Endophytic *Bacillus subtilis* MJMP2 from Kimchi inhibits *Xanthomonas oryzae* pv. *oryzae*, the pathogen of Rice bacterial blight disease

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Abstract An endophytic bacterial strain was isolated from kimchi, a Korean traditional fermented *Brassica campestris* and identified as *Bacillus subtilis* MJMP2 based on the 16S rRNA sequence. This strain showed strong antagonistic activity against *Xanthomonas oryzae* pv. *oryzae* (Xoo) KACC10331, the pathogen of bacterial rice blight disease, as well as activity against some other rice phytopathogenic fungi. The active compound was purified through size-exclusion chromatography and preparative High-performance liquid chromatography. The molecular weight was determined as m/z 1043 by mass spectroscopy, which is identical to that of iturin A. Furthermore, a crude extract from the culture supernatant of *Bacillus subtilis* MJMP2 showed inhibitory activity against rice blight disease in both a rice leaf explant assay and a pot assay. The crude extract also enhanced the length of roots of *Arabidopsis thaliana*. These results suggest that the strain *Bacillus subtilis* MJMP2 could be used as a biological agent to control rice blight disease.

Keywords *Bacillus subtilis* · Bacterial blight disease · Biocontrol · Iturin A · *Xanthomonas oryzae*

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Introduction

Bacterial rice blight is an endemic disease that affects rice cultivars in most tropical Asian countries. This disease is characterized by greyish-white lesions on leaf veins, indicating that it is a vascular disease. A severely affected field usually loses about 50 % of its total rice yield. *Xanthomonas oryzae* pv. *oryzae* (Xoo) has been identified as a major causative agent of bacterial blight on rice (*Oryza sativa* L.) (Ezuka and Kaku 2000).

Since the revolution in agriculture, to achieve better productivity and quality of agricultural products, farmers have been relying ever more heavily on chemical fertilizers and pesticides. Excessive use and misuse of such compounds has led to adverse effects including a reduction in the growth potential of fields, loss of essential microbiota in the soil, and environmental pollution. Thus, there is a need to develop novel methods or compounds to replace chemically synthesized products (Paul et al. 2012). Several microorganisms, known as biological control agents, have been used to control pests and diseases. The U.S. National Research Council defines biological control as “Use of natural or modified organisms, genes or gene products to reduce the effects of undesirable organisms and support the growth of desirable organisms which includes crops, beneficial insects and essential microbes.”

Interactions between plants and microorganisms affect plant health in several ways; such interactions include mutualism, proto-cooperation, commensalism, neutralism, competition, amensalism, parasitism, and predation. Microorganisms that affect plant health by controlling diseases are categorized as competitive saprophytes, facultative plant symbionts, or facultative hyperparasites. Previous reports have shown that compounds including antibiotics, enzymes, and various metabolites produced from microbes are the key to their protective effects (Whipps 1997; Weller et al. 2002).

Many species from the *Bacillus* genus have been identified as successful candidates for biocontrol because they have features like excellent colonization ability (Kloepper et al. 2004) and sporulation ability (Schallmeyer et al. 2004). Previous findings suggest that *Bacillus* strains have a great capacity to synthesize compounds

such as lipopeptide surfactants by employing large multi-enzyme complexes in a non-ribosomal pathway (Asaka and Shoda 1996). These natural surfactants are endowed with broad-spectrum antimicrobial activities (Emmert and Handelsman 1999) and are involved in plant disease control (Maget-Dana and Peypoux 1994). Lipopeptides have cyclic structures in which a peptide moiety has been conjugated with a β -amino or β -hydroxy fatty acid, making them amphiphilic. One widely-studied *Bacillus* lipopeptide is iturin. Lipopeptides in the iturin family include iturin A, mycosubtilin, and bacillomycin. These molecules have strong antifungal activities (Thimon et al. 1995; Duitman et al. 1999; Moyne et al. 2004).

In this study, an endophytic *Bacillus* strain with strong inhibitory activity against *Xoo* was isolated from kimchi, and tested for their efficacy against bacterial blight disease in rice. Furthermore, the active compound was isolated and characterized.

Materials and Methods

Isolation of microorganisms from kimchi

Microbes were isolated from kimchi, a Korean traditional fermented *Brassica*, by serial dilution. Kimchi fermented for 1 month was purchased from a local market and homogenized using a stainless steel blender. One g of homogenized kimchi was suspended in 10 mL 0.8 % saline and serially diluted. One hundred μ L of appropriately diluted suspension was plated on a Luria-Bertani agar plate (Difco, Detroit, MI, USA), and the plates were incubated at 37 °C for 24 h until bacterial colonies appeared. Single colonies were picked and cultured by streaking on LB agar plates. The bacteria were preserved at –80 °C in 20 % glycerol stocks. *Xanthomonas oryzae* pv. *oryzae* KACC10331 and other phytopathogenic fungi, *Fusarium oxysporum* KACC 40037 and *Rhizoctonia solani* AG-2-2 (IIIB) KACC 40151, were obtained from the Korean Agriculture Culture Collection (KACC), Jeonju, Korea, and grown on potato dextrose agar (PDA) (Acumedia, Lansing, MI, USA) at 25–28 °C.

Screening of antimicrobial bacteria

The antagonistic activities of bacterial isolates were assayed using *Xoo* as an indicator strain. Briefly, bacterial isolates were grown on LB agar at 37°C for 24 hours, and then grown in LB broth one day prior to the test. After complete incubation, the culture broth was loaded onto paper disks and placed on PDA plates inoculated with fungal pathogens. Mycelial growth inhibitory activities of the isolates were evaluated after 3 days of incubation. The isolate with the strongest inhibitory activity was selected for further study.

Identification of antimicrobial bacteria

Identification of microorganisms was done by 16S rRNA sequences. Extraction of bacterial genomic DNA was accomplished using phenol (Maniatis et al. 1982). Two universal primers, fD1 and rP2,

were used to amplify 16S rRNA by polymerase chain reaction (PCR). The sequences of fD1 and rP2 are 5'-AGAGTTTGAT CCTCCCTCTCAG-3' and 5'-ACGGCTACCTTGTTACGACTT-3', respectively (Weisburg et al. 1991). The amplification protocol was as follows: one cycle of denaturation for 3 min at 94 °C, 35 cycles at 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 2 min, and then one cycle at 72 °C for 7 min. After completion of the gene amplification process, the product was purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and sequencing was accomplished with an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Wellesley, MA, USA).

Lipopeptide extraction and purification

The selected strain was cultivated in M9 broth at 30°C and the lipopeptide-containing fraction was collected after removal of bacterial cells by centrifugation at 6,000 $\times$ g for 20 min followed by aseptic filtration (0.45 μ m). The supernatant was extracted with same volume of butanol, and the butanol extract was dried by vacuum evaporation. The dried extract was dissolved in butanol, and filtered with a 0.2- μ m nonpyrogenic hydrophilic membrane (Sartorius AG, Goettingen, Germany). These filtrates were fractionated by size exclusion chromatography using a Sephadex LH-20 column (16 mm ID $\times$ 70 cm L; Amersham Biosciences, Uppsala, Sweden) with butanol as an elution solvent and analysed by measuring absorbance at 215 nm. Fractions were collected and concentrated with a vacuum evaporator and their antimicrobial activity was assessed against the fungal pathogens. The fraction with antimicrobial activity was then loaded on a Zorbax SB-C18 column (9.4 mm ID $\times$ 25 cm L, 5 μ m particle diameter; Agilent Technologies Co.) and separated by reverse phase High-performance liquid chromatography (HPLC) (Waters, USA). The mobile phase components were (A) 0.1 % trifluoroacetic acid (TFA) in 10 % acetonitrile and (B) 0.1 % TFA in 100 % acetonitrile. The flow rate was set at 4 mL/min with a linear gradient of solvent B. The elution profile was analysed by measuring absorbance at 215 nm. The collected fractions were prepared for further ESI-MS.

ESI-MS analysis of active fraction

Mass spectral data were obtained with an LC-MS-8030 system (Shimadzu, Kyoto, Japan) enabled with a triple quadrupole mass analyser coupled with an ESI source set at positive full scan mode. The interface voltage was kept at 4.5 kV (ESI+) and the detector voltage was set to 1.2 kV. The desolvation gas temperature and heat block temperature were kept at 250 and 400 °C, respectively. Nitrogen gas was used as the nebulizer at a flow rate of 3.0 L/min and dry gas was set to flow at 15 L/min. Argon gas was used as the collision gas at a pressure of 230 kPa for CID and the collision energy was optimized for each precursor ion, i.e., –40 eV for iturin.

Assessment of inhibitory activity of crude extract from *Bacillus subtilis* MJMP2 against bacterial blight disease

Inhibition of bacterial blight disease was assessed as follows. *X.*

Fig. 1 Neighbour-joining tree based on 16S rRNA sequences showing relationships between the strains. Numbers at nodes indicates levels of bootstrap support (%) based on neighbor-joining analysis 1,000 resampled datasets. Bar, 0.005 substitutions per nucleotide position

Fig. 2 Structure of iturin A isolated from *Bacillus subtilis* MJMP2

Fig. 3 Effect of butanol extract treatment on bacterial blight disease in rice leaf explant (A) and in rice plant (B). (A) Explant infected by *Xanthomonas oryzae* by butanol extracts from *Bacillus subtilis* MJMP2. Rice leaf explant was infected with *Xoo* (a), and b, c, d were treated with butanol extract on the concentration of 10, 50, and 100 µg/mL before infection. (B) Effect of butanol extract from *Bacillus subtilis* MJMP2 on bacterial blight in rice plant. Rice leaf without any treatment (e), rice leaf treated with *Xoo* (f), BuOH extract was added before *Xoo* infection (g)

isolates, the MJMP2 strain showed the strongest activity. This strain showed the typical morphology of a *Bacillus* species. The colony on nutrient agar is round to irregular with an entire margin, and its colour is white to creamy. Single cells appear rod-shaped under a microscope (data not shown). Phylogenetic analysis based on the 16S rRNA sequence showed that this strain is closely related to *B. subtilis* subsp. *subtilis*, with the similarity of more than 99 %. The phylogenetic tree based on the neighbour joining method is shown in Fig. 1.

Purification and characterization of lipopeptides

Among the fractions collected by Sephadex LH-20 chromatography, fractions 5–8 showed strong activity against *Xoo*. These fractions were combined and further purified by Prep-HPLC. Fractions were collected every 2 min and lyophilized under reduced pressure conditions. The fraction that showed the strongest activity was selected for analysis through ESI-MS direct injection in negative and positive full scan mode. ESI-MS analysis revealed four main signals at m/z 1042.6, 1056.6, 1072.7 and 1083.8. The molecular

Fig. 4 Plant growth promoting activity of BuOH extract from *Bacillus subtilis* MJMP2. A: 7 days seedling of *Arabidopsis*; B: 7 days seedling after treatment with BuOH extract from *Bacillus subtilis* MJMP2

weights corresponded to iturin A containing an acyl chain with C14, C15, C16, and C17, respectively. In positive mode, three main signals at m/z 1066, 1080.6, and 1160.8 were observed, which correspond to C14, C15, and C17 fatty acyl chains in iturin A variants (Fig. 2).

Inhibitory activities of butanol extract from *Bacillus subtilis* MJMP2 against bacterial blight disease in rice leaf explant and pot assays

The inhibitory activity of butanol extracts from *Bacillus subtilis* MJMP2 against bacterial blight disease was assessed in both leaf explant and pot assays. In the leaf explant assay, leaf explants treated with *Xoo* suspension turned white, which is symptomatic of bacterial blight disease. After treatment with crude extract from *Bacillus subtilis* MJMP2, this symptom was alleviated (Fig. 3A). At a concentration of 10 µg/mL extract (Fig. 3B), the experimental and control plants looked the same (both turned white) (Fig. 3A). However, the whiteness was eliminated completely at concentrations of 50 and 100 µg/mL (Fig. 3C, D). In the pot assay, treatment with *Xoo* induced bacterial blight disease (Fig. 3f), while pretreatment with 50 µg/mL butanol extract from *Bacillus subtilis* MJMP2 prevented the disease (Fig. 3G).

Table 1 Antagonistic activity of *Bacillus subtilis* MJMP2 against various rice pathogens

Strain name	Activity	Pathogen
<i>Xanthomonas oryzae</i>	+++	Rice blight disease
<i>Rhizotonia solani</i>	++	Sheath blight of rice
<i>Fusarium oxysporum</i>	++	Root rot

+++ , strong inhibitory activity with inhibition zone of more than 10 mm

++ , moderate inhibitory activity with inhibition zone of 5–10 mm

Growth-promoting activity of crude extract from *Bacillus subtilis* MJMP2

The growth-promoting activity of the crude extract from *Bacillus subtilis* MJMP2 was studied *in vitro* using *Arabidopsis* seedlings. The root length of *A. thaliana* was enhanced after supplementation with crude extract in the agar plate. The length of roots reached 25 mm after crude extract treatment, while it was only 15 mm in the untreated group (Fig. 4).

Antimicrobial spectrum of crude extract from *Bacillus subtilis* MJMP2

Bacillus subtilis MJMP2 also showed strong inhibition of other rice pathogens, including *Rhizoctonia solani* KACC 40151 and *Fusarium oxysporum* KACC 40037, which are the pathogens responsible for rice sheath blight disease and rice root rot disease, respectively (Table 1).

Discussion

For a long time, chemical agents have been widely available for the control of various plant diseases; they are very popular in the agricultural sector. However, chemical products cause heavy damage to the fields, soil microbiota, and the environment. Severe negative effects on human health are also seen after such chemicals are used for a long time agriculturally. To ameliorate such problems, natural products and natural organisms such as microorganisms have become more important and earned a degree of commercial success; many studies on the use of various microorganisms as biocontrol agents are ongoing (Hoitink and Boehm 1999; Weller et al. 2002).

Previous studies reported the production of iturin by *Bacillus subtilis*, and its antagonistic activity against several phytopathogens is well documented (Ohno et al. 1993; Yu et al. 2002). *Bacillus amyloliquefaciens* has also proven its ability to produce iturin and prevent various fungal pathogens (Yoshida et al. 2001; Yu et al. 2002; Arrebola et al. 2010). All these studies indicate that production of iturin A is very important in the control of fungal pathogens by *Bacillus* species. Iturin A disrupts fungal cytoplasmic membranes, leading to formation of transmembrane channels that then permit the leakage of vital ions such as K^+ from fungal cells (Hsieh et al. 2008).

Besides iturin, *Bacillus* species are also known to produce surfactin. Recently, a surfactin homologues lipopeptide antagonistic to *Xoo* was obtained from the culture supernatant of *Bacillus amyloliquefaciens* B014. This lipopeptide, named as AXLP14 not only inhibited the growth of *Xoo*, but also negatively affected the motility and the biofilm formation of *Xoo*. Moreover, AXLP14 also regulated the gene expression which is involved in the rice defence mechanism (Li et al. 2015). In addition, endophytic *Bacillus* strains isolated from plant sources significantly reduced the severity of bacterial leaf blight for 40 % compared with control under glasshouse conditions. Treatment of *Bacillus* species highly induced some defence related enzymes like peroxidase, polyphenol oxidase, and resulted in higher accumulation of total phenols compared with control plants (Nagendran et al. 2013). Furthermore, *Bacillus subtilis* CMB32 was reported to produce biosurfactant lipopeptides iturin A, Fengycin, and surfactin, and showed good activity against *Colletotrichum gloeosporioides* (Kim et al. 2010). However, *Bacillus* strains showing inhibitory activity against *Xoo* through iturin A production has not been reported.

In this study, several *in vitro* assays have been performed against *Xoo* and some fungal pathogens. The active compounds was purified and identified by mass spectrometry. The mass spectra data were found to be similar to the data from previous studies on lipopeptides of *Bacillus* species, which confirmed the identity of our compound as iturin A (Kowall et al. 1998; Schneider et al. 1999). Furthermore, the butanolic extract of cell-free supernatant also inhibited the mycotic pathogens in several pot assays. When rice leaf explants infected with *Xoo* were treated with butanol extract of MJMP2 in a dose-dependent manner, the results clearly showed fungal growth inhibition. At a concentration of 10 $\mu\text{g/mL}$ about 40 % of explants were found to be clear or uninfected. As the doses were increased to 50 and 100 $\mu\text{g/mL}$, explants were found to be protected from infection by *Xoo*. In pot assay, rice leaves were treated with butanol extract and then infected with *Xoo* and the appearance of rice leaves was similar to that of uninfected leaves, whereas there was a clear difference from untreated infected leaves (Fig. 3). Figure 4 shows the growth-promoting activity of butanol extract. Treatment of seedlings prior to infection with pathogen not only saves these seedlings from infection but also helps in complete growth of the seedlings. Hence, butanol extract of MJMP2 can be used as an antibiotic as well as a plant growth promoting agent. The lipopeptides isolated from microbes are more effective against phytopathogens and have fewer side effects than do agrochemicals. These results established *Bacillus subtilis* MJMP2 as a potent biocontrol agent, and its efficacy in elimination of phytopathogens in field conditions should be exploited.

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