J. Microbiol. Biotechnol. (2004), **14**(4), 872–876

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Inhibitory Effect of Bacteriophage EPS-Depolymerase on Growth of Asian Pear Blight Pathogen *Erwinia pyrifoliae*

KIM, IL-GI[1], MYUNG-SHIN LEE[2], TAE-EUN JIN[1], BYUNG-KOOK HWANG[2], JIN-HYUNG LEE[1], SUK-CHUL SUH[3], AND SEONG-LYUL RHIM[1]*

[1]*Department of Genetic Engineering, Hallym University, Chuncheon 200-702, Korea*
[2]*Laboratory of Molecular Plant Pathology, College of Life and Environmental Sciences, Korea University, Seoul 136-701, Korea*
[3]*National Institute of Agricultural Biotechnology, Rural Development Administration, Suwon 441-707, Korea*

Received: October 7, 2003
Accepted: March 10, 2004

Abstract The plant pathogen *Erwinia pyrifoliae* was infected with bacteriophage PEa1(h), which produced a translucent halo plaque when grown on a lawn of *E. pyrifoliae*. To investigate the function of an exopolysaccharide (EPS)-depolymerase in the growth of *E. pyrifoliae*, an EPS-depolymerase gene was synthesized using the PCR method and sequenced. The synthesized gene was then transferred to *E. pyrifoliae*. The transformed *E. pyrifoliae* did not produce any ooze, and its growth was inhibited. However, the EPS-depolymerase did not appear to induce cell death. Accordingly, the present results suggest that an EPS-depolymerase may be effective in inhibiting the cell growth or infection of the pathogen *E. pyrifoliae*.

Key words: Asian pear blight, *Erwinia pyrifoliae*, exopolysaccharide, EPS-depolymerase

We have isolated *Erwinia pyrifoliae* for the first time and registered it in Korea. It is a novel pathogen that causes Asian pear blight and produces an exopolysaccharide (EPS) [16]. Bacterial EPS is generally produced in a capsular form or as free slime in pathogenic bacteria and plant pathogens. It often plays an essential role in plant-bacteria interactions, including the protection of pathogens against recognition by plant defense reactions, preserving water to keep the cells in a moist state, and holding the ions and nutrients released from damaged plant cells [12, 13]. As such, the synthesis of such capsules and slime is a common feature of Gram-negative bacteria [21]. The chemical structure of the EPS produced by *E. pyrifoliae* is similar to that of the EPS produced by *Erwinia amylovora*, an amylovoran, as ascertained by methylation, ESI-MS, and NMR analysis [15]. Yet, the major difference is that the EPS of *E. amylovora* includes glucose residues, whereas the EPS of *E. pyrifoliae* does not [11].

Certain bacteriophages liberate an enzyme that can degrade bacterial EPS. This enzyme exists in part as a freely diffusible protein and in part as a form firmly attached to the phage particles [1]. Depolymerases (dpo) for EPS have already been demonstrated in various phage-infected bacteria, including *Pseudomonas aeruginosa* [2], *Klebsiella* sp. [20, 22], *Alcaligenes faecalis* [23], *Enterobacter aerogenes* [26], *Rhizobium trifolii* [7], and *E. amylovora* [5, 24]. The dpo binds to the capsular EPS (secondary receptor) and degrades the polymer until a phage reaches the cell surface, where it binds to an outer-membrane receptor (primary receptor) and remains in a lytic or lysogenic state [14]. The EPS from *E. amylovora* can be degraded by the viral EPS-depolymerase of phage PEa1(h) [9], which cleaves the amylovoran between two galactose residues at its β-1→3 linkage [3]. As such, the current study investigated whether the EPS from *E. pyrifoliae* could be degraded by the EPS-depolymerase of phage PEa1(h) to determine whether the Asian pear blight caused by *E. pyrifoliae* could be controlled using an EPS-depolymerase.

All restriction enzymes, the T4 DNA ligase, RNase A, and DNase I were purchased from Roche (Mannheim, Germany). The yeast extract, NA, and NB were purchased from Difco (U.S.A.) and the glucose, anhydrous sodium bicarbonate, DMSO, and polyethylene glycol (PEG) 8000 from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The *Taq* DNA polymerase was purchased from Takara Bio Inc. (Otsu, Japan) and the PCR purification kit from Qiagen (QIAquick; Hilden, Germany). The other biochemicals were of reagent grade.

E. pyrifoliae strain Ep1/96 was isolated in 1996 from diseased Asian pear trees exhibiting symptoms similar to

*Corresponding author
Phone: 82-33-248-2113; Fax: 82-33-242-8090;
E-mail: slrhim@hallym.ac.kr

fire blight in orchards near Chuncheon in Korea [16]. The bacteriophage PEa1(h) [17] was obtained from the American Type Culture Collection (ATCC) in the U.S. (Cat. No. 29780-B1, http://www.atcc.org). The media used to culture *E. pyrifoliae* strain Ep1/96 were 2.0% (w/v) NA containing 0.5% (w/v) glucose (NAG medium) and 0.8% (w/v) NB with 0.5% (w/v) glucose (NBG medium). The bottom and top layers were composed of 15–20 ml of NAG and 3 ml of 0.7% (w/v) NA, 0.5% (w/v) glucose, and 0.2% (w/v) yeast extract, respectively. When necessary, the media were supplemented with 100 μg/ml of ampicillin. *E. pyrifoliae* strain Ep1/96 was grown with vigorous shaking at 28°C.

The double-layer agar technique [17] was used for the phage infection in standard 90-mm diameter plastic petri dishes. *E. pyrifoliae* strain Ep1/96 was precultured in 2 ml of NBG medium at 28°C for 20 h, then the precultured cells were transferred into the same medium and incubated for 8 h. One-hundred μl of the cells was added to 3 ml of the top layer that had been warmed. The cell and top layer mixture was then poured onto the bottom layer. After the top layer had solidified, 5 μl of phage PEa1(h) was dropped onto the central section of the plate, and the plate incubated overnight at 28°C.

The isolation of phage PEa1(h) from the top layer was performed using a modified version of the procedure developed by Ritchie and Klos [18]. As such, the lysates were obtained from the plates by a confluent lysis, where 3 ml of an ice-cold sterile 20 mM potassium phosphate buffer (pH 6.8) was added to each plate. The top agar was then removed with a glass rod, chloroform added to the solution to a final concentration of 1.0% (v/v), and the tube vortexed. Any debris was removed by centrifugation at 12,000 ×*g* for 10 min, then the supernatant was filtered (Millipore 0.45-μm pore size) and the filtrates stored in over 1% (v/v) chloroform extracted with anhydrous sodium bicarbonate at 4°C.

The phage lysate was concentrated with 10% (w/v) PEG 8000 using a modified version of the procedure developed by Yamamoto and Alberts [25]. As such, pancreatic DNase I and RNase A at respective final concentrations of 1 μg/ml were added to the phage lysates isolated from the top layer. The lysates were then incubated for 30 min at room temperature, solid NaCl added to a final concentration of 0.5 M, and the lysates left for another 1 h on ice. Any debris was removed by centrifugation at 12,000 ×*g* for 10 min at 4°C and the supernatant transferred into a sterile Corex glass tube. PEG 8000 was added to the supernatant to give a final concentration of 10% (w/v), dissolved by inverting at room temperature, then the tube was stored for at least 1 h at 4°C. The precipitate was pelleted by centrifugation at 12,000 ×*g* for 10 min and resuspended in 20 mM potassium phosphate buffer (pH 6.8). The PEG and residual cell debris were extracted from the phage PEa1(h) by adding an equal volume of chloroform. The organic and aqueous phases were separated by centrifugation at 3,000 ×*g* for 15 min at 4°C. The precipitated phage PEa1(h) was then stored at 4°C in over 1% (v/v) extracted chloroform, while long-term storage was at −70°C in over 7% (v/v) DMSO.

The nucleotide sequence of the dpo gene with accession number AJ278614 was obtained from the European Molecular Biology Laboratory (EMBL) nucleotide sequence database. The dpo gene was amplified using the PCR method in a volume of 50 μl with 5 μl of the genomic DNA of phage PEa1(h), 100 pmol of each primer (Depol-1, 5'-GGAAG-CTTCA**AGGGAGG**CATA*ATG*CC-3'; and Depol-2, 5'-CCGGATCCTCGTTACGTGTTAGATAC-3'; where the ribosome binding site is represented in bold, the start codon in italics, and the *Hin*dIII and *Bam*HI sites underlined), 200 μM dNTPs, and 5 U of *Taq* DNA polymerase in the 10× buffer provided by Takara. The reaction mixture was denatured at 95°C for 5 min and subjected to 30 cycles of 93°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec with a 2 sec elongation time every cycle in an Applied Biosystems GeneAmp PCR System 2400 Thermal cycler [8]. The PCR product was purified with a PCR purification kit, digested with *Bam*HI and *Hin*dIII, and analyzed on a 0.8% agarose gel. The PCR fragment was eluted from the gel and ligated into a pBluescript KS+ vector. The DNA fragment was then sequenced by Bionex Ltd. in Korea.

The transformation of *E. pyrifoliae* was performed using a modified version of the method developed by Kim *et al*. [11], where a single colony of *E. pyrifoliae* from NAG medium was inoculated in 2 ml of NBG medium. The culture was then incubated overnight at 28°C with vigorous aeration in a rotary shaker. One-hundred μl of the precultured medium was incubated in 100 ml of NBG medium until the OD_{600} reached between 0.4–0.6. The

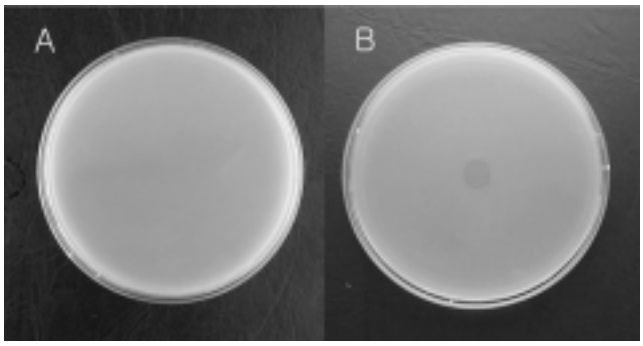

Fig. 1. Lysis and EPS degradation of *Erwinia pyrifoliae* strain Ep1/96 by bacteriophage PEa1(h).
(A) Control. (B) 5 μl droplets of phage PEa1(h) was added to mixture of 100 μl cultured cells and 3 ml of top layer. The plates were incubated overnight at 28°C.

culture was kept on ice for 15–30 min, then the cells were washed twice in 25 ml of ice-cold sterile water and harvested by centrifugation at 6,500 ×*g* for 10 min at 4°C. The supernatant was carefully decanted, and resuspended in 3 ml of ice-cold sterile water and 1 ml of ice-cold 40% glycerol. The resuspended cells were then aliquoted at 250 µl/tube into autoclaved eppendorf tubes and stored at –70°C. To transform *E. pyrifoliae* strain Ep1/96 using the electroporation method, 100 ng of the plasmid DNA was mixed with the pretreated competent cells and incubated on ice for 10 min. The chilled mixture was then transferred to a cold 2-mm electroporation cuvette (Bio-Rad, CA, U.S.A.) and electroporated at a 40 µF capacitance, 2.5 kV, and 201 Ω resistance in an electroporation apparatus, EASYJET One (EquiBio, Belgium). The electroporated cells were then incubated for 1 h at 28°C in 2 ml of NBG medium. Finally, the cells were spread on LB agar plates containing 100 µg/ml of ampicillin and incubated for 2 days at 28°C, then the transformed Ep1/96 cells were observed under a fluorescent microscope (Olympus BX50F-3, Japan).

E. pyrifoliae strain Ep1/96 transformed by the electroporation method was inoculated using sterile toothpicks into the NAG medium supplemented with 100 µg/ml of ampicillin and the bacteria were grown for 5 days at 28°C.

The bacteriophage PEa1(h) was previously demonstrated to infect the plant pathogen *E. amylovora* based on the production of clear plaques when plated in soft agar layers [6, 17]. Thus, to determine whether or not phage PEa1(h) could lyse *E. pyrifoliae*, *E. pyrifoliae* was infected with phage PEa1(h) using a double-layer technique. As a result, phage PEa1(h) formed a translucent halo plaque on a lawn of *E. pyrifoliae* strain Ep1/96 (Fig. 1B), indicating that *E. pyrifoliae* strain Ep1/96 was infected by phage PEa1(h), seemingly reflecting the high structural similarity between the EPS of *E. pyrifoliae* and *E. amylovora*. Although bacteriophages have recently been isolated from *E. amylovora* and characterized [19], none have yet been found from *E. pyrifoliae*.

The EPS-depolymerase gene from phage PEa1(h) [5, 9] was amplified by PCR without its viral promoter using the primers Depol-1 and -2. The amplified 2.0 kb DNA fragment was then inserted into the *Bam*HI and *Hin*dIII sites after the *lac* promoter of a pBluescript KS+ vector, and the resulting plasmid was called pBlue-Dpo (Fig. 2A). When the inserted DNA was sequenced as described above, it was revealed that the sequence was identical to

Fig. 2. (A) Construct of pBlue-Dpo.
Plac: promoter of β-galactosidase gene. Dpo: dpolymerase gene. Ap^r: ampicillin-resistant gene. Ori: replication origin. The arrow indicates the transcription direction. (B) DNA sequence of depolymerase gene. Ribosome binding site, translation start site, and stop site are all indicated.

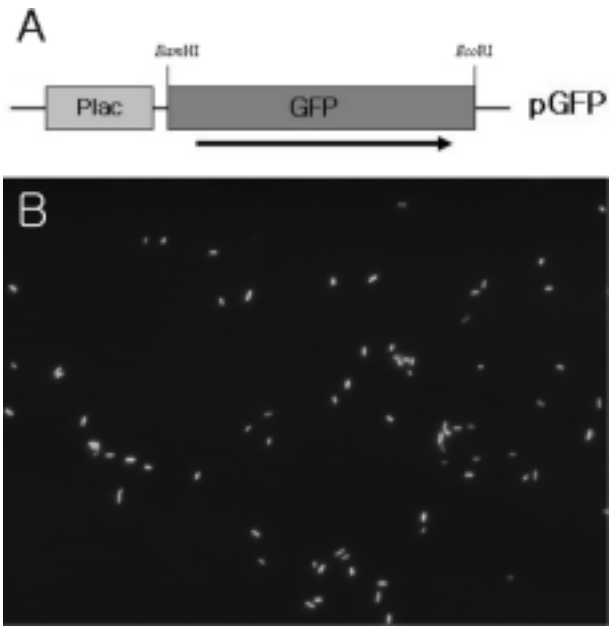

Fig. 3. Construct of plasmid DNA pGFP containing the GFP gene (A) and expression of the GFP gene in *Erwinia pyrifoliae* strain Ep1/96. The transformed cells were visualized under a fluorescent microscope (B).

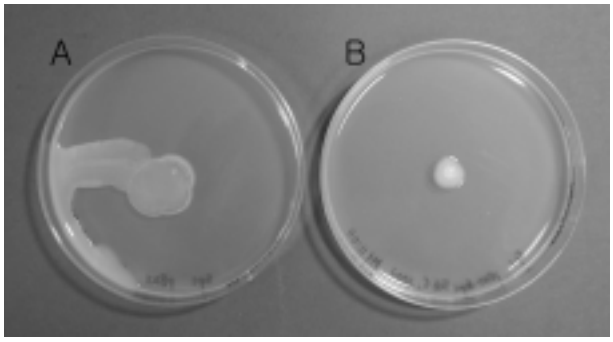

Fig. 4. Colony morphology of *Erwinia pyrifoliae* Ep1/96 (pBluescript KS+) (A) and Ep1/96 (pBlue-dpo) (B).
Each strain was grown on an NA plate containing 0.5% glucose (NAG medium) for 5 days at 28°C.

that from the EMBL database, and that the DNA fragment contained a ribosome binding site and translational start and stop codon (Fig. 2B). Before the transformation of *E. pyrifoliae* with pBlue-Dpo, it was tested whether the dpo gene could be transcribed under the control of the *lac* promoter in *E. pyrifoliae.* For this test, a GFP (green fluorescence protein)-gene under the control of the *lac* promoter in pBluescript KS+ (Fig. 3A) was inserted into *E. pyrifoliae* Ep1/96 using electroporation. All the Ep1/96 strains selected on ampicillin expressed GFP (Fig. 3B), indicating that the *lac* promoter successfully transcribed the dpo gene in *E. pyrifoliae* and that the electroporation method could be used as a transformation method for *E. pyrifoliae*. Thus, to examine the inhibitory effect of dpo on cell growth, *E. pyrifoliae* strain Ep1/96 was transformed with pBlue-dpo using electroporation. As a result, the *E. pyrifoliae* harboring pBlue-dpo was not found to produce any slime or ooze (Fig. 4B), whereas slime and ooze were produced by the strain transformed with only the pBluescript KS+ vector (Fig. 4A). Therefore, these results indicate that the EPS liberated from *E. pyrifoliae* was degraded by the depolymerase. Bacterial EPS aids the movement of bacteria inside plant tissues, plugs xylem vessels, and blocks water flow, thereby causing the wilting of diseased plants [12]. Accordingly, based on the current findings, an EPS-depolymerase may be an effective agent for suppressing the Asian pear blight caused by *E. pyrifoliae*. In addition, the development of transgenic plants that express the dpo gene may be useful in suppressing the symptoms caused by *E. pyrifoliae*.

Acknowledgments

This work was supported by the Hallym University Research Fund, 2003 (HRF-2003-35) of Hallym University and Crop Functional Genomics Center at Seoul National University.

REFERENCES

1. Adams, M. H. and B. H. Park. 1956. An enzyme produced by a phage-host cell system II. The properties of the polysaccharide depolymerase. *Virology* **2:** 719–736.
2. Bartel, P. F., G. K. Lam, and T. E. Orr. 1968. Purification and properties of polysaccharide depolymerase associated with phage infected *Pseudomonas aeruginosa*. *J. Biol. Chem.* **243:** 2077–2080.
3. Bellemann, P., S. Bereswill, S. Berger, and K. Geider. 1994. Visualization of capsule formation by *Erwinia amylovora* and their biochemical characterization. *Int. J. Biol. Macromol.* **16:** 290–296.
4. Bereswill, S., S. Jock, P. Bellemann, and K. Geider. 1998. Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. *Plant Dis.* **82:** 158–164.
5. Hartung, J. S., D. W. Fulbright, and E. J. Klos. 1988. Cloning of a bacteriophage polysaccharide depolymerase gene and its expression in *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* **1:** 87–93.
6. Hartung, J. S., P. B. Rosenthal, D. W. Fulbright, and E. J. Klos. 1982. Capsular depolymerase from bacteriophage infected *Erwinia amylovora*. *Phytopathology* **72:** 945.
7. Higashi, S. and M. Abe. 1978. Phage induced depolymerase for exopolysaccharide of *Rhizobiaceae*. *J. Gen. Appl. Microbiol.* **24:** 143–153.
8. Kim, T. K., H. D. Shin, M. C. Seo, J. N. Lee, and Y. H. Lee. 2003. Molecular structure of PCR cloned PHA synthase genes of *Pseudomonas putida* KT2440 and its utilization for medium-chain length polyhydroxyalkanoate production. *J. Microbiol. Biotechnol.* **13:** 182–190.
9. Kim, W.-S. and K. Geider. 2000. Characterization of a viral EPS-depolymerase, a potential tool for control of fire blight. *Phytopathology* **90:** 1263–1268.
10. Kim, W.-S., L. Gardan, S.-L. Rhim, and K. Geider. 1999. *Erwinia pyrifoliae* sp. nov., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia* Nakai). *Int. J. Syst. Bacteriol.* **49:** 899–906.
11. Kim, W.-S., M. Schollmeyer, M. Nimts, V. Wray, and K. Geider. 2002. Genetics of biosynthesis and structure of the capsular exopolysaccharide from the Asian pear pathogen *Erwinia pyrifoliae*. *Microbiology* **148:** 4015–4024.
12. Leigh, J. A. and D. L. Coplin. 1992. Exopolysaccharides in plant-bacterial interactions. *Annu. Rev. Microbiol.* **46:** 307–346.
13. Lim, H. S., J. M. Jung, and S. D. Kim. 2002. A plant growth-promoting *Pseudomonas fluorescens* GL20: Mechanism for disease suppression, outer membrane receptors for ferric siderophore, and genetic improvement for increased biocontrol efficacy. *J. Microbiol. Biotechnol.* **12:** 249–257.
14. Lindberg, A. A. 1977. Bacterial surface carbohydrates and bacteriophage adsorption, pp. 289–356. *In* I. W. Sutherland (ed.), *Surface Carbohydrates of the Prokaryotic Cell*. Academic Press, London.
15. Nimts, M., A. Mort, V. Wray, T. Domke, Y. Zhang, D. L. Coplin, and K. Geider. 1996. Structure of amylovoran, the

capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora*. *Carbohydr. Res.* **287:** 59–76.
16. Rhim, S.-L., B. Volksch, B. L. Gardan, J.-P. Paulin, C. Langlots, W.-S. Kim, and K. Geider. 1999. *Erwinia pyrifoliae*, an *Erwinia* species, different from *Erwinia amylovora*, causes a necrotic disease of Asian pear trees. *Plant Pathology* **48:** 514–520.
17. Ritchie, D. F. and E. J. Klos. 1977. Isolation of *Erwinia amylovora* bacteriophage from aerial parts of apple trees. *Phytopathology* **67:** 101–104.
18. Ritchie, D. F. and E. J. Klos. 1979. Some properties of *Erwinia amylovora* bacteriophages. *Phytopathology* **69:** 1078–1083.
19. Schnabel, E. L. and A. L. Jones. 2001. Isolation and characterization of five *Erwinia amylovora* bacteriophages and assessment of phage resistance in strains of *Erwinia amylovora*. *Appl. Environ. Microbiol.* **67:** 59–64.
20. Sutherland, I. W. 1976. Highly specific bacteriophage-associated polysaccharide hydrolases for *Klebsiella aerogenes* type 8. *J. Gen. Microbiol.* **94:** 211–216.
21. Sutherland, I. W. 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharide. *Annu. Rev. Microbiol.* **39:** 243–270.
22. Sutherland, I. W. and J. F. Willkinson. 1965. Depolymerase for bacterial exopolysaccharide obtained from phage-infected bacteria. *J. Gen. Microbiol.* **39:** 373–383.
23. Tanio, T., T. Fukui, Y. Shirakura, T. Saito, K. Tomita, and T. Kaiho. 1982. An extracellular poly(3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*. *Eur. J. Biochem.* **124:** 71–77.
24. Vandenbergh, P. A., A. M. Wright, and A. K. Vidaver. 1985. Partial purification and characterization of a polysaccharide depolymerase associated with phage-infected *Erwinia amylovora*. *Appl. Environ. Microbiol.* **49:** 994–996.
25. Yamamoto, K. R. and B. M. Alberts. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40:** 734–744.
26. Yurewicz, E. C., M. A. Ghalambor, D. H. Duckworth, and E. C. Heath. 1971. Catalytic and molecular properties of a phage-induced capsular polysaccharide depolymerase. *J. Biol. Chem.* **246:** 5607–5616.