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RESEARCH ARTICLE

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Characterization of Nonaflatoxigenic *Aspergillus flavus/oryzae* Strains Isolated from Korean Traditional Soybean Meju

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ABSTRACT

Filamentous fungi that could be classified into *Aspergillus flavus/oryzae* were isolated from traditionally fermented meju commercially available in Korea. The samples were analyzed for aflatoxin B1 and ochratoxin A contamination by HPLC; however, no toxin was detected. In addition, fungal and bacterial metagenomic sequencing were performed to analyze the microbial distribution in the samples. The results revealed that the distribution and abundance of fungi and bacteria differed considerably depending on the production regions and fermentation conditions of the meju samples. Through morphological analysis, ITS region sequencing, and assessment of the aflatoxin-producing ability, a total of 32 *A. flavus/oryzae* strains were identified. PCR analysis of six regions with a high mutation frequency in the aflatoxin gene cluster (AGC) revealed a total of six types of AGC breaking point patterns. The *A. flavus/oryzae* strains did not exhibit the high amylase activity detected in the commercial yellow koji strain (starter mold). However, their peptidase and lipase activities were generally higher than that of the koji isolates. We verified the safety of the traditionally fermented meju samples by analyzing the AGC breaking point pattern and the enzyme activities of *A. flavus/oryzae* strains isolated from the samples. The isolated strains could possibly be used as starter molds for soybean fermentation.

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Aflatoxin; *Aspergillus flavus*; *Aspergillus oryzae*; fermentation; meju

1. Introduction

Fungi can live anywhere with organic matter; most of them are saprophytes and grow on the corpses of animals and plants or organic detritus. Therefore, they play an important role as subdecomposers in the ecosystem. However, some fungi are parasitic in living organisms and can cause infectious diseases in the host. Many pathogenic fungi are known to cause diseases in animals and plants. Some *Aspergillus* species can cause invasive aspergillosis in immunocompromised humans [1,2]. Recently, as cases of COVID-19-associated pulmonary aspergillosis (CAPA) caused by fungi have increased, the treatment of severe COVID-19 patients has become more difficult and complicated [3,4]. In addition, fungi produce a wide variety of secondary metabolites, which are known to be factors associated with mechanisms such as growth and development, nutrient acquisition, response and defense against external stressors, and survival [5–7].

However, the functions and roles of fungal secondary metabolites in microbial ecosystems are mostly unknown [8]. Nevertheless, the utility and value of primary and secondary metabolites produced by fungi are very high. Fungi themselves and/or their metabolites have been used since a long time in the production and processing stages of various industrial fields [9,10]. On the other hand, some secondary metabolites produced by fungi can cause serious damage to humans and livestock. A typical example is mycotoxins, which are toxic secondary metabolites produced by fungi. Aflatoxin is the most potent mycotoxin. In particular, in *Aspergillus* section *Flavi* species, four types of aflatoxins, B1, B2, G1, and G2, are synthesized through the polyketide biosynthesis metabolic pathway [11]. In *Aspergillus flavus* and *Aspergillus parasiticus*, 25 genes responsible for aflatoxin biosynthesis are clustered in a region of about 75 kB at the end of chromosome 3 [12,13].

Comparative genome analysis performed in a previous study revealed that this aflatoxin gene cluster (AGC) was highly conserved in most *Aspergillus* section *Flavi* species, but some species, such as *Aspergillus tamarii*, had completely or partially lost AGC regions [14]. However, the presence of AGC in the fungal genome does not mean that the fungus is capable of producing aflatoxin. *Aspergillus aflatoxiformans*, *A. parasiticus*, and *Aspergillus nomiae*

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produce both B- and G-type aflatoxins, while *Aspergillus togoensis*, *A. flavus*, and *Aspergillus pseudotamarii* only produce B-type aflatoxin. However, *A. tamarii*, *Aspergillus oryzae*, *Aspergillus sojae*, and *Aspergillus caelatus* do not produce aflatoxin [15]. The difference in the aflatoxin-producing ability of *Aspergillus* species is expected to be related to the normal functioning of AGC genes. *A. tamarii* has completely lost AGC in the genome, while *A. caelatus* has truncated AGC, with only a few genes remaining in the polyketide biosynthetic metabolic pathway [14]. Moreover, in *A. oryzae*, deletions, frame shifts, and base pair substitutions have been reported in many AGC regions [16–18]. *Aspergillus* species that produce only G-type aflatoxin have not been discovered so far. Therefore, it is suggested that B-type aflatoxin-producing *Aspergillus* species have evolved from B- and G-type aflatoxin-producing ancestors by loss-of-function mutation of a gene required for G-type aflatoxin production [19].

A. oryzae, *A. sojae*, and *A. tamarii*, which do not produce aflatoxin, have been used for centuries in food fermentation and in the production of industrial enzymes. On the other hand, *A. flavus* produces toxic metabolites, such as aflatoxin and cyclopiazonic acid, and has a very good ability to colonize plants. This fungal population is highly genetically diverse and contains numerous vegetative compatibility groups [20]. Moreover, it shows morphological variations (S and L strains) depending on the size of the sclerotium and can be divided into aflatoxigenic and nonaflatoxigenic strains depending on the ability to produce aflatoxin [21]. *A. flavus* and *A. oryzae* are presumed to have diverged from the same ancestor because of their similar genome composition as well as morphological identity. *A. oryzae* strains selected for human food fermentation were hypothesized to be domesticated from a pool of nonaflatoxigenic *A. flavus* variants already present in nature [22]. However, a recent comparative genome analysis interestingly revealed that *A. oryzae* is more closely related to *A. aflatoxiformans* than to *A. flavus* [14]. In any case, given the similarity between *A. oryzae* and aflatoxigenic *Aspergillus* species, aflatoxin contamination may occur if sufficiently strict quality control is not maintained during the production process of fermented foods.

Meju is a fermented and dried soybean block that is used as a raw material for doenjang (soybean paste) and ganjang (soy sauce), the basic ingredients in Korean food. Meju produced in the traditional manner is naturally fermented by various microorganisms, such as bacteria, yeast, and mold, without the need for specific starter microorganisms. Therefore, meju has a very complex and unique microbial ecosystem depending on the process of fermentation. Fungi play an important role in decomposing the high molecular components of soybeans into micronutrients using various hydrolytic enzymes during the fermentation process. Although it varies depending on the fermentation period, the fungi involved in the fermentation of meju mainly genera *Aspergillus*, *Mucor*, *Geotrichum*, *Rhizopus*, and *Penicillium* [23]. Of these, *Aspergillus* species are most prevalent in the fermentation process. In particular, fungi belonging to *Aspergillus* section *Flavi* and section *Nigri* appear in large numbers during high-temperature fermentation at the end of the fermentation process [24]. Hong et al. [24] reported *A. oryzae* to be the most isolated strain among 533 *Aspergillus* species isolated from meju collected in Korea, followed by *Aspergillus pseudoglaucus* (*Eurotium pseudoglaucus*) and *Aspergillus chevalieri* (*E. chevalieri*). In addition, *A. flavus* and *A. parasiticus* were isolated in small numbers from some meju samples. Aflatoxin or aflatoxigenic mold contamination is not uncommon in traditionally fermented Korean soybean products, such as Doenjang and meju [25,26]. This is also related to the fermentation method of traditional Korean meju, which ferments naturally without the use of a starter mold (yellow koji).

In the present study, the distribution of microorganisms was analyzed through metagenome analysis on meju produced by natural fermentation method, and filamentous fungi were isolated. In addition, the activity of the hydrolase involved in soybean fermentation was investigated for the strains identified as *A. flavus*/*oryzae* among the isolated strains, and the characteristics of their AGC region were analyzed. Through this work, we tried to find the possibility of using selected strains as a fungal starter to produce safe meju from mycotoxin contamination.

2. Materials and methods

2.1. Meju collection and metagenomic analysis

Five types of traditionally fermented meju samples were collected from local markets in Seongnam, Gyeonggi Province (one type); Wanju, Jeonbuk Province (three types); and Gurye, Jeonnam Province (one type) in Korea. After collecting a piece penetrating the deep part from the surface of the meju, it was crushed and used as a sample for extracting the total genomic DNA. The total genomic DNA was extracted by the ethanol precipitation method using 10 M lithium chloride [27] or using the HiGene^{TM} Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea). A sequencing library for analyzing the bacterial and fungal communities present in the meju samples was prepared after tagmentation by the random fragmentation of genomic

DNA. The 16S rRNA primer set and ITS primer set were used for bacterial and fungal amplification by PCR, respectively [28,29]. After converting the sequencing data into raw data for analysis, raw images were produced using sequencing control software for system control and base calling through integrated primary analysis software. BCL (base call) binaries were converted to FASTQ and analyzed using Illumina package bcl2fastq. Sequencing and data analysis were performed at Macrogen (Daejeon, Korea).

2.2. Isolation and identification of fungal strains from the meju samples

By cutting the middle part of the meju samples, fungal strains were isolated from both the surface and the deep parts of the samples. Filamentous fungi were isolated according to the shape of the conidiophore head and branching characteristics by observing the conidiophore aggregate formation area on the surface of the meju samples under a stereoscopic microscope. From the deep part samples, conidia aggregates or vegetative mycelia observed on the cut surface were isolated. The media used for fungal isolation were potato dextrose agar (PDA), Czapek Dox agar (CDA), and dichloran glycerol 18% agar (DG18). The growing filamentous fungi were isolated by culturing for 20 days or more in an incubator at 25 °C. The cultured fungi were re-inoculated with asexual spores to isolate a single colony. Alternatively, mycelia were directly subcultured. In addition, using PDA, *A. flavus/oryzae* strains were isolated from yellow koji used for making meju obtained from four companies on the market. A piece of yellow koji was placed on PDA and CDA plates and cultured for three days in an incubator at 25 °C. Following this, a single colony was isolated by subculturing asexual spores of the growing strain. The identification of filamentous fungi isolated from the meju and yellow koji samples was performed by analyzing the nucleotide sequence of the internal transcribed spacer (ITS) region of the rRNA gene. The isolated strains were preferentially classified according to their morphological characteristics, and the genomic DNA of the strains in each classification group was extracted. ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers were used for PCR amplification of the ITS region, and the nucleotide sequences of the analyzed ITS regions were used for identification by searching for the nucleotide sequence showing 100% identity with the target sequence from the "ITS from fungi type and reference material" database in the NCBI BLAST server (National Center for Biotechnology Information, USA).

2.3. Analysis of mycotoxins

Aflatoxin was analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). For analyzing ochratoxin A, only HPLC was used. For aflatoxin analysis, the meju samples were pulverize and made into powder. Following this, 10 ml of chloroform was added to 5 g of meju powder. The chloroform solution was then extracted by shaking for 30 min. The extracted sample was concentrated to 0.5 ml, filtered using a 0.2 µm syringe filter, and used for TLC and HPLC analyses. For TLC analysis of aflatoxin, 20 µl of the sample was loaded onto an aluminum TLC silica gel 60 plate (F254) using a capillary tube. Acetone:chloroform (1:9, v:v) was used as the developing solution. After development, the spot of aflatoxin was analyzed by irradiating with 365 nm UV. For HPLC analysis, each dried extract sample was dissolved in aqueous methanol (aflatoxin) or aqueous acetonitrile (ochratoxin A). HPLC analysis was performed using an HPLC system equipped with the HITACHI Chromaster 5110 Binary Pump System (HITACHI, Japan) coupled with a 5210 auto-sampler and column compartment. Chromatographic separation was performed on a TSK-gel 100 V column (4.6 mm × 150 mm, 5 µm, TOSOH, Japan) at a 1 ml/min flow rate.

2.4. Mutation analysis inside AGC

AGC mutation patterns were analyzed in *A. flavus/oryzae* strains isolated from the meju samples, in which aflatoxin is not produced, and in *A. oryzae* strains isolated from the commercial yellow koji samples. Based on the results of PCR analysis of *A. flavus* AGC reported by Chang et al. [30], we analyzed the breaking point patterns at six sites (the C1 repeat flanking, *norB-cypA* inter-gene, *aflR* ORF, *ver1* 5′-UTR, *verA-avnA* inter-gene, and *omtA* SNP regions in chromosome 3) with a high mutation frequency in AGC using gene-specific primers by PCR (Table 1). The nucleotide sequence of the gene site-specific primer set used for the analysis is provided in Supplementary Table 1.

Table 1. Breaking point patterns of *Aspergillus flavus/oryzae* AGC according to the gene-specific PCR assay results.

C1	*norB-cypA*	*aflR*	*ver1*	*verA*	*omtA*	Deletion type
O	0.3 kb	O	O	O	O	A
X	0.3 kb	O	O	O	O	B
O	0.3 kb	X	X	X	O	C
O	0.8 kb	O	O	O	O	D
X	X	X	O	O	O	E
X	X	X	X	O	O	F
X	X	X	X	X	O	G
X	X	X	X	X	X	H

Note: O: PCR amplified. X: No PCR amplification.

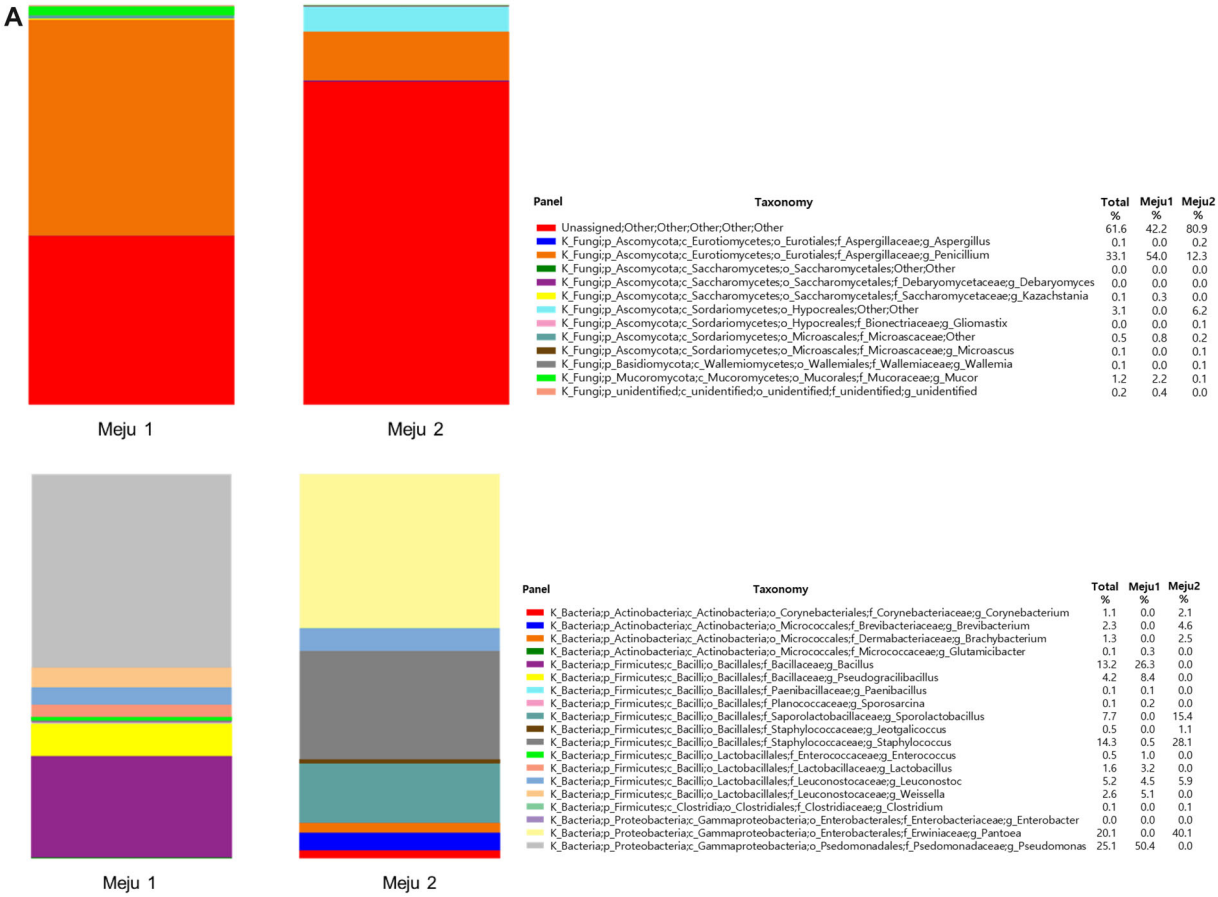
2.5. Analyses of amylase, lipase, and peptidase activities

The amylase, peptidase, and lipase activities of the *A. flavus*/*oryzae* isolates were assessed in the culture media. The evaluation was based on the production of each enzyme in solid media. In brief, amylase activity was assessed by modifying the methods of Ceska [31] and Hankin and Anagnostakis [32]. After adding a final concentration of 2% soluble starch to complete agar medium (CM) excluding glucose, the strain was point inoculated and incubated at 30 °C for four days. Then, an iodine–potassium iodide solution was added in plate to induce a blue–violet iodine–starch reaction at room temperature for 30 min. Following this, the width of the translucent zone at the end of the mycelium, which was hydrolyzed by amylase, was measured. Peptidase activity was assessed by modifying the method of Hankin and Anagnostakis [32]. After adding skim milk to 2% in CM that did not contain glucose, the strain was point inoculated and cultured at 30 °C for four days. Then, the width of the translucent zone at the end of the mycelium was measured when the skim milk was decomposed. Lipase activity was assessed by modifying the method of Mourey and Kilbertus [33], which measures the hydrolysis rate of 1% tributyrin. After performing sterilization by adding 1% glyceryl tributyrate to CM, emulsion was formed using a sonicator to prepare the medium. The strain was point inoculated into the prepared medium and cultured at 30 °C for four days to measure the width of the translucent zone of the mycelial end. The enzyme activity was determined using the method of Hankin and Anagnostakis [32], with the enzymatic index (EI) being expressed as EI = R/r, where R is the degradation halo (the translucent zone) diameter and r is the colony diameter. The halo around the colony was measured using a ruler in millimeters. The evaluation was performed in three dishes for each fungus. The results are expressed as the average of the triplicates.

3. Results

3.1. Metagenomic analysis of the meju samples

Fungal and bacterial communities present in meju samples collected from Seongnam in Gyeonggi



Panel	Taxonomy	Total %	Meju1 %	Meju2 %
	Unassigned;Other;Other;Other;Other;Other	61.6	42.2	80.9
	K_Fungi;p_Ascomycota;c_Eurotiomycetes;o_Eurotiales;f_Aspergillaceae;g_Aspergillus	0.1	0.0	0.2
	K_Fungi;p_Ascomycota;c_Eurotiomycetes;o_Eurotiales;f_Aspergillaceae;g_Penicillium	33.1	54.0	12.3
	K_Fungi;p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;Other;Other	0.0	0.0	0.0
	K_Fungi;p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Debaryomycetaceae;g_Debaryomyces	0.0	0.0	0.0
	K_Fungi;p_Ascomycota;c_Saccharomycetes;o_Saccharomycetales;f_Saccharomycetaceae;g_Kazachstania	0.1	0.3	0.0
	K_Fungi;p_Ascomycota;c_Sordariomycetes;o_Hypocreales;Other;Other	3.1	0.0	6.2
	K_Fungi;p_Ascomycota;c_Sordariomycetes;o_Hypocreales;f_Bionectriaceae;g_Gliomastix	0.0	0.0	0.1
	K_Fungi;p_Ascomycota;c_Sordariomycetes;o_Microascales;f_Microascaceae;Other	0.5	0.8	0.2
	K_Fungi;p_Ascomycota;c_Sordariomycetes;o_Microascales;f_Microascaceae;g_Microascus	0.1	0.0	0.1
	K_Fungi;p_Basidiomycota;c_Wallemiomycetes;o_Wallemiales;f_Wallemiaceae;g_Wallemia	0.1	0.0	0.1
	K_Fungi;p_Mucoromycota;c_Mucoromycetes;o_Mucorales;f_Mucoraceae;g_Mucor	1.2	2.2	0.1
	K_Fungi;p_unidentified;c_unidentified;o_unidentified;f_unidentified;g_unidentified	0.2	0.4	0.0

Panel	Taxonomy	Total %	Meju1 %	Meju2 %
	K_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Corynebacteriales;f_Corynebacteriaceae;g_Corynebacterium	1.1	0.0	2.1
	K_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Micrococcales;f_Brevibacteriaceae;g_Brevibacterium	2.3	0.0	4.6
	K_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Micrococcales;f_Dermabacteriaceae;g_Brachybacterium	1.3	0.0	2.5
	K_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Micrococcales;f_Micrococcaceae;g_Glutamicibacter	0.1	0.3	0.0
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Bacillus	13.2	26.3	0.0
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Pseudogracilibacillus	4.2	8.4	0.0
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Paenibacillaceae;g_Paenibacillus	0.1	0.1	0.0
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Planococcaceae;g_Sporosarcina	0.1	0.2	0.0
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Saporolactobacillaceae;g_Sporolactobacillus	7.7	0.0	15.4
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Staphylococcaceae;g_Jeotgalicoccus	0.5	0.0	1.1
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Bacillales;f_Staphylococcaceae;g_Staphylococcus	14.3	0.5	28.1
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae;g_Enterococcus	0.5	1.0	0.0
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus	1.6	3.2	0.0
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Leuconostocaceae;g_Leuconostoc	5.2	4.5	5.9
	K_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Leuconostocaceae;g_Weissella	2.6	5.1	0.0
	K_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Clostridium	0.1	0.0	0.1
	K_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Enterobacteriaceae;g_Enterobacter	0.0	0.0	0.0
	K_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Erwiniaceae;g_Pantoea	20.1	0.0	40.1
	K_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas	25.1	50.4	0.0

Figure 1. Analysis of microbial community distribution in the meju samples. Meju samples 1 and 2 fermented using the traditional method were analyzed, and their fungal and bacterial communities were compared. (A) The graph shows the distribution ratio of fungi and bacteria in each meju sample at the genus level; (B) The clustering heatmap shows the relative comparison of the distribution of microbial communities between meju samples 1 and 2. In fungi, the ITS sequence of the rRNA gene was analyzed, and in bacteria, the 16S rRNA gene was used. "Other" stands for unclassified.

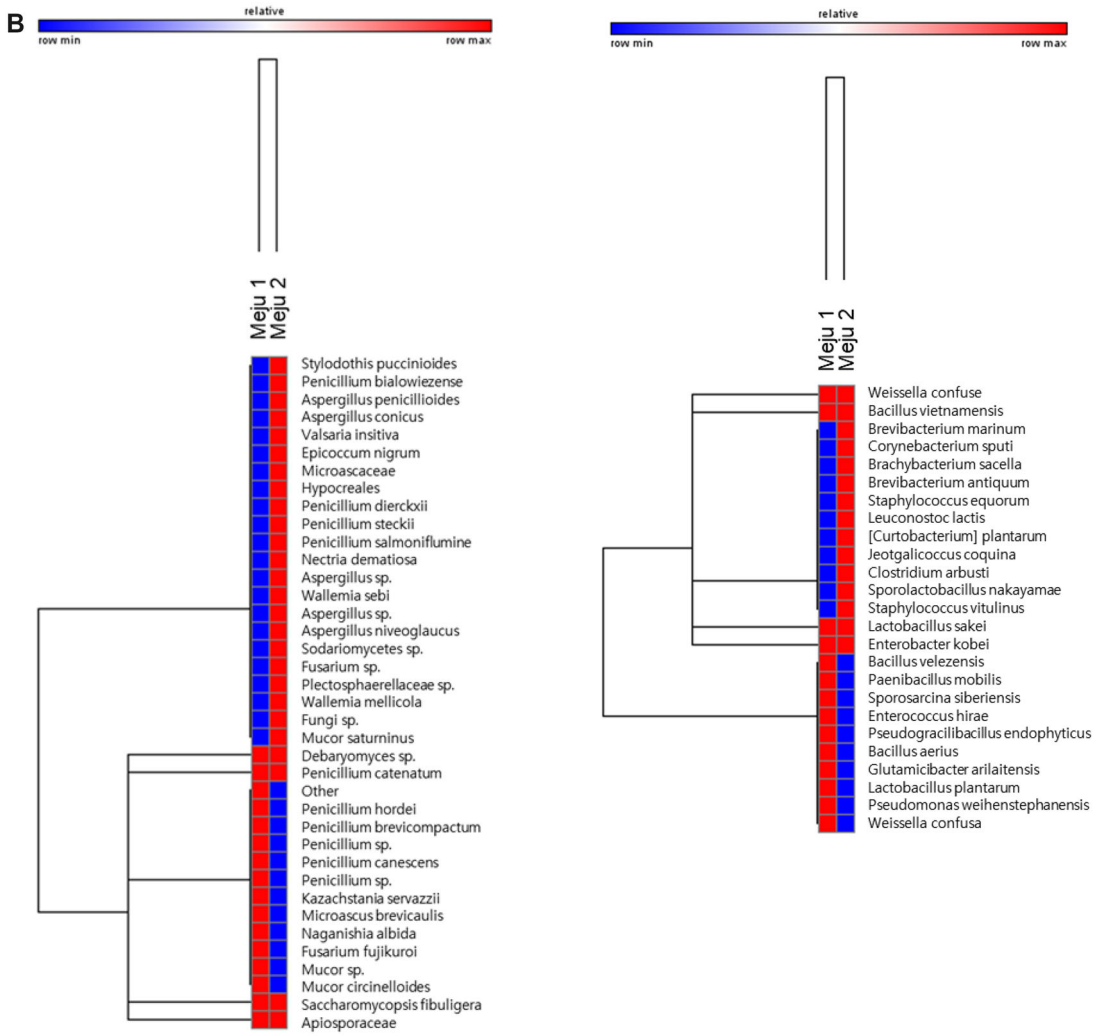


Figure 1. Continued.

Province (sample 1) and Gurye in Jeonnam Province (sample 2) were assessed. The total read count of the ITS library for meju sample 1 was 123,413 and the OTU count was 21.0, while that for meju sample 2 was 151,222 and the OTU count was 32.0. In the 16S library, sample 1 had a total read count of 77,381 and an OTU count of 17.0, while sample 2 had a total read count of 87,049 and an OTU count of 14.0. Regarding the distribution of fungi, it was confirmed that unidentified taxa accounted for the largest proportion (61.6%) in all the samples, followed by the genus *Penicillium* (33.1%). However, the genus *Aspergillus* had a prevalence of 0.1%, showing a lower distribution ratio than expected (Figure 1(A)). Assessment of the distribution ratio of bacteria using the 16S library revealed the highest prevalence of the genus *Pseudomonas* (25.2%), followed by *Pantoea* (20.1%), *Staphylococcus* (14.3%), *Bacillus* (13.2%), and *Sporolactobacillus* (7.7%) (Figure 1(B)). Both fungi and bacteria showed very different distribution patterns in different meju samples.

3.2. Isolation and identification of filamentous fungi from the meju samples

In total, 44 fungal strains were isolated from meju sample 1 collected from Seongnam, Gyeonggi Province, while 114 strains were isolated from meju sample 2 collected from Gurye, Jeonnam Province. In addition, 28, 34, and 40 fungal strains were isolated from meju samples 3-1, 3-2, and 4, respectively, collected from Wanju, Jeonbuk Province. A total of 260 strains of filamentous fungi were thus isolated and cultured. Filamentous fungi isolated from the meju samples could be grown at temperatures ranging from 25 °C to 30 °C using PDA, CDA, or DG18 medium. The 260 isolated strains were preferentially classified according to their morphological characteristics. Among them, 32 strains of

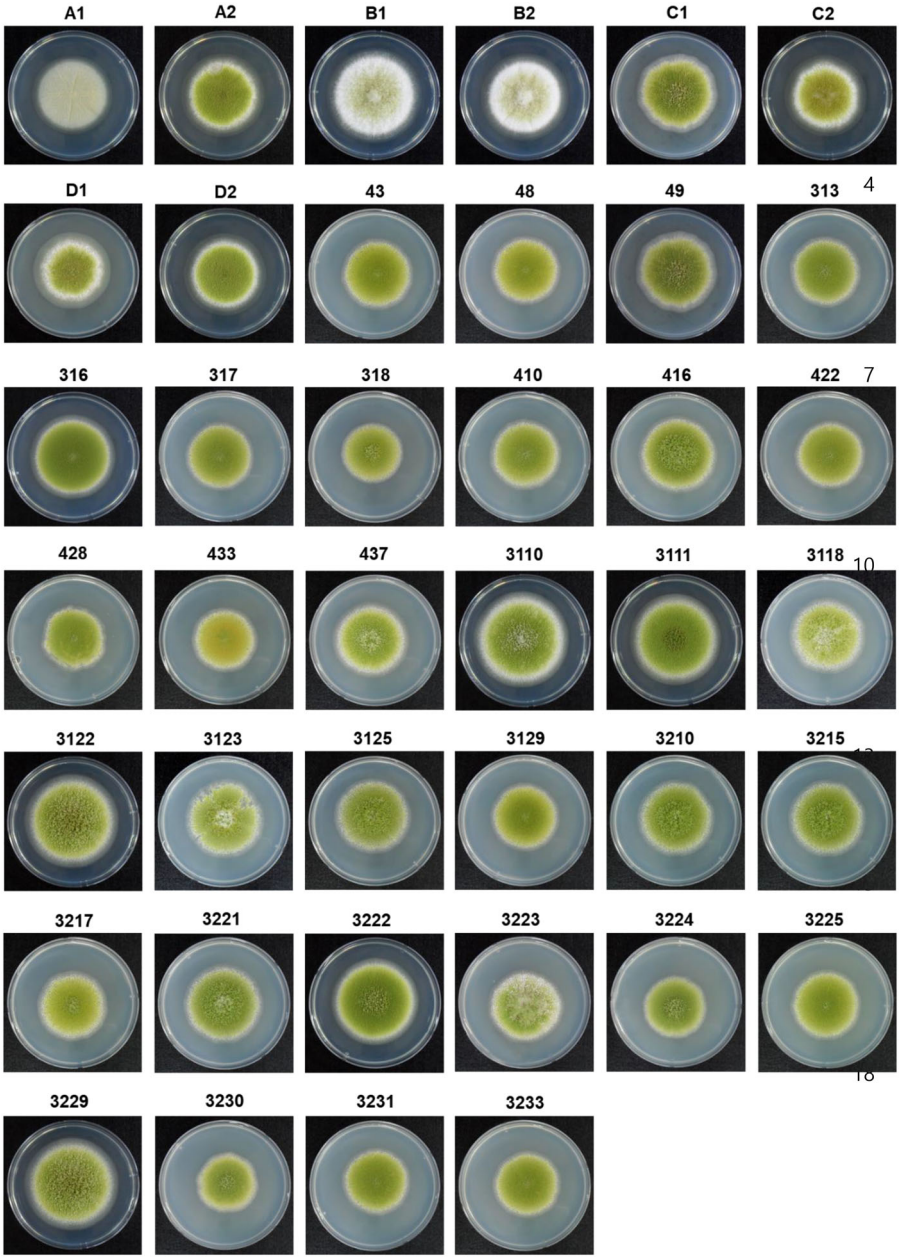


Figure 2. *Aspergillus flavus/oryzae* strains isolated from the meju. Thirty-two *A. flavus/oryzae* strains were identified through ITS sequencing among filamentous fungi isolated from meju sample. A1-D2 are *A. oryzae* strains isolated from commercial yellow koji for making meju. These strains did not produce aflatoxin as a result of TLC and HPLC analysis, and most did not produce sclerotium, or some produced L-type form. And also these strains confirmed a total of six types of deletion pattern through AGC's breaking point analysis (see Table 2). The strains were cultured on PDA medium at 30 °C for four days.

Aspergillus section *Flavi* were isolated. As *A. flavus* and *A. oryzae* cannot be distinguished by ITS sequencing, all strains analyzed as the two types have been denoted as *A. flavus/oryzae*.

3.3. AGC breaking point pattern and characteristics of A. flavus/oryzae strains

Of the strains identified as *A. flavus/oryzae* were analyzed for breaking point patterns at six sites inside AGC (Figure 2). HPLC analysis confirmed that all these strains were nonaflatoxigenic *A. flavus/oryzae* strains (data not shown). All eight *A. oryzae* strains isolated from commercially available yellow koji samples used for making meju (koji samples A1–D2) were confirmed to have variants of the type B breaking point pattern, in which mutations exist in the *ver1* 5′-UTR region. On the other hand, 32 *A. flavus/oryzae* strains isolated from the meju samples had B-type (three strains), D-type (one strain), E-type (18 strains), and B-type variants (one strain) inside AGC. In addition, the E-type variant without a mutation in the C1 repeat flanking region (eight strains) and the A-type variant with a mutation in the *aflR* ORF (one strain) were newly identified. Thus, a total of six types of breakpoint patterns were confirmed in the meju isolates (Figure 3, Table 2). Moreover, eight *A. oryzae* strains isolated from

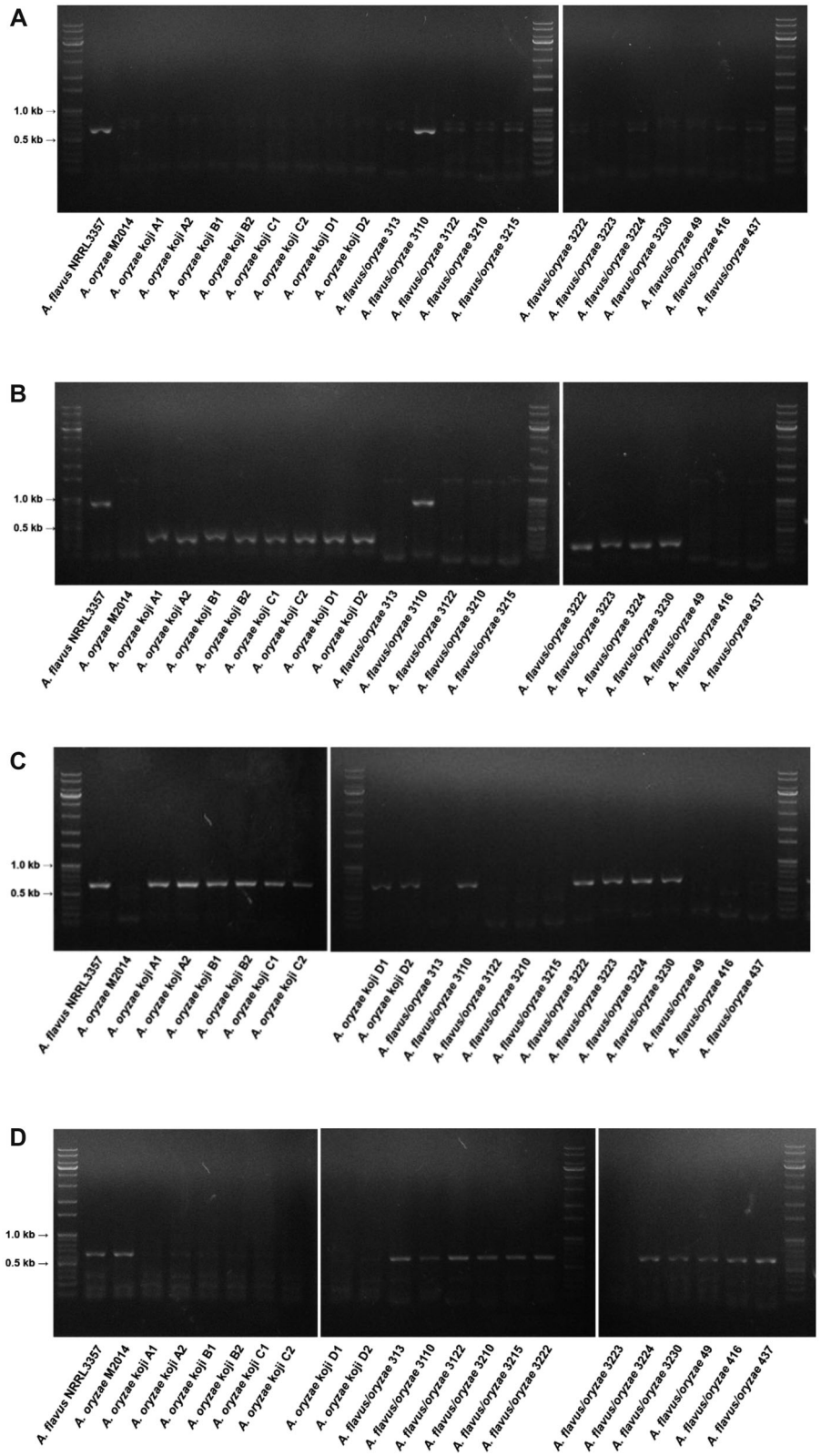


Figure 3. Analysis of breaking point patterns inside the aflatoxin gene cluster. The breaking point pattern of the C1 repeat flanking region (A); *norB-cypA* inter-gene region (B); *aflR* ORF (C); and *ver15*′-UTR region (D) inside AGC for the nonaflatoxigenic strain was analyzed by PCR amplification. PCR amplification was performed using a gene-specific primer. Thirty-two *Aspergillus flavus*/*oryzae* strains isolated from meju samples and eight *A. oryzae* strains isolated from yellow koji samples were analyzed. PCR analysis of the *verA-avnA* inter-gene region and the *omtA* SNP region showed no difference between the strains; only some of the results are presented.

the yellow koji samples did not produce sclerotium when cultured on PDA and CM. However, some *A. flavus*/*oryzae* strains isolated from the meju samples produced sclerotium and some did not; all the strains that produced sclerotium were found to be L strains (Table 2).

Table 2. Characteristics and AGC breaking point pattern types in meju and yellow koji isolate strains.

Strain	C1	*norB-cypA*	*aflR*	*ver1*	*verA*	*omtA*	Deletion pattern	Aflatoxin	Sclerotium
A. oryzae koji A1	X	0.3 kb	O	X	O	O	B variant	–	–
A. oryzae koji A2	X	0.3 kb	O	X	O	O	B variant	–	–
A. oryzae koji B1	X	0.3 kb	O	X	O	O	B variant	–	–
A. oryzae koji B2	X	0.3 kb	O	X	O	O	B variant	–	–
A. oryzae koji C1	X	0.3 kb	O	X	O	O	B variant	–	–
A. oryzae koji C2	X	0.3 kb	O	X	O	O	B variant	–	–
A. oryzae koji D1	X	0.3 kb	O	X	O	O	B variant	–	–
A. oryzae koji D2	X	0.3 kb	O	X	O	O	B variant	–	–
A. flavus/oryzae 313	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 316	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 317	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 318	X	0.3 kb	O	O	O	O	E	–	–
A. flavus/oryzae 3110	O	0.8 kb	O	O	O	O	D	–	+
A. flavus/oryzae 3111	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 3118	X	X	X	O	O	O	E	–	+
A. flavus/oryzae 3122	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 3123	X	X	X	O	O	O	E	–	+
A. flavus/oryzae 3125	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 3129	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 3210	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 3215	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 3217	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 3221	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 3222	X	0.3 kb	O	O	O	O	B	–	–
A. flavus/oryzae 3223	X	0.3 kb	O	X	O	O	B variant	–	+
A. flavus/oryzae 3224	X	0.3 kb	O	O	O	O	B	–	–
A. flavus/oryzae 3225	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 3229	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 3230	X	0.3 kb	O	O	O	O	B	–	–
A. flavus/oryzae 3231	O	0.3 kb	X	O	O	O	A variant	–	–
A. flavus/oryzae 3233	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 43	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 48	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 49	X	X	X	O	O	O	E	–	+
A. flavus/oryzae 410	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 416	X	X	X	O	O	O	E	–	+
A. flavus/oryzae 422	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 428	O	X	X	O	O	O	E variant	–	–
A. flavus/oryzae 433	X	X	X	O	O	O	E	–	–
A. flavus/oryzae 437	X	X	X	O	O	O	E	–	–

Note: O: PCR amplified. X: no PCR amplification; +: produced; –: not produced.

3.4. Amylase, peptidase, and lipase activities

The activities of amylase, peptidase, and lipase enzymes involved in soybean decomposition and saccharification were analyzed and compared between *A. flavus/oryzae* strains isolated from the meju samples and *A. oryzae* strains isolated from the commercial yellow koji samples. The amylase activity of *A. oryzae* strains isolated from the koji samples was significantly higher than that of *A. flavus/oryzae* strains isolated from the meju samples (Figure 4). The amylase activity of the meju isolates was on average only about 20% of the amylase activity of the koji isolates. However, the difference in amylase activity between individual strains was very large. *A. flavus/oryzae* 3122 and 3223 strains showed little amylase activity under the experimental conditions. However, the *A. flavus/oryzae* 3222 strain exhibited nearly twofold higher enzyme activity than the *A. oryzae* M2014 control strain. On the other hand, the peptidase activity was found to be slightly higher in *A. flavus/oryzae* strains isolated from the meju samples than in those isolated from the koji samples. However, the peptidase activity largely differed between individual strains. The lipase activity did not differ significantly between the meju and koji isolates. However, it was generally higher than that of *A. flavus* NRRL3357 or *A. oryzae* M2014 (control strains).

4. Discussion

In total, 260 strains of filamentous fungi were isolated by collecting five types of meju samples prepared by the traditional fermentation method from three regions and four markets in Korea. Among the strains, the genus *Aspergillus* had the highest prevalence (48.1%, 63 strains). In the genus *Aspergillus*, section *Aspergillus* containing *Eurotium* species and section *Flavi* were prevalent. Other strains belonging to section *Nigri* and section *Fumigati* were also isolated. These results are similar to those of the distribution of *Aspergillus* strains isolated from meju samples reported by Hong et al. [24]. On the other hand, when assessing aflatoxin and ochratoxin A contamination by HPLC analysis of all five meju samples, mycotoxin was not detected (data not shown). However, the clear differences noted in filamentous fungi isolated and cultured from the five types of meju samples are considered

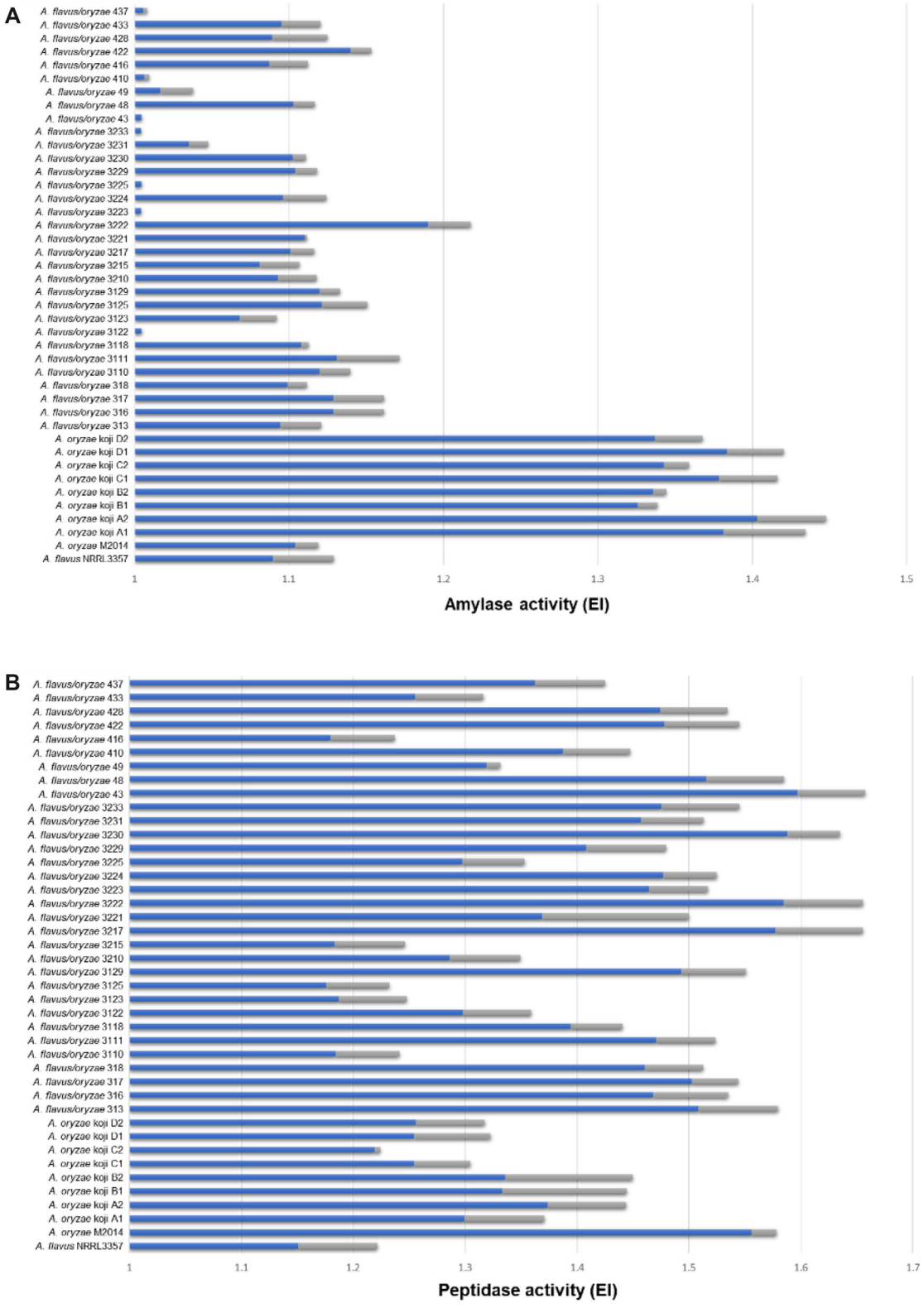


Figure 4. Analysis of amylase, lipase, and peptidase activities of the meju and koji isolates. The activities of amylase (A); peptidase (B); and lipase (C); which are enzymes associated with soybean decomposition, were assessed in the isolated *Aspergillus flavus*/*oryzae* strains. After inoculating the fungus on an agar plate containing each enzyme substrate and culturing for four days, the activity was calculated by measuring the halo caused by the hydrolysis of the substrate. The enzyme activity was measured thrice; the average value is presented. *A. flavus* NRRL3357 and *A. oryzae* M2014 strains were used as control strains. EI: enzymatic index (see Section 2), Blue bar: mean value of enzyme activity, Grey bar: standard deviation.

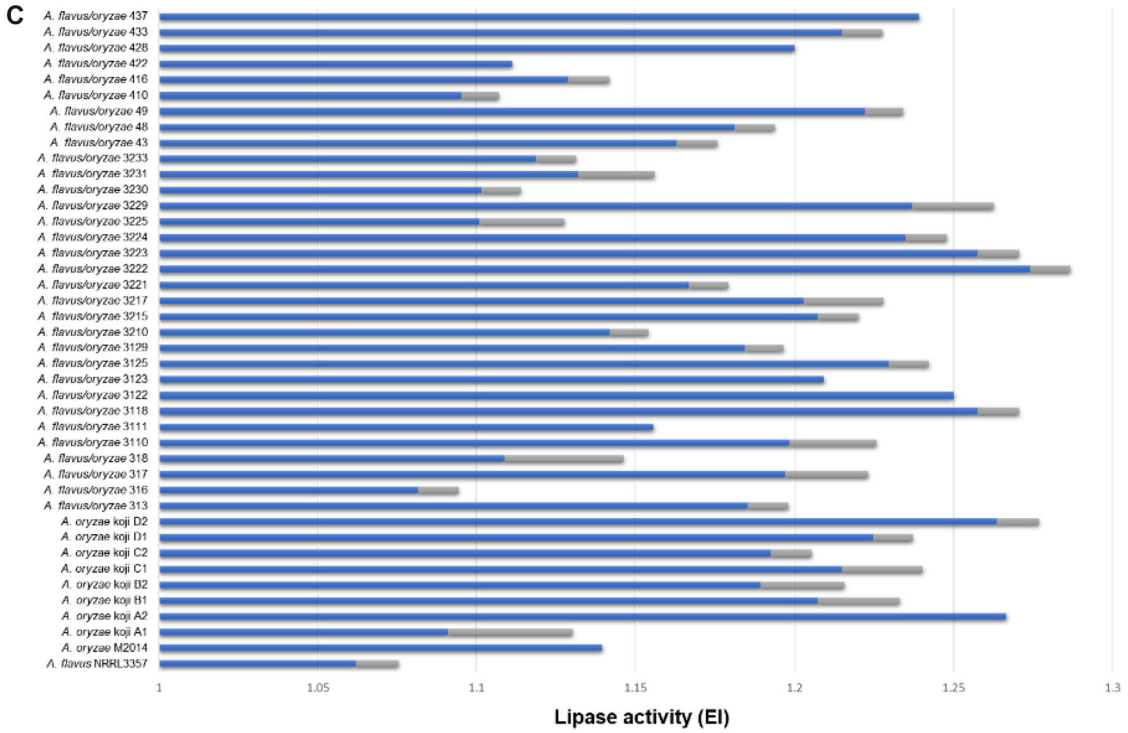


Figure 4. Continued.

to be attributed to the production regions, fermentation periods, and fermentation conditions of meju. Many strains belonging to the genus *Cladosporium* were isolated from meju sample 2, which had a lot of moisture at the time of collection. *Cladosporium*, a common flora in the air, is presumed to grow by attaching to the humid surface of meju at the beginning of fermentation after the making of meju. As the fermentation of soybeans progresses, meju is gradually dried up, and then it is thought that strains belonging to the genus *Aspergillus* or *Penicillium* gradually become predominant [34,35]. However, in the case of meju samples 1, 3-1, 3-2, and 4, it was predicted that the fermentation of meju had significantly progressed, considering that the surface part was dry and fungal growth was much progressed in the deep part (the exact time is unknown). In addition, only four strains were simultaneously identified in the fungal communities distributed in meju samples 1 and 2 by metagenomic analysis: *Debaryomyces* species, *Penicillium catenatum*, *Apiosporaceae* species, and *Saccharomycopsis fibuligera*. The fungal community distribution significantly differed between the two samples. Similarly, the distribution of bacterial communities was very different between the two meju samples. The types and distribution of bacteria were unique in each meju sample. Only four types of bacteria, *Weissella confusa*, *Bacillus vietnamensis*, *Lactobacillus sakei*, and *Enterobacter kobei*, were simultaneously detected in both the samples, while the rest were specific to each meju sample. The profiling of microbial communities and metabolites is widely used to analyze changes in the metabolic status of fermented soybean products, including doenjang [36], cheonggukjang [37], and gochujang [38], in addition to meju [39]. Meju, a soybean-derived fermented food, affects the type and content of metabolites according to the distribution of microorganisms, so differences may appear in the fermentation process. Therefore, it is necessary to confirm the diversity of microbial communities and comprehensive metabolites in various traditionally fermented meju samples.

In the present study, all 32 *A. flavus*/*oryzae* strains isolated from traditionally fermented meju samples did not produce aflatoxin. Morphological or ITS sequence analysis cannot distinguish the two representative species of section *Flavi*, namely *A. flavus* and *A. oryzae*. Moreover, some *A. flavus* strains are unable to produce aflatoxin. Therefore, the exact distinction between the two species can be possible only through a comprehensive physiological and biochemical comparative analysis and a comparative genomic analysis of the whole genome. Chang et al. [30] grouped AGC into eight breaking point patterns (A–H) by PCR analysis using aflatoxin gene-specific primers targeting nonaflatoxigenic *A. flavus* strains. The analysis of 32 *A. flavus*/*oryzae* strains isolated from the meju samples using an aflatoxin gene-specific primer confirmed that there were three new breaking point patterns (A-type, B-type, and E-type variants) different from the existing eight breaking point patterns. This result is not surprising.

As reported previously, the deletion of AGC from *A. flavus* strains is not uncommon and the pattern varies. Interestingly, several nonaflatoxigenic *A. flavus*/*oryzae* strains showing various AGC breaking point patterns were isolated from a single meju sample. Alshannaq et al. [40] reported that an *A. oryzae* strain isolated from meju could effectively inhibit the growth of and aflatoxin production by *A. flavus*, an aflatoxin-producing fungus, suggesting that it could be used as a biocontrol agent. Strains with a deletion in AGC appear to have ecological competitiveness against aflatoxin-producing strains. However, the physiological and genetic mechanisms underlying this action remain unclear. The competitiveness of nonaflatoxigenic *A. flavus*/*oryzae* strains according to the diversity of AGC breaking point patterns also remains to be confirmed. Given the inherent diversity of *A. flavus* and *A. oryzae* populations, it is more difficult to understand their biocontrol strategies as each population has a different ability to produce aflatoxins and other secondary metabolites. In addition to the diverse AGC breaking point patterns, the activity of hydrolytic enzymes varied in *A. flavus*/*oryzae* strains isolated from the meju samples. Fungal strains with various enzyme activities coexist in meju. This can affect the fermentation process and can consequently be a factor influencing the flavor of fermented meju. The amylase activity of *A. oryzae* strains isolated from the commercially available yellow koji samples was found to be particularly higher than that of other strains. This can predict the possibility of the strains originating from white koji (nuruk) in brewing, where the process of saccharification is important.

In conclusion, microbial metagenomic sequencing were performed using meju samples commercially available in Korea in order to identify the fungal and bacterial microflora. A total of 260 filamentous fungi were isolated from commercially available meju, and 32 strains of nonaflatoxigenic *A. flavus*/*oryzae* were identified by performing ITS sequencing and assessing the aflatoxin-producing ability. By analyzing the mutation type in AGCs of the *A. flavus*/*oryzae* strains, their characteristics and safety were determined; the results were compared with those of *A. oryzae* strains isolated from commercial yellow koji. We selected nonaflatoxigenic *A. flavus*/*oryzae* strains isolated from meju and are conducting whole genome analysis for accurate species identification. The strains that have been analyzed are expected to suggest the possibility of being used as an efficient and safe starter for meju fermentation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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