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# Isolation and Characterization of Histamine-Producing Bacteria from Fermented Fish Products

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**Histamine is mainly produced by microorganisms that are found in fermented foods, and is frequently involved in food poisoning. Two histamine-producing bacteria were isolated from fermented fish products, anchovy sauce, and sand lance sauce by using a histidine decarboxylating medium. The species were identified as *Bacillus licheniformis* A7 and *B. coagulans* SL5. Multiplex PCR analysis showed the presence of the conserved histidine decarboxylase (*hdc*) gene in the chromosome of these bacteria. *B. licheniformis* A7 and *B. coagulans* SL5 produced the maximum amount of histamine (22.3±3.5 and 15.1±1.5 mg/L, respectively). As such, they were determined to be potential histamine-producing bacteria among the tested cultures.**

***Keywords***: histamine, histidine decarboxylase gene (*hdc*), multiplex PCR, *Bacillus licheniformis*, *Bacillus coagulans*

Biogenic amines (BAs) are low molecular weight nitrogenous compounds that are mainly formed by amino acid decarboxylation (Brink *et al*., 1990; Halász *et al*., 1994). BAs most frequently involved in food intoxication is histamine, produced from the precursor amino acids histidine by the histidine decarboxylase (*hdc*) (Santos, 1996). Histamine is produced and degraded as part of normal cellular metabolism; however, high intake may cause vasoactive or psychoactive health problems, including cutaneous (primarily facial flush), gastrointestinal, haemodynamic (hypotension) and/or neurologic symptoms (Rice *et al*., 1976; Brink *et al*., 1990; Halász *et al*., 1994). It is also of concern because they are an index of hygienic quality; the presence of high levels of histamine in food is indicative of microbial spoilage (Roig-Sagúes *et al*., 2002). Histamine is synthesized in a variety of fermented foods, including fish (Moon *et al*., 2010b), meat (Maijala *et al*., 1993), dairy (Stratton *et al*., 1991), soybean products (Chin and Koehler, 1986), wine (Lehtonen *et al*., 1992), beer (Dumont *et al*., 1992), and vegetables (Taylor *et al*., 1978).

*Aekjeot* and *Jeotkal* are respectively fermented fish-sauce and fish-paste produced through the fermentation of various fishes by salts and naturally occurring microorganisms. They have been consumed for centuries in Korea as protein sources and seasoning ingredient. Those sauces and pastes contain relatively high concentrations of amino acids degraded from fishes and may be a source for histamine synthesis. The presence of histidine decarboxylase activity has been described in different microbial groups, such as *Pseudomonas* (López-Sabater *et al*., 1994), *Vibrios* (Niven *et al*., 1981), sporulated microorganisms (Rodriguez-Jerez *et al*., 1994), and lactic acid bacteria (Joosten and Nunez, 1996). Despite this, insufficient research data is available on the growth of histamine-producing microorganisms in fermented fish products.

This study was performed to isolate and characterize histamine-producing bacteria in Korean fermented fish foods. In total, 24 samples of fermented fish products, which included 8 anchovy sauces, 8 sand lance sauces, 4 squid pastes, 2 clam pastes, and 4 shrimp pastes, were purchased from retail stores. All fermented fish products were immediately transported to the laboratory and kept at 4°C before analysis. Each sample (1 g) was mixed with 9 ml of sterile physiological saline [0.85% (w/v) NaCl], homogenized in a stomacher (AES Laboratoire, France) for 2 min, and then further diluted in saline at 1:10 dilution. The diluted sample solutions were spread on HD medium (Niven *et al*., 1981) to qualitatively assess the histamine-producing capacity of the bacterial strains. The HD medium contained 0.125% tryptone, 0.125% yeast extract, 0.75% $(NH_4)_2SO_4$, 0.5% NaCl, 0.1% glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot 4H_2O$, 0.004% $FeSO_4 \cdot 7H_2O$, 0.05% Tween 80, 0.02% cresol red, 3% agar, and 0.5% (w/v) of L-histidine monohydrochloride (Sigma Chemical Co., USA). The plates were incubated at 25°C for 7 days under anaerobic and aerobic conditions. After 7 days of incubation, halos around colonies were formed on the HD medium. Two Gram-positive, rod-shaped organisms were isolated: A7 from anchovy sauce and SL5 from sand lance sauce.

To confirm the presence of specific genes in these isolated

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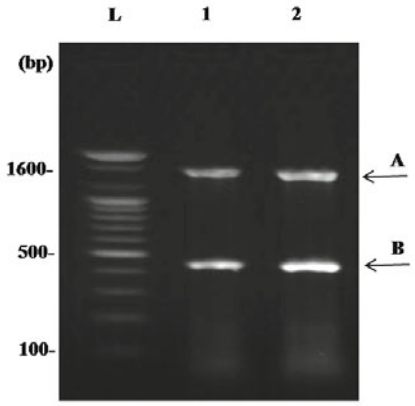


**Fig. 1. Multiplex PCR-based detection of *hdc*$^+$ strains**. Lanes: L, ladder; 1, A7 isolate obtained from anchovy sauce; 2, SL5 sand lance sauce; (A) 16S rRNA (1520 bp) internal control; (B) histidine decarboxylase gene (*hdc*, 441 bp).

organisms, multiplex PCR analysis was performed for the 2 isolates using procedures described by Coton and Coton (2005). Genomic DNA was isolated from 2-mL cultures using the Genomic DNA Extraction kit (Bioneer, Korea). For the immediate detection of the *hdc* and 16S rRNA genes, 2 sets of primers were used: HDC3 (5′-GATGGTATTGTTTCKT ATGA-3′) and HDC4 (5′-CAAACACCAGCATCTTC-3′) for *hdc* gene fragments, the universal primers 27F (5′-AGAG TTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCT TGTTACGACTT-3′) for 16S rRNA genes (Lane, 1991). The multiplex PCR experiments were performed using the iQ5 thermal cycler (Bio-Rad, USA) using the following program: 95°C for 5 min, 32 cycles of 95°C for 45 sec, 48°C for 45 sec, and 72°C for 75 sec, and a final extension of 72°C for 5 min. PCR reactions performed on DNA from the isolates showed an amplified band of 441 bp (Fig. 1, band B), which corresponds to an *hdc* gene fragment. Biochemical tests of A7 and SL5 isolates were also carried out using the API 50CHB/E test kit (Biomerieux, USA), and the results indicated that the A7 isolate had 97% identity with *Bacillus licheniformis*, while the SL5 isolate had 96% identity with *Bacillus coagulans.* Subsequently, genetic identification of the strains was performed by sequencing the 16S rRNA and *hdc* genes of the 2 isolates. Species identification of the isolates was accomplished by comparing the isolated sequences with those of related reference strains in the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp) using the Fasta program. The sequence of the A7 isolate showed 99% homology to *B. licheniformis*, and the sequence of the SL5 isolate showed 99% homology to *B. coagulans*. The 16S rRNA gene sequences of strains A7 and SL5 have been deposited in the DDBJ under accession numbers AB553280 and AB557590, respectively. In addition, the partial *hdc* sequences of *B. licheniformis* A7 and *B. coagulans* SL5 were deposited in the DDBJ under accession numbers AB553282 and AB553281, respectively.

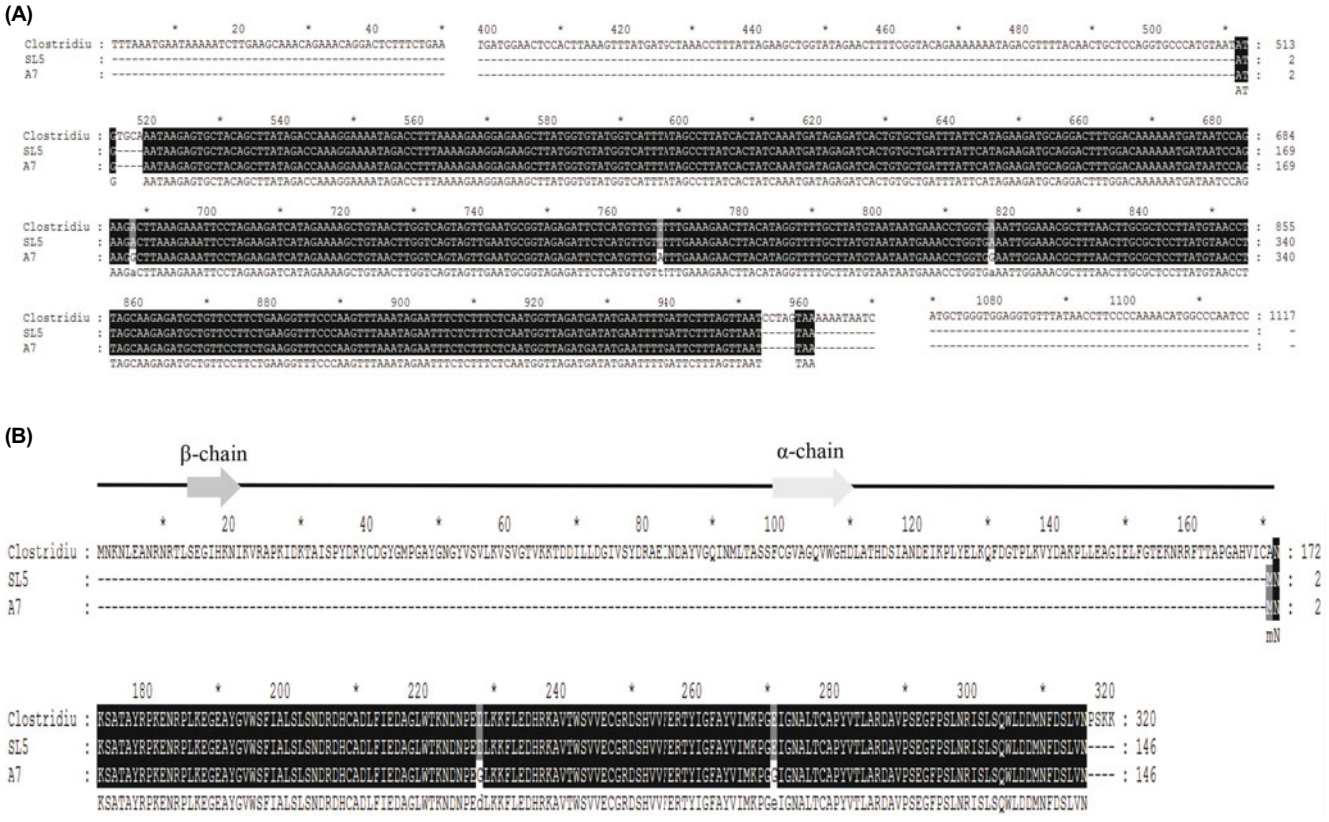


**Fig. 2. Partial alignments of *hdc* gene sequences (A) and amino acids (B) of *B. licheniformis* A7, *B. coagulans* SL5, and *C. perfringens* using the GeneDoc program.**

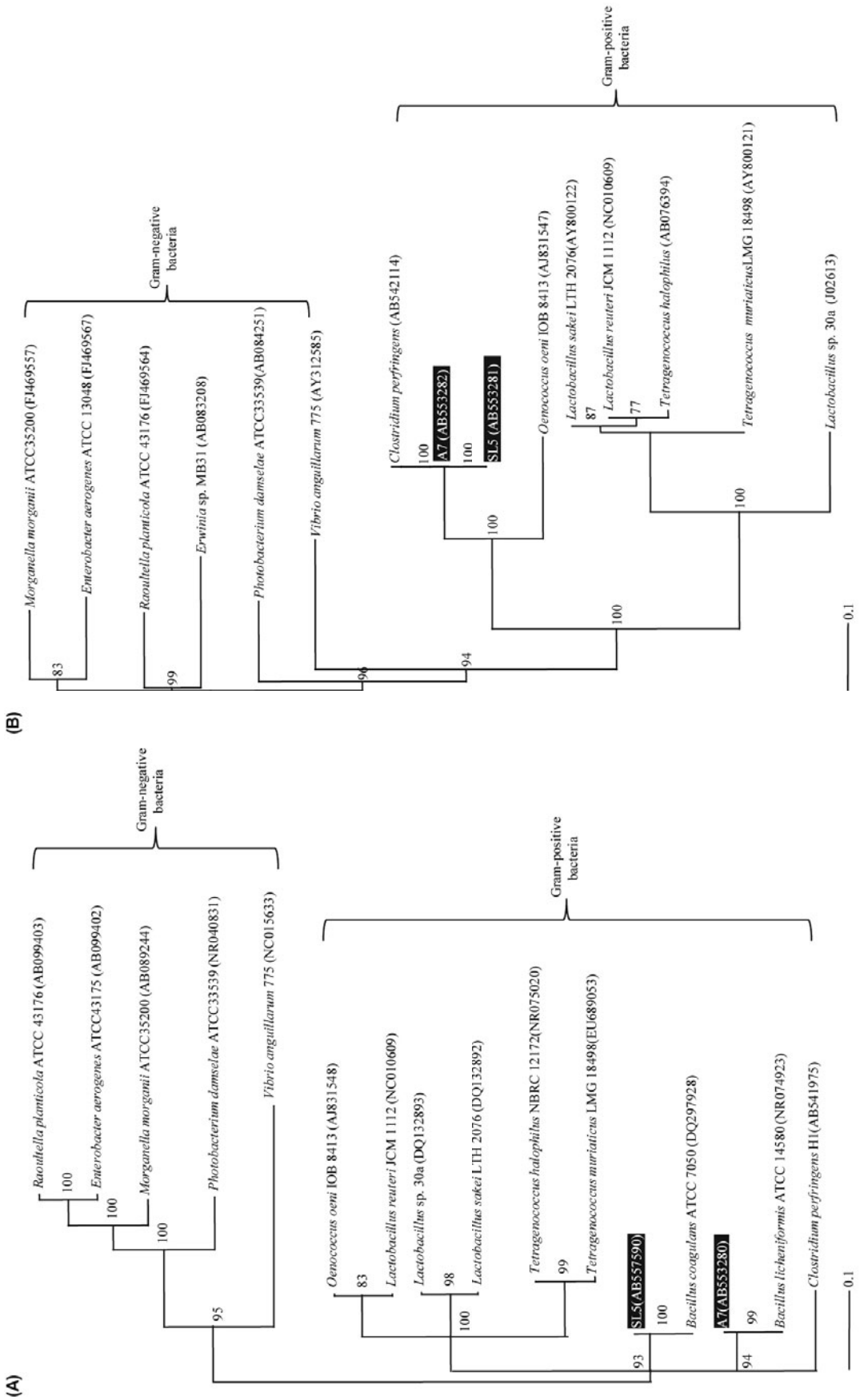


**Fig. 3. Phylogenetic trees of strains of histamine producers based on 16S rRNA gene sequences (A) and partial *hdc* gene sequences (B).** The trees were constructed using the neighbor-joining method. The genetic distances were calculated using the Kimura's two-parameter method. Each bar represents 10% estimated phylogenetic divergence. The corresponding bootstrap values (100 replications) are shown on each branch.

Sequence analysis of amino acids showed that 2 *hdc* partial genes from isolates were similar to the previously reported pyruvoyl-dependent *hdc* of *Clostridium perfringens* ATCC 13124 (Fig. 2A). Notably, the amplification of 2 *hdc* partial genes from the isolates was located at the N-terminus of the α-chain. The above result shows that a molecular method using PCR can be used for detection and identification of histamine-producing bacteria instead of or simultaneously with the conventional culture technique.

Phylogenetic trees of the isolated strains based on the newly determined sequences of partial *hdc* genes and 16S rRNA sequences were compared (Fig. 3A and 3B). The bootstrap scores observed for all the nodes are indicated. The phylogenetic tree of the *hdc* genes show that the A7 and SL5 isolates are genetically closely related with *C. perfringens* ATCC 13124 and *Oenococcus oeni* IOB8413 (Fig. 3B). Overall, the phylogenetic tree determined by the partial *hdc* gene was similar to that determined by the 16S rRNA sequence. In both trees, groups of Gram-negative and Gram-positive strains showed high bootstrap values (99% and 98% on 16S rRNA and partial *hdc* gene trees, respectively). The phylogenetic distance matrix tree of *hdc* showed that the *hdc* fragment evolved similarly to the 16S rRNA gene and may provide a basis for identification of Gram-positive histamine producers. Along with three known bacterial *hdc-Tetragenococcus muriaticus* LMG 18498(EU689053), *O. oeni* IOB 8413(AJ831548), *Lactobacillus* 30a (DQ 132893), and *C. perfringens* ATCC 13124-partial *hdc* (J02880) sequences from the 4 strains contained the conserved sequence α-chain (Phe-Cys-Gly-X-Val-Ala-Gly-Gln) in the pyruvoyl group (detected as glutamine). These sequence similarities suggest that the 4 enzymes have evolved from a common ancestral protein with similar catalytic mechanisms. While the *hdc* gene cannot be used for the universal phylogenetic analysis of gram-positive bacteria, the partial *hdc* sequence is sufficient for identifying gram-positive histamine producers that have *hdc*, similar to the phylogenetic trees generated from 16S rRNA. These data are in agreement with the findings of studies on the evolution of pyruvoyl-dependent decarboxylases (Konagaya *et al.*, 2002). Significant and extensive similarities of pyruvoyl-dependent decarboxylases suggest an ancient and common origin for all pyruvoyl-dependent decarboxylases.

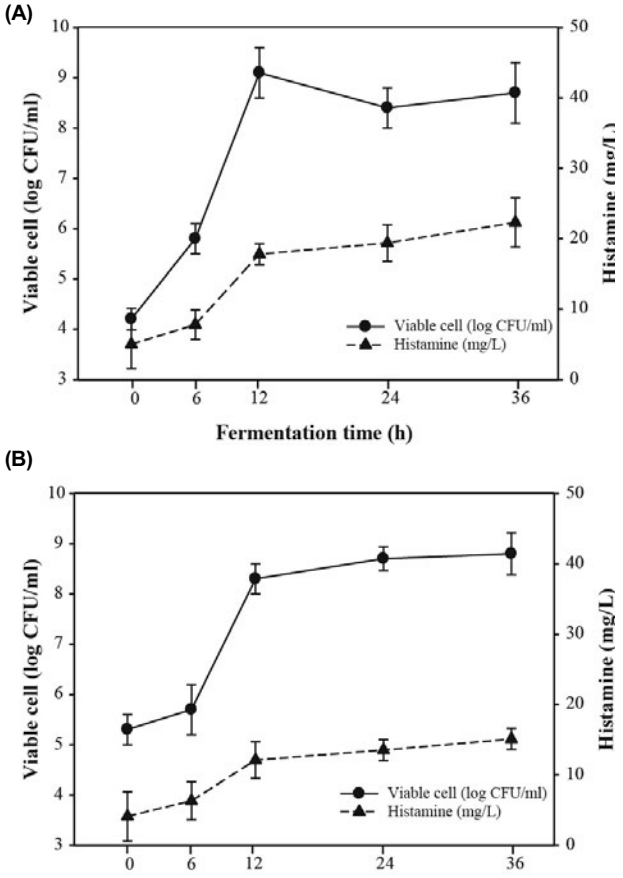


**Fig. 4. Synthesis of (A) histamine by *B. licheniformis* A7 and (B) histamine by *B. coagulans* SL5 in decarboxylating broth containing 0.5% histidine.**

Histamine production ability of the isolated microbial cells was confirmed via qualitative and quantitative assays as shown in Fig. 4. The isolates, *B. licheniformis* A7 and *B. coagulans* SL5, were cultivated in decarboxylating medium containing 0.5% histidine, and the HPLC unit (Waters 2695) equipped with a Waters 2996 photodiode array detector was utilized for histamine analysis (Garcia-Garcia *et al.*, 2000). After 36 h of cultivation, A7 and SL5 isolates produced the maximum amounts of histamine (22.3±3.5 mg/L and 15.1 ±1.5 mg/L, respectively). Meanwhile, *Clostridium perfringens* H1, which was cultured as a positive control for histamine production (Moon *et al.*, 2010a) produced 25.4±2.8 mg/L, while *Leuconostoc mesenteroides* ATCC 8293, which is a negative control for histamine production, produced no detectable amount of histamine.

It is well known that *Bacillus* species (often *Bacillus subtilis* spp.) continue to be the dominant microbial participant in food fermentations, particularly for protein-rich soya ; they contribute to important biochemical changes, pH, and flavor development. While the occurrence of *B. cereus* in foods at numbers of $10^3$–$10^5$ CFU/g or mL is considered unsafe due to the ability of *B. cereus* to cause food poisoning, *B. subtilis* and *B. licheniformis* generally are regarded as safe by European Food Safety Authority (EFSA, 2005). However, food-borne *B. licheniformis* outbreaks have been reported to be predominantly associated with cooked meats and vegetables (Rosenkvist and Hansen, 1995), and a toxin-producing *B. licheniformis* was isolated from raw milk and baby food products (Salkinoja-Salonen *et al.*, 1999).

*B. coagulans* is often used in veterinary applications, especially as a probiotic in pigs, cattle, poultry, and shrimp. There are many references to the use of this bacterium in humans, especially for improving vaginal flora, improving abdominal pain and bloating in Irritable Bowel Syndrome (IBS) patients, and increasing the immune response to viral challenges (Hun *et al.*, 2009). Our results indicate that *B. licheniformis* and *B. coagulans* are potential histamine producers. Potential histamine-producing bacteria have been isolated from various salted fish products, including salted sardines, salted Spanish anchovies, and fish sauce; they were *Staphylococcus* spp., *Vibrio* spp., and *Pseudomonas* III/IV-NH (Yatsunami and Echigo, 1991, 1992), *S. epidermidis*, *S. xylosus*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *P. cepaciae*,

and *Bacillus* spp. (Rodriguez-Jerez *et al*., 1994; Hernandez-Herrero *et al*., 1999), and *T. muriaticus*, halophilic lactic acid bacterium (Kimura *et al*., 2001).

The existence of contaminating microorganisms in fermented foods may be considered an indicator of the quality or grade of sanitation of the raw material applied during food production (Halász *et al*., 1994). Foods can also be contaminated during raw material preparation, particularly during the natural incubation period. These data suggest that safe starter strains and hygienic conditions should be used for the production of fermented fish products. The starter strains frequently used in commercial products should be tested for the absence of *hdc* genes in their genome and the inability to produce histamine during growth in amino acid-rich medium. Application of the culture-dependent and independent methods used in this study will permit broad monitoring of histamine-producing bacteria in various fermented foods. 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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## References

**Brink, B., Damirik, C., Joosten, H.M.L.J., and Huis in't Veld, J.H.J.** 1990. Occurrence and formation of biologically active amines in foods. *Int. J. Food Microbiol.* **11**, 73–84.

**Chin, K.D.H. and Koehler, P.E.** 1986. Effect of salt concentration and incubation temperature on formation of histamine, phenethylamine, tryptamine and tyramine during miso fermentation. *J. Food Prot.* **49**, 423–427.

**Coton, E. and Coton, M.** 2005. Multiplex PCR for colony direct detection of Gram-positive histamine- and tyramine-producing bacteria. *J. Microbiol. Methods* **63**, 296–304.

**Dumont, E., De Geeter, H., and Huyghebaert, A.** 1992. Presence and formation of biogenic amines in local Belgian beers. *Med. Fac. Landbauww. Univ. Gent.* **57**, 423–427.

**EFSA.** 2005. Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs. *EFSA J.* **175**, 1–48.

**Garcia-Garcia, P., Brenes-Balbuena, M., Hornero-Mendez, D., Garcia-Borrego, A., and Garrido-Fernandez, A.** 2000. Content of biogenic amines in table olives. *J. Food Prot.* **63**, 111–116.

**Halász, A., Baráth, A., Simon-Sarkadi, L., and Holzapfel, W.** 1994. Biogenic amines and their production by microorganisms in food. *Trends Food Sci. Technol.* **5**, 42–49.

**Hernandez-Herrero, M.M., Roig-Sagues, A.X., Rodriguez-Jerez, J.J., and Mora-Ventura, M.T.** 1999. Total volatile basic nitrogen and other physicochemical and microbiological characteristics as related to ripening of salted anchovies. *J. Food Sci.* **64**, 344–347.

**Hun, L.** 2009. *Bacillus coagulans* significantly improved abdominal pain and bloating in patients with IBS. *Postgrad. Med.* **121**, 119–124.

**Joosten, H. and Nunez, M.** 1996. Prevention of histamine formation in cheese by bacteriocin-producing lactic acid bacteria. *Appl. Environ. Microbiol.* **62**, 1178–1181.

**Kimura, B., Konagaya, Y., and Fujii, T.** 2001. Histamine formation by *Tetragenococcus muriaticus*, a halophilic lactic acid bacterium isolated from fish sauce. *Int. J. Food Microbiol.* **70**, 71–77.

**Konagaya, Y., Kimura, B., Ishida, M., and Fujii, T.** 2002. Purification and properties of a histidine decarboxylase from *Tetragenococcus muriaticus*, a halophilic lactic acid bacterium. *J. Appl. Microbiol.* **92**, 1136–1142.

**Lane, D.J.** 1991. 16S/23S rRNA sequencing, pp. 115–175. *In* Stackbrandt, E. and Goodfellow, M. (eds.), Nucleic acid techniques in bacterial systemmatics. Wiley, Chichester, UK.

**Lehtonen, P., Saarinen, M., Vesanto, M., and Riekkola, M.L.** 1992. Determination of wine amines by HPLC using automated precolumn derivatisation with o-phthalaldehyde and fluorescetree detection. *Z. Lebensm. Unters. Forsch.* **194**, 434–437.

**López-Sabater, E.I., Rodríguez-Jerez, J.J., Hernández-Herrero, M., and Morana-Ventura, M.T.** 1994. Evaluation of histidine decarboxylase activity of bacteria isolated from sardine (Sardina pilchardus) by an enzymic method. *Lett. Appl. Microbiol.* **19**, 70–75.

**Maijala, R.L., Eerola, S.H., Aho, M.A., and Hirn, J.A.** 1993. The effect of GDL-induced pH decrease on the formation of biogenic amines in meat. *J. Food Prot.* **56**, 125–129.

**Moon, J.S., Cho, S.K., Choi, H.Y., Kim, J.E., Kim, S.Y., Cho, K.J., and Han, N.S.** 2010a. Isolation and characterization of biogenic amine-producing bacteria in fermented soybean pastes. *J. Microbiol.* **48**, 257–261.

**Moon, J.S., Kim, Y.J., Jang, K.I., Cho, K.J., Yang, S.J., Yoon, G.M., Kim, S.Y., and Han, N.S.** 2010b. Analysis of biogenic amines in fermented fish products consumed in Korea. *Food Sci. Biotechnol.* **19**, 1689–1692.

**Niven, C.F. Jr., Jeffrey, M.B. Jr., and Corlett, D.A. Jr.** 1981. Differential plating medium for quantitative detection of histamine-producing bacteria. *Appl. Environ. Microbiol.* **41**, 321–322.

**Rice, S.L., Eitenmiller, R.R., and Koehler, P.E.** 1976. Biologically active amines in foods. A review. *J. Milk Food Technol.* **39**, 353–358.

**Rodriguez-Jerez, J.J., Mora-Ventura, M.T., Lopez-Sabater, E.I., and Hernandez-Herrero, M.M.** 1994. Histidine, lysine and ornithine decarboxylase bacteria in spanish salted semi-preserved anchovies. *J. Food Prot.* **57**, 784–787.

**Roig-Sagúes, X., Molina, A.P., and Hernándes-Herrero, M.M.** 2002. Histamine and tyramine-forming microorganisms in Spanish traditional cheeses. *Eur. Food Res. Technol.* **215**, 96–100.

**Rosenkvist, H. and Hansen, Å.** 1995. Contamination profiles and characterization of *Bacillus* species in wheat bread and raw materials for bread production. *Int. J. Food Microbiol.* **26**, 353–363.

**Salkinoja-Salonen., M.S., Vuorio, R., Andersson, M.A., Kämpfer, P., Andersson, M.C., Honkanen-Buzalski, T., and Scoging, A.C.** 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. *Appl. Environ. Microbiol.* **65**, 4637–4645.

**Santos, M.H.S.** 1996. Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* **29**, 213–231.

**Schallmey, M., Singh, A., and Ward, O.P.** 2004. Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* **50**, 1–17.

**Stratton, J.E., Hutkins, R.W., and Taylor, S.L.** 1991. Biogenic amines in cheese and other fermented foods: a review. *J. Food Prot.* **54**, 460–470.

**Taylor, S.L., Leatherwood, M., and Lieber, E.R.** 1978. Histamine in sauerkraut. *J. Food Sci.* **43**, 1030–1032.

**Yatsunami, K. and Echigo, T.** 1991. Isolation of salt tolerant histamine-forming bacteria from commercial rice-bran pickle sardine. *B. Jpn. Soc. Sci. Fish* **57**, 1723–1728.

**Yatsunami, K. and Echigo, T.** 1992. Occurrence of halotolerant and halophili histamine-forming bacteria in red meat fish products. *B. Jpn. Soc. Sci. Fish* **58**, 515–520.