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Endophytic bacterial diversity in Korean *kimchi* made of Chinese cabbage leaves and their antimicrobial activity against pathogens

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ABSTRACT

The diversity of bacterial endophytes associated with Chinese cabbage (CC) leaves from two cultivation areas in Korea, namely, Seosang-gun (SS) and Haenam-gun (HN), and from a laboratory transgenic plant (TP) was investigated. A total of 5.76 log CFU/g isolates were identified from the interior leaves of the three cultivars of CC, which were grouped into 5.08, 5.36, and 5.34 log CFU/g from SS, HN, and TP samples, respectively. Phylogenetic analysis based on the 16S rRNA sequences indicated that the isolates belonged to four major phylogenetic groups: high-G + C Gram-positive bacteria (HGCGPB), low-G + C Gram-positive bacteria (LGCGPB), *Proteobacteria*, and *Bacteroidetes*. The most predominant group of species in the leaves of the SS, HN and TP CC cultivars were LGCGPB (50%), LGCGPB (65.2%), and HGCGPB (43%), respectively. A total of 23 bacterial genera were identified from the three cultivars of Chinese cabbage. Most extracellular hydrolytic enzyme-producing colonies among the isolates belong to the genus *Bacillus* those were predominant in the HN cultivars. In addition, some isolates especially *Bacillus* sp. (TPL08), *Bacillus subtilis* (SSL16, HNL10) exhibited potential activity as biocontrol agents against food-borne pathogenic bacteria and phytopathogenic fungi tested in this study. This study first revealed the endophytic bacterial communities in leaves of Chinese cabbage (main ingredients of *kimchi*) grown in Korea.

1. Introduction

Chinese cabbage (*Brassica campestris* L.) has been one of the most important vegetable crops in eastern Asia for many centuries. This cabbage is a major raw material of Korean traditionally fermented *kimchi* (Park, 1995). In addition to Korea, the popularity of Chinese cabbage *kimchi* is increasing globally. The consumption of Chinese cabbage *kimchi* may improve health because of its anti-carcinogenic secondary metabolites, e.g., glucosinolates and numerous ingredients, such as vitamin A, vitamin C, folic acid, calcium, potassium, and iron (Bender & Bender, 1995). The leaves of Chinese cabbage are generally used to prepare fermented *kimchi*. The fermentation pattern of *kimchi* was divided into three stages on the basis of pH (An, Lew, & Lee, 1999; Jung, Lee, & Jeon, 2014): 1) Initial stage- *Aeromonas* spp., *Erwina* spp., *Pleiosomonas* spp., *Xenohabdus* spp., *Bacillus* spp., 2) developmental stage- *Leuconostoc* spp., *Streptococcus* spp., *Pediococcus* spp., *Lactobacillus* spp., and 3) terminal stage- *Hansenulla* spp., *Brettanomyces* spp., *Torulopsis* spp., *Rhodotorula* spp., *Pichia* spp., *Klebsiella* spp., *Leuconostoc* spp., and *Lactobacillus* spp. were considered as major fermentation microbes, and selected to monitor change of microflora during *kimchi* fermentation. Because Chinese cabbage leaves are finally processed to consumable *kimchi* using several bacterial strains, the endophytic bacterial diversity of the vegetable crops should be revealed to improve its processing and to elucidate the presence of any pathogens. To date, the endophytic bacterial diversity of Chinese cabbage leaves has not been investigated.

Plants provide a nutrient-rich niche for the growth and development of microorganisms, particularly bacteria (de Melo Pereira, Magalhaes, Lorenzetii, Souza, & Schwan, 2012). Each plant supports a suite of microorganisms known as endophytes (Strobel & Daisy, 2003). Most endophytes live in association with the host plant in a dormant or active state but do not cause overt symptoms in their host plant (Bacon & White, 2000). Bacterial endophytes have focused roles within plants regarding nutrition (Dalton et al., 2004), stress or defense responses (Cho et al., 2002), invading phytopathogens (Furnkranz et al., 2012), growth promotion (Anand, Grayston, & Chanway, 2013), and pollutant catabolism

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(Siciliano et al., 2001). The populations of endophytic bacteria present significant differences associated with species, age, tillage, and environmental conditions (Assigbetse, Gueye, Thioulouse, & Duponnois, 2005; Germida, Siciliano, de Freitas, & Seib, 1998; Graner, Persson, Meijer, & Alstrom, 2003; Islam et al., 2010; Zhang, Gao, Cao, Ma, & He, 2013). However, there is a need for comparative investigations to elucidate the relationships between plant genotypes and colonization of endophytes (Graner et al., 2003). Moreover, the diversity among the endophytic populations of crop plants has been monitored by PCR-based techniques, and the results have revealed a range of organisms that belong to several distinct phylogenetic groups (Lkeda et al., 2013). An analysis of the endophytic bacteria that colonize ginseng roots discovered 13 different bacterial genera among 63 bacterial isolates classified into three major groups based on 16S rRNA gene analysis: Proteobacteria, high-G + C Gram-positive bacteria (HGCGPB), and low-G + C Gram-positive bacteria (LGCGPB) (Cho et al., 2007).

Bacterial endophytes penetrate into the plant root, stem or leaves using their extracellular hydrolytic enzymes. The extracellular cellulase, xylanase, pectinase, and protease enzyme activities in endophytic isolates from ginseng root and balloon flower have been investigated (Cho et al., 2007; Islam et al., 2010; Seo et al., 2010). Importantly, many bacterial endophytes have antibacterial activity against bacterial pathogens belonging to the genera *Erwinia* and *Xanthomonas* (Sessitsch, Reiter, & Berg, 2004). In addition, some endophytic bacteria have shown strong inhibitory action against food-borne pathogenic bacteria, (Seo et al., 2010) and phytopathogenic fungi (Cho et al., 2007; Islam et al., 2010).

This study first examined the population structures of endophytic bacteria from the leaves of Chinese cabbage cultivars in several areas of Seosang-gun and Haenam-gun and in a transgenic plant grown in the laboratory. A total of 5.76 log CFU/g viable cells were isolated from the leaves of Chinese cabbage cultivars. The 16S rRNA sequencing placed these Chinese cabbage leaves endophytes into 23 different bacterial genera. The extracellular enzymatic activity from 5.76 log CFU/g endophytic isolates was investigated. In addition, the inhibitory activity against foodborne-pathogenic bacteria and phytopathogenic fungi of these endophytes was also investigated. Importantly, some of these endophytes exhibited potential activity as biocontrol agents against food-borne pathogenic bacteria and phytopathogenic fungi.

2. Materials and methods

2.1. Microorganisms, plasmids, and media

The endophytic bacteria were isolated from Chinese cabbage (CC) and cultured at 28 °C or 37 °C in tryptic soy (TS) medium, and Number 3 medium (No. 3; 10 g of polypeptone, 10 g of glucose, 1 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$ per 1 L), (pH 6.8) was used for antibiotic production. *Escherichia coli* DH5α and recombinant *E. coli* cells were cultured at 37 °C in Luria–Bertani (LB) medium or LB medium supplemented with the appropriate antibiotics. The food-borne pathogenic bacteria used were *Escherichia coli* KCTC 1682, *Pseudomonas aeruginosa* KCTC 1750, *Salmonella enterica* KCTC 12456, *Salmonella enteritidis* KCTC 12400, *Salmonella typhimurium* KCTC 1925, *Shigella flexineri* KCTC 2008, *Shigella sonnei* KCTC 2518, *Bacillus cereus* KCTC 1012, *Listeria innocula* KCTC 3586, *L. ivanovii* KCTC 3444, *Listeria monocytogenes* KCTC 3569, and *Staphylococcus aureus* KCTC 1621. The pathogenic bacteria were collected from the Korean Collection for Type Culture (KCTC). These bacteria were grown on TS medium at 37 °C. The phytopathogenic fungi *Rhizoctonia solani*, *Pythium ultimum*, *Phytophthora capsici*, and *Fusarium oxysporum* were kindly provided by the Laboratory of Phytopathology, Gyeongnam Agricultural Research and Extension Services, Jinju, Korea. The plant pathogenic fungi were maintained on potato dextrose agar (PDA) medium and were cultured at 28 °C. The antibiotic ampicillin was purchased from Sigma and used at a concentration of 50 μg/ml. The LB, TS, and PDA media were purchased from Difco (Becton Dickinson Co., Sparks, MD, USA). The pGEM-T Easy vector (Promega, MI, USA) was used for cloning and sequencing.

2.2. Isolation of Chinese cabbage endophytic bacteria

Endophytic bacteria were isolated from CC leaves. A total of ten plants of Chinese cabbage were randomly collected from each cultivars grown in Seosang-gun (SS, *B. campestris* L. ssp. *Perkinensis* cv. Geyodong), Haenam-gun (HN, *B. campestris* L. ssp. *perkinensis* cv. Dongpung) and from a transgenic plant (TP, *B. campestris* L. ssp. *perkinensis* cv. Kenshin) grown in a laboratory at the Gyeongnam Agricultural Research and Extension Services (GARES) in the Jinju area of Korea. The leaf surfaces of CC were disinfected with 1% sodium hypochlorite for 10 min. The external portion of the leaves from approximately 0.5 cm from the margin was removed with a sterile blade. After then the leaf tissue of CC plants were triturated in sterile porcelain mortar with sterile 10 mM phosphate buffer (pH 7.2). To identify the maximum genera of endophytes from the same area, the ten CC plants leaves from the same area (cultivar) were mixed together before triturated in sterile porcelain mortar. Actually, 10 g of leaf tissue from each cultivar was separately homogenized in to 100 ml of the buffer. Aliquots of 1 ml were serially diluted ten-fold using the buffer and 100 μl leaf extracts were spread on TS agar and incubated at 28 °C and 37 °C for 48 h. The bacterial colonies were initially screened and grouped by colony color and morphological characteristics. The number of viable cells was expressed as log CFU/g of Chinese cabbage leaf tissue sample.

2.3. DNA isolation, amplification, sequencing, and recombinant technique

The isolated endophytic bacteria were cultured and centrifuged at 14,000× g and 4 °C for 5 min. The pellet was subjected to DNA extraction using the G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Suwon, Korea). The PCR primers used to amplify the 16S rRNA gene fragments were the universal primers (forward, 5′-CGGAGAGTTTPATCCTPG-3′; reverse, 5′-TACGGCTACCTTPTTAGC-GAC-3′). The 16S rRNA genes were amplified by PCR using the extracted DNA, Super-Therm DNA polymerase (JMR, Side Cup, Kent, UK), 1.5 mM $MgCl_2$, 2 mM dNTP, and primers in a final volume of 50 μl over thirty cycles (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min 30 s) followed by a final incubation at 72 °C for 10 min. The anticipated PCR product was isolated and cloned, and its sequence was analyzed according to Cho et al. (2007). Plasmid DNAs were isolated using a Plasmid DNA Purification Kit (iNtRON Biotechnology, Suwon, Korea). Standard procedures for restriction endonuclease digestion, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were used as described by Sambrook and Russel (2001). Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, MD, USA) and Promega (USA). All other chemicals were purchased from Sigma Chemical Co. (Louis, MO, USA).

2.4. Extracellular hydrolytic enzyme activity assay

The agar diffusion method was used for the detection of the extracellular hydrolytic enzyme activity of the isolated CC endophytic bacteria. The isolates were grown on different enzyme activity indicator media, such as cellulase, xylanase, mannanase,