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Isolation and Characterization of Biogenic Amine-Producing Bacteria in Fermented Soybean Pastes

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Biogenic amines (BAs) are produced primarily by microorganisms found in fermented foods and are often implicated in food poisoning. BA-producing bacteria found in fermented soybean pastes were isolated and characterized using a decarboxylating medium and multiplex PCR analysis. Two BA-producing bacteria were isolated from traditional soybean pastes: one was a histamine-producing *Clostridium* strain, and the other was a tyramine-producing *Pseudomonas* strain. The *Clostridium* strain was determined to be a potent histamine producer among the cultures tested. Synthesis of tyramine by *Pseudomonas* sp. T1 was observed for the first time in this study.

***Keywords*:** biogenic amines, histamine, tyramine, multiplex PCR, *Clostridium* sp., *Pseudomonas* sp.

Biogenic amines (BAs) are primarily produced via decarboxylation of amino acids by substrate-specific enzymes generated by microorganisms found in fermented foods (Brink *et al*., 1990; Halász *et al*., 1994). Histamine and tyramine are the most frequently studied BAs because of their implication in food poisoning (Santos, 1996). Histamine and tyramine are produced and degraded as part of normal cellular metabolism; however, intake of these BAs in high quantities can cause vasoactive and psychoactive health problems, including headache, nausea, hot flushes, respiratory distress, sweating, heart palpitation, bright red rash, burning sensations in the mouth, and hyper- or hypotension (Rice *et al*., 1976; Brink *et al*., 1990; Halász *et al*., 1994; Shalaby, 1996). Microorganisms with decarboxylase activity are considered either starter (Fernández-García *et al*., 1999) or contaminating microorganisms in food fermentation processes (Roig-Sagúes *et al*., 2002).

Most fermented foods are subjected to conditions that enable BA synthesis. The amount of the different amines formed is highly dependent on the nature of the foods and the microorganisms present in these foods (Brink *et al*., 1990). BAs are present in a wide range of fermented food products; for example, fish (Shalaby, 1996), meat (Maijala *et al*., 1993), dairy (Stratton *et al*., 1991), and soybean products (Chin and Koehler, 1986), as well as wine (Lehtonen *et al*., 1992), beer (Dumont *et al*., 1992), and vegetables (Taylor *et al*., 1978). Soybean paste, or *doenjang*, is a traditional Korean food produced through the fermentation of soybeans by naturally occurring bacteria and fungi, and has been consumed for centuries as a protein source and flavoring ingredient in Korea. This paste contains a relatively high concentration of amino acids degraded from soybeans and may be a source for BA formation. Decarboxylase activity has been described in several microbial groups, including *Bacillus*, *Citrobacter*, *Clostridium*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Photobacterium*, *Lactobacillus*, *Pediococcus*, and *Streptococcus* (Rice *et al*., 1976; Brink *et al*., 1990; Huis in't Veld *et al*., 1990). Despite this, very little information is available on the growth of BA-producing microorganisms in soybean paste.

The aim of this study was to isolate and characterize BA-producing bacteria in traditional Korean fermented soybean pastes. Five fermented soybean paste products were used; among them, three (A1-A3) were factory-made products purchased from a local market, and two (A4 and A5) were made by traditional procedures. A small amount of each paste sample (5 g) was mixed with 9 ml of sterile physiological saline [0.85% (w/v) NaCl], homogenized in a stomacher (Easy Blender, AES, France) for 2 min and then further diluted in physiological saline at 1:10 dilutions. The diluted sample solutions were spread on a decarboxylating agar medium (Niven *et al*., 1981; Mah *et al*., 2001) to qualitatively assess the BA-producing capacity of the bacterial strains. The decarboxylating agar medium contained 0.125% (w/v) tryptone,

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Table 1. Primers used in multiplex PCR analysis

Target gene[a]	Primer name	Sequence (5′→3′)	Amplicon size (bp)	Reference
hdc	HDC3(F)	GATGGTATTGTTTCKTATGA	436	Coton and Coton (2005)
	HDC4(R)	CAAACACCAGCATCTTC		
tyrdc	TD2(F)	ACATAGTCAACCATRTTGAA	1182	Coton *et al*. (2004)
	TD5(R)	CAAATGGAAGAAGAAGTAGG		
16S rRNA	27F(F)	AGAGTTTGATCCTGGCTCAG	~1500	Lane (1991)
	1492R(R)	GGTTACCTTGTTACGACTT		

[a] *hdc*, histidine decarboxylase; *tyrdc*, tyrosine decarboxylase

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% of the corresponding precursor amino acids (tyrosine disodium salt or histidine monohydrochloride). The pH of the medium was adjusted to 5.3, and it was sterilized at 121°C for 10 min. The plates were incubated at 25°C for 7 days under anaerobic or aerobic conditions. During this time, halos formed around dozens of colonies on either the histamine medium or tyramine medium on a yellowish background. The colonies of these halos were transferred to new plates, and the morphology of the subsequent colonies was observed under a microscope following Gram staining. All colonies from the histamine medium showed a Gram-positive rod-shaped organism; all colonies from the tyramine medium showed a Gram-negative rod-shaped organism.

To confirm the existence of specific genes in these organisms, multiplex PCR analysis was performed for two isolates, utilizing the procedures described by Coton and Coton (2005). Purified genomic DNA from the individual colonies was used as a template for this process. Genomic DNA was isolated from 2-ml cultures by using the Genomic DNA Extraction kit (Bioneer, Korea) according to the manufacturer's instructions. For detection of specific genes, three sets of primers were used (Table 1). PCR mixtures were prepared using a 2× Multiplex mix (Solgent, Korea) and primer concentrations of 10 pmol for the HDC3, HDC4, TD2, and TD5 primers and 5 pmol for the 27F and 1492R primers. The amplification program was as follows: 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. All multiplex experiments were performed using the Thermal Cycler (iQ5 cycler; Bio-Rad, USA). PCR samples were analyzed using agarose gel electrophoresis; 1% (w/v) agarose gels (Duchefa Biochemie, Netherlands) with 1× TBE buffer were run at 100 V for 40 min, and DNA bands were then visualized with ethidium bromide staining (Fig. 1). PCR reactions performed on DNA from the H1 isolate from the histamine-detection medium showed an amplified band of 436 bp (Fig. 1, band C), which corresponds to an *hdc* gene fragment (see positive control, HC lane, Fig. 1); PCR reactions performed on DNA from the T1 bacterial isolate showed a band of 1,182 bp (Fig. 1, band B), corresponding to the *tyrdc* gene (Fig. 1, see positive control, TC lane). *Oenococcus oeni* and *Enterococcus faecalis* were also used as positive controls, since they are known to possess the *hdc* (Coton *et al*., 1998) and *tyrdc* genes (Coton *et al*., 2004), respectively. PCR analysis of *O. oeni* and *E. faecalis* resulted in amplification of the expected lengths of DNA bands (about 440 bp and 1,100 bp, respectively).

Biochemical testing of the H1 isolate with the API 20A kit (bioMérieux, USA) indicated that the H1 isolate had 99% identity with *Clostridium perfringens*; the same test using the API 20NE kit for Gram-positive anaerobic bacteria had no significant database match for the T1 isolate. Subsequently, genetic identification of strains was performed by sequencing 16S rRNA and the *hdc* and *tyrdc* genes of two isolates. Species identification of the isolates was accomplished by comparing the isolate sequences with the sequences 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 histamine-producing bacterium (H1 isolate) showed 99% homology to *C. perfringens* ATCC 13124, and the sequence of the tyramine-producing bacterium (T1 isolate) showed 99% homology to *Pseudomonas entomophila*. The 16S rRNA gene sequences of strains H1 and T1 have been deposited in DDBJ under accession numbers AB541974 and AB541975, respectively. Next, for construction of the phylogenetic tree, the 16S rRNA gene sequences of the isolates and closely related type cultures were aligned using CLUSTAL X software (Thompson *et al*., 1997). The evolutionary distance was computed using Kimura's two-parameter method (Kimura, 1980), and the phylogenetic tree was constructed with the PHYLIP program (Felsenstein, 1993) using neighbor-joining (Saitou and Nei, 1987). The tree presented in Fig. 2 shows the relationship

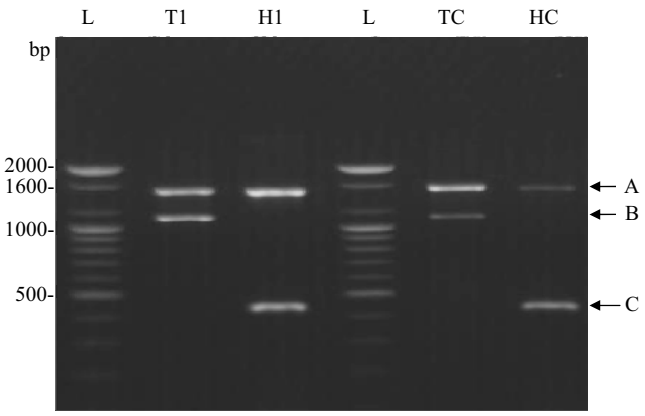

Fig. 1. Multiplex PCR-based detection of $hdc^+/tyrdc^+$ strains isolated from fermented soybean paste. Lanes: L, ladder (100 bp); T1, T1 isolate; H1, H1 isolate; TC, tyramine-producing strain (positive control), *E. faecalis* KCCM 11729; HC, histamine-producing strain (positive control), *O. oeni* KCTC 3927. (A) 16S rRNA (~1,500 bp), (B) *tyrdc* (1,180 bp), (C) *hdc* (436 bp).

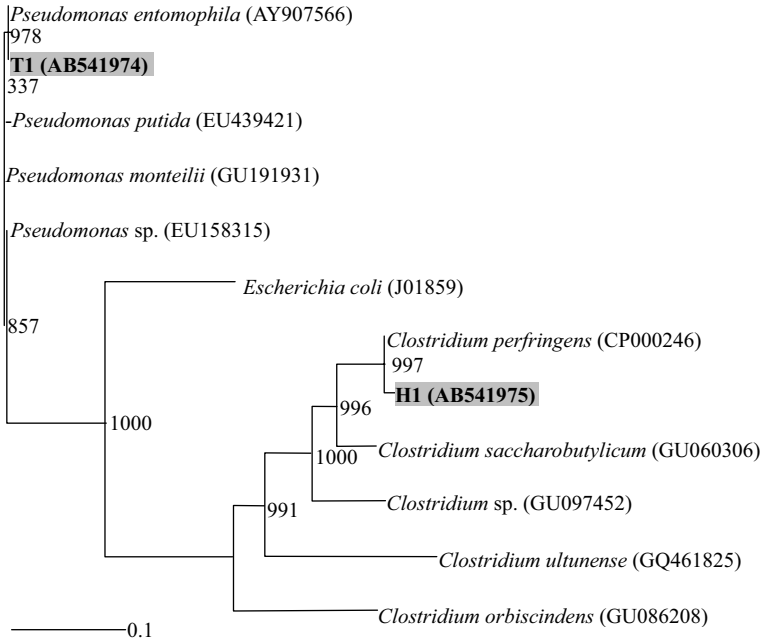

Fig. 2. Neighbor-joining phylogenetic tree of BA-producing bacteria based on the 16S rRNA gene sequences. Each bar represents 10% estimated phylogenetic divergence. The corresponding bootstrap values (1,000 replications) are shown on each branch. *E. coli* (J01859) was used as the outgroup.

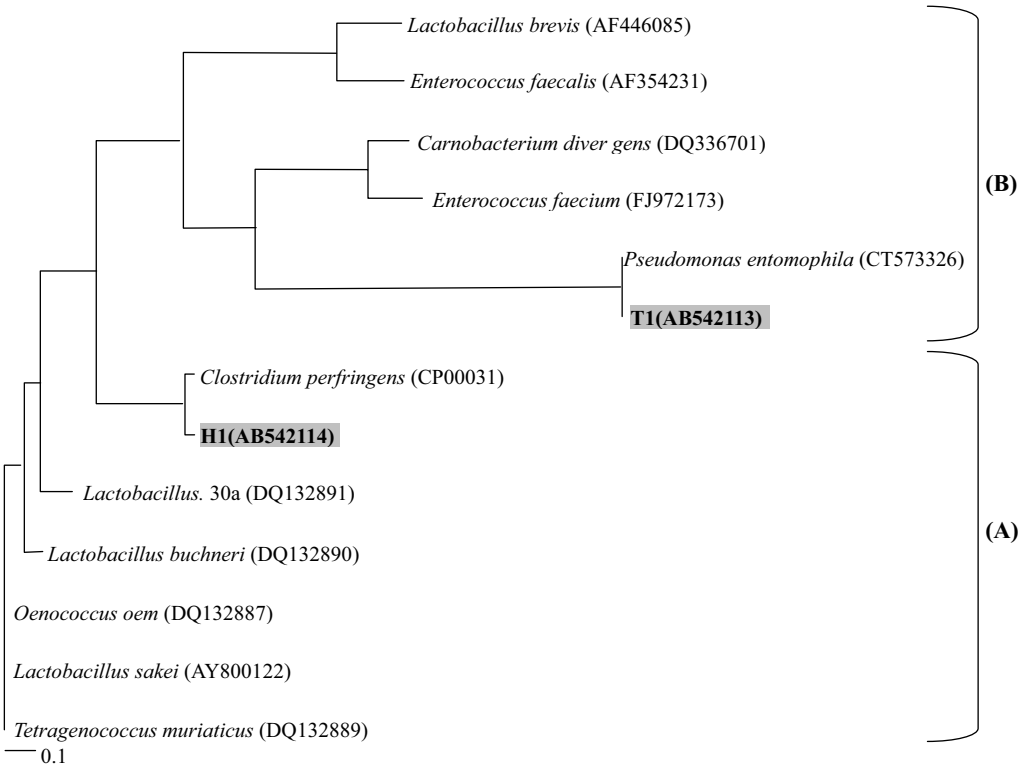

Fig. 3. Neighbor-joining phylogenetic tree of the (A) *hdc* and (B) *tydc* sequences showing the relationship of H1 and T1 strains among the hdc and tyrdc bacteria groups. Each bar represents 10% estimated phylogenetic divergence.

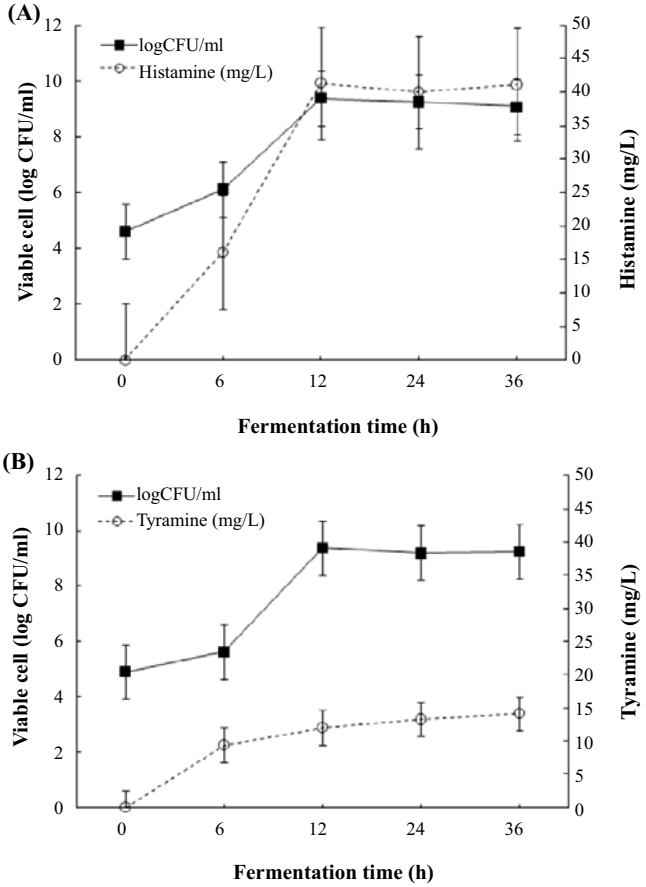

Fig. 4. Synthesis of (A) histamine by *C. perfringens* H1 and (B) tyramine by *P. entomophila* T1 in decarboxylating broth containing 0.5% histidine and 0.5% tyrosine.

among the H1 and T1 isolates and representative organisms from other genera. Topologies of the tree were evaluated using bootstrap analysis (Felsenstein, 1985) based on 1,000 resamplings. Additionally, amplicons of the *hdc* and *tyrdc* genes obtained by multiplex PCR (Fig. 1) were sequenced and aligned with known sequences of other decarboxylase genes. Partial alignment of the *hdc* gene sequences of isolate H1 and *C. perfringens* (CP000312) indicated five base pair variations among the 436 bp of the gene, and the phylogenetic tree revealed that the H1 strain was genetically distinct from *Lactobacillu*s and *Oenococcus* spp. (Fig. 3A). Partial alignment of the *tyrdc* gene sequences of T1 and *P. entomophila* (CT573326) showed seven base pair variations among the 1182 bp of the gene, and the phylogenetic tree revealed that the T1 isolate was genetically distinct from *Enterococcus faecium*, *E. faecalis*, *Carnobacterium divergens*, and *Lactobacillus brevis* (Fig. 3B). The *hdc* and *tyrdc* gene sequences of the isolates were registered in DDBJ (accession numbers AB542114 and AB542113, respectively). Tyramine and histamine production ability of the isolated microbial cells was confirmed via qualitative and quantitative assays. The isolates, *Clostridium* sp. H1 and *Pseudomonas* sp. T1, were cultivated in decarboxylating medium containing 0.5% of both histidine and tyrosine and synthesis of BAs was analyzed. Methods utilized for the preparation of samples and HPLC analysis were a modification of the procedure described by Garcia-Garcia *et al*. (2000). An HPLC unit (Waters 2695) equipped with a Waters 2996 photodiode array detector and Millennium 2010 software was utilized for analysis. A Shiseido UG-120 (5 μm, 4.6×250 mm) column was used, with water (solvent A) and acetonitrile (solvent B) as the mobile phases and a flow rate of 1 ml/min. The program was set for a linear gradient starting at 50% of solvent B to reach 90% of the solvent at 19 min. The sample volume injected was 20 μl, and the sample was monitored at 254 nm. As shown in Fig. 4, after 36 h of cultivation, the H1 isolate produced the maximum amount of histamine (41.1 mg/L, Fig. 4A), and the T1 isolate produced the maximum amount of tyramine (14.1 mg/L, Fig. 4B). *O. oeni*, the positive control for histamine production, produced 15.4 mg histamine/L; *E. faecalis*, the positive control for tyramine production, produced 22.1 mg tyramine/L; *Leuconostoc mesenteroides*, the negative control for BAs, produced no detectable amount of any BA (data not shown). These results indicate that among the cultures tested, *Clostridium* sp. H1 is a potent histamine producer.

C. perfringens grows in protein-rich media and cannot survive in media that lacks essential amino acid supply (Shimizu *et al*., 2002). Accordingly, this bacterium is often detected in amino acid-rich environments, including protein-fermented foods like *sufu*, a traditional Chinese fermented soybean curd (Han *et al*., 2001). Synthesis of histamine by this bacterium has also been reported in skipjack tuna (Yoshinaga and Frank, 1982). Our study is the first to report that a bacterial strain, *Clostridium* sp., can produce histamine in fermented foods.

In this study, *Clostridium* sp. H1 and *Pseudomonas* sp. T1 were isolated by their ability to produce BAs in soybean pastes. Surprisingly, these pastes were made utilizing traditional methods and ingredients. *Meju* is a major ingredient in a traditional soybean paste; this ingredient may play an important role as a solid medium to cultivate starter microorganisms that have been inoculated either naturally or deliberately. In *meju*, various bacteria, fungi, and yeast can readily grow and consume soybean protein. The *meju* is placed with brine in pottery jars and left for further fermentation, during which time protease-producing bacteria, such as *Bacillus subtilis*, hydrolyze proteins into amino acids, and often, yeast metabolize into aromatic compounds. While traditional home-made soybean paste uses *meju* after natural inoculation, most factory-made soybean pastes are currently made of starter-inoculated *meju*, using microorganisms that are safe for consumption, such as *B. subtilis* or *Aspergillus oryzae*. 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; Valsamaki *et al*., 2000). Foods can also be contaminated during raw material preparation, particularly during the natural incubation period of *meju*. This suggests that safe starter strains and hygienic conditions should be used for the production of soybean paste. The starter strains frequently used in commercial products should be tested for the absence of decarboxylase genes in their genome and the inability to produce BAs during growth in amino acid-rich medium. Application of the culture-dependent and -independent

methods used in this study will permit broad monitoring of BA-producing bacteria in various fermented foods.

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