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A novel lysozyme from *Xanthomonas oryzae* phage ϕXo411 active against *Xanthomonas* and *Stenotrophomonas*

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Abstract

In this study, a bacteriophage of *Xanthomonas oryzae* pv. oryzae designated as ϕXo411 was isolated. Random sequencing of its genome revealed that it is closely related to another *X. oryzae* phage, Xp10. A cloned fragment carries the lysozyme gene, *lys411*. The deduced protein, Lys411, shares 92% identity with Xp10 lysozyme, which contains an extra 46 aa at the N-terminus. Lys411 shows over 40% identities to several other phage lysozymes. The His-tagged protein, Lys411H, expressed in *Escherichia coli* largely formed as inclusion bodies. The insoluble protein was solubilized in urea and purified by passing through a His-bind column, and the lytic activity was then restored by a refolding process. The optimal assay conditions determined for Lys411H are in 0.1 M potassium phosphate buffer, pH 6.6 containing 1 mM $CuCl_2$ at 25 °C. Lysis assays using different bacterial cells as the substrates indicate that Lys411H is the first lysozyme active against both *Xanthomonas* and *Stenotrophomonas maltophilia*. This suggests that Lys411 can be a candidate to be developed into a therapeutic agent for treating *S. maltophilia* infections, in addition to the potential use in control of the plant diseases caused by *Xanthomonas*. By analogy to the situation in Xp10, we predict that ϕXo411 has no holin, the protein required for lysozyme export, and the N-terminal signal-arrest-release sequence of Lys411 can accommodate its own export to the periplasm.

Keywords: *Xanthomonas*; *Stenotrophomonas*; Bacteriophage; Lysozyme

Genus *Xanthomonas* consists of over hundreds of bacteria causing diseases in plants, resulting in tremendous loss in agriculture. For examples, *Xanthomonas campestris* pv. campestris and *X. oryzae* pv. oryzae are the causative agents of black rot in crucifers and bacterial leaf blight in rice, respectively [1,2]. Therefore, prevention of the diseases caused by this genus has been an important issue of agriculture.

Bacteriophages are the viruses that infect and in most cases lyse bacteria with host specificity. In recent years, owing to the overuse of antibiotics, bacteria resistant to most or all available antibiotics are causing increasingly serious problems and therapy with bacteriophages has been a possible alternative for the treatment of bacterial infections [3,4]. Similar to the possible use in medical purposes, use of phages for biological control of plant diseases has been demonstrated [5,6].

Phage lysins are capable of degrading bacterial cell wall peptidoglycan. Based on the bond specificity, four types of phage lysins are grouped: lysozyme-like enzymes (muramidases), lytic transglycosylases, amidases or endopeptidases [7–10]. Muramidases can be divided further into four types: chicken egg type lysozymes, goose type lysozymes, phage type lysozymes, and fungal type lysozymes [11–14]. Lysozymes have a variety of applications [15–19]. Among others, they can be used (1) as a food additive and food preservation, (2) in production of cheese and wine, (3) in medical uses such as eye drops and oral drops, (4) in household chemicals such as toothpaste and cosmetics, and (5) in

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agriculture to improve the variety of crops and develop resistance against diseases in fruits and vegetables.

In this study, we isolated a phage (ϕXo411) specifically infecting *X. oryzae* pv. oryzae. As the first step towards application of the lysozyme encoded, we isolated the gene (*lys411*) coding for the lysozyme by random sequencing of the phage genome. The recombinant lysozyme protein, Lys411H, expressed in *E. coli* was characterized and the results are reported in this communication.

Materials and methods

Bacterial strains and growth conditions

Xanthomonas oryzae pv. oryzae and *X. campestris* pv. campestris strains were cultivated in TYG medium (1 liter contained: tryptone, 10 g; yeast extract, 6 g; 1 mM MgSO4, and 0.5% glucose) or TYG Agar at 28 °C and *E. coli* was grown in LB broth or LB agar [20] at 37 °C. LB was also used to grow *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Pseudomonas fluorescens*, and *S. maltophilia* at 30 °C. Cell concentrations were measured turbidimetrically, OD_{550} for *Xanthomonas* and OD_{600} for other bacteria. Ampicillin (50 μg/ml) was added when necessary.

Bacteriophage isolation

To isolate the phage, surface soil samples were taken from a rice paddy near National Chung Hsing University, in which the rice plants had been infected by *X. oryzae* pv. oryzae and showed serious symptoms of leaf blight. Ten gram of each soil sample was mixed with 40 ml of TYG in a 250-ml flask, followed by incubating the mixture at 28 °C. After 4 h, the upper layers were decanted into the centrifuge tubes and centrifuged at 8000*g* for 10 min. To the supernatants, 0.5 ml of chloroform was added and vortexed vigorously. The aqueous phases were filtered through a 0.45-μm membrane filter and spotted onto the double-layered TYG agar with the indicator host, *X. oryzae* pv. oryzae strain 21 (Xo21),[1] being included in the top agar (0.75%). Formation of a clearing zone on the spotted areas indicated the possible presence of bacteriophage. The clearing zones were picked up with a toothpick and subjected to three rounds of single plaque isolation.

Plaque assay

Phage lysates were diluted serially with sterilized deionized water. One hundred μl of a phage suspension and 100 μl of the cells from an overnight culture of Xo21 were mixed with 3 ml of the molten soft TYG agar and poured on the surface of a regular TYG agar plate. The numbers of the plaque were counted after incubating the plates overnight.

Purification of phage particles

A high titer lysate of ϕXo411 (400 ml, ca. 1.0×10^{10} PFU/ml) was centrifuged at 6,000 rpm for 20 min at 4 °C (Sovall RC 5C, GS3 rotor). The supernatant was passed through a 0.45-μm membrane filter, then DNAse I and RNAase were added (1 μg/ml final concentration). After incubation for 30 min at 37 °C, the mixture was centrifuged at 8400 rpm for 2 h at 4 °C with the same rotor. The pellet was suspended in 1.0 ml of SM buffer (one liter contained 5.8 g of NaCl, 2.0 g of $MgSO_4 \cdot 7H_2O$, 0.01% gelatin and 0.05 M Tris–HCl, pH 7.5), to which CsCl was added (0.5 g/ml of bacteriophage suspension). The mixture was carefully loaded onto a CsCl step gradient, followed by ultracentrifugation at 28,000 rpm for 2 h at 4 °C with a TH641 rotor (Sorvall OTD Combi). The fraction containing the phage particles was recovered, dialyzed against SM buffer, and then stored at 4 °C until used [21] .

Isolation and restriction enzyme digestion of the phage DNA

The phage particles purified by ultracentrifugation were treated with sodium dodecyl sulfate (SDS, 1% final concentration) and 20 U of proteinase K at 58 °C. After 1 h, an equal volume of phenol/chloroform (1:1) was added to remove the proteinaceous materials. The extraction was repeated twice, and the DNA was precipitated according to the standard procedures [21]. Restriction enzyme digestions of the phage DNA were carried out following the instructions provided by the suppliers.

Random sequencing of ϕXo411 genome

The sequence of ϕXo411 DNA was determined using a shotgun cloning strategy. The purified phage DNA was treated by sonication and the fragments of 1.0–3.0 kb in size were isolated and ligated into pBluscript. The transformants were randomly picked for plasmid isolation and some of the inserts were sequenced from both ends using the dideoxy chain termination method.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The protein samples were analyzed by SDS–PAGE with 12% (w/v) polyacrylamide and 0.1% SDS. Protein bands were visualized by staining the gel with Coomassie brilliant blue (Bio-Rad) [21].

Cloning, expression, and purification of the recombinant Lys411H protein

The entire coding region of *lys411* gene was PCR-amplified using the ϕXo411 genomic DNA as the template with

[1] *Abbreviations used:* Xo21, *X. oryzae* pv. oryzae strain 21; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Xc17, *X. campestris* pv. campestris strain 17; orf, open reading frames; TMD, transmembrane domain; SAR, signal-arrest-release.