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Isolation and Characterization of *Streptomyces* sp. KACC 91027 Against *Plasmodiophora brassicae*

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Abstract Club root caused by *Plasmodiophora brassicae* is found in crucifers. Among the over hundreds of *Streptomyces* isolated from soil in Korea. One strain showed prominent activity against *P. brassicae*. The strain was identified based on 16S rDNA sequencing and the morphology by a method of scanning electron microscopy. An active compound in the fermented broth obtained from the strain was separated. Even though the complete assignments of the compound remain for future work, the results regarding the isolation and characterization of the strain with a certain activity against *P. brassicae* are shown in this paper.

Key words: *In vivo* antifungal activities, *Plasmodiophora brassicae*, *Streptomyces*, club root

Club root caused by *Plasmodiophora brassicae* is found in crucifers such as cabbage, broccoli, and turnip. In Korea, it is one of the major cabbage diseases known today, since resting spores of *P. brassicae* can survive in soil for seven to eight years, despite the fact that the best way to control them is through crop rotation. During the cultivation process of other crops, they can be disseminated by the transport of the infected soil [2]. Therefore, it is very difficult to completely prevent resting spores from soil. Fluazinam (3-chloro-5-trifluoromethyl-2-pyridyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine) and flusulfamide (2',4-dichloro-α,α,α-trifluoro-4'-nitro-*m*-toluenesulfonailide) are used for club root control, but due to consumer rejection of chemical synthetic pesticides, an attempt was made to discover biopesticides from microorganisms. Among the over hundreds of *Streptomyces* isolated from soil in Korea, one strain showed prominent activity against *P. brassicae*. The strain was identified and an active compound in the fermented broth obtained from the strain was separated.

Soil samples were collected during 2002 at several sites in Ilgamho, Seoul, Korea. The detailed procedures for isolating strains were followed as previously described [2]. To inhibit fungal growth, cyclohexamide was added to starch-casein agar that was used as an isolation medium at a concentration level of 50 μg/ml. Sterilized heat-labile nystatin was also added, and the pH level was adjusted to 7.2. Approximately 1 g of soil was ground and heated at 60°C for 1.5 h in a dry oven. It was transferred to sterilized bottles and 10 ml of sterile distilled water was added. The soil suspensions were mixed vigorously and left to stand for 30 min. The supernatant was innoculated onto a starch-casein agar plate which was incubated at 28°C for 14 days. Colonies showing the typical characteristics of actinomycetes were selected and transferred to Bennett agar medium (Difco, Sparks, MD, U.S.A., pH 7.2). The colonies were cultured for ten days at 28°C. Each colony was inoculated into 30 ml of Bennett's broth in a 100-ml capped tube and cultured in a shaking incubator at 28°C for ten days. The fermented broths were collected and centrifuged. Supernatants were concentrated under reduced pressure and filtered. Remnants were dissolved in dimethyl sulfoxide and final concentrations were adjusted to 50 μg/ml. They were actually used for activity tests [3, 5, 8].

The crop used for testing was *Brassica campestris* subsp. napus var. pekinensis, cv Hukjinju and pathogenic microorganism was *Plasmodiophora brassicae* Woron. To infect with club root, resting spores (1×10^6 spores/ml) of *P. brassicae* were mixed with soil which was placed in the plastic pot containing the second leaf stage crop. Ten ml of the prepared sample as mentioned above was transfered to

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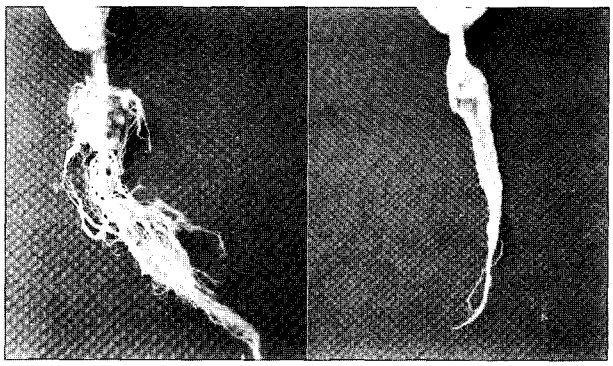
the pot. Test crops were grown in a greenhouse at 25 (±5)°C for three weeks. The disease severity was determined based on the following index: 0=no clubs, 1= small galls formed on lateral roots, 2=galls formed on lateral roots or small galls formed on the main roots, 3= many big galls formed on lateral and main roots. The index 3 was the same as the control. The sample showed 100% control value. Fluazinam, one of two chemical pesticides used for club root control, was compared with a reference which showed 67% control value at 125 ppm, and 100% control value at 500 ppm. Figure 1 shows the results of the treatment by the sample, where the left one is the root without the treatment and the right one, with treatment by the sample. As shown in Fig. 1, the root without the treatment is swollen, while that with the treatment is not [2, 4].



(A) (B)

Fig. 1. The roots of the crop, *Brassica campestris* subsp. napus var. pekinensis, cv Hukjinju, infected by *P. bracissicae* without the treatment of the fermented broth of KACC91027 (A) and with the treatment (B).

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K  GACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATAGAGTTTGATCCTGG 60
S  ------------------------------------------------------------G 1

K  CTCAGGACGAACGCTGGCGGCGTGCTTAGCACATGCAAGTCGAACGGTGAAGCCCTTCGG 120
S  CTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGTGAAGCCCTTCGG 61

K  GGTGGATCAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACA 180
S  GGTGGATCAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACA 121

K  AGCCCTGGAAACGGGGTCTAATACCGGATATGACCTTCCTCCGCATGGGGGTTGGTGGAA 240
S  AGCCCTGGAAACGGGGTCTAATACCGGATATGACCTTCCTCCGCATGGGGGTTGGTGGAA 181

K  AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTA 300
S  AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTA 241

K  CCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACAC 360
S  CCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACAC 301

K  GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT 420
S  GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGAT 361

K  GCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGGAG 480
S  GCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAG 421

K  AAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG 540
S  AAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG 481

K  TAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCC 600
S  TAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAG--CTCGTAGGCGGCC 539

K  TGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGG 660
S  TGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGG 599

K  CTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAG 720
S  CTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAG 659

K  GAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGC 780
S  GAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGC 719

K  GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGG 840
S  GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGG 779

K  TGTTGGCGACATTCCACCTCGTCGTTTCCGCACCTAACGCATTAAGTTCCCCGCCTGGGG 900
S  TGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGG 839

K  AGTACGCCCGCAAGGCTAAAACTCTAAG-AATTGACGGGGGCCCGCACAAGCAGCGGAGC 959
S  AGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGC 899

K  ATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATGCCGGAAAC 1019
S  ATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATGCCGGAAAC 959

K  ATCCAGAGATGGGTGCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGTTGTCGTCAGCT 1079
S  ATCCAGAGATGGGTGCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGTTGTCGTCAGCT 1019

K  CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCA 1139
S  CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCA 1079

K  GCGAGTAATGTCGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGAC 1199
S  GCGAGTAATGTCGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGAC 1139

K  GACGTCAAATCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGTCGGTAC 1259
S  GACGTCAAATCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGTCGGTAC 1199

K  AAAGGGCTGCGATGCCGTGAGGCGGAGCGAATCCCAAAAAGCCGGCCTCAGTTCGGATTG 1319
S  AAAGGGCTGCGATGCCGTGAGGCGGAGCGAATCCCAAAAAGCCGGCCTCAGTTCGGATTG 1259

K  GGGTCTGCAACTCGACCCCATGAAGTTGGAGTTGCTAGTAATCGCAGATCAGCATGCTGC 1379
S  GGGTCTGCAACTCGACCCCATGAAGTTGGAGTTGCTAGTAATCGCAGATCAGCATGCTGC 1319

K  GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACAC 1439
S  GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACAC 1379

K  CCGAAGCCGGTGGCCTAACCC-TCTGGGATGGAGCCGTCGAAGGTGGGACCAGCGATTGG 1498
S  CCGAAGCCGGTGGCCTAACCCGTAAGGGATGGAGCCGTCGAAGGTGGGACCAGCGATTGG 1439

K  GACGAAGTCGTAACAAGGTAGCCGTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGA 1558
S  GACGAAGTCGTAACAAGGTAGCCGTA---------------------------------- 1465

K  CCATATGGGAGAGCTCCCAACGCGTTGGATGC 1590
S  -------------------------------
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Fig. 2. The result obtained by BLASTN (K: KACC91027, S: *Streptomyces aureofaciens*).

In order to identify the strain that produced the test sample, 16S rDNA analysis was carried out. The strain was fermented in Bennett's medium for seven days and the fermented broth was centrifuged for the extraction of DNA. It was carried out by using the Genomic DNA Extraction Kit (Intronbiotechnology, Kyungkido, Korea). An amplification of DNA was performed by using PCR kit. The primers were fD1 5'-AGA GTT TGA TCC TGG CTC AG-3' and rP2 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The thermal cycle profile commenced with an initial denaturation at 94°C for 1 min; 3 cycles, of denaturation at 94°C for 5 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. In the last cycle, the reaction mixture was kept at 72°C for 10 min and cooled to 4°C. The PCR products were cloned by using the pGEM-T cloning kit (Promega, Madison, WI, U.S.A.). The sequences of the PCR products were determined by four internal primers (p510r 5'-TAT TAC CGC GGC TGC TG-3', p364f 5'-GGC AGC AGT GGG GAA TAT TG-3', p783f 5'-TAG ATA CCC TGG TAG TCC AC-3', and p1037f 5'-TCG TCA GCT CGT GTC GTG AG-3') [10]. The 16S rDNA sequence is shown in Fig. 2. It was analyzed by using BLAST (National Center for Biotechnology Information). The result shows that the strain has 98% homology (1452/1467) with *Streptomyces aureofaciens*. The strain was deposited in Korean Agricultural Culture Collection (KACC) at Suwon, Korea. Its registration number is KACC91027.

In the analysis of the morphology of strain KACC91027, scanning electron microscopy (SEM, JSM 5410LV, JEOL, Peabody, U.S.A.) was used. Strain KACC91027 was cultured on a small Petri dish containing Bennett's agar medium at 28°C for seven days. The sample was fixed in an osmotically balanced solution of 2% gluteraldehyde in 0.05 M sodium of cacodylate buffer (pH 7.2) at 25°C for three hours. It was washed three times at 4°C for ten minutes with 0.05 M of sodium cacodylate buffer (pH 7.2), and post-fixed in 1% aqueous osmium tetroxide in 0.05 M of sodium cacodylate buffer (pH 7.2) for two hours at 4°C. And it was washed twice with distilled water. The prepared sample was dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 100, 100, and 100), and were dried in liquid CO_2 by using the Balzers CPD 010 (Balzers Instruments, Liechtenstein, Germany). Finally, the sample was mounted on aluminum stubs and coated with gold/palladium by using the Polaron SEM Coating Unit E5100 (Thermo VG Scientific, Beverly, MA, U.S.A.). The image obtained in this study is shown in Fig. 3.

The evolutional distance was calculated by the Jukes and Cantor method and a phylogenic tree was created by using the neighbor-joining method (Fig. 4) [1, 9].

The strain was cultured to identify the active compound. Three liters of the fermented broth was prepared. The same volume of 50% isopropanol was added into the broth. The mixture solution was centrifuged and an aqueous solution

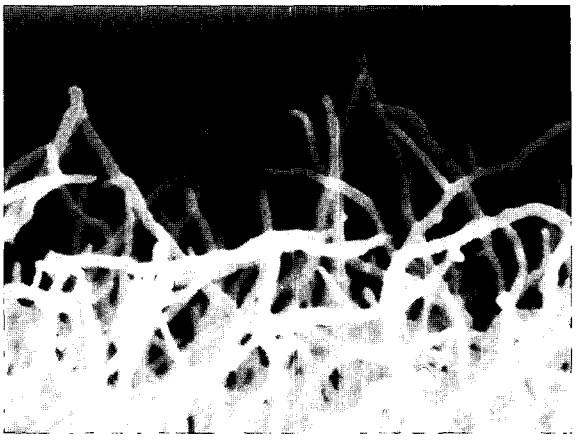

Fig. 3. Morphological observation of strain KACC91027 obtained by using the scanning electron microscopy after growth on Bennett's medium at 28°C for 7 days.

was collected and filtered. The column chromatography packed with HP20 was used, where the eluents contained mixtures of water and isopropanol in various ratios (10:0, 3:7, 5:5, 7:3, and 0:10). Among these, 5:5 mixture showed the best activity so that the collected solution was concentrated *in vacuo*. The reversed phase column chromatography packed with Lichroprep C18 was used again with the eluent containing 45% isopropanol in water. The fraction isolated from the eluent containing 30% isopropanol in water showed better activity than any other eluent conditions. The remnant obtained from freeze-drier was chromatographed on a Prep-HPLC (Waters, Milford, MA, U.S.A.) using acetonitrile in water. The flow rate of 20 ml/min and the injection volumn of 1 ml were used. The column and detector were SymmetryPrep C18 (19× 300 mm) and photodiode array. The fraction with the retardation time of 9 min showed the best activity and it was confirmed to be a single compound based on the

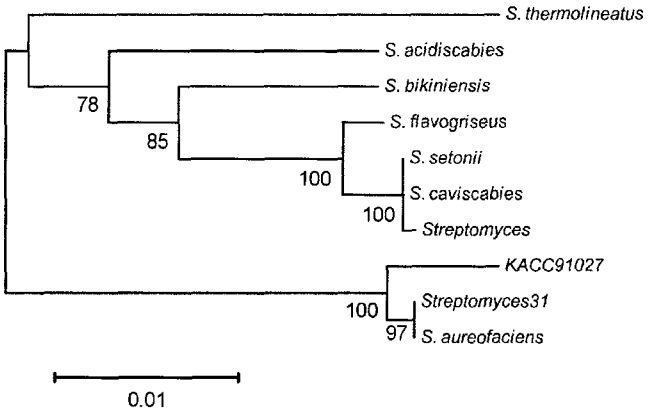

Fig. 4. The evolutional distance calculated by the Jukes and Cantor method and a phylogenic tree was created by using the neighbor-joining method.

two-dimensional chromatogram obtained from photodiode array [7].

Even though the complete assignments of the compound remain for future work, we report the results regarding the isolation and characterization of the strain showing an activity against *Plasmodiophora brassicae.*

Acknowledgments

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References

1. Jukes, T. H. and C. R. Cantor. 1969. Evolution of protein molecules, pp. 21-132. *In* H. N. Munro (ed.) *Mammalian Protein Metabolism*, Academic Press, New York, U.S.A.
2. Kim, B.-J., G. J. Choi, K. Y. Cho, H. Yang, C. Shin, C.-H. Lee, and Y. Lim. 2002. Antifungal activities against *Plasmodiophora brassicae* causing club root. *J. Microbiol. Biotechnol.* **12:** 1022-1025.
3. Kim, B.-J., M. Cho, J.-C. Kim, K. Y. Cho, G. J. Choi, C.-H. Lee, and Y. Lim. 2001. Streptomyces showing antifungal activities against six plant pathogenic fungi. *J. Microbiol. Biotechnol.* **11:** 1120-1122.
4. Kim, J., G. Choi, J. Park, H. Kim, and K. Cho. 2001. Activity against plant pathogenic fungi of phomalactone isolated from *Nigrospora sphaerica. Pest Manag. Sci.* **57:** 554-559.
5. Lee, C.-H., B.-J. Kim, G. J. Choi, K. Y. Cho, H. Yang, C. Shin, S. Min, and Y. Lim. 2002. Streptomyces with antifungal activity against rice blast causing fungus, *Magnaporthe grisea. J. Microbiol. Biotechnol.* **12:** 1026-1028.
6. Lee, J., Y. Lyoo, D. Park, J. Jung, C.-H. Lee, M. Kim, and Y. Lim. 2001. Therapeutic agents against bacteria causing porcine pneumonia. *J. Microbiol. Biotechnol.* **11:** 1102-1105.
7. Lyoo, Y., D. Park, S. Lee, Y. Choi, J. Jung, T. Jun, H. Ahn, C.-H. Lee, and Y. Lim. 2001. Antibacterial compound against *Pasturella multocida* and *Actinobacillus pleuropneumoniae* causing porcine pneumonia. *J. Microbiol. Biotechnol.* **11:** 173-176.
8. Park, U., J. Suh, and S. Hong. 2000. Genetic analysis of *abs*R, a new *abs* locus of *Streptomyces coelicolor. J. Microbiol. Biotechnol.* **10:** 169-175.
9. Saitou, N. and M. Mei. 1987. The neighbor-joining method: A new method for reconstructing phylogenic trees. *Mol. Biol. Evol.* **4:** 406-425.
10. Ueda, K., T. Seki, T. Kudo, T. Yoshida, and M. Kataoka. 1999. Two distinct mechanisms cause heterogeneity of 16S rRNA. *J. Bacteriol.* **181(1):** 78-82.