J Microbiol. Biotechnol. (2000), **10**(2), 272–274

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Implications of Exonuclease Activity of Bacteriophage P2 Old Protein for Lambda Exclusion

KIM, KWANGHO, CHANHEE PARK, HYEONJOO YEO, YOUNGHOON KEE, JUNGCHAN PARK, AND HEEJOON MYUNG*

Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Yongin, Kyunggi-Do 449-850, Korea

Received: January 3, 2000

Abstract Temperate bacteriophage P2 has a nonessential gene called *old* (*o*vercoming *l*ysoginization *d*efection). In the presence of *old*, the growth of the host (*Escherichia coli*) with *recBC*$^-$ genotype is inhibited, and another bacteriophage, lambda, cannot superinfect. The Old protein has been shown to possess an exonuclease activity. Three mutant P2s (*old* 1, *old* 17, *old* 49) which did not exhibit lambda exclusion were obtained and each *old* gene was cloned into expression vectors to produce hexahistidine-tagged proteins. The proteins were affinity-purified and shown to lose its exonuclease activity on both double-stranded and single-stranded DNA substrates. Thus, it was concluded that the lambda exclusion was related to Old's exonuclease activity.

Key words: Bacteriophage P2, Old exonuclease, mutant protein, purification, Spi selection

The Old protein produced from bacteriophage P2 inhibits the superinfection of another bacteriophage, λ, and the growth of the host strain with *recBC*$^-$ genotype [1, 5, 6, 8]. Whether in a prophage or a plasmid state, the DNA replication, transcription, and protein synthesis of λ were inhibited. This phenomenon is called Spi (sensitivity to P2 interference) and has been used for the selection of the recombinant λ vectors [4, 13]. From a nucleotide sequence analysis, it has been shown that the Old protein had motifs belonging to the *uvr*A-related ATPase superfamily [7, 10]. The Old protein fused to a maltose binding protein (MBP) was previously purified to near homogeneity and shown to have an exonuclease function [12]. A double-stranded DNA, single-stranded DNA, DNA with a nick, DNA with a gap, and RNA were all degraded by P2 Old. When Old existed in the host cell, λ could not grow. But no direct evidence on the relationship between exonuclease activity and λ exclusion has been presented. Accordingly, to test this possibility, we cloned and purified Old from 3 mutant P2 strains which had lost their λ exclusion phenotype. To minimize the number of extra amino acids fused to Old, hexahistidine tagging [2] instead of MBP [12] was used in this work.

Three mutant P2s (designated *old*1, *old*17, and *old*49, selected for loss of phenotype after UV mutagenesis and kindly provided by R. Calendar) and the wild-type P2 were used in this work. The following two primers were used to amplify the *old* gene from the phage genomic DNA: forward-5'AGGAGGATCCATGACTGTACGTCTTGC3', reverse-5'TCAGAAGCTTTTAAATCCATTTTATGAAA-TC3'. The forward primer had a *Bam*HI site in its 5' end and the reverse primer had a *Hin*dIII site in its 5' end. After PCR amplification, the products were isolated, cut with two restriction enzymes, and subsequently cloned into pQE30 (Qiagen) to express N-terminally hexahistidine tagged proteins. The resulting three plasmids expressing mutant Olds and the one expressing the wild-type were named pKQ1, pKQ17, pKQ49, and pKQW, respectively. Each plasmid was used to transform *E. coli* SG13009 (pREP4). The transformed strains were grown in the presence of kanamycin (45 μg/ml) and ampicillin (10 μg/ml) to the early exponential phase and then induced with 1 mM IPTG for 2 h. The cells were harvested and subjected to a French press to produce crude extracts. Thereafter, the extracts were loaded on Ni-NTA columns (Qiagen) for affinity purification. The purified proteins were analyzed by SDS-PAGE. The nucleotide sequences of the mutant *old*s were analyzed using dideoxy chain termination methods [14]. The nuclease assay was described in Myung and Calendar [12]. Briefly, bacteriophage T7 was grown in a host *E. coli* B1 strain in the presence of [^{3}H] thymidine (Amersham) and the phage DNA was purified using a Wizard lambda DNA purification Kit (Promega). The resulting DNA was used as a double-stranded DNA substrate. The single stranded DNA was obtained first by heating the double-stranded T7 DNA at 90°C for 10 min and then putting it into ice directly. The assay mixture

*Corresponding author
Phone: 82-335-330-4098; Fax. 82-335-333-1696;
E-mail: hjmyung@maincc.hufs.ac.kr

was as follows; 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 0.1% BSA, 300 μM ATP, 50 U [11] of purified protein, 2 μg of [^{3}H] thymidine-labeled T7 DNA, and distilled water to make up a reaction volume to 100 μl. The reaction mixture was incubated at 37°C for 4 h, 50 μl of 20% trichloroacetic acid (TCA) was then added and the mixture was put into ice for 10 min. After centrifugation at 10.000 ×g for 5 min, the supernatant was isolated and the radioactivity was measured in a liquid scintillation counter (Beckman).

From the nucleotide sequence analysis, the following amino acid substitutions were found; 269 arginine to serine for *old*1, 573 isoleucine to lysine for *old*17, and 36 glycine to valine for *old*49 (the entire Old protein has 583 amino acids, [8]). The 36th amino acid residue was located in the motif 1 (which is designated as NTP-binding) of the *uvr*A-related ATPase superfamily [10]. Although Old exhibited exonuclease activity in the absence of ATP *in vitro* [12], it is probable that it requires ATPase activity for one or more of the substrates *in vivo*. These substrates need to be identified. The 269th (in the middle) and 573rd (in the C-terminus) amino acid residues did not belong to any of the ATPase motifs. Accordingly, these regions are thought to be involved in the degradation function.

The partially purified wild-type and mutant Old proteins are shown in Fig. 1. The 65 kDa bands corresponded to the estimated protein size. For the mutant Olds (lanes 2, 3, and 4), contaminating bands were observed. The smaller

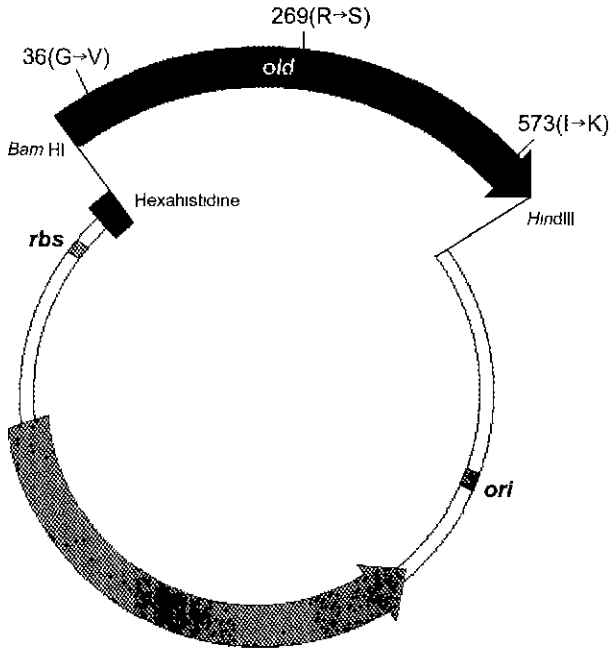

Fig. 1. Construction of plasmids expressing N-terminally hexahistidine-tagged Old proteins.
pKQW, which harbors the wild-type *old*, is shown. All the mutant constructs are exactly the same except for the indicated amino acid changes

Table 1. Relative activities of wild-type and three mutant Old proteins on the double-stranded and the single-stranded DNA substrates.

Type of Old	Relative activity	
	Double-stranded DNA	Single-stranded DNA
wild-type	100.0	100.0
Old 1	0.9	0.9
Old 17	0.7	1.0
Old 49	<0.1	<0.1

bands were thought to be degraded Old products, while larger bands were thought to be cellular proteins with multihistidine residues. Nevertheless, the 65 kDa band was the major band in each lane. The nuclease assay results are shown in Table 1. All the mutant Olds showed a minimized exonuclease activity on both the double-stranded and the single-stranded DNA substrates. The possibility that the contaminating proteins inhibited the exonuclease activity was minimal when considering the significant amount of activity in the crude extracts containing the wild-type Old [12]. The wild-type Old had a specific activity of 4641 U/mg for the linear double-stranded DNA substrate, which was considered to be 100% activity.

The result presented in this work shows only indirect evidence that the Old's exonuclease activity is responsible for λ exclusion. Nevertheless, it is clear that the Old's exonuclease activity is related to the exclusion phenomenon. The replication of λ DNA in the host cell takes place in two stages [3]. First, it is replicated in the θ form, followed by the rolling circle replication. The θ form replication intermediate DNAs which harbor nicks and gaps are thought to be attacked by Old. The rolling circle replication also produces single and double-stranded DNAs which can be degraded by Old.

The phenomenon of λ exclusion is widely used for the recombinant selection of λ-based vectors (e.g. λ2001,

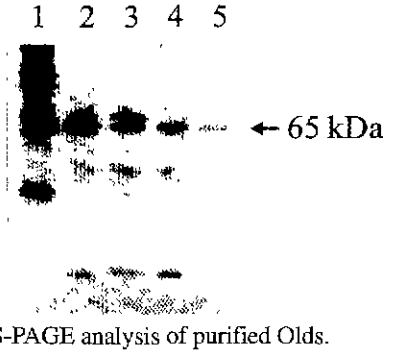

Fig. 2. SDS-PAGE analysis of purified Olds.
Lane 1, Protein molecular weight marker (212 kDa, 116 kDa, 97.4 kDa, 66.2 kDa, 57.5 kDa, and 40 kDa, from the top to the bottom); lane 2, mutant Old expressed from pKQ1; lane 3, mutant Old expressed from pKQ17, lane 4, mutant Old expressed from pKQ49; lane 5, wild-type Old expressed from pKQW.

λDASH, and λFIX, 13), known as Spi selection [9, 13]. In the presence of P2 prophage, these vectors cannot be reproduced without harboring an insert DNA. The molecular mechanisms underlying this cloning strategy have not yet been elucidated. However, it can be concluded from this work that the exonuclease activity of Old does play a role in exclusion, thereby enabling Spi selection.

Acknowledgments

This work was supported by a grant from KOSEF (961-0502-019-1).

REFERENCES

1. Bregegère, F. 1976. Bacteriophage P2-lambda interference II. Effects on the host under the control of lambda genes O and P. *J. Mol. Biol.* **104:** 411-420.
2. Chung, B., C. Chu, Y. Chang, J. Sohn, S. Rhee, and Y. Park. 1993. Immobilized metal ion affinity chromatography of genetically engineered hirudin variants. *J. Microbiol. Biotechnol.* **3:** 161-167.
3. Enquist, L. W. and A. Shalka. 1973. Replication of bacteriophage lambda DNA dependent on the function of host and viral genes. *J. Mol. Biol.* **75:** 185-212.
4. Finkel, S., C. Halling, and R. Calendar. 1986. Selection of lambda Spi⁻ transducing phages using the P2 *old* gene cloned onto a plasmid. *Gene* **46:** 65-69.
5. Ghisotti, D., S. Zangrossi, and G. Sironi. 1983. An *Escherichia coli* gene required for bacteriophage P2-lambda interference. *J. Virol.* **48:** 616-626.
6. Goldmark, P. J. and S. Linn. 1972. Purification and properties of the RecBC DNase of *Escherichia coli* K-12. *J. Biol. Chem.* **247:** 1849-1860.
7. Gorbalenya, A. and E. Koonin. 1990. Superfamily of *uvrA* related NTP-binding proteins. *J. Mol. Biol.* **213:** 583-591.
8. Haggård-Ljunquist, E., E. V. Barreiro, R. Calendar, D. M. Kurnit, and H. Cheng. 1989. The P2 phage *old* gene: sequence, transcription, and translational control. *Gene* **85:** 25-33.
9. Karn, J., H. Matthes, M. Gait, and S. Brenner. 1984. A new selective phage cloning vector, λ2001, with sites for *Xba*I, *Bam*HI, *Hin*dIII, *Eco*RI, *Sst*I, and *Xho*I. *Gene* **32:** 217-225.
10. Koonin, E. V. and A. E. Gorbalenya. 1992. The superfamily of *uvrA* related ATPases includes three more subunits of putative ATP-dependent nuclease. *Prot. Seq. Data Anal.* **5:** 43-45.
11. Lindahl, G., G. Sironi, H. Bialy, and R. Calendar. 1970. Bacteriophage lambda: Abortive infection of bacteria lysogenic for phage P2. *Proc. Natl. Acad. Sci. USA* **66:** 587-594.
12. Myung, H. and R. Calendar. 1995. The *old* exonuclease of bacteriophage P2. *J. Bacteriol.* **177:** 497-501.
13. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York, U.S.A.
14. Sanger, F., S. Nicklen, and A. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463-5468.
15. Taylor A. and G. Smith. 1992. RecBCD enzyme is altered upon cutting DNA at a Chi recombination hotspot. *Proc. Natl. Acad. Sci. USA* **89:** 5226-5230.