The Journal of Microbiology, June 2005, p.308-312 Vol. 43, No. 3

NOTE

A Putative Early Response of Antifungal *Bacillus lentimorbus* WJ5 Against the Plant Pathogenic Fungus, *Colletotrichum gloeosporioides*, Analyzed by a DNA Microarray

Young-Keun Lee*, Yu-Sin Jang, Hwa-Hyoung Chang, Seok Won Hyung and Hye-Young Chung

Radiation Application Research Division, Korea Atomic Energy Research Institute, Sinjeong, Jeongeup 580-185, Republic of Korea

(Received November 5, 2004 / Accepted April 14, 2005)

The global RNA transcription profiles of *Bacillus lentimorbus* WJ5 under an *in vitro* co-culture with *Colletotrichum gloeosporioides* were analyzed in order to study the antagonistic bacteria-fungi interactions. Using a filter membrane system, *B. lentimorbus* WJ5 was exposed to the spores of *C. gloeosporioides* at the late exponential stage. The transcription profiles of the *B. lentimorbus* WJ5, both with and without a challenge from *C. gloeosporioides*, were analyzed using custom DNA chips containing 2,000 genome fragments. A total of 337 genes were expressed, with 87 and 47 up- and down-regulated, respectively. Of these, 12 genes, which were involved in central carbon metabolisms, and 7 from minor catabolism were relatively highly up-regulated (> 10 fold) and down-regulated (< 0.2 fold), respectively. Nine genes, which were thought to be related to the antifungal activity, were also up-regulated, but their levels were not so high (2.0 - 9.7 folds). From the results, during the early stage of the co-culture of *B. lentimorbus* WJ5 and *C. gloeosporioides*, nutrient competition seemed to occur; therefore, the genes from central carbon metabolisms could be up-regulated, while those from minor catabolism could be down-regulated.

Key words: Antifungal activity, bacteria-fungi interaction, *Bacillus lentimorbus* WJ5, *Colletotrichum gloeosporioides*, nutrient competition

There have been many studies regarding the use of antagonistic microbes as an alternative to synthetic chemical pesticides in biocontrol systems, as the latter could be a potential cause of public pollution in the future (Whipps, 1997; Lee *et al.*, 2001b, 2003a). Many bacteria and fungi have been reported as antagonistic microbes against phytopathogenic fungi (Howell, 1998; Bonsall *et al.*, 1999; Lee *et al.*, 2001a).

Most of the interactions between antagonistic and phytopathogenic microbes have been summarized as deriving from the inhibition of the pathogen by antimicrobial materials (Raaijmakers *et al.*, 2002), competition for nutrients (Mondal and Hyakumachi, 2000), the inactivation of pathogen germinating factors (Whipps, 1997) and degradation of the pathogenicity factor (Steijl *et al.*, 1999), etc. It has also been suggested that these mechanisms are not necessarily mutually exclusive within an interaction system between a bacterium and a phytopathogenic fungus (Whipps, 2001); therefore, research on each mechanism as well as their interrelationship are required.

A co-culture assay of antagonistic microbes has previously been carried out to investigate the microbe interactions. Meyer *et al.* (2003) directly cultured *Aspergillus giganteus* with *Fusarium oxysporum* for the determination of an elevated antifungal protein titer.

However, the global gene expressions due to antagonist-fungus interactions remain to be studied. DNA microarrays could offer a simultaneous method for the identification of all genes expressed due to antagonist-fungus interactions (Ye *et al.*, 2001).

In this work, custom DNA microarrays of the antagonistic bacterium, *B. lentimorbus* WJ5, were constructed and the RNA transcription profiles from the *in vitro* co-culture of *B. lentimorbus* WJ5 against *C. gloeosporioides* observed, in order to study the antagonist-fungus interaction.

An antifungal bacterium, *B. lentimorbus* WJ5, was isolated from soil (Uljin province, Korea) and identified by an analysis of the 16S rDNA gene sequences and FAME composition (Lee *et al.*, 2003a). The assay for the anti-

* To whom correspondence should be addressed.
(Tel) 82-63-570-3300; (Fax) 82-63-570-3309
(E-mail) yklee@kaeri.re.kr

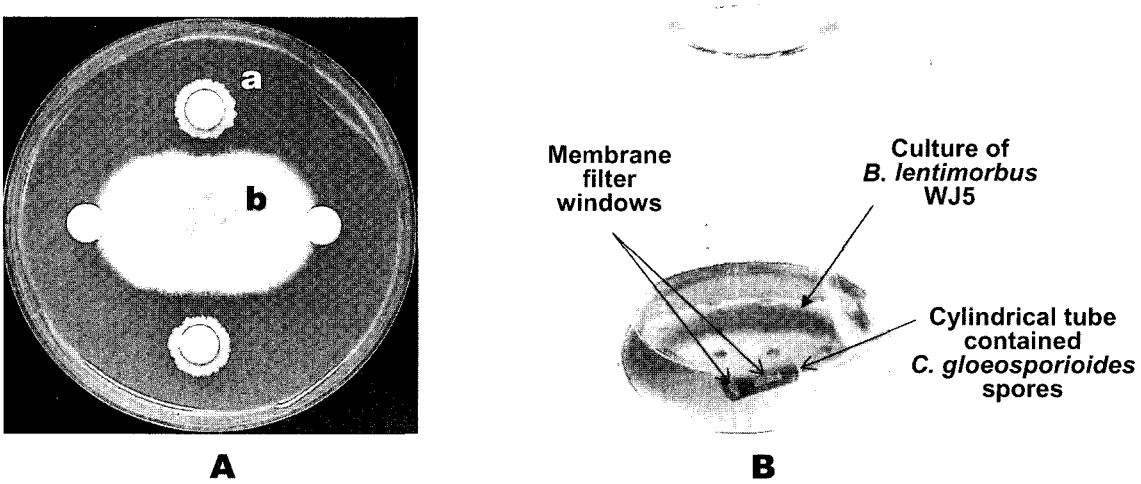

Fig. 1. Co-culture of *B. lentimorbus* WJ5 and *C. gloeosporioides*. (A) Antifungal spectra of *B. lentimorbus* WJ5 against the plant pathogenic fungus *C. gloeosporioides*. a; *B. lentimorbus* WJ5, b; *C. gloeosporioides*. (B) Schematic representation of the homogeneous co-culture of *B. lentimorbus* WJ5 and *C. gloeosporioides*.

fungal activity was carried out in a Petri dish containing 15 ml of potato dextrose agar. Paper discs (diameter = 6.25 mm), with bacterial samples, were loaded 15 mm from the mycelia of the phytopathogenic fungus, *C. gloeosporioides* KACC 40804. After incubation at 27°C for 5 days, an inhibition zone, referred to as the antifungal activity, formed around the paper disc (Fig. 1A).

The *B. lentimorbus* WJ5 was cultured at 37°C, with agitation, in 500 ml of nutrient broth (NB, Difco, USA). Ten milliliters of *C. gloeosporioides* spores (5.0×10^5 spores/ml) were placed in a sterilized cylindrical polyethylene tube (diameter; 25 mm, height; 30 mm), in which 4 outlets for the membrane filters (upper and bottom; 25 mm, side; 15 mm, pore size; 0.45 μm) had already been made. Seven hrs after initially culturing the *B. lentimorbus* WJ5, that is, 1 h before the late exponential stage, the co-culture was carried out by the administration of the tube containing the spore into the culture broth of the *B. lentimorbus* WJ5, which was incubated for a further 1 h (Fig. 1B).

Total RNA was extracted from the bacteria using spin columns (RNeasy plant mini kit, Qiagen, Germany). Cultured cells were precipitated by centrifugation at 5,000 × g for 5 min at 4°C. The precipitate was resuspended in a TE buffer containing lysozyme (5 mg/ml). After lysis, the samples were purified on spin columns, according to the recommendation of the manufacturer, and eluted in nuclease-free water (Promega, USA). After digestion with 1 U/μl of DNase I, the samples were purified using RNeasy spin columns and finally eluted in nuclease-free water (Promega, USA). The quality and concentration of the RNA samples were estimated by agarose gel electrophoresis and spectrophotometry. The samples were stored at -80°C until use.

The DNA microarray was constructed at GenomicTree, Inc. (Korea), with random clones, as the complete genome sequence of *B. lentimorbus* was not available. Genomic DNA was extracted from the bacterial culture using AccuPrep spin columns (Bioneer, Korea), according to the recommendations of the manufacturer. The genomic DNA of the *B. lentimorbus* WJ5 was restricted with both *EcoR*I and *Sau3A*I. Two thousand genomic DNA fragments (500-1,000 bp) were ligated with pBluescript SK(+), which were digested with both *EcoR*I and *BamH*I, transformed into the *E. coli* host XL1-Blue and amplified using a PTC-200 thermal cycler (MJ Research, USA). Amplified DNAs were spotted onto silanized glass slides (CMT-GAPS, Corning, USA), using an OmniGrid microarrayer (GeneMachines, USA). Each slide was cross-linked with 300 mJ of short wave ultraviolet (UV) using a Stratalinker (Stratagene, USA).

The RNA of the *B. lentimorbus* WJ5 was labeled with cyanine 3-dUTP (Cy3-dUTP, NEN Life Science Products, USA) and used as the control probe. The RNA of the *B. lentimorbus* WJ5 challenged with the *C. gloeosporioides* spores was labeled with cyanine 5-dUTP (Cy5-dUTP, NEN Life Science Products, USA). The labeled cDNA probes were then purified through a microcon-30 column and resuspended in 80 μl of a hybridization solution (3 × SSC and 0.3% SDS). The target mixture was denatured at 100°C for 2 min and applied to the DNA microarrays at 65°C for 16 h in a humidified chamber. The hybridized slides were washed in 2 × SSC for 2 min, 0.1 × SSC/0.1% SDS for 5 min, and 0.1 × SSC for 5 min and then spin-dried prior to scanning at room temperature.

Microarray scanning and normalization of the data were performed using a GenePix 4000B scanner and the GenePix Pro 3.0 software package (Axon, USA). Poor quality spots (sum of median < 300) were filtered from the raw data before analysis. The microarray experiments were repeated twice, and the average of the two gene expres-

sion values for each gene used for the analysis. For each hybridized spot, the gene expression value (ratio of the median in the GenePix Pro 3.0), the ratio of the Cy5 fluorescence intensity minus the Cy5 background intensity to that of the Cy3 fluorescence intensity minus the Cy5 background intensity, was calculated. This represented the gene expression fold change for each gene. Hierarchical clustering was performed using the Cluster software (EisenSoftware) and visualized using the TreeView software (EisenSoftware). The expression profile found in the DNA microarray analysis was confirmed by RT-PCR analysis.

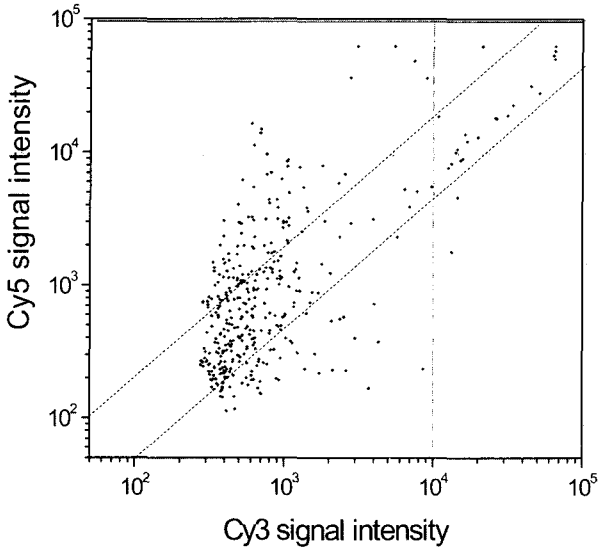

Fig. 2. Comparison of the gene expression profiles during the interactions of *Bacillus lentimorbus* WJ5 against the *Colletotrichum gloeosporioides* spores. The intensity of Cy5 represents the expression of the co-cultured *B. lentimorbus* WJ5 compared to the expression of the control (*B. lentimorbus* WJ5, Cy3).

Of the 2000 genes, 337 were expressed in *B. lentimorbus* WJ5, either with or without the *C. gloeosporioides* challenge, of which 87 were increasingly expressed and 47 suppressed (Fig. 2).

Among the 87 highly transcribed genes, 18 expressed 10 fold increases in their expressions, and were analyzed for the wide range antagonistic interaction (Table 1). Five genes (*tkt, tpi, pgk, citG* and *ahpC*) were involved in a central carbon metabolism: the pentose phosphate pathway, glycolysis, gluconeogenesis, the Kreb's cycle or the reduction of organic peroxides. Five genes (*yxeP, metB, ilvD, trpA* and *infA*) were involved in either an amino acid or polypeptide metabolism: hydrolysis of L-acylamino acids, methionine biosynthesis, valine and isoleucine biosyntheses, tryptophan biosynthesis and protein biosynthesis. Two genes (*ecs* and *yxeO*) were involved in transportation. For the 6 newly discovered genes (K233, K1215, K1688, K1547, K1757 and K1221), database searches revealed no significant homologies with any other known functions.

Several enzymes of the glycolytic pathway (pyruvate decarboxylase, triose phosphate isomerase, and aldolase, enolase and glucose phosphate isomerase) were increased appreciably in response to abiotic stresses in *Saccharo-*

Table 1. Genes highly up-regulated (>10 fold) during the interactions of *B. lentimorbus* WJ5 and *C. gloeosporioides* spores

Chip ID	Gene	Function	Ratio of the Median (challenged/unchallenged)
K1769	*tkt*	Transketolase	38.0
K1214	*citG*	Fumarate hydratase	32.7
K223		No similarity	31.0
K1755	*tpi*	Triose phosphate isomerase	28.8
K207	*pgk*	Phosphoglycerate kinase	23.0
K36	*ahpC (perR)*	Alkyl hydroperoxide reductase (small subunit)	19.4
K1215		No similarity	18.8
K1863	*yxeP*	Similar to aminoacylase	17.0
K1688		No similarity	14.3
K1547		No similarity	13.5
K1762	*ecsA, ecsB*	ABC-type hypothetical EcsA, EcsB protein	12.6
K533	*infA*	Initiation factor 1	12.4
K1851	*yxeO*	Homologous to GlnQ glutamine transport ATP-binding protein of *Bacillus stearothermophilus*	11.6
K113	*metB*	Homoserine O-succinyltransferase (methionine biosynthesis)	11.3
K1757		No similarity	10.7
K1221		No similarity	10.5
K1763	*trpA, hisH*	TrpA, HisH	10.9
K1602	*ilvD*	Dihydroxy-acid dehydratase (valine/isoleucine biosynthesis)	10.7

Table 2. Genes down-regulated (< 0.2 fold) during the interactions of *B. lentimorbus* WJ5 and *C. gloeosporioides* spores

Chip ID	Gene	Function	Ratio of the Median (challenged/unchallenged)
K1620	*tdh*	Threonine 3-dehydrogenase (threonine catabolism)	0.19
K61	*rel*	LSU ribosomal protein L11P	0.17
K1307	*pucR*	Transcriptional regulator (regulation of purine degradation)	0.17
K325		No similarity	0.16
K469	*acoL*	Dihydrolipoamide dehydrogenase (E3 component of the acetoin dehydrogenase enzyme system)	0.14
K28	*gld*	Glycerol dehydrogenase	0.12
K1580		No similarity	0.08
K1531		No similarity	0.07
K1326	*mmsA*	Methylmalonate-semialdehyde dehydrogenase (myo-inositol catabolism)	0.07
K906	*malA*	6-phospho-alpha-glucosidase (splits maltose-6-P)	0.02

myces cerevisiae, *Candida albicans* and rice (Deepika *et al.*, 1999; Leverrier *et al.*, 2004). In our study, 5 genes (*tkt, tpi, pgk, citG* and *ahpC*) involved in a carbon metabolism were up-regulated, which seemed to have resulted from the stress interaction between the bacterium and the fungus (Table 1). Nutrient competition and antibiosis could be the major control mechanisms for the homeostasis of both the bacteria and the fungi. In our study, the expression levels of the 5 genes from a central carbon metabolism and the 5 from an amino acid or polypeptide metabolism increased markedly (Table 1). These results seemed to have been derived from the mutual nutrient competition between the *B. lentimorbus* WJ5 and *C. gloeosporioides*. Also, the 2 over expressed genes related to transportation (*ecs,* and *yxeO,* Table 1) could take part in the nutrient competition, influencing the production of extracellular proteins and the competence development as a kind of regulatory protein secretion (Kunst *et al.*, 1997; Leskelä *et al.*, 1999). However, further study on the functions of the 6 unknown genes (K233, K1215, K1688, K1547, K1757 and K1221) appears to be required to establish any relationship to nutrient competition.

In our laboratory, previous attempts were made to establish the down-regulated genes of the antifungal activity using a microarray in *B. lentimorbus* WJ5m12, an antifungal activity deficient mutant induced by gamma radiation (Lee *et al.*, 2003b). Among the above down-regulated genes, only 9; *ptsG, ftsY, yqfZ, tdk, spo0A, recN, ypvA, yabO* and *rncS,* were observed to be up-regulated in this co-culture experiment. These 9 genes expressed relatively low levels for the ratio of the median (2.0 - 9.7 folds) and are thought to be either directly or indirectly related to the antifungal activity (Trowsdale *et al.*, 1978; Lee *et al.*, 2003b). We expected our results to be those of early interactions, which could occur under co-culture conditions, as samples were obtained at the late exponential stage, and this is thought to be the stage when a bacterium reveals specific functions, such as antifungal activity. It seems that the cell process related to the antifungal activity had not been fully evoked at this stage, but that of the nutrient competition activity had.

Among the 47 genes transcribed less than 0.5 fold, 10 were transcribed less than 0.2 fold (Table 2). Gene products of *acoL*, *gld* and *malA* were involved in a minor carbon metabolism, whereas those of *mmsA*, *tdh* and *pucR* were involved in valine, threonine and purine degradations, respectively. A gene product of *rel* controlled the intracellular proteolysis, which is normally triggered by amino acid starvation. The other three genes (K325, K1580 and K1531) had no significant homologies with any other known functions. It seems that during the interaction of a bacterium and a fungus, a central rather than a minor metabolism could be the essential process for a bacterium to compete with a fungus for efficient nutrient utilization, such as an amino acid catabolism or nucleotide degradation.

From the results of our experiments, nutrient competition seemed to occur at an early stage of the co-culture of *B. lentimorbus* WJ5 and *C. gloeosporioides,* and in advance of antibiosis, so that the genes from a central carbon metabolism could be up-regulated and those from a minor metabolism could be down-regulated.

In this study, a modified co-culture method, using a cylindrical spore reservoir, on which membrane filters were windowed and the gene expression profiles analyzed by a microarray analysis, could be useful in the debate on the interaction between the antifungal bacterium, *B. lentimorbus* WJ5, and the phytopathogenic fungus, *C. gloeosporioides.*

This study was carried out under the Nuclear R&D Program of the Ministry of Science and Technology (MOST), Republic of Korea.

References

Bonsall, R.F., D.M. Weller, and L.S. Tomashow. 1999. Quantification of 2,4-diacetylphloroglucinol produced by fluorescent *Pseudomonas* spp. *in vitro* and in the rhizosphere of wheat. *Appl. Environ. Microbiol.* 63, 951-955.

Deepika, M. and G. Anil. 1999. Transcript levels of genes encoding various glycolytic and fermentation enzymes changes in response to abiotic stresses. *Plant Sci.* 146, 41-51.

Howell, C.R. 1998. The role of antibiosis in biocontrol, p. 173-184. *In* G.E. Harman and C.P. Kubicek (eds.), Trichoderma and Gliocladium, Vol. 2. Enzymes, biological control and commercial applications, Tayler & Francis, London.

Kunst, F., N. Ogasawara, I. Moszer, A.M. Albertini, G. Alloni, V. Azevedo, M.G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S.C. Brignell, S. Bron, S. Brouillet, C.V. Bruschi, B. Caldwell, V. Capuano, *et al.* 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* 390, 249-256.

Lee, Y.-K., J.-S. Kim, B.I. Jang, Y.-S. Jang, and H.Y. Lee. 2003a. Biological control of *Pythium* root rot by radiation induced mutant of *Bacillus lentimorbus* WJ5a17. *Korean J. Environ. Biol.* 21, 276-285.

Lee, Y.-K., J.-S. Kim, H.-Y. Chung, Y.-S. Jang, and B.I. Jang. 2001a. Two-dimensional electrophoresis analysis of antifungal activity related proteins in *Bacillus licheniformis* DM3. *Korean. J. Environ. Agric.* 22, 203-209.

Lee, Y.-K., J.-S. Kim, I.-G. Song, H.-Y. Chung, and H.-H. Chang. 2001b. Characteristics of antifungal bacterium, *Bacillus subtilis* YS1 and its mutant induced by gamma radiation. *Kor. J. Microbiol.* 37, 305-311.

Lee, Y.-K., J.-S. Kim, Y.-S. Jang, K.S. Cho, and H.-H. Chang. 2003b. DNA microarray analysis of gene expression in antifungal bacterium of *Bacillus lentimorbus* WJ5. *Kor. J. Microbiol.* 39. 141-147.

Leskelä, S., E. Wahlström, H.-L. Hyyryläinen, M. Jacobs, A. Palva, M. Sarvas, and V.P. Kontinen. 1999. Ecs, an ABC transporter of *Bacillus subtilis*: dual signal transduction functions affecting expression of secreted proteins, as well as their secretion. *Mol. Microbiol.* 31, 533-543.

Leverrier, P., J.P. Vissers, A. Rouault, P. Boyaval, and G. Jan. 2004. Mass spectrometry proteomic analysis of stress adaptation reveals both common and distinct response pathways in *Propionibacterium freudenreichii*. *Arch. Microbiol.* 181, 215-230.

Meyer, V. and U. Stahl. 2003. The influence of co-cultivation on expression of the antifungal protein in *Aspergillus giganteus*. *J. Basic Microbiol.* 43, 68-74.

Mondal, S.N. and M. Hyakumachi. 2000. Soil factors affecting carbon loss and pathogenicity of oospores of *Pythium aphanidermatum*. *Soil Biol. Biochem.* 32, 111-118.

Raaijmakers, J.M., M. Vlami, and J.T. de Souza. 2002. Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek* 81, 537-547.

Steijl, H., G.J. Niemann, and J.J. Boon. 1999. Changes in chemical composition related to fungal infection and induced resistance in carnation and radish investigated by pyrolysis mass spectrometry. *Physiol. Mol. Plant Pathol.* 55, 297-311.

Trowsdale, J., S.M.H. Chen, and J.A. Hoch. 1978. Genetic analysis of phenotype revertants of *spo0A* mutants in *Bacillus subtilis*: a new cluster of ribosomal genes, p. 131-135. *In* G. Chambliss and J.C. Vary (eds.), Spores VII. American Society for Microbiology, Washington, D.C.

Whipps, J.M. 1997. Developments in the biological control of soilborne plant pathogens. *Adv. Bot. Res.* 26, 1-134.

Whipps, J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52, 487-511.

Ye, R.W., T. Wang, L. Bedzyk, and K.M. Croker. 2001. Applications of DNA microarrays in microbial systems. *J. Microbiol. Meth.* 47, 257-272.