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Antifungal Activity of *Bacillus* sp. Against *Phytophthora infestans*

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Abstract Because of consumer rejection of chemical pesticides and the appearance of microorganisms that are resistant to fungicides, we tried to discover biopesticides. Of 13 microorganisms isolated from Shrimp-*jeotkal*, a *Bacillus* sp. showed strong activity against tomato late blight caused by *Phytophthora infestans*. Its activity was tested both *in vivo* and *in vitro*. The identification of the strain was carried out based on 16S rDNA analysis and the morphology by scanning electron microscopy.

Key words: *Bacillus* sp., tomato late blight, *Phytophthora infestans*, Shrimp-*jeotkal*

Tomato late blight (TLB) is caused by *Phytophthora infestans*, which is the most important pathogen of the tomato and potato, infecting stems and leaves. It is known as a fungus, but was reclassified as *Chromista* because of its zoospore. *P. infestans* is reproduced sexually and asexually. Sexual structures contain oogonia and antheridia, and the asexual structure includes zoospores that can infect susceptible tissue of plants [4, 5, 8, 14].

TLB can be controlled by cultural and chemical strategies. The former requires irrigation and moisture management, because *P. infestans* is a water mold. Even though many pesticides including dimethomorphs against TLB are known, the occurrence of resistant strains against some chemicals begs the need for development of other chemicals. As a result, a novel pesticide against TLB is being expected. Because of consumer rejection of chemical pesticides, biological pesticides have been discovered from microorganisms [10, 12]. Many microorganisms have been isolated from soil, but it is difficult to use them as biological pesticides, because of their safety problems. We attempted to isolate microorganisms, showing an activity against *P. infestans*, from a Korean salt-fermented fishery product, Shrimp-*jeotkal*. The microflora found in *jeotkal* are *Achromobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Halobacterium*, *Micrococcus*, *Pediococcus*, *Pseudomonas*, *Staphylococcus*, and *Saccharomyces* [11]. It was reported that these microorganisms in *jeotkal* produce bacteriocin, biogenic amines, and proteases [9, 11]. Because *jeotkal* is a fermented food, the products obtained from them can be relatively safer than those from soil microorganisms.

Authors have tried to isolate the *Bacillus* genus from Shrimp-*jeotkal*, because *Bacillus thuringiensis* is one of the major biopesticides [6, 13]. Shrimp-*jeotkal* tested in this work was manufactured and fermented for 4 years in Kangwha-do, Korea. The sample was stored at 4°C until use and it was diluted with distilled water (approximately 10^{-3}). One-hundred μl of the diluted solution was spread on nutrient agar in a Petri dish and incubated at 30°C for 3 days. Colonies showing the typical characteristics of *Bacillus* were selected at random from the plates, and they were then transferred to new nutrient agar medium at 30°C for 3 days. Samples of each colony from the agar were inoculated into LB medium in a 100-ml capped tube and cultured in a shaking incubator at 30°C for 3 days. Thirteen strains were isolated, and fermentation broths were centrifuged. The supernatants were collected and kept for the activity test against TLB at 4°C.

The plant used for *in vivo* test was *Lycopersicon esculentum Mill*, cv *Seokwang*, which was grown in vinyl pots (4.5-cm diameter) in a greenhouse at 25 (±5°C) for 3 weeks [7]. Tomato plants at the third leaf stage were sprayed with each test solution until run-off and allowed to stand for 24 h. The solution was prepared by adding 0.25 mg of Tween 20 per ml of the supernatant. Control plants were treated with Tween 20 solution (0.25 mg/ml). For the development of TLB, the treated tomato plants were inoculated with *P. infestans* by spraying a zoospore suspension released

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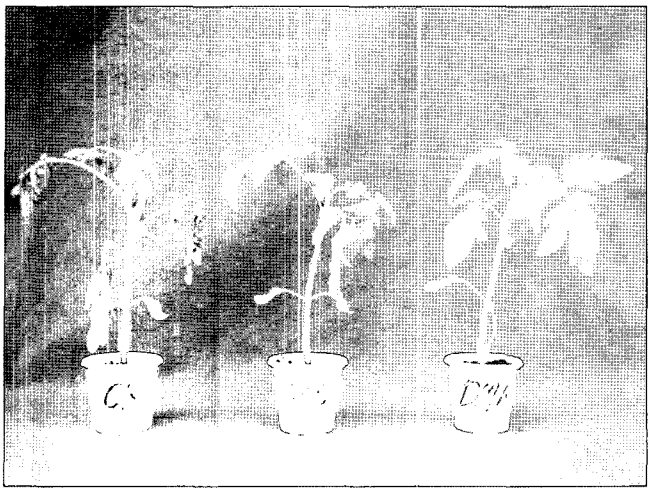

Fig. 1. *In vivo* activity of BA34 against tomato late blight. (left: control; center: treatment of BA34; right: treatment of dimethomorph).

from a sporangial suspension (5×10^4 sporangia/ml) of the fungus. They were incubated in a moist chamber for 2 days at 20°C and then transferred to a growth chamber (20°C and 70% humidity). The disease severity was assessed 3 days after inoculation. Data represent the result of three trials. The mean value for each treatment was converted into a percentage of fungal control using the equation:

$$\% \text{ Control}=100\ [(A-B)/A]$$

where A=area of infection (%) on leaves sprayed with Tween 20 solution alone, and B=area of infection (%) on treated leaves. Analysis of variance was performed on the data with the PROC GLM procedure (SAS Institute, Cary, NC, U.S.A.). If P>F was less than 0.01, means were separated with the least significant different (LSD) test at the P=0.05 level [7]. To compare the activity of the broth with the chemical pesticide, dimethomorph (10 mg/ml) was used. Of 13 samples tested, BA34 showed 88 control% and dimethomorph showed 95%. Figure 1 shows an activity of BA34 against TLB (left: control; center: BA34; right: dimethomorph).

In vitro test was performed using disks containing BA34 fermented broth. The disks were prepared by the culture of *P. infestans* on V-8 juice agar medium (20% V-8 juice, 80% distilled water, 0.3% $CaCO_3$, 1.5% agar). The broth of BA34 was obtained from the fermentation on LB medium for 3 days. Twenty μl of the solution of the BA34 broth was dropped on the disk containing *P. infestans*. As shown

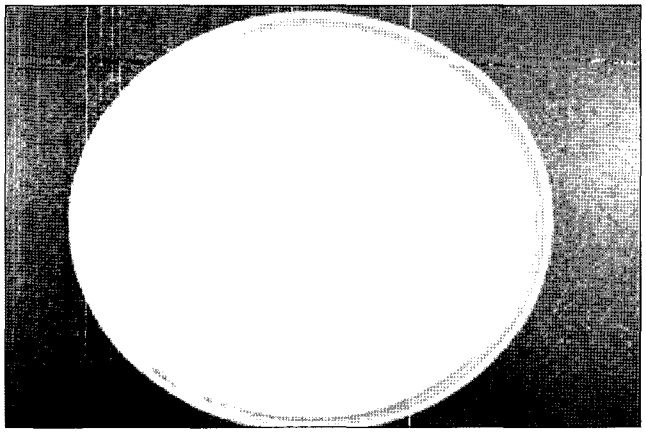

Fig. 2. *In vitro* assay of BA34 against *Phytophthora infestans*. (left, *P. infestans*; right, broth of BA34).

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A       GGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCA
B       GGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCA

A       CCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGAC
B       CCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGAC

A       TGCGATCCGAACTGAGAATAGATTTGTGGGATTGGCTTGACCTCGCGGTTTCGCTGCCCT
B       TGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCT

A       TNGTTCTGCCCATNGTAGCACGTGTGTAGCCNAGGTCATAAGGGGCATGATGATTTGACG
B       TTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACG

A       TCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGC
B       TCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGC

A       TGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
B       TGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG

A       AGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAG---ACGTCCTATCTCTA
B       AGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTA

A       GGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCAC
B       GGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCAC

A       ATGCTCCACCGCTTGTGCGG-CCCCCGTCAATTCCTTTGAGTTTNAGTCTTGCGACCGTA
B       ATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTA

A       CTCCCCAGGCGGAGTGCTTAATGCGTTA-CTGCAGCACTAAGGGGCGGAAACCCCCTAAC
B       CTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAAC

A       ACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCA
B       ACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCA

A       CGCTTTCGCTCCTCACGCTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCT
B       CGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCT

A       CCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAA
B       CCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAA

A       GTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGGCTTTCACATCAGACTNAAG
B       GTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAG

A       AAACCGCCTGCCAGCCCTTNACGCCCAANAATTCCGGACAACGCTNGCCACCTACGTATN
B       AAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATT

A       ACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCNAGGTNCCG
B       ACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCG

A       CCCTATTTGAACGGCACTTGTTCTTCCCTAACAACANAGCTTTACGATCCGAAAACCTTC
B       CCCTATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTC

A       ATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCT
B       ATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCT

A       GCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGT
B       GCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGT

A       CGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGG
B       CGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGG

A       TCCATCTCTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAAG
B       TCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAACCATGCGGTTCAAACAAC

A       CATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACG
B       CATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACG

A       TGTTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTT
B       TGTTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTT

A       GCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTCTC 1427
B       GCATGTATTAGGCACGCCGCCAGCGTTCGTCCTGAGCCAGGATCAAACTCTA 1432
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Fig. 3. The result obtained by BLASTN. (A: *Bacillus amyloliquefaciens*; B: BA34).

in Fig. 2, *P. infestans* was unable to access the region where BA34 broth was dropped.

In order to identify BA34, the partial sequences containing 16S rDNA from the strains were analyzed [1, 3]. Template DNAs were prepared by using a genomic DNA extraction kit (iNtRON, Kyongi-do, Korea), and stored at −20°C. The primer sequences were forward: 5'-AGAGTTTGATCCTGGCTCAG-3', and downward: 5'-ACCGCTACCTTGTTACGACTT-3'. The sequence of each primer was selected from the conserved region. PCR reaction was performed in a 20 μl reaction mixture tube (Qiagen, Hilden, Germany). The reaction mixture was subjected to 30 cycles for amplification (60 sec at 94°C, 60 sec at 55°C, and 60 sec at 72°C) followed by a 5 min extension at 72°C. After electrophoresis, the PCR product was purified using a PCR product purification kit (iNtRON, Kyungki-do, Korea). The purified PCR product was ligated to T-vector for determination of the sequences. 16S rDNA sequences of strain BA34 identified from GenBank by the BLAST program showed the highest homology (99% identity) with *Bacillus amyloliquefaciens* (Fig. 3).

The evolutional distance was calculated by the Jukes and Cantor method, and a phylogenetic tree was created using the neighbor-joining method (Fig. 4). Strain BA34 was found to belong to the genus *Bacillus* with a high bootstrap value, based on an analysis of the phylogenetic tree. Strain BA34 was also identified to be closely related to *Bacillus amyloliquefaciens* in the phylogenic tree [2].

In the morphology study of strain BA34, scanning electron microscopy was used. Strain BA34 was cultured on LB medium at 30°C for a day. The sample was fixed in 2% gluteraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) and post-fixed in 1% aqueous osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2). The prepared sample was then dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 100, 100, and 100%), and mounted on aluminum stubs coated with gold (Polaron SEM Coating Unit E5100, Thermo VG Scientific, MA, U.S.A.). The image obtained is shown in Fig. 5.

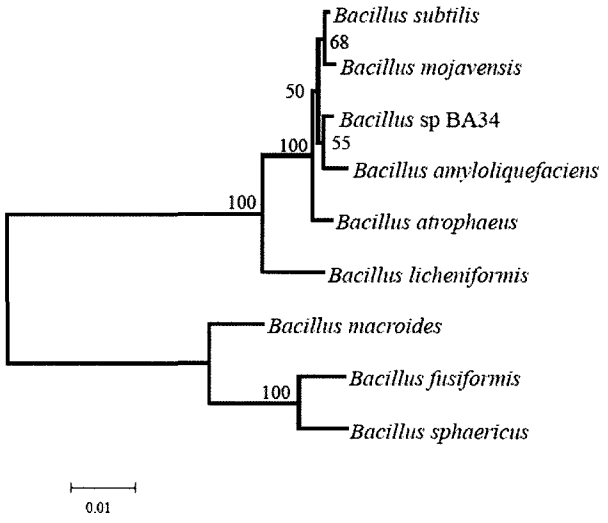

Fig. 4. A phylogenetic tree of *Bacillus* species 16S rDNA, based on the neighbor-joining method. The value at the internal nodes indicates the level of bootstrap support with 1,000 replications.

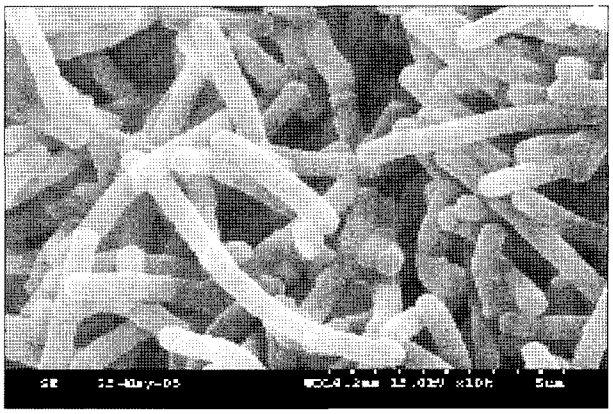

Fig. 5. Morphology of BA34, observed by using scanning electron microscopy.

In conclusion, BA34 isolated from Shrimp-*jeotkal* showed an activity against TLB caused by *P. infestans*. Of course, its activity cannot be compared directly with dimethomorph used for TLB control because of its concentration. However, under the current experimental conditions, the BA34 broth showed 88% of control *in vivo*, whereas dimethomorph at 10 μg/ml showed 95%. Therefore, BA34 appears to be a promising biopesticide.

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