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보 문

Molecular diversity of endobacterial communities in edible part of King oyster mushroom *(Pleurotus eryngii)* based on 16S rRNA

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16S rRNA 기초 새송이 버섯*(Pleurotus eryngii)*의 식용가능 부위 내생세균 군집 다양성

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ABSTRACT: The diversity of endobacteria in the edible part (cap and stipe) king oyster mushroom *(Pleurotus eryngii)* was investigated using 16S rRNA sequence analysis. The bacterial 16S rRNA libraries were constructed from the body cap (BC) and the body stipe (BS) of the king oyster mushroom. The twenty sequenced BC clones were divided into four groups and the largest group was affiliated with the Firmicutes (40% of clones). While, the twenty sequenced BS clones could be divided into six groups and the largest group was affiliated with the Actinobacteria (40% of clones). The predominant bacterial family from both the cap and stipe of the mushroom was corresponded with the Gram positive bacteria (62.5%).

Key words: *Pleurotus eryngii*, 16S rRNA gene, bacteria diversity, endobacteria, phylogenetic tree

The edible king oyster mushroom (*Pleurotus eryngii*) is cultivated throughout the world and *Pleurotus* fungi as a group are linked to several agro-industrial activities of great economic importance, *e.g.*, conversion of lignocellulosic residues to food and feed, biocontrol of plant diseases, degradation of noxious pollutants, and production of enzymes and medicinal compounds (Ruiz-Duenas and Martinez, 1996; Wasser and Weis, 1999; Philippoussis *et al.*, 2001; Wang and Ng, 2004; Kang and Cho, 2014).

Intracellular symbiosis is a widespread and biologically important phenomenon. Although host cells usually provide a suitable shelter and supply important material needs for endosymbionts, the cytoplasm of a host cell may be considered to be a hostile environment for an invading organism (Morioka and Ishikawa, 1993; Mota *et al.*, 2005). Thus endosymbionts must still overcome many difficulties to survive in the host. Nevertheless, microorganisms can live in different tissues of healthy plants without causing symptoms of plant damage and give benefit to plants (Perotti, 1926; Gao *et al.*, 2005; Hashiba and Narisawa, 2005). The root system of plants, in particular, is known to offer different microhabitats for microbial growth.

Bacterial endosymbiosis with fungi, both parasitic and mutualistic types, is characterized by the intracellular localization of the bacteria (Moran and Wernegreen, 2000). Examples reported thus far are mainly for species belonging to the phylum *Glomeromycota* (Schußler *et al.*, 2001). Moreover,

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except for the cyanobacteria colonizing *Geosiphon pyriforme* (Schußler and Kluge, 2000), known intra-fungal bacteria are nonculturable. Despite this difficulty, the bacterial nature of these endosymbionts has been confirmed using molecular methods. Previously, endobacteria (e.g. *Burkholderia* sp.) was identified from several species of endomycorrhizal fungi such as *Gigaspora* sp. and *Scutellospora* sp. (Bianciotto *et al.*, 1996). Meanwhile, intracellular bacteria from wild types of *Laccaria bicolor* were detected using fluorescence *in situ* hybridization (FISH) in combination with confocal laser scanning microscopy (Bertaux *et al.*, 2003, 2005). In fact, the bacterium was identified as *Paenibacillus* sp. by using a 16S rRNA-directed oligonucleotide probe (Bertaux *et al.*, 2003). While, Barbieri *et al.* (2000) identified bacteria belonging to *Flexibacter-Cytophaga-Bacteroides* (FCB) phylogroup of Bacteroidetes phylum from the cell wall of hyphae of *Tuber borchii* (ectomycorrhizal fungus) using FISH analyses. A molecular study aimed at surveying potential endobacteria within the mycelium of the ectomycorrhizal fungus *Tuber borchii* Vittad. revealed the presence of novel uncultured bacteria (Gazzanelli *et al.*, 1999; Barbieri *et al.*, 2000). In addition, the phylogenetic analysis placed the bacterial sequences in a single new rRNA branch in the *Sphingobacterium* subgroup of the Bacteroidetes phylum (Steyn *et al.*, 1998; Barbieri *et al.*, 2002, 2005). Despite the above progress identifying fungal endosymbionts, little is known about the diversity and functions of bacterial populations living in the edible mushroom. Studies on the genetic and physiological diversity of the endomicrobial populations in edible mushrooms are important for understanding of their ecological role and for the development of biotechnological applications aimed at utilizing beneficial strains (Frey *et al.*, 1997; Timonen *et al.*, 1998).

The structure of mushroom have a stem (stipe), a cap (pileus), and gills (lamellae, sing. lamella) on the underside of the cap. These gills produce microscopic spores that help the fungus spread across the ground or its occupant surface. The goal of this study is to examine the endobacterial community in edible part (cap and stipe) of king oyster mushroom using analysis of 16S rRNA sequences in a culture-independent manner. To date, this study first reveals the endobacterial community in edible part of king oyster mushroom.

Materials and Methods

Microbial strains and growth conditions

Escherichia coli DH5α and recombinant *E. coli* cells were cultured in LB containing ampicillin (50 μg/ml). The king oyster mushroom (*P. eryngii*) used in this study was kindly provided by the Laboratory of Mushroom, Gyeongnam Agricultural Research and Extension Services (GARES), Jinju, Republic of Korea.

Growth of mushroom

The king oyster mushroom is usually cultivated on a sawdust medium in a plastic bottle. Sawdust from poplar was used and rice bran (30%) was supplemented to promote mycelial growth. The final moisture content of substrate materials was 65%. Prepared and moisture-conditioned sawdust mixture was loaded into the bottle feeder. Bottles were sterilized at 121°C for 90 min. After the bottles were removed from the autoclave, they were cooled to 20°C in the cooling room. Inoculated bottles were hauled to an incubation room, where temperature and humidity were maintained at 22 to 24°C and 65 to 68%, respectively. When the substrate in the bottles was colonized, the bottles were transferred to a cultivation room for 20 days to obtain fruiting bodies. The formation of fruiting body was induced at low temperature (about 15°C) and high humidity (about 90 to 95%).

Total DNA extraction

The surface of mushroom was disinfected with 1% sodium hypochlorite for 10 min. The external fruiting body of mushroom was removed approximately 0.5 cm from the margin of fruiting body with sterile razor blades. The fruiting body tissue was triturated by pestle in sterile 10 mM phosphate buffer (pH 7.2). The triturate was subjected to DNA extraction using the DNAzol Extraction Kit (MRC Inc.). The mixture of the extracted DNA from the ten samples was used as template for PCR to amplify 16S rDNA.

Polymerase chain reaction

The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers, 5′-CGGAGAGTTTGATCCTGG-

3′ (forward) and 5′-TACGGCTACCTTGTTACGAC-3′ (reverse) (Lane, 1991). Ribosomal DNAs were amplified by PCR using the extracted DNA, Super-Therm DNA polymerase (JMR), 1.5 mM $MgCl_2$, 2 mM dNTP, and primers in a final volume of 50 μl. Fifteen cycles (denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 1 min 30 sec) were followed by a final incubation at 72°C for 10 min. The anticipated product of approximately 1,500 bp was isolated after agarose gel electrophoresis of the amplified mixture using a gel extraction kit (NucleoGen).

Cloning and sequencing

PCR products were directly cloned into the pGEM-T Easy vector (Promega) and recombinant colonies were randomly (20 colonies) picked from approximately 40 colonies. The recombinant plasmids were extracted using plasmid DNA isolation kit (iNtRON). Samples for nucleotide sequencing were prepared by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp.). The samples were analyzed in both directions and two repeats with an automated DNA sequencer (model 3100; Applied Biosystems). Assembly of the nucleotide sequences was performed with the DNAMAN analysis system (Lynnon Biosoft).

Sequence analysis

All reference sequences were obtained from the GenBank and Ribosomal Database Project (RDP) databases (Maidak *et al.*, 2000). The sequences were analyzed using the CHECK_CHIMERA program (Maidak *et al.*, 2000) to exclude sequences from chimeric rDNA clones. The similarity search against database entries was done using online BLAST search (Madden *et al.*, 1996). Sequences were aligned using the multiple sequence alignment program CLUSTAL W versions 1.6 (Tompson *et al.*, 1994). Phylogenetic analysis was performed using the neighbor-joining method (Saitou and Nei, 1997). Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Bootstrap analysis was performed on data resampled 1,000 times using the DNAMAN analysis system (Lynnon Biosoft).

Nucleotide sequence accession numbers and nomenclature

The 16S rDNA gene sequences of endobacteria isolated from the king oyster mushroom have been deposited in the GenBank database under the accession numbers AY838556, AY838458-AY838495. The names of the 16S rDNA gene library from body cap (BC) and body stipe (BS) of mushroom begins with the letters BC and BS (e.g., BC1 for clone 1 of the cap library).

Results

Cloning and detection of bacterial rDNA

The PCR amplification from the total DNAs by the cap and the stipe samples of king oyster mushroom with the bacteria-specific primers produced a single band of approximately 1.5 kb. The products were purified from an agarose gel and cloned in *E. coli* DH5α in the pGEM-T Easy vector. Twenty clones were obtained from the body cap library (BC clones) and twenty from the body stipe library (BS clones).

Similarity with database sequences

All clones from the two libraries were subjected to sequence analysis followed by online homology searches using two databases: GenBank which implements the BLAST algorithm, and the RDP database which implements the SIMILARITY_RANK program (Table 1). Although there are no exact 16S rDNA similarity limits for defining specific taxa such as genus and species, in general, species definition requires sequence similarities greater than 97%. Fourteen sequences (35%) from the 40 clones in our libraries (6 from BC clones, 8 from BS clones) can be identified as belonging to Actinobacteria. About 15% of the sequences have a similarity level with database sequences in the range of 90–97% (Table 1). Twenty clones of the BC library were analyzed for DNA similarity. Among them, eighteen clones had the highest similarities with cultured bacteria and two clones had the highest similarities to uncultured rumen bacteria. Of the 20 clones of the BS library that were analyzed, eight were highly similar to each other and gave hits in the same similarity range (Table 1).

Table 1. **Similarity value of 16S rDNA sequences retrieved from mushroom cap and stipe**

Group	Clone (Accession NO.)	Phylum	Nearest relative[a] (Accession NO.)	Similarity (%)
Cap				
BCI	BC01 (AY838556)	Bacteroidetes	Uncultured rumen bacterium (AY244965)	90
BCII	BC02 (AY838458), BC10 (AY838466)	Bacteroidetes	*Sphingobacterium mizutaii* DSM 11724^T (AJ438175)	97–99
BCIII	BC03 (AY838459)	Firmicutes	*Staphylococcus epidermidis* ATCC 12228 (AE016751)	99
BCIV	BC04 (AY838460), BC07 (AY838463), BC09 (AY838465), BC11 (AY838467) BC19 (AY838475)	Firmicutes	*Streptococcus sanguinis* ChDC OS38 (AF543290)	99
BCV	BC05 (AY838461)	Actinobacteria	*Propionibacterium* sp.V07-12348 (Y17821)	99
BCVI	BC06 (AY838462) , BC12 (AY838468) BC13 (AY838469), BC15 (AY838471) BC20 (AY838476)	Actinobacteria	*Propionibacterium acnes* (AB097215)	99
BCVII	BC08 (AY838464), BC14 (AY838470)	Firmicutes	*Staphylococcus* sp. LMG 21006 (AJ316320)	99
BCVIII	BC16 (AY838472), BC18 (AY838474)	β-Proteobacteria	CDC Group Ivc-2 str. JHH 1448 (AF067657)	99
BCIX	BC17 (AY838473)	Bacteroidetes	Uncultured rumen bacterium (AY244919)	92
Stipe				
BSI	BS01 (AY838477), BS02 (AY838478) BS07 (AY838483) , BS10 (AY838486) BS13 (AY838489), BS14 (AY838490) BS16 (AY838492), BS20 (AY838495)	Actinobacteria	*Propionibacterium acnes* (AB097215)	99
BSII	BS03 (AY838479), BS06 (AY838482) BS18 (AY838557)	Firmicutes	*Salinicoccus alkaliphilus* (AF275710)	96
BSIII	BS04 (AY838480), BS05 (AY838481) BS08 (AY838484), BS17 (AY838493)	Bacteroidetes	*Sphingobacterium mizutaii* DSM 11724^T (AJ438175)	99
BSIV	BS09 (AY838485), BS15 (AY838491) BS19 (AY838494)	β-Proteobacteria	*Delftia acidovorans* (AB020186)	99
BSV	BS11 (AY838487)	α-Proteobacteria	*Methylobacterium nodulans* ORS2060 (AF220763)	96
BSVI	BS12 (AY838488)	γ-Proteobacteria	Bacterium G5 (AY160686)	99

[a] Accession of the nearest relative. When more than one sequence had the same similarity, only the accession number of the first sequence is given.

Phylogenetic placement of sequences from the two libraries

The results of phylogenetic analysis of the BC library are shown in Fig. 1A. In this library, the majority of the sequences were placed within the Actinobacteria phylum. Two sequences, BCI and BCIX, were related to the typical rumen bacteria of the Bacteroidetes, which indicates the symbiotic relationship between this family and the host mushroom. The eight clones in the Firmicutes phylum were clustered with the *Streptococcus* and *Staphylococcus.*

The results of phylogenetic analysis of the BS library are shown in Fig. 1B. The majority of the sequences in this library belonged to the Actinobacteria phylum. Eight clones were clustered with the type strain of *Propionibacterium acnes*.

Discussion

Compared with other ecosystems, there have been very few studies examined bacteria in mushroom. The relative lack of information available on endobacteria in mushroom may be due to difficulties in isolation, culture, or maintenance. We have examined the bacterial diversity in the edible mushroom using PCR-based 16S rRNA gene analysis. This culture-independent approach offers the possibility of characterizing microbial ecosystems independent of isolating, maintaining, and propagating bacteria under laboratory conditions. In fact, this study examined the bacterial community in the *Pleurotus eryngii* by analysis of bacterial 16S rDNA libraries constructed from the body cap (BC) and body stipe (BS) of king oyster mushroom. A total of twenty BC clones were divided into four groups and the

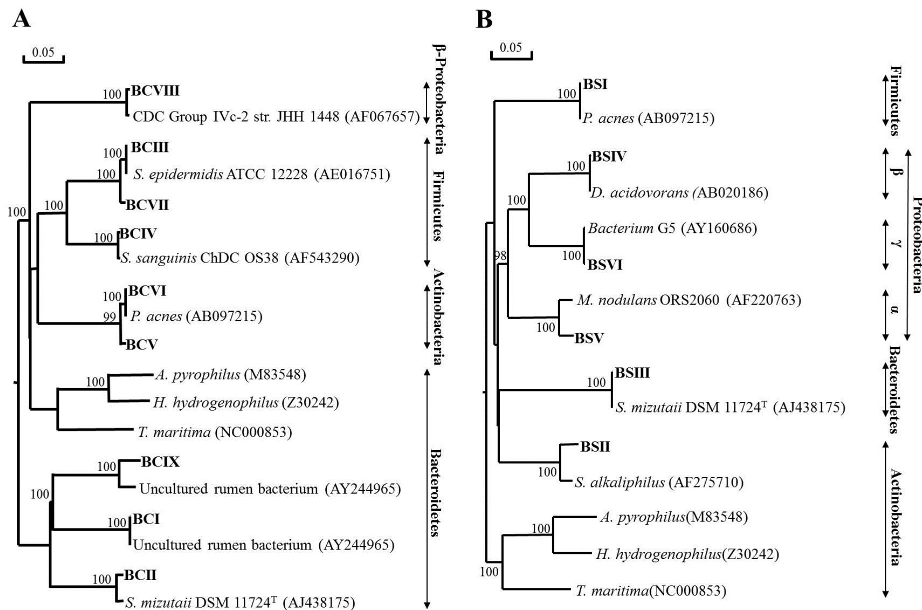

Fig. 1. Phylogenetic placement of 16S rRNA sequences from the fraction of BC (A) and BS (B) from king oyster mushroom. Numbers above each node are confidence levels (%) generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. Only bootstrap values of greater than 90% are shown. BC, body cap fraction of king oyster mushroom; and BS, body stipe fraction of king oyster mushroom.

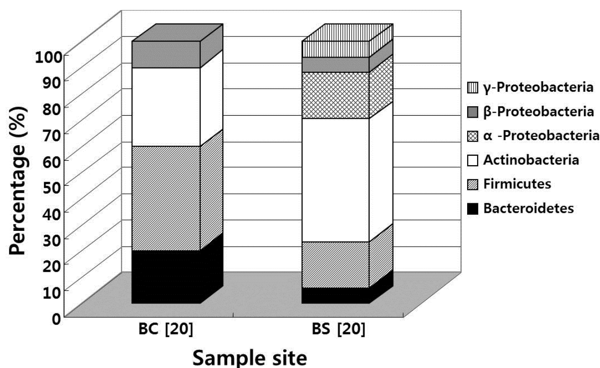

Fig. 2. Bacterial distribution of the fraction of BC and BS. Numbers in square brackets give the total number of clones in that library. BC, body cap fraction of king oyster mushroom; and BS, body stipe fraction of king oyster mushroom.

largest group was affiliated with the Firmicutes phylum (40% of clones). On the other hand, a total of twenty BS clones were divided into six groups and the largest group was affiliated with the Actinobacteria phylum (40% of clones) (Fig. 2).

One striking characteristic of the PCR-retrieved sequences was that the majority of them showed high similarity with known bacterial isolates and could be placed within a specific genus and species. The number of hits with database entries is shown in Table 1. The BS library had a high number of hits with isolates cultured from mushroom in comparison with the BC libraries (38% vs. ~8%). Interestingly, thirteen sequences of all forty clones were closely belonged to the *P. acnes* strains, while six sequences of all forty clones were corresponded with *Sphingobacterium mitutaii*. The predominance of sequences corresponded with *Propionibacterium* sp. and *Sphingobacterium* sp. suggests its role in the structure and function of the mushroom community. However, the current study did not coverage the functional role of these identified endobacteria in mushroom. Yet there is no available study shows these endobacterial functional relationships with these identified strains. Therefore, it is difficult to explain of any functional relationship of these endobacteria with mushroom. A possible reasonable explanation can be drawn that the identified endobacteria may play symbiotic role like endophytic bacteria in plants.

A second striking feature is that the majority of bacteria identified in these libraries from mushroom are Actinobacteria and Firmicutes (62.5%). Firmicutes were predominant in the mushroom cap while Actinobacteria were predominant in the stipe. In particular, the BCI and BCIX clones are similar to

uncultured rumen bacterium. The phylogenetic clustering of microorganism groups is a more useful guide for defining taxa than a specific similarity value (Fell *et al*., 2000). However, when the similarity values for a majority of sequences are too low to allocate them with a reasonable degree of confidence to particular taxa, then phylogenetic analysis be performed to clarify their taxonomic position (Paster *et al*., 1991).

A third feature is that the species distributions were different in the cap *vs*. the stipe. This suggests that these are likely to represent different microbial niches (Fig. 2). For example, the stipe samples contained sequences related to *Salinicoccus alkaliphilus* and *Delfia acidovorans* (sequences BSII and BSIV), but sequences related to neither were detected in the BC library. Two novel clones representing uncultured bacteria (BC01 and BC17) were found only in the cap samples. Approximately half of the LGCGPB-related sequences were affiliated with the *Propionibacterium* sp. group in both cap and stipe isolates. Representatives of known bacteria were more abundant in the BC library. A clone similar to *Staphylococcus epidermidis* was present in the sequences from cap. This atypical sequence may result from transient microbial contamination of the environment.

Studies on the bacteria associated with the edible mushroom will shed light on the relative importance of the bacterial species as a significant partner in fungus symbiosis. *In vivo* investigations are needed to assess whether any of the strains identified in this work can be used as fungal helper bacteria, promoting the establishment of the symbiosis between bacteria and its mushroom. The functional roles of endobacteria in the fungi are not clear and are needed to be described. However, the relationship between fungi and bacteria is unlikely to be casual. The trophic relationships between fungi and bacteria are likely to be complex. If these complex relationships are essential then it is necessary to ensure that all the components are present to stable infections of mushrooms with potential helper bacteria (Sbrana *et al.*, 2002; Hall *et al.*, 2003).

How do endobacteria differ from pathogens? Why does endomicrobial colonization not lead to disease? The status of the "endomicrobe" must be regulated at several well-evolved steps. With regard to the example of fungi-plant intracellular interactions, Schulz *et al.* (2002) hypothesized that the fungal endophyte-plant host interaction is characterized by a finely tuned equilibrium between fungal virulence and plant defense. If this balance is disturbed by either a decrease in plant defense or an increase in fungal virulence, diseases are developed. Also, endophytic penetration of the host plant was *via* the stomata and along the anticlinal epidermal cells; the pathogen, in contrast, was also able to penetrate directly through the cell wall. Interestingly bacteria, in particular *Pseudomonas* sp., have also been isolated from tuber fruiting bodies (Frey-Klett *et al.*, 2005), and are involved in the suppression of competing ectomycorrhizal fungi (Sbrana *et al.*, 2000). Most interesting is the antimycotic activity of *Pseudomonas* sp. against fungal contaminants from ascocarps of *T. borchii* (Bedini *et al.*, 1999; Sbrana *et al.*, 2000). Yet several bacteria have been reported playing role to mushroom included: *Pseudomonas* spp. (antibiotic producing), *Burkoholderia gladioli* pv. *agaricicola* (soft rots), *Pseudomonas aeruginosa* (mummy disease), *Pseudomonas agarici* (drippy gill disease), *Pseudomonas gingerii* (lighter discoloration on mushroom surfaces), and *Ewingella americana* (stipe necrosis). However, none of these bacteria are corresponded with those of endobacteria in the *P. eryngii* tested. It is probably occurred due to study on the sterile and healthy oyster mushroom in this study. Therefore, it is supposed that the endobacteria identified in this study may have friendly symbiotic relationship with the edible part of *Pleurotus eryngii.*

In this study, we revealed the diversity of endobacteria in edible part of king oyster mushroom (*Pleurotus eryngii*) by using 16S rRNA gene sequence analysis. In fact, we sampled from two parts of the mushroom such as cap and stipe. As a matter of fact, total 40 clones were identified and sequenced from the cap and stipe of mushroom. Since the mushrooms cap and stipe are used as foods, therefore, we focused the endobacterial diversity of these edible parts in this study. More endobacterial strains could be identified if we could analyze the roots of the mushroom tested. In future, a study should be conducted to reveal the whole diversity of endobacteria in the king oyster mushroom. Moreover, the role of these intracellular bacteria and their possible impact on the fugal symbiosis has to be studied thoroughly. Future studies to determine the effects of varying bacterial flora for the growth of the mushroom, and the impact of environmental conditions such as pH, moisture, and temperature on those effects will also be important.

적 요

세균 16S rRNA 유전자 특이 프라이머를 기초로 하여 중합효소연쇄반응을 이용하여 *Pleurotus eryngii* (큰느타리 버섯)의 내부에 존재하는 세균의 다양성을 조사하였다. 세균 16S rRNA의 라이브러리는 버섯 갓(BC, body cap)와 줄기(BS, body stipe)로 구성하였다. BC 20 클론은 네 그룹으로 구별되었고 가장 큰 그룹은 Firmicutes (클론의 40%) 있었다. 그러나, BS 20 클론은 여섯 그룹으로 나뉘어졌고 가장 큰 그룹은 Actinobacteria (클론의 40%) 있었다. 전체 버섯 내에 존재하는 세균 그룹은 그람양성 세균(62.5%) 있었다.

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