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Identification and Molecular Characterization of Three Isoforms of Iturin Produced by Endophytic *Bacillus* sp. CY22

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Endophytic *Bacillus* sp. CY22 was previously isolated from the interior of balloon flower root and showed strong antifungal activity against phytopathogenic fungi such as *Rhizoctonia solani*, *Fusarium oxysporum*, and *Phythium ultimum*. Many *Bacillus* strains produce antifungal compound such as iturin, fengycin, and mycosubtilin. We isolated and identified antifungal compound from cell supernatant of the endophytic strain. By the MALDI-TOF mass result, the antifungal compound was similar to the known antifungal lipopeptide iturin. It was found that the purified iturin had three isoforms with protonated masses of m/z 1,043.39, 1,057.42, and 1,071.42 and different structures in combination with Na^+ ion using MALDI-TOF MS. The *ita22* gene, which transacylase gene is associated with production of antifungal iturin, had an open reading frame (ORF) of 1,200 bp encoding 400 amino acids. Results of deduced amino acids sequence homology search, Ita22 was homologous with FenF (BAB69697) of *Bacillus subtilis* 168.

Key words – Endophytes, *Bacillus* sp., Iturin isoforms, MALDI-TOF mass

Bacillus species have the interesting property of producing secondary metabolites with a wide spectrum of antibiotic activity and very diverse structures. Therefore, they have been very valuable for medical and agricultural applications. Especially, many *Bacillus subtilis* strains produce a small peptides[10] with a long fatty moiety, the so-called lipopeptide antibiotics and peptide portions of these compounds contain α-amino acids with a D configuration. On the basis of the structural relationships, the lipopeptides that have been identified in *B. subtilis* are generally classified into three groups: the surfactin group [21], the plipastatin-fengycin group[10,14,16,32,33], and the iturin group. The structure of the iturin group consists of two major parts: a peptide ring composed of seven α-amino acids residues and an 11-12 carbons hydrophobic tail. This structure clearly suggests a marked amphiphilic character for this compound, thus pointing to the cellular membranes as the most probable site of its action. In general, all these biological actions have been ultimately explained as the result of the interaction of the lipopeptide with the membrane of its target cells, which induces a leakage of K^+ ions and other vital constituents, in parallel with cell death. These antifungal peptides were originally isolated from the culture medium of a strain of *Bacillus* sp. and inhibit the growth of a large number of fungi, including *Aspergillus*, *Penicillium*, and *Fusarium* species[20], as well as some yeasts[27]. Therefore, strains of *Bacillus* sp. have been studied as biocontrol agents of phytopathogens[6]. Only a few antibiotics have been isolated and purified, and their activity as biocontrol agents has been reported[2].

Biosurfactants including iturin group have many advantages compared with chemical surfactants: low toxicity, high biodegradability, environmentally friendly characteristics[8,19] and low foaming[34]. Furthermore, biosurfactants are able to protect the cell from attacks of other microorganisms[11].

Iturin is synthesized by large multienzyme complexs. Such peptide synthetases can be found in prokarytes as well as eukaryotes[7,12,13,18]. Recently, the operons that encode surfactin, plipastatin-fengycin, and mycosubtilin[7, 29], which is a member of the iturin A group, have been sequenced and characterized. Genetic and biochemical analyses of peptide synthetases have revealed a modular structure of these multifunctional proteins[7,12,13,17,18, 24,25]. A module is defined as the unit that catalyzes the incorporation of a specific amino acid into the peptide product. The arrangement of the modules of a peptide synthetase is usually colinear with the amino acid sequence of

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the peptide. The modules can be further subdivided into different domains that are characterized by a set of short conserved sequence motifts[17]. The core of each modules is an amino acid adenylation domain that recognizes and activates a specific amino acid[31]. The thiolation domain, located at the C terminus of the adenylation domain, contains an invariant serine residue essential for the binding of a 4'-phosphopantetheine cofactor[23]. An N-terminal condensation domain is required for the coupling of two consecutively bound amino acids. In addition, modules can be supplemented with domains that catalyze the modification of the activated amino acid, such as N-methylation, and epimerization from the L-configuration to the D-configuration[9]. The conserved modular organization of peptide synthetases provides the means for the creation of peptide synthetases by genetic engineering strategies. In this way, the exchange of different modules in the surfactin synthetase has been successfully used to produce new lipopeptide compounds[25,26]. The prospect of creating numerous bioactive peptides by the engineering of existing peptide synthetases has stimulated the search for additional peptide synthetases.

In this study, we report on the preparative separation of the iturin mixture isolated from endophytic *Bacillus* sp. CY22 and on the cloning of an transacylase gene which is associated with production of iturin production, named as *ita22*. The iturin isoforms were fractionated by reversed-phase HPLC and charaterized by its MALDI-TOF mass.

Materials and Methods

General methods and materials

Molecular cloning and PCR procedures were used as described by Sambrook *et al.*[22]. *Bacillus* sp. CY22 was originally isolated from the interior of balloon flower root (*Platycodon grandiflorum*) as previously described[3]. The *Rhizoctonia solani* and *Escherichia coli* were kindly provided by the Laboratory of Phytopathology, Gyeongnam Agricultural Research and Extension Services, Chinju, Korea. Media for growth of *Bacillus* sp. CY22 and *Escherichia coli* have been previously described[4,5]. The solvents, chemicals, and silica gel plates DC-60 were products of Merck (Darmstadt, FRG).

Isolation of iturin fraction

Isolation of iturin produced by *Bacillus* sp. CY22 was essentially performed as described by Cho *et al.*[5]. Cells were grown in number 3 medium (No. 3; 10 g polypeptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, pH 6.8) at 28℃. After 4 days of cultivation, the supernatant was collected by centrifugation and adjusted to pH 2.0 by using concentrated HCl. The precipitate was collected by centrifugation and extracted three times with methanol. The methanolic extracts were concentration and then dissolved in methanol.

TLC

For the further analysis, the methanolic extracts were separated using silica gel 60-plates (Merck). Chloroform/methanol/water (65:25:5, v/v/v) was used as developing solvent. The various spots were visualized by charring after spraying with concentrated sulfuric acid. For preparative isolation of the iturin fraction, the corresponding spots were scratched out from the thin-layer chromatograms, and the silica gel material was extracted with methanol. For further purification, the extract (1 mg) was subjected to reversed phase high-performance liquid chromatography (RP-HPLC; LC-908, JAIGEL-1H column, Japan Analytical Industry, Japan) and eluted with as follow condition: flow rate of 2.5 ml/min, acetonitrile/water (1:1) as mobile phase.

Mass spectrometric analysis of iturin fraction

The iturin fraction was analyzed by using fast atom bombardment (FAB) mass spectrometry (data not shown) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF, Vg-Instruments, Manchester, UK) mass spectrometry. Sample (4 to 5 μg) was dissolved in dimethyl sulfoxideglycerol and introduced on copper probe tip using a mixture of glycerol and triglycerol as matrix. A saturated solution of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% trifluoroacetic acid (v/v) was mixed with an equal volume sample for the MALDI-TOF mass analysis. The 1 μl of the sample (2 to 3 ρmol) was deposited on a sample plate and air-dried. Ions were accelerated with a voltage of 20 kV. The positive-ion and reflector mode was applied.

Antifungal assay of an iturinn fraction

The purified iturin fraction was dissolved in methanol and added to the paper disk (8 mm, Advantec, Germany) at concentrations ranging from 0 to 1.021 μg/ml. The pa-

per disk was placed in the margin of PD agar plates with various plant pathogen fungi, and then incubated at 28℃. After 5 days, inhibitory activity was measured as the width of the clear zone between the iturin fraction and fungal pathogen. For the control, the paper disk contained only methanol without iturin fraction.

Cloning of an transacylase gene associated with antifungal iturin production

To isolate of transacylase gene associated with antifungal iturin production Hurin production, PCR amplifications were performed with endophytic *Bacillus* sp. CY22 genomic DNA using degenerate primers. The degenerate primers were designed the basis of highly conserved region between antifungal compound producing genes of *Bacillus* species. The forward primer was 5′-GGH GAA TAY TCA GCR CTK G-3′ and the reverse primer was 5′-GCD GWR ACA TTY GAR ATG-3′. Purified DNA was used as a template in 50 μl PCR reaction containing 1 × PCR buffer, 10 mM dNTPs, 20 pmol primer, and 0.5 U Super-Therm DNA polymerase (JMR, Side Cup, Kent, UK). Thermal cycling conditions were: 2 min denaturation at 95℃ followed by 30 cycles at 94℃ for 20 sec, 54℃ for 20 sec and 72℃ for 1 min. The PCR product was purified using Gel Extraction Kit (NucleoGen, Seoul, Korea), sequenced, and confirmed by BLAST search. From these initially sequenced DNA regions we then amplified upstream (*pbp* gene) and downstream (*myc*A gene) outside DNA by primer walking using degenerated primers 5′-GSA TGM RMG RYA CBS YYG CMS ARG-3′ and 5′-GAT GRG TTA RYA TKA CTC CYT TCG-3′, respectively. The PCR fragments were sequenced and cloned into pGEM-T Easy vector (Promega, USA). All nucleotide sequencing was done by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, USA). The samples were analyzed with an automated DNA sequencer (model 310; Applied Biosystems, Foster City, CA, USA). Assembly of the nucleotide sequences and the amino acid sequence analysis were performed with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada).

DNA sequence accession number

The DNA sequences reported here have been deposited in the GenBank database under accession number AF534917 (*ita*22).

Results

Isolation and detection of three isoforms of iturin produced by endophytic *Bacillus* sp. CY22

The culture supernatant of *Bacillus* sp. CY22 was precipitated with concentrated HCl and then extracted with methanol. The methanolic extracts were detected by thin layer chromatography (TLC) on silica gel DC 60 plates using chloroform/methanol/water 65:25:5 (v/v/v) as the mobile phase. The antifungal iturin was detected by charring with concentrated 10% sulfuric acid and showed a broad spot with R_f of 0.36 (Fig. 1A). The spot was ex-

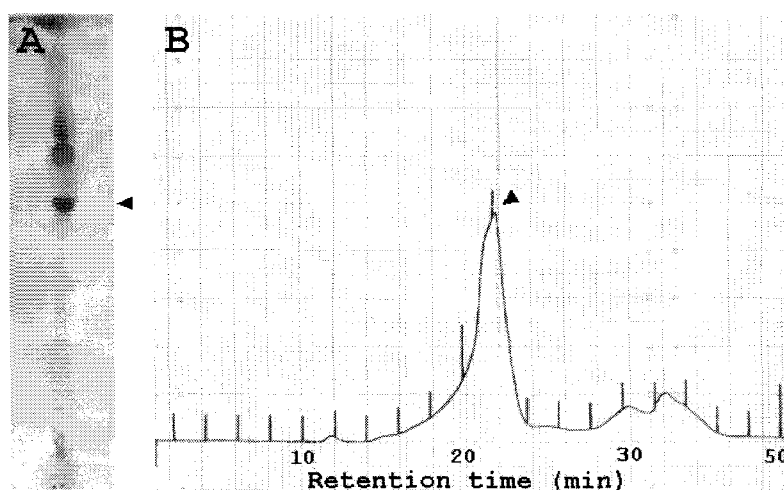

Fig. 1. Thin-layer chromatography (A) and HPLC spectrogram (B) of antifungal compound isolated from *Bacillus* sp. CY22. A, Developing solvent: $CHCl_3/MeOH/H_2O$=65:25:5, Visualization: 10% H_2SO_4 in water; B, Eluent solvent: CH_3CN/H_2O=1:1, Flow rate: 2.5 ml/min. Absorbance: 250 nm.

tracted with methanol and purified using reversed phase HPLC for further analysis (Fig. 1B).

The purified iturin fraction was identified and analyzed by MALDI-TOF mass spectrum (Fig. 2). The MALDI-TOF mass spectrum displayed $[M+H]^+$ peaks at *m/z* 1,043.39, 1,057.42 and 1,071.42, and these peaks were separated by *m/z* 14. Comparison with the mass database indicated that the peak at m/z 1,043.39 was similar to the known lipopeptide iturin, and at m/z 1,057.42 and 1,071.42 isoforms were deduced according to the number of carbon atoms of their fatty acid side chains. A series of ion was found at *m/z* 1,065.42 and 1,079.42 deducing sodium adducts according to *m/z* 14 lower, respectively.

Antifungal activity

The iturin exhibited a moderate antibiotic activity against *Rhizoctonia solani, Phytium ultimum,* and *Fusarium oxysporum.* The MIC_{50} of *R. solani* was 36 μg/ml and I_{50} of *P. ultimum* and *F. oxysporum* had similar sensitivities (Table 1).

Table 1. Antifungal activity of iturin against several phythopathogen fungi.

Fungus Tested	MICs (μg/ml)*
Rhizoctonia solani	36
Fusarium oxysporum	45
Phytium ultimum	66

*MICs are defined as the minimal concentration of iturin causing 50% (I_{50}) inhibition of fungal growth on PDA after 7 days.

Amplification and cloning of the transacylase gene region

For the isolation of the transacylase gene associated with production of antifungal iturin, we amplified 550 bp fragment using degenerate primers derived from high conserved consensus sequences[HFNI-X(1)-SHS] and [IK-X(2)-GKG]. From the results of a sequenced and a BLAST search, the fragment showed highly homology with the sequence of the *fen*F gene, which is known to the malonyl-CoA transacylase. From these initially sequences, the 'outside' region of upstream (*pbp* gene) and downstream (*myc*A gene) were amplified by primer walking using degenerate primers for each region. The PCR product of 2,114 bp in the downstream was amplified and then cloned into pGEM-T Easy vector and sequenced. From upstream, PCR product of 400 bp was amplified and sequenced.

Nucleotide sequence analysis of transacylase gene associated with production of antifungal compound

The inserted 2,114 bp fragment in pGEM-T Easy vector was sequenced using the dideoxy-chain termination method. It contains one complete open reading frame (ORF). Figure. 3 depicts the structural transacylase gene with its flanking regions. The open reading frame contains 1,200 nucleotides and encodes a protein of 400 amino acid residues. The ATG initiation codon at nucleotide position 378 is preceded by a putative Shine-Dalgarno sequence, GGAGGA. The open reading frame ends with the stop condon TAA at position 1,578. The resulting gene is named as *ita22*.

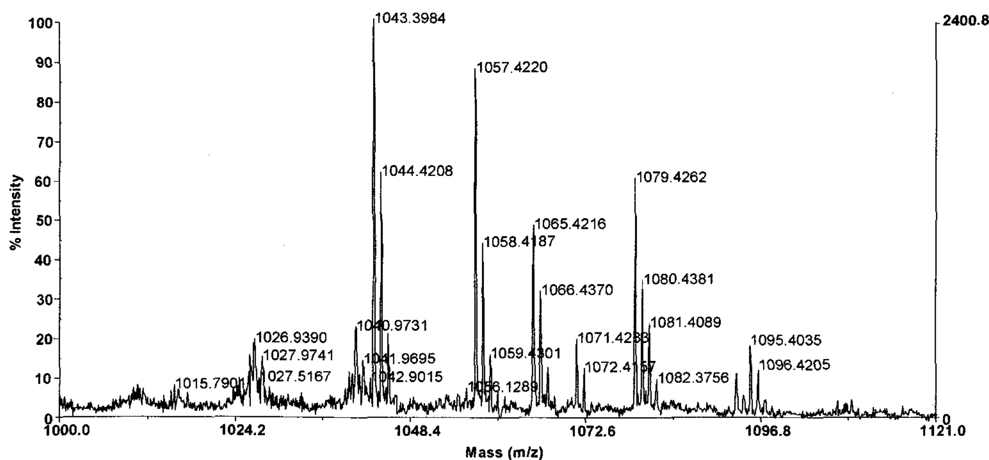

Fig. 2. MALDI-TOF mass spectrum of iturin produced by *Bacillus* sp. CY22. The iturin appeared as a complex mixture of several isoforms.

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1     ATCTATAATCAGCCACAATCAGTTTACAATCCTTCACAGATATACAAATGATTTTTTACA
61    ATAAACAGAAAATATGTAATTTCTGACACAATTATGCCGATAGCCCTAAAACATATGAAA
121   CATAAAGAGCGCGTTCTAAGTGAAGAAGAGCCGGTAACAGGCTCCACGCCGGCTTTCATC
181   ATCTCTCTTTAAGACTGATTTTCGGTGAAACCTCCATGTTTTATTTTATGAATAAATATA
241   TCTAATTCGTTTGTAAAATCGAACAATAACTCCTCCGAAAGTATTCTATACACATTTGTT
301   ATAATCATGCTAGGATGTTAGATAAAGGGATATTTTGTAGGGTATTGTATCCTGTTCAAT
361   ATGATCGGAGGAATCTCATGAACAATCTTGCCTTTTTATTTCCTGGACAAGGGTCTCAAT
1           SD          M  N  N  L  A  F  L  F  P  G  Q  G  S  Q
421   TTGTAGGAATGGGCAAACCTTTTTGGAATGATTTTGTGCTCGCAAAGAGATTGTTTGAAG
15    F  V  G  M  G  K  P  F  W  N  D  F  V  L  A  K  R  L  F  E
481   AAGCGAGCGATGCGATCTCCTTGGATGTAAAAAAACTGTGTTTTAACGGAGATATGAATG
35    E  A  S  D  A  I  S  L  D  V  K  K  L  C  F  N  G  D  M  N
541   AATTGACAAAGACAATGAACGCGCAGCCCGCTATTTTAACGGTCAGTGTGATTGCTTTTC
55    E  L  T  K  T  M  N  A  Q  P  A  I  L  T  V  S  V  I  A  F
601   AAGTGTATATGCAGGAAATAGGGGTGAAGCCCCGCTTCCTGGCAGGCCATAGCTTAGGCG
75    Q  V  Y  M  Q  E  I  G  V  K  P  R  F  L  A  G  H  S  L  G
661   AATATTCAGCGCTTGTCTGTGCCGGCGCCCTTTCTTTTCAGGATGCCGTTACACTTGTAA
95    E  Y  S  A  L  V  C  A  G  A  L  S  F  Q  D  A  V  T  L  V
721   GGGAGCGGGGAATTCTTATGCAAAATGCGGATCCCAAGCAGCAGGGGGCGATGGCCGCCG
115   R  E  R  G  I  L  M  Q  N  A  D  P  K  Q  Q  G  A  M  A  A
781   TGACTCACCTCTCTCTTCAAACGTTGCAGGAAATATGTTCGAAAGTGTCGACGGAAGACT
135   V  T  H  L  S  L  Q  T  L  Q  E  I  C  S  K  V  S  T  E  D
841   TTCCGGCAGGAGTAGCCTGCATGAATTCAGAACAGCAGCATGTGATTTCCGGACACCGGC
155   F  P  A  G  V  A  C  M  N  S  E  Q  Q  H  V  I  S  G  H  R
901   AAGCTGTGGAACGTGTCATCAAGATGGCGGAGGAAAAGGGTGCGGCATACACTTATTTGA
175   Q  A  V  E  R  V  I  K  M  A  E  E  K  G  A  A  Y  T  Y  L
961   ATGTCAGTGCGCCTTTTCACAGTTCGATGATACGATCAGCCTCCGAACAATTCCAGACTG
195   N  V  S  A  P  F  H  S  S  M  I  R  S  A  S  E  Q  F  Q  T
1021  TATTACAACGGTATTCCTTCCGGGATGCCGCATGGCCGATCATTTCAAATGTCACCGCGC
215   V  L  Q  R  Y  S  F  R  D  A  A  W  P  I  I  S  N  V  T  A
1081  GCCCTTACAGCAGCGGAAATTCGATCAGCGAACACCTCAAGCAGCACATGATGATGCCGG
235   R  P  Y  S  S  G  N  S  I  S  E  H  L  K  Q  H  M  M  M  P
1141  TAAGATGGACAGAATCGATGCATTATTTGCTTTTACACGGAGTGACGGAAGTCATCGAAA
255   V  R  W  T  E  S  M  H  Y  L  L  L  H  G  V  T  E  V  I  E
1201  TGGGTCCGAACAATGTCCTAGCCGGTCTGCTGAGAAAAACAACGAATCACATTGTACCTT
275   M  G  P  N  N  V  L  A  G  L  L  R  K  T  T  N  H  I  V  P
1261  ATCCCTTAGGACAGACATCCGATGTTCCCCCGCTTTCCAATTCAGCGGAAAGAAAGAAAC
295   Y  P  L  G  Q  T  S  D  V  P  P  L  S  N  S  A  E  R  K  K
1321  ATATTGTCCATTTACGCAAAAAACAACTGAATAAATTGATGATTCAATCCGTCATTGCGC
315   H  I  V  H  L  R  K  K  Q  L  N  K  L  M  I  Q  S  V  I  A
1381  GAAATTACAACAAGGATTCAGCGGCTTATTCCAATATGACGACGCCATTATTTACGCAAA
335   R  N  Y  N  K  D  S  A  A  Y  S  N  M  T  T  P  L  F  T  Q
1441  TCCAAGAGCTGAAAGAGAGAATGAAAAGGCATGAAGACGTGCTTTCAGAACAAGAACTCG
355   I  Q  E  L  K  E  R  M  K  R  H  E  D  V  L  S  E  Q  E  L
1501  AACATTCGATCCATTTATGCAAATTAATTTGTGAAGCTAAACAGCTTCCGGCTTGGGAAG
375   E  H  S  I  H  L  C  K  L  I  C  E  A  K  Q  L  P  A  W  E
1561  AATTGCGGATTTTAAAATAAAGCGCCCAGGAGGGGACCTATGTATACCAGTCAATTCCAA
395   E  L  R  I  L  K  -
1621  ACCTTAGTAGATGTCATTCGGGAAAGAAGCAATATCTCTGATCGCGGGATCCGTTTTATC
1681  GAATCCGATAAAAACGAGACGGTTGTCTCTTATCGCCAATTGTTTGAAGAGGCGCAAGGG
1741  TATCTTGGCTATTTACAGCATCTCGGCATTAAGCCGAAGCAGGAAATTGTATTTCAAATC
1801  CAAGAAAACAAATCATTTGTCGTTGCTTTTTGGGCTTGTATATTAGGAGGAATGATCCCG
1861  GTGCCGGTCAGTATCGGAGAAGATGATGACCATAAGCTGAAGGTCTGGCGCATTTGGAAT
1921  ATATTAAATCATCCGTTTCTGATTGCCTCTGAAAAAGTATTGGACAAAATAAAGAAATAC
1981  GCTGCAGAACACGATTTACAGGATTTCCATCATCAATTAAACGAAAAATCTGACGTCATT
2041  CAAGATCAAACCTACGATTACCCCGCTTCGTTTTATGAACCTGATGCGGATGAACTCGCC
2101  TTTATCCAATTTTC
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Fig. 3. Nucleotide and deduced amino acid sequence of the transacylase gene of *Bacillus* sp. CY22 with its flanking regions. Shine-Dalgarno sequence was underlined.

Comparison of amino acid sequence between Ita22 and other transacylase proteins

Sequence analysis revealed that the cluster of the ORF, designated *ita22*, belonged to a iturin synthetase operon (Fig.4). The *ita22* encodes a protein for the production of iturin with a 70% similarity to transacylase. Also, the deduced amino acid sequence of *ita22* (AF534917) was shown to be similar to that of the following proteins: FenF of *Bacillus*

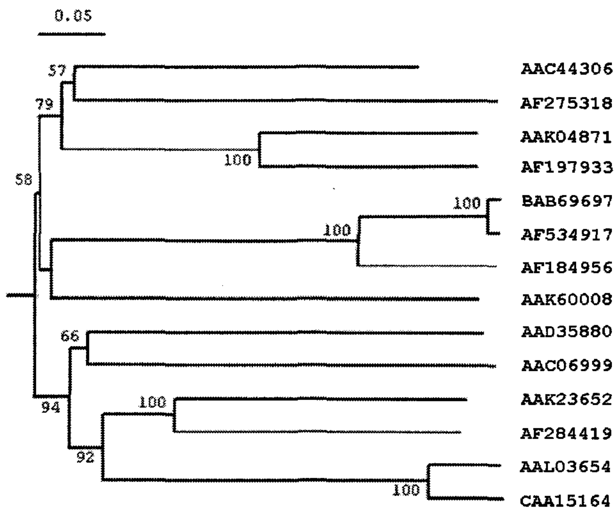

Fig. 4. Phylogenetic tree showing the evolutionary relationships of Ita22 and other closely related amino acid sequences. The estimated genetic distance between sequences is proportional to the lengths of the horizontal lines connecting one sequence to another. This is an unrooted tree. Bootstrap values of the major branch points are shown; they represent the number of times the group consisting of the species to the right of that branch occurred among 100 trees. The sequences are of the follow protein: AF184956, *Bacillus subtilis* ATCC6633, FenF; AAC44306, *Bacillus subtilis* 168, FabD; AF197933, *Streptococcus pneumoniae* R6, FabD; AF275318, *Staphylococcus aureus*, FabD; AAK04871, *Lactococcus latis* subsp. *Lactis* IL1403, FabD; BAB69697, *Vibrio cholerae* N1691, Vc2022; AF284419, *Sinorhizobium meliloti*, FabD; AAK23652, *Caulobacter crescentus*, Cc1674; CAA15164, *Rickettsia prowazekii* MadridE, Rp735; AAL03654, *Rickettsia prowazekii* Malish7, FabD; AAK60008, *Streptomyces aureofaciens* CCM3239, Aur1M; AAC06999, *Aquifex aeolicus* VF5, FabD; AAD35880, *Thermotoga maritima*, TM0798.

subtilis ATCC6633 (AF184956), FabD of *Bacillus subtilis* 168 (AAC44306), FabD of *Streptococcus pneumoniae* R6 (AF197933), FabD of *Staphylococcus aureus* (AF275318), FabD of *Lactococcus latis* subsp. *lactis* IL1403 (AAK04871), VC2022 of *Vibrio cholerae* N1691 (BAB69697), FabD of *Sinorhizobium meliloti* (AF284419), CC1674 of *Caulobacter crescentus* (AAK23652), RP735 of *Rickettsia prowazekii* MadridE (CAA15164), FabD of *Rickettsia prowazekii* Malish7 (AAL03654), AUR1M of *Streptomyces aureofaciens* CCM3239 (AAK60008), FabD of *Aquifex aeolicus* VF5 (AAC06999) and TM0798 of *Thermotoga maritima* (AAD35880). We constructed a phylogenetic tree of the transacylase protein by DNAMAN analysis system using above sequences, as shown in Figure 4.

Discussion

Among endophytic bacteria, *Bacillus* sp. are stable in soil as spores, and this is advantageous for the use of this bacterium as a biocontrol agent mainly because of the spore stability, ease of handling and production of the antifungal lipopeptide. Furthermore, some *Bacillus subtilis* are known to produce antimicrobial and biosurfactant substances, such as surfactin[21], fengysin[14,32,33], and iturin[10, 16]. Endophytic *Bacillus* sp. CY22 could produce an iturin. However, production of an antifungal and biosurfactant substance by any strain of endophytic *Bacillus* sp. has not been reported to date. To our knowledge, this is the first report of the production of iturin by endophytic *Bacillus* sp. We described the identification of a partial iturin synthetase operon specifying the synthesis of this iturin and a three isoform of iturin with different carbon length of fatty acid chain. Iturin is a cyclolipopeptide containing seven residues of α-amino acids (L-Asn, D-Tyr, D-Asn, L-Gln, L-Pro, D-Asn, and L-Ser) and one residue of a β-amino acid[1]. The biological activity of iturin from *B. subtilis* depends both on the amino acid composition and the sequence of their peptide ring as well as on the nature of their lipid moiety. The biological activity of iturin is enhanced with increasing number of carbon atoms of their fatty acid side chains presumably due to stronger interactions with biomembranes[14].

The root of the balloon flower is widely cultivated and used as a remedy for asthma and vegetable in East Asia. Especially, over-ten-years-old balloon flower root is one of the most cultivated medicinal crops, but 3 to 5 years old balloon flower root is generally prone to well known disease, such as root-rot caused by *Rhizotonia solani*[4]. Therefore, developing of biological control agent against balloon flower root rot was very important. Excessive use of chemical pesticides has caused a number of environmental problems. Because of this, the use of biological control agents has been promoted. Many applications of fungus and bacteria for plant disease control have been attempted.

In this paper, we isolated and identified three isoforms of iturin which is antifungal lipopeptide produced by endophytic *Bacillus* sp. CY22. Previously, we reported isolation and identification of three isoforms of surfactin produced by endophytic *Bacillus* sp. CY22 using MALDI-TOF mass and ESI-MS/MS spectrometry[5]. In this study, we carried out ESI-MS/MS analysis to identify three isoform of iturin. But we failed to get the results because first amino acid of surfactin is connected to a β-hydroxyl fatty acid,

and the carboxy-terminal amino acid forms a lactone ring to the β-OH group of the lipophilic part of the molecule[1,8]. In contrast to the surfactin, structure of iturin has too strong to broken by electric current.

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초록 : 식물 내생균 *Bacillus* sp. CY22가 생성하는 iturin isoform의 분리 및 특성

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식물 내생균 *Bacillus* sp. CY22는 식물병원균 *Rhizoctonia solani*, *Fusarium oxysporum* 및 *Phythium ultimum*에 대해 강한 항균력을 나타내었다. 일반적으로 많은 *Bacillus*속 균주들은 iturin, fengycin, mycosubtulin과 같은 항균물질을 분비한다. 본 연구에서는 식물내생균 *Bacillus* sp. CY22의 배양액으로부터 항균물질을 분리, 정제하였으며 MALDI-TOF mass로 분자량을 확인하였다. MALDI-TOF mass spectrum 분석 결과 분리된 항균물질은 *Bacillus*속 균주가 생성하는 항균물질로서 잘 알려져 있는 iturin의 분자량과 거의 일치하였으며, m/z 1043.4, 1057.4, 1071.4에서 molecular ion peak를 나타내었다. 이들은 각각 m/z 14차이를 가진 iturin의 isoform으로 추정되며 이것은 iturin을 구성하고 있는 지방산의 탄소수 차이로 생각되며 m/z 1065.4, 1079.4 peak는 sodium adduct로서 추정된다. 또한 항균물질 iturin을 생성하는데 관여하는 transacylase 유전자를 크로닝하여 *ita*22 유전자로 명명하고, 그 특성으로 *ita*22 유전자는 400 개의 아미노산을 인지하는 1,200 bp의 open reading frame (ORF)을 가지며, 아미노산의 상동성을 조사한 결과 *Bacillus subtilis* 168의 FenF (BAB69697)와 가장 유사하였다.