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Two-Dimensional Electrophoresis Analysis of Proteins; *Bacillus subtilis* LTD and Its Antifungal Activity Deficient Mutant

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Abstract - To investigate the antifungal activity related protein in pesticidal bacteria, a bacterial strain LTD was isolated from soil collected at Gimje in Jeonbuk province, Korea, and identified as *Bacillus subtilis* LTD based on a API50 CHB kit and 16S rDNA sequencing. It has an antifungal activity against 9 plant pathogenic fungi in a paper disc assay. The antifungal activity-deficient mutant, *B. subtilis* mLTD was induced at a 5 kGy dose of ^{60}Co gamma radiation. Using the two-dimensional electrophoresis and the matrix assisted laser desorption ionization time-of-flight mass spectrometry, the comparison analysis of proteins between the wild and mutant were performed. A major intracellular serine proteinase IspA (MW: 32.5 kDa), a NAD (P) H dehydrogenase (MW: 20.0 kDa), and a stage II sporulation protein AA, SpoIIAA (MW: 14.3 kDa) were detected only in the *B. subtilis* LTD. These results suggested that the functions of these proteins found only in the *B. subtilis* LTD could be closely related to the antifungal activity against plant pathogenic fungi.

Key words : antifungal activity, *bacillus subtilis* LTD, gamma radiation, mutant, 2-DE

INTRODUCTION

A large amount of synthetic chemical pesticides is used every year to prevent plant diseases and control insect pests. The overuse of chemical pesticides has caused soil and environment pollution and has harmful effects to human being and animal (Baker 1987). To reduce the use of synthetic chemicals, pesticidal microorganisms especially bacteria which has three useful criteria are used: a high antagonism to plant pathogens, an easy application and the low cost of mass production (Shoda 2000). The bacteria that provide benefits to plants are usually termed plant growth-promoting rhzobacteria (PGPR) (Kloepper *et al.* 1989), or yield-increasing bacteria (YIB) (Tang 1994). A number of different bacteria including *Bacilli* have been considered to be PGPR (Glick 1995). Gram-positive bacteria has a natural formation advantage over their Gram-negative counterpart: spore. *Bacillus* species especially *Bacillus subtilis* has been considered as effective biocontrol agents to plant pathogen and a stimulator of plant growth (Fravel 1988; Emmert and Handelsman 1999). They have been researched in depth and emphasized because their genetic and biochemical analyses and mass production are easier than those of fungi (Tang 1994). They have the ability to control the specific diseases involved their production of lipopeptide (Phae and Shoda 1991), siderophores (Leong 1986), and several kinds of antibiotics (Fravel 1988).

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Gamma radiation causes various types of damage to DNA in cells (Hutchinson 1985), requiring a concerted action of a number of DNA repair enzymes to restore the genomic integrity (Thacker 1999). And Lee *et al.* (2000) reported that gamma radiation could induce specific activity deficient mutants. Therefore, the gamma radiation induced mutant is a useful strain to obtain some more information on the proteins related to the antifungal activity of a pesticidal bacteria.

Two-dimensional electrophoresis (2-DE) has been known to be the most effective tool as well as one of the simplest methods for separating the complex protein mixtures (O'Farrell 1975). Isolated proteins by 2-DE can be identified and characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Nordhoff *et al.* 2001).

In this study, antifungal bacterium *B. subtilis* LTD was newly isolated from soil and the antifungal activity deficient bacterium *B. subtilis* mLTD was induced by gamma radiation. Protein expression profiles of the wild and mutant type were compared on the base of a 2-DE analysis and the different proteins were analyzed by MALDI-TOF MS to investigate the proteins which are related to the antifungal activity of a pesticidal bacterium.

MATERIALS AND METHODS

1. Isolation and identification of antifungal bacterium

Soil samples collected at Gimje in Jeonbuk province, Korea were diluted in a 0.85% NaCl solution. Extract solutions were smeared on to a nutrient agar (NA) medium and incubated at 37°C for 3 days. The revival bacterial strains were tested for antifungal activity against 10 plant pathogenic fungi which were obtained from the Korea Agricultural Culture Collection (*Alternaria alternata* KACC 40020, *Alternaria solani* KACC 40570, *Botrytis cinerea* KACC 40574, *Colletotrichum gloeosporioides* KACC 40804, *Colletotrichum higginsianum* KACC 40193, *Fusarium oxysporum* KACC 40239, *Phythium ultimum* KACC 40705, *Rhizoctonia solani* KACC 40124) and the National Institute of Agricultural Science and Technology (*Botryosphaeria dothidea* and *Mycosphaerella melonis*). The antifungal bacteria were isolated (Lee *et al.* 2003a) and identified by an API 50 CHB kit (BioMerieux, France) and 16S rDNA sequencing. Sequencing primers were 16-F (*E. coli* numbering 42-63 : 5′-CAGGCCTAACACATGCAAGTC-3′) and 16-R (*E. coli* numbering 1386-1404 : 5′-GCGCG GWGTGTACAAGGC-3′).

2. Mutant induction by gamma radiation (^{60}Co)

Isolated antifungal bacteria were cultivated in a nutrient broth (NB) medium in a 37°C shaking incubator for 12 hr and then each 1 mL was aliquoted in a 1.5 mL Eppendorf tube. After irradiation with a 95% lethal dose of gamma radiation (5 kGy, ^{60}Co, 60,000 Ci of capacity, Atomic Energy of Canada Ltd., dose rate: 920 Gy hr^{-1}), the antifungal activity deficient mutant was isolated (Lee *et al.* 2003b). The dose rate was measured with a 5 mm diameter alanine dosimeter (Bruter Instruments, Rjeomstettem, Germany). And the free-radical signal was measured using a Bruker EMS 104 EPR analyzer. The actual dose was within ±2% of the target dose.

3. Two-dimensional electrophoresis analysis

The wild and mutant strain were cultured in a 500 mL NB medium for 12 hr and then centrifuged at 1,600 × g for 20 min. The cells were transferred to a pre-chilled mortar and ground with a pestle in liquid nitrogen to a fine powder. The powder was homogenized in 12.5 mL of a protein extraction buffer containing 0.5 M Tris-HCl pH 8.3, 2% Nonidet P-40, 20 mM $MgCl_2$, 2% 2-mercaptoethanol and 1% phenylmethylsulfonylfluoride (PMSF). After centrifugation at 1,600 × g for 20 min at 4°C, the proteins in the supernatant were precipitated by adding four volumes of cold acetone at −20°C for 30 min for the analysis of the intracellular protein by 2-DE. After centrifugation at 1,600 × g for 20 min, the pellets were air-dried and applied to IEF (Kim *et al.* 2001). IEF was carried out in glass tubes, 5 mm inner diameter and 15 cm in length. The bottom of each tube was sealed with parafilm. The IEF gel mixture consisted of 4.5% w/v acrylamide solution, 9.5 M urea, 2% v/v NP-40, and 2.5% v/v ampholytes (pH 3-10 : pH 4-6.5 : pH 5-8 = 1 : 3 : 3). Each sample was mixed with a sample buffer and then loaded. IEF was performed at 250 V, 300

V and 400 V for 30 min respectively, 600 V for 10 hr, 800 V for 5 hr and then 1,000 V for 2.5 hr (O'Farrell 1975). Each focused gel was put into a 20 mL screw-cap tube with 5 mL of an equilibration buffer that contained 10% v/v glycerol, 2.5% w/v SDS, 125 mM Tris-HCl (pH 6.8), 5% v/v 2-mercaptoethanol, and 0.1 mg mL^{-1} bromophenol blue. It was then agitated gently at room temperature for 30 min. SDS-PAGE in the second dimension was carried out as described by Laemmli (1970). The 2-DE gels were stained by 0.3% w/v Coomassie brilliant blue R-250, 50% v/v methanol and 10% v/v acetic acid.

4. MALDI-TOF MS analysis

Protein spots obtained from the 2-DE gel were analyzed by MALDI-TOF MS (Applied Biosystems 4700 Proteomics Analyzer, USA) of the Korea Basic Science Institute. Proteins were identified with the mascot database (http://www.matrixscience.com/) (Perkins *et al.* 1999).

RESULTS AND DISCUSSION

1. Identification of the antifungal bacterium

An antifungal bacterium LTD was isolated from the soil sample at Gimje in Jeonbuk province. LTD strain was identified as *Bacillus subtilis* based on a API 50 CHB kit and 16S rDNA sequencing. *B. subtilis* LTD has a positive antifungal activity against 9 plant pathogenic fungi and a negative antifungal activity against *Fusarium oxysporum* (Table 1). *B. subtilis* is one of the biocontrol agents that has been emphasized recently because of its benefits. It has been used for many years in attempts to control plant pathogens and increase plant growth (Herish *et al.* 1998).

2. Antifungal activity deficient mutant induction by gamma radiation (^{60}Co)

Isolated *B. subtilis* LTD strain was irradiated at the dose of 0, 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 kGy of gamma ray (^{60}Co). The irradiated *B. subtilis* LTD was cultured on a NA medium to determine the radiation sensitivity. The D_{10} value was 2.29 kGy. The D_{10} value means the total radiation per one log titer reduction of the population (Woods and Pikaev 1994). Antifungal activity tests of the irradiated microbes in a paper disc assay were carried out. An antifungal activity deficient mutant was obtained from strain *B. subtilis* LTD irradiated at 5 kGy (95% lethal dose) of gamma radiation (^{60}Co) and named *B. subtilis* mLTD (Fig. 1). Table 1 shows that the *B. subtilis* mLTD has negative antifungal activities against all of the 10 plant pathogenic fungi. The gamma radiation induced mutant, *B. subtilis* mLTD could be useful

Table 1. Antifungal spectra of the *B. subtilis* LTD and *B. subtilis* mLTD against plant pathogenic fungi

Plant pathogenic fungi	Antifungal activities	
	LTD	mLTD
Alternaria alternata	+	−
Alternaria solani	+	−
Botrytis cinerea	+	−
Botryosphaeria dothidea	+	−
Colletotrichum gloeosporioides	+	−
Colletotrichum higginsianum	+	−
Fusarium oxysporum	−	−
Mycospharella melonis	+	−
Pythium ultimum	+	−
Rhizoctonia solani	+	−

+: positive, −: negative.

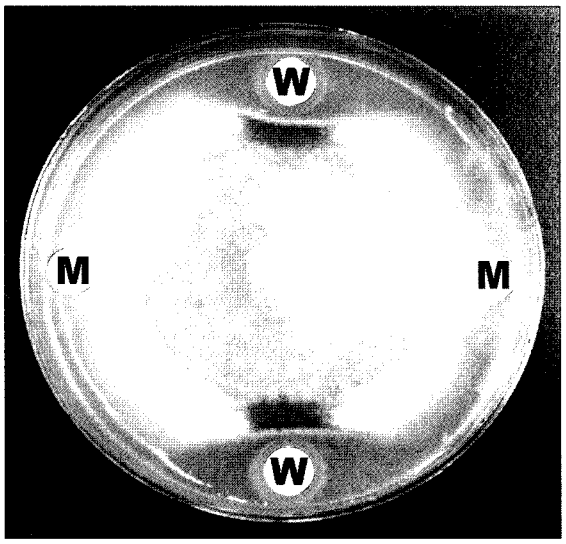


Fig. 1. Antifungal activity of a radiation induced mutant (*Bacillus subtilis* mLTD) against *Colletotrichum higginsianum*. W: *B. subtilis* LTD, M: *B. subtilis* mLTD.

for obtaining some information of the proteins related to the antifungal activity.

3. Three unique spots in the *Bacillus subtilis* LTD from 2-DE analysis

Intracellular proteins of the *B. subtilis* LTD and its antifungal activity deficient mutant *B. subtilis* mLTD were separated on to the 2-DE gels. The results are given in Fig. 2. Protein spots were detected on the Coomassie Brilliant Blue R-250 stained gel in the range of pI separation: from 4.22 to 6.78 and molecular weight from 10 to 225 kDa. The overall patterns of the protein spots were broadly similar to each other in molecular weight and isoelectric point. There were 3 protein spots obtained only in the *B. subtilis* LTD. Spot 1 has a pI of 5.03, a MW of 32.5 kDa. Spot 2 has a pI of 5.61, a MW of 20.0 kDa. Spot 3 has a pI of 5.97, a MW of 14.3 kDa. It is suggested that these 3 unique proteins could be expected to be related directly and/or indirectly to the antifungal activity.

4. MALDI-TOF MS analysis

The 3 protein spots that were obtained only in the strain *B. subtilis* LTD from the 2-D gel were analyzed by MALDI-TOF MS. The results are shown in Table 2. Spot 1 was a homologous protein, major intracellular serine proteinase IspA (*Bacillus subtilis*) with a mascot database: accession number in NCBInr: gi 2118110,

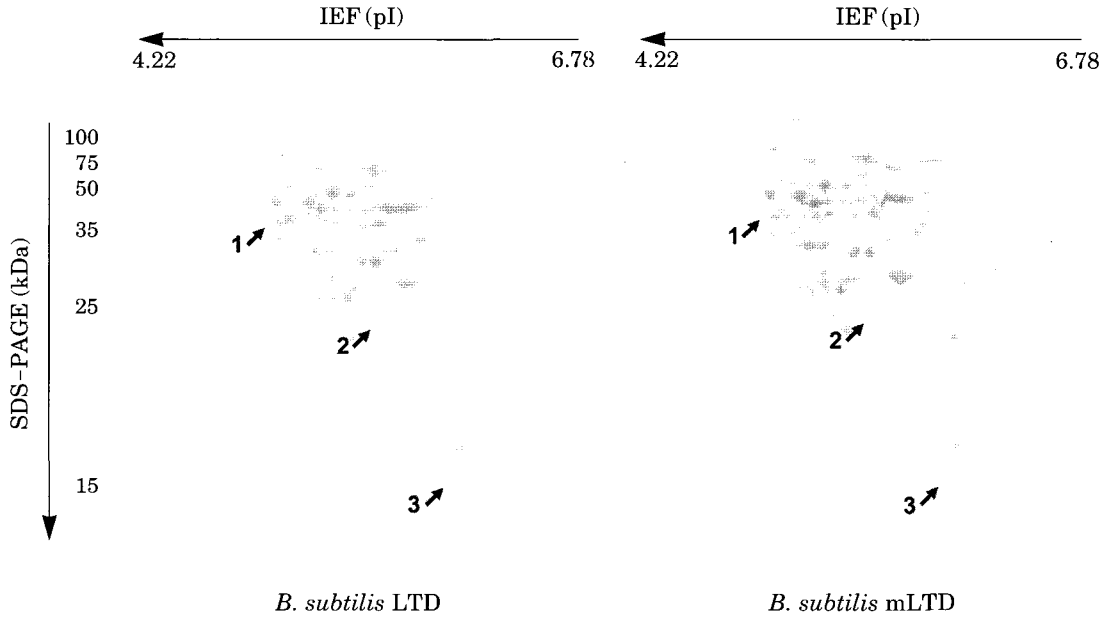


Fig. 2. Comparison of the 2-dimensional electrophoresis patterns of the intracellular proteins between *B. subtilis* LTD (AF+) and *B. subtilis* mLTD (AF−). After IEF (pI 4.22-6.78, 15 cm height) and SDS-PAGE (13% acrylamide gel, 15 cm × 16 cm), the gel was stained by 0.3% Coomassie brilliant blue R-250. Numbered arrows indicated the protein spots which were detected only in the *B. subtilis* LTD.

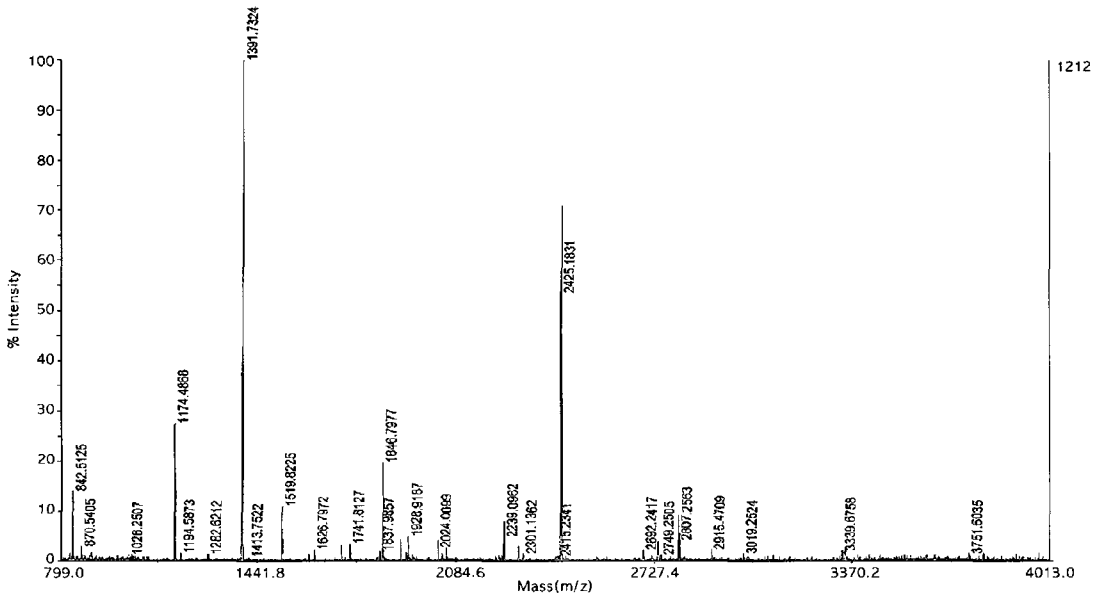
Table 2. Proteins identified from the 2-DE of *B. subtilis* LTD and *B. subtilis* mLTD by MALDI-TOF MS

Spot No.[a]	2-DE		Mascot database search results				
	pI	MW (kDa)	Accession[b]	Mass (kDa)	Homologous protein	Score[c]	Coverage (%)[d]
1	5.03	32.5	gi2118110	33.815	Major intracellular serine proteinase, IspA	136	56
2	5.61	20.0	gi16080407	23.257	NAD(P)H dehydrogenase	72	49
3	5.97	14.3	gi1075920	13.200	Stage II sporulation protein AA, SpoIIAA	44	49

[a]The numbering corresponding to the 2-DE image in Fig. 2; [b] Accession number in NCBInr; [c]Score is -10*Log (P), where P is the probability that the observed match is a random event; Protein scores greater than 66 are significant ($p < 0.05$); [d]Percentage of the protein sequence covered by the matched peptides.

MW: 33.815 kDa, match score: 136. Its 2-DE map by MALDI-TOF MS is shown in Fig. 3. Spot 2 was a homologous protein, NAD(P)H dehydrogenase (*Bacillus subtilis*) with a mascot database: accession number in NCBInr: gi 16080407, MW: 23.257 kDa, match score: 72, percentage of the protein sequence covered by the matched peptides: 56, percentage of the protein sequence covered by the matched peptides: 49, respectively. Spot

(A)



(B) 1. gi 2118110 **Mass**: 33815 **Score**: 136
microbial serine proteinase (EC 3.4.21. -), intracellular - *Bacillus subtilis*

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
1174.49	1173.48	1173.49	-0.01	261-	269	0	SYEEESFQR
1391.73	1390.73	1390.75	-0.02	271-	282	0	LSESEVFAQLIR
1519.82	1518.82	1518.84	-0.03	270-	282	1	KLSESEVFAQLIR
1741.81	1740.81	1740.84	-0.03	46-	61	0	VAVLDTGCDTSHPDLK
1837.99	1836.98	1837.01	-0.03	242-	260	0	LTGTSMAAPHVSGALALIK
1845.81	1844.80	1844.83	-0.03	169-	186	0	NGVLVVCAAGNEGDGDER
1923.00	1921.99	1922.04	-0.04	220-	237	0	EIDLVAPGENILSTLPNK
2023.01	2022.00	2022.03	-0.03	292-	310	0	TLAGNGFLYLTAPDELAEK
2051.09	2050.08	2050.13	-0.05	220-	238	1	EIDLVAPGENILSTLPNKK
2285.12	2284.12	2284.15	-0.03	143-	164	0	VDIISMSLGGPSDVPELEE
2301.14	2300.13	2300.15	-0.02	143-	164	0	VDIISMSLGGPSDVPELEE
2415.23	2414.23	2414.24	-0.01	7-	27	0	LIPYVTNEQIMDVNELPEG
2415.18	2424.18	2424.22	-0.04	187-	209	0	TEELSYPAAYNEVIAVGSV

No match to : 868.52, 874 46, 904.31, 913.72, 918.07, 986.84, 1026.25, 1037.05, 1194.

(C)

1	MNGEIR**LIPY**	**VTNEQIMDVN**	**ELPEGIKV**IK	APEMWAKGVK	GKNIK**VAVLD**	**TGCDTSHPDL**
61	KNQIIGGKNF	SDDDGGKEDA	ISDYNGHGTH	VAGTIAANDS	NGGIAGVAPE	ASLLIVKVLG
121	GENGSGQYEW	IINGINYAVE	**QKVDIISMSL**	**GGPSDVPELE**	**EAVK**NAVK**NG**	**VLVVCAAGNE**
181	**GDGDERTEEL**	**SYPAAYNEVI**	**AVGSVSVARE**	LSEFSNANK**E**	**IDLVAPGENI**	**LSTLPNKK**YG
241	KL**TGTSMAAP**	**HVSGALALIK**	**SYEEESFQRK**	**LSESEVFAQL**	**IR**RTLPLDIA	**KTLAGNGFLY**
301	**LTAPDELAEK**	AEQSHLLTL				

Fig. 3. Analysis of spot 1 on the *B. subtilis* LTD 2-DE map by MALDI-TOF MS. (A) MALDI-TOF MS peptide mass fingerprint spectrum obtained from a crude peptide mixture after an in-gel tryptic digest of spot 1. (B) The list of the matching peptides between the experimental and theoretical values. (C) The sequence of the major intracellular serine proteinase, IspA identified. The matched peptides are shadowed in the sequence.

3 was a homologous protein, stage II sporulation protein AA, SpoIIAA (*Bacillus subtilis*) with a mascot database: accession number in NCBInr: gi 1075920, MW: 13.200 kDa, match score: 44, percentage of the protein sequence covered by the matched peptides: 49. Molecular weight and the databases for the peptide mass matches have made high-throughput protein identification possible. Peptide mass finger-prints, acquired by MALDI mass spectrometry, enabled unambiguous protein identifications to be made where full gene sequence information was available (Cohen *et al.* 2002).

Antifungal activity deficient mutant, *B. subtilis* mLTD didn't express an intracellular serine protease (IspA), stage II protein, (SpoIIAA) and an energy generating protein, (NAD(P)H dehydrogenase). In the stationary phase, it is reported that *B. subtilis* expressed various molecules related to the intracellular and extracellular functions including the antifungal activity and sporulation (Strauch 1993; Cho *et al.* 2003).

In our experiment, *B. subtilis* mLTD didn't express IspA which has been reported as a endotoxin related protein in *B. thuringiensis* (Chen *et al.* 2003). It has also been reported that in *B. thuringiensis*, endotoxin could be produced by the regulators of sigma E and sigma K after activation of the pro-sigma K by IspA (Baum and Malvar 1995). It seems that IspA could be related with the antibiotics, which are secreted inside or outside the cell.

According to the SpoIIAA which was expressed only in the *B. subtilis* LTD, Phillips and Strauch (2002) reported that SpoIIAA was one of the sporulation regulators coded from the cistronic gene of the sigma F operon in *B. subtilis*. So, we are not sure whether the metabolic processes between the antifungal activity and sporulation could be partly similar or not.

The last disappearing spot in *B. subtilis* mLTD was a NAD(P)H dehydrogenase, which is one of the energy generating members of the Krebs cycle as an electron-transport system (Kerscher 2000). In *B. subtilis* mLTD, the NAD(P)H dehydrogenase was not expressed as much as in the *B. subtilis* LTD. It seems that some metabolic activities including antifungal bioactive material synthesis could be arrested in the *B. subtilis* mLTD.

As a result, it is suggested that all of the 3 unique spots in the *B. subtilis* LTD could be directly and/or indirectly related to the antifungal activity. Further studies will follow in order to determine how much these 3 proteins are involved in the metabolic processes of the antifungal activity.

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