detected 4 or 8 h earlier than HPLC detection of histamine in 7 samples. In the other three samples, the detection of *hdc* by PCR and that of histamine by HPLC occurred at the same sampling time. In the other three samples in which *hdc* was not amplified, histamine was not detected throughout the incubation period.

SSCP analysis of histamine producers. All amplification products digested with *Hinf*I yielded three or four fragments of the expected sizes. SSCP analysis of them demonstrated a total of 21 SSCP types of *hdc* in 36 isolates (see Table 3). Among the SSCP types, seven were observed in 13 strains of *M. morganii* as anticipated from the sequencing data. Likewise, three SSCP types were observed in 9 strains of *R. planticola*. Two SSCP types were observed in three strains of *P. damsela*. Two SSCP types were observed in four strains of *P. phosphoreum*. Other strains demonstrated different SSCP patterns, respectively. In all isolates investigated, no shifts in SSCP-derived banding patterns were observed among isolates that shared an identical SSCP type.

SSCP analysis of 10 fish samples from supermarkets dem-

onstrated eight SSCP types. Among the 10 samples, eight had profiles identical to reference strains and were therefore positively identified by SSCP analysis (Fig. 4): six samples were identified as *M. morganii*, one sample as *P. damsela*, and one sample as *Enterobacter amnigenus*. It was not possible to identify two other samples because the band patterns shared no similarity to the reference strains. In all of the samples tested, the SSCP patterns were the same, regardless of incubation time (Fig. 4). In this experiment, strains were not identified because many studies employing direct molecular analysis have demonstrated the discrepancy between cultivated and naturally occurring species, which underscores the biases of our view of microbial diversity obtained by conventional culture methods (1, 19). Instead, the identification by SSCP analysis was confirmed by direct sequencing of the original PCR product bands. All identified strains showed high similarities (>98.5%) to the reference strains, while sequence analysis of two samples unidentifiable by SSCP band pattern analysis showed no similarity (<96%) to the reference strains.

DISCUSSION

Detection of histamine-producing bacteria by conventional culture techniques is often tedious and unreliable. To our knowledge, this is the first report describing a molecular method for detection and identification of gram-negative histamine producers. In this study, two oligo-mixed primers were used to specifically amplify a 709-bp fragment in gram-negative histamine-producing bacteria, and with the exception of a strain identified as *Citrobacter braakii*, all strains were positive in PCR assays. Strains that did not exhibit HDC activity in HPLC assays also did not produce PCR products. After optimization of the DNA extraction protocol and PCRs, 709-bp amplification products were obtained from pure cultures of bacteria when viable counts were higher than 10^4 CFU/ml. Likewise, amplification products were detected from fish samples with viable counts of histamine producers higher than 10^4 to 10^5 CFU/g of fish tissue. In this study, fish DNA extractions were performed using commercial kits following confirmation (data not presented) that indicated equivalent success and similar sensitivity in PCR as guanidine isothiocyanate DNA extractions using a protocol previously applied to various fish samples (30). The lower sensitivity of PCR in this study compared to PCR of other bacteria of between 10^2 to 10^3 CFU/ml in pure cultures (10) is presumably because we used oligo-mixed primers to detect a wide range of histamine producers. Despite the moderately low sensitivity of our PCR, it was still possible to detect histamine producers via PCR before histamine was detected by HPLC in most of the fish samples tested (Table 2; Fig. 3).

The failure to amplify DNA from the strain of *C. braakii* was most likely due to the remarkable difference the HDC sequence, because hybridization of a DNA probe made from the amplification product of *M. morganii* also failed (data not shown). This strain was originally isolated as a histamine-producing strain from fish in routine screenings of histamine producers in our laboratory. This strain produced a moderate amount of histamine (310 mg/liter), which was about 1/10 of the activity of other powerful histamine producers, such as *M. morganii*, *R. planticola*, and *E. aerogenes* (Table 1). Several

FIG. 4. SSCP band patterns of PCR products of the *hdc* amplified from reference strains (A) and fish samples held at 30°C (B). Identification was performed by comparing the band patterns of the fish samples with those of the reference strains (from species database, only those used for identification of the fish samples are shown in this figure). Lanes: m, 100-bp ladder size marker; a, *E. amnigenus* strain 23a06; b, *M. morganii* strain 4b19; c, *M. morganii* strain AP28; d, *P. damsela* strain 6a20; e, *M. morganii* strain 30a04; f, *M. morganii* strain 5c15. Fish samples correspond to the same samples as in Table 2. Lanes: m, 100-bp ladder size marker; 1, tuna 1 (16 h); 2, tuna 1 (20 h); 3, tuna 3 (16 h); 4, tuna 3 (20 h); 5, tuna 4 (16 h); 6, tuna 4 (20 h); 7, tuna 5 (16 h); 8, tuna 5 (20 h); 9, tuna 7 (20 h); 10, tuna 7 (24 h); 11, sardine 1 (12 h); 12, sardine 1 (16 h); 13, mackerel (20 h); 14, young yellowtail (24 h); 15, horse mackerel (16 h); 16, horse mackerel (20 h); 17, yellowtail (16 h); 18, yellowtail (20 h). Identical band patterns were indicated by arrows.

strains of *C. braakii* have also been isolated by Kim et al. (28) as histamine producers from fresh and temperature-abused albacore. Further study is required to identify the HDC gene of this and other *C. braakii* strains.

No false-positive PCR results were found among 470 non-histamine-producing strains. These results suggest that, among

the non-histamine-producing bacteria we tested, none had either mutated or disrupted HDC genes. Thus, the reported strain or species specificity in the HDC activity (18, 43) is more likely the result of a large chromosomal deletion rather than minor mutation or disruption of HDC gene. The relationship between the dynamic deletion of large sections of DNA and

TABLE 2. Results of *hdc*-specific PCR and histamine analysis in various fish samples during storage at 30°C

Fish sample	Analysis	Result at incubation time (h)						
		0	4	8	12	16	20	24
Tuna 1	PCR	—	—	—	—	+	+	+
	Histamine ^a	ND ^b	ND	ND	ND	ND	16.5	148.5
Tuna 2	PCR	—	—	—	—	—	—	—
	Histamine	ND	ND	ND	ND	ND	ND	ND
Tuna 3	PCR	—	—	—	+	+	+	+
	Histamine	ND	ND	ND	ND	ND	77.6	297.7
Tuna 4	PCR	—	—	—	—	+	+	+
	Histamine	ND	ND	ND	ND	ND	96	307.4
Tuna 5	PCR	—	—	—	+	+	+	+
	Histamine	ND	ND	ND	ND	6	100.4	195.7
Tuna 6	PCR	—	—	—	—	—	—	—
	Histamine	ND	ND	ND	ND	ND	ND	ND
Tuna 7	PCR	—	—	—	—	—	+	+
	Histamine	ND	ND	ND	ND	ND	3.6	27.7
Sardine 1	PCR	—	—	+	+	+	+	+
	Histamine	ND	ND	ND	22.9	72.9	276.5	287
Sardine 2	PCR	—	—	—	—	—	—	—
	Histamine	ND	ND	ND	ND	ND	ND	ND
Mackerel	PCR	—	—	—	—	—	+	+
	Histamine	ND	ND	ND	ND	ND	10.4	41.3
Young yellowtail	PCR	—	—	—	—	—	+	+
	Histamine	ND	ND	ND	ND	ND	ND	12.7
Horse mackerel	PCR	—	—	—	—	+	+	+
	Histamine	ND	ND	ND	ND	5.7	29.5	40
Yellowtail	PCR	—	—	—	+	+	+	+
	Histamine	ND	ND	ND	ND	ND	7.3	9.6

^a Measured in mg/100 g.^b ND, not detected.

enzyme activity has been demonstrated in recent studies (9). Day et al. (9) demonstrated that in each species of *Shigella*, the chromosome containing the lysine decarboxylase gene (*cadA*) has been independently deleted during the evolution of shigellae from *E. coli* leaving lysine decarboxylase activity uniformly absent in *Shigella* strains but present in 90% of *E. coli* strains. Also, Kanki et al. (24) report that all the *R. planticola* (formerly *Klebsiella planticola*) strains produce histamine while all the *Klebsiella pneumoniae* strains do not. The authors confirmed by both PCR and Southern blot hybridization that all the strains of *K. pneumoniae* do not have the HDC gene.

Since both powerful and weak histamine producers in pure cultures were amplified by PCR (Table 1), fish with weak histamine production may be positively identified based on PCR assay while not producing large amounts of histamine. However, in this study, histamine accumulation always occurred when PCR amplification of *hdc* was positive (Table 2). Similar experiments were repeated, and no false-positive reactions were obtained (data not shown). The reasons why the PCR method did not produce false positive results is not clear, but it is known that HDC as well as other bacterial amino acid decarboxylase activity is induced by substrate availability as well as low pH (2). In fish flesh such as tuna and mackerel, large amounts of histidine are uniformly present (61). Hence, it is possible that in such conditions, either powerful histamine-producing bacteria dominate, or at least the populations of weak histamine producers cannot reach the detection limit of PCR. These data are in agreement with previous culture studies concluding that most of bacteria responsible for high levels

of histamine in fish stored at temperatures abuse almost exclusively belong to *Enterobacteriaceae* (27, 28, 37, 38, 51, 62). *M. morganii* is generally the most prevalent and potent histamine producer in fish (25, 27, 28, 37, 38, 40, 51). The histamine producers isolated in this study—*M. morganii*, *P. vulgaris*, *R. planticola*, and *P. damsela*—had been previously reported by other workers.

If characterization of HDC genes amplified by PCR were possible, we could identify contamination sources and species. Comparison of 16S rRNA sequences is currently the most powerful and accurate technique for determining phylogenetic relationships between microorganisms (67). However, the analysis of 16S rRNA sequences requires isolation of bacterial strains by conventional culture methods, which does not meet the goals of this study to develop a direct characterization method for gram-negative histamine-producing bacteria. The phylogenetic distance matrix tree of *hdc* shows that the *hdc* fragment evolved similarly to 16S rRNA gene (Fig. 2) and may provide a basis for identification and typing of gram-negative histamine producers. Along with three known bacterial HDCs—*M. morganii* (ATCC 35200) (64), *E. aerogenes* (ATCC 43175) (23), and *R. planticola* (ATCC 43176) (23)—partial HDC sequences from 11 strains (six species) contained the conserved sequence Ser-X-His-Lys in the reduced active site peptides from PLP-dependent HDC. These sequence similarities suggest that the 14 enzymes have evolved from a common ancestral protein with similar catalytic mechanisms. Unlike the 16S rRNA gene, which is necessary for the survival of the bacteria, some nonessential protein-coding genes were completely deleted in lineages at early stages of divergence (9). This is true for HDC, and it is well known that histamine production is specific to species or strain (18, 43). While the HDC gene cannot be used for the universal phylogenetic analysis of gram-negative bacteria, the partial *hdc* sequence is sufficient for identifying gram-negative histamine producers which have HDC, as the similar phylogenetic trees generated from 16S rDNA and partial *hdc* sequences suggest that HDC was present in the common ancestor of the domain *Bacteria*. This is in agreement with the findings of studies on the evolution of PLP-dependent decarboxylases (20, 42). Significant and extensive similarities among prokaryotic and eukaryotic PLP-dependent decarboxylases suggest an ancient and common origin for all PLP-dependent decarboxylases (20, 42).

Several authors have suggested that there is no correlation between 16S rRNA gene and protein-coding genes when the latter is more dependent on selection pressure (17) or had been horizontally transferred across groups at an earlier stage of divergence (7). However, we could not find evidence of the horizontal transfer of this gene based on the branching order of *hdc* in this study. Phylogenetic trees based on HDC genes from a larger number of gram-negative bacteria should further establish the evolutionary relationships and hierarchical order among important histamine producers.

In this study, SSCP analysis (49) was performed to differentiate the HDC genes amplified from fresh fish. This method is highly suitable for food testing laboratories without access to more sophisticated and costly typing methods, such as sequencing or Southern hybridization. Although multiple banding patterns were obtained within the same species (Table 3) in pure culture experiments, repeatability in PCR-SSCP was ob-

TABLE 3. Summary of SSCP results for representative strains of gram-negative histamine producers

Species	No. of strains tested	No. of SSCP types
<i>Morganella morganii</i>	13	7
<i>Klebsiella planticola</i>	9	3
<i>Enterobacter aerogenes</i>	2	2
<i>Enterobacter amnigenus</i>	1	1
<i>Erwinia</i> sp.	1	1
<i>Proteus vulgaris</i>	2	2
<i>Photobacterium damsela</i>	3	2
<i>Photobacterium phosphoreum</i>	4	2
<i>Hafnia alvei</i>	1	1
Total	36	21

served 100% of the time within the same strain. Thus, the variation in band patterns is due to the minute variation of *hdc* sequences within the same species. In the present study, we could differentiate by SSCP analysis strong histamine producers such as *M. morganii* from marine histamine producers such as *P. damsela* (29) or *P. phosphoreum* (14). We could match the band to a single reference species in 8 (6 identified as *M. morganii*, 1 identified as *E. amnigenus*, and 1 identified as *P. damsela* by SSCP analysis) out of 10 fish samples. In the samples analyzed, SSCP band patterns were identical with pure cultures and showed no extra bands (Fig. 4). Moreover, throughout the experiments, patterns obtained from samples did not change with incubation time (Fig. 4). These results suggest that a single species of histamine-producing bacteria is responsible for the accumulation of histamine in fish. Alternatively, multiple HDC genes may not have been amplified from the same fish samples due to the moderately low sensitivity of PCR (between 10^4 and 10^5 CFU histidine decarboxylating bacteria per g of fish samples). Unlike other SSCP methods using universal primers amplifying 16S rDNA of multiple bacteria (58), our PCR is highly specific, amplifying only bacteria with HDC. Two samples that could not be identified by SSCP analysis were successfully sequenced by direct sequencing, suggesting that the failure of identification was not due to the amplification of multiple HDC genes, but due to the lack of reference sequences. For future identifications, more reference strains should be included in the PCR-SSCP analysis of HDC gene. Overall, the probability of simultaneously amplifying the DNA of HDCs of multiple bacteria from a single fish sample seems to be low, although this possibility cannot be ruled out. When more complex patterns are obtained by PCR-SSCP analysis due to amplification of multiple HDC genes, denaturing gradient gel electrophoresis (47) could be applied before SSCP analysis.

The results of this study support the practical application of PCR-SSCP analysis of HDC gene. It has been well documented that the histamine is a health hazard if present in fish muscle at levels higher than 50 mg/100 g (34). Although standards or guidelines for permissible concentrations of histamine in fish products have not been established in Japan, the U.S. Food and Drug Administration sets the limit at 5 mg of histamine/100 g of muscle for scombroid fish (13). While many diverse analytical procedures have been published for histamine

in unprocessed and canned fish (34), many studies (26, 27, 39) have shown that detectable amounts of histamine accumulated only after fish were completely decomposed or after aerobic plate counts reached $>10^7$ CFU/g in fish muscle. This was also confirmed in our study (Fig. 3B). Histamine has been shown to accumulate only in the late stationary phase of bacteria in pure cultures in this study (Fig. 3A) as well as in other studies (18, 25, 31). Therefore, the ability to detect histamine producers before histamine accumulates will be an important tool for hazard analysis and critical control point monitoring in fish processing plants. In our protocol, preenrichment steps are not necessary making it is possible to detect the presence of gram-negative histamine producers at the level of 10^4 to 10^5 CFU per g of fish tissue from samples taken from the processing line within 4 h.

An intrinsic disadvantage of PCRs is the detection of non-viable cells (44). The ability to distinguish between viable and nonviable organisms is crucial when PCR is used for risk assessment of histamine accumulation such as in raw fish processing plant. Although microorganisms are most likely alive in such processing plants, we may overestimate the risk when frozen fish meat is used for processing. Different workers (3, 11, 15) have showed that freezing inactivates gram-negative histamine producers. On the other hand, the ability of PCR to detect dead microorganisms may be beneficial in frozen, salted, or smoked fish sample when we attempt to identify the species responsible for histamine accumulated before such processing treatments that killed the histamine-producing bacteria. This can help us identify the cause of contamination and limit contamination to the lowest possible level during processing.

This study is the first step in developing the molecular methods for detecting and identifying gram-negative histamine producers. The molecular information presented here opens the way to further studies on developing more sophisticated molecular methods for detecting and identifying these bacteria.

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