The Journal of Microbiology, April 2007, p. 175-178
Vol. 45, No. 2
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NOTE

Recombinant Expression and Purification of Functional *Xor*II, a Restriction Endonuclease from *Xanthomonas oryzae* pv. *oryzae*

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(Received November 6, 2006 / Accepted March 5, 2007)

An endonuclease from *Xanthomonas oryzae pathovar oryzae* KACC 10331, *Xor*II, was recombinantly produced in *Escherichia coli* using a T7 system. *Xor*II was purified using a combination of ion exchange and immobilized metal affinity chromatography (IMAC). An optimized washing protocol was carried out on an IMAC in order to obtain a high purity product. The final amount of purified *Xor*II was approximately 2.5 mg/L of LB medium. The purified recombinant *Xor*II was functional and showed the same cleavage pattern as *Pvu*I. The enzyme activity tested the highest at 25°C in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol at a pH of 7.9.

Keywords: *Xor*II, restriction endonuclease, *Xanthomonas oryzae* pv. *oryzae*

Restriction endonucleases (EC 3.1.21.4) are the key components of restriction modification systems that defend bacteria against invading viruses by cleaving foreign DNA that is not methylated at N4 or C5 of cytosine or at N6 of adenine within the recognition sequence (Bickle and Kruger, 1993). Proper methylation serves to protect the host DNA against such cleavage. These restriction endonucleases are classified into four different types: I, II, III, and IV (Roberts *et al.*, 2003). Type II restriction endonucleases are homodimeric or tetrameric, cleave DNA within or close to the specific recognition sites, and require Mg^{2+} ions for catalysis (Roberts *et al.*, 2003).

Xanthomonas oryzae pv. *oryzae* (*Xoo*) belongs to the γ-subdivision of the *Proteobacteria* and causes bacterial blight on rice (Ezuka and Kaku, 2000). Bacterial blight is a vascular disease resulting in white lesions along the leaf veins. Severe infections of the disease can result in yield losses as high as 50%. The *Xoo* KACC10331 genome project was undertaken and recently completed in an effort to better understand the properties of *Xoo* and to minimize the losses caused by this pathogen (Lee *et al.*, 2005). Other genome sequences of related *Xanthomonas* strains have also been reported (Wang *et al.*, 1980; da Silva *et al.*, 2002; Ochiai *et al.*, 2005; Qian et al., 2005; Thieme *et al.*, 2005). However, the restriction/modification system of the KACC 10331 strain was so active that the genetic analysis of this strain had not yet been successful. We thought it essential to first understand the restriction/modification system of this strain before attempting to overcome this challenge.

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In this work, we focused on *Xor*II, one of the endonucleases from *Xoo*. The DNA sequences of its cognate methyl transferase, M.*Xor*II, was previously reported (Choi and Leach, 1994). This enzyme was known to be an isoschizomer of *Pvu*I (Wang *et al.*, 1980). However, in their report, *Xor*II was directly produced from *Xoo* and purified only to a low degree, thus it was not possible to perform a detailed analysis. Here we report the recombinant production of functional *Xor*II as a starting point for the detailed analysis.

The genomic DNA of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) KACC 10331 was prepared as described in Lee *et al.* (2005). Since the genome sequences of *Xoo* were previously reported, we analyzed the open reading frames (ORFs) to search for the genes coding for the type II restriction endonucleases. A candidate gene, XOO0608, was located between sequences 636095 and 636892, although it was annotated only as "endonuclease." As illustrated in Fig. 1, the protein from this ORF shares 64% (169/265) sequence identity with afa22MI-R of *Acidocella facilis* (GenBank accession no. AB117613). We searched the REBASE database (http://rebase.neb.com/rebase/rebase.html) and found that afa22MI-R was an isoschizomer of *Pvu*I, which was another clue in our search for *Xor*II. Because the RM system is mobile, and the genes coding for an endonuclease and its cognate methylase are located in close proximity to each other on the chromosome (Kobayashi, 2001), we expected to find a methylase gene nearby. Fortunately, a modification methylase gene (XOO0607) was found right next to the *XorII* gene in the *Xoo* sequence. This finding was the third clue in our search, and it increased the probability that this candidate was actually the sought-after *XorII*. This gene was amplified by PCR from the *Xoo* genome. The forward

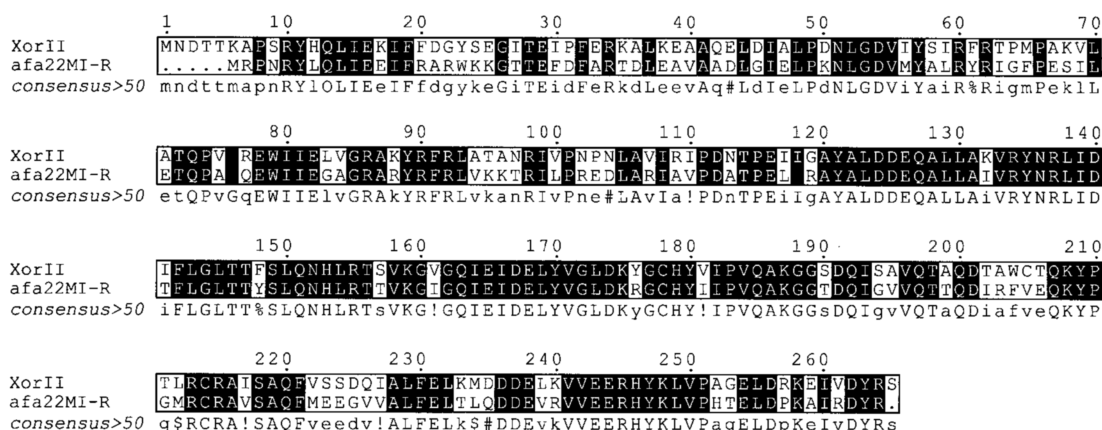

Fig. 1. Alignment of sequences from *XorII* and afa22MI-R. The alignment was performed at the MultAlin site (http://prodes.toulouse.inra.fr/multalin/multalin.html). The boxed plot was created at the ESPript site (http://espript.ibcp.fr/ESPript/ESPript/).

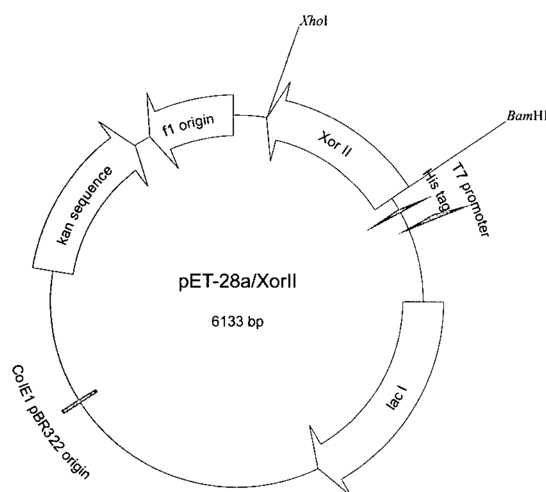

Fig. 2. Plasmid map of pET-28a/XorII. The $(His)_6$-tagged *Xor*II gene is under the control of the T7 promoter.

primer was 5'-GGG CCC GGA TTC GTG AGC TTG CCT CCC TAC GTC-3', and the reverse primer was 5'-GGG CCC CTC GAG TTA AAC GCC GTG CAT CAA CGT-3'. The PCR product was treated with *BamHI* and *XhoI* and subsequently inserted into pET-28a as shown in Fig. 2. It was expected that the use of these cloning sites would result in a protein with 34 additional amino acid residues added to the N-terminus of the natural protein. The resulting plasmid was named pET -28a/XorII (Fig. 2), and it was brought into the expression host, BL21(DE3) (Novagen, USA), in order to produce the protein.

We began the protein production process by using a single colony to inoculate 100 ml of LB medium supplemented with 50 μg/ml of kanamycin and 34 μg/ml of chloramphenicol. The culture was grown overnight at 37°C in a shaking incubator and mixed the next morning with a fresh liter of LB medium supplemented with the same antibiotics. The culture was continuously grown at 30°C until an OD_{600} reading of 1.0 was obtained. IPTG was then added to the final concentration of 0.5 mM to induce protein production. The culture was then grown for another 3 h and harvested by centrifugation at 5000 rpm for 10 min at 4°C. The harvested cells were resuspended in 50 ml of 10 mM TrisHCl pH 8.0 and frozen at -20°C.

We initially grew the cells at 37°C for the protein production process; however, the cells started to die when forced to produce *Xor*II. This was probably because the cells had suffered chromosomal damage from the *Xor*II attack because it did not contain the cognate methyltransferase, M.*Xor*II. We can speculate that this cell death might have been prevented if *Xor*II had been coexpressed with M.*Xor*II; however, this could also result in a reduction in the expression level of *Xor*II. This problem was partially circumvented when the temperature was lowered to 30°C, probably because the expression rate of the *Xor*II gene was reduced. We also tried using lower temperatures, but did not choose one of those temperatures because the cell growth became very slow, and there was no increase in the final yield. We also varied the timing of the induction, but the amount of protein produced showed little difference when the protein production was induced at an OD_{600} in the range of 0.7 to 1.4 (data not shown). The most intriguing observation in our experiment was that if the growth medium was inoculated from the frozen cell stock stored at -70°C, the cells did not grow well following the addition of IPTG. That is, after protein production was induced at OD_{600}-1.0, the cells only grew to a small extent (OD_{600}-1.5) and then began to die, as shown by the decreasing OD_{600}. We had to bring the expression plasmid into the host cell and pick a colony for inoculation each time we produced and purified *Xor*II. The cells grew much better in terms of the optical density when fresh transformation was performed.

The harvested cells were lysed by freeze and thaw, and the DNA was fragmented by ultrasonication. The soluble fraction was retained after centrifugation at 15,000 rpm for 20 min and filtered through a HiTrap SP HP column (5 ml, GE Healthcare, USA). The filtered fraction was loaded onto a HiTrap Chelating HP column (5 ml) charged with Ni^{2+} (GE Healthcare, USA). The column was washed sequentially with 50 ml of Buffer A (10 mM sodium phosphate buffer

pH 7.4, 10 mM imidazole, 300 mM NaCl), 50 ml of Buffer A containing 20% ethanol, 50 ml of Buffer A containing 20% glycerol, 50 ml of Buffer A containing 10% Buffer B (10 mM sodium phosphate buffer; pH 7.4, 400 mM imidazole, 300 mM NaCl), and 50 ml of Buffer; A containing 40% Buffer B. The bound fraction was eluted with 30 ml of

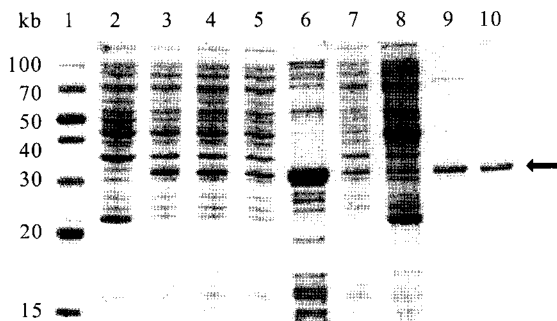

Fig. 3. The 12% SDS-PAGE gel used in the purification process. Lane 1, size marker; lane 2, whole cell lysate before induction; lane 3, whole cell lysate after induction; lane 4, soluble fraction of lane 3; lane 5, flow-through fraction of lane 4 from the HiTrap SP HP column; lane 6, bound fraction that was eluted from the HiTrap SP HP column; lane 7, flow-through fraction of lane 6 from the HiTrap Chelating HP column; lanes 8, 10% Buffer B wash fraction of lane 6 from the HiTrap Chelating HP column; lane 9, 40% Buffer B wash fraction of lane 6 from the HiTrap Chelating HP column; lane 10, bound fraction that was eluted from the HiTrap Chelating HP column. The arrow indicates the *Xor*II band.

Buffer B. The eluted fraction was then concentrated and buffer-exchanged with 100 mM TrisHCl pH 8.0 containing 200 mM NaCl, 2 mM DTT, 0.2 mM EDTA, and 0.1% Triton X-100 by Amicon Ultra (Millipore, USA). The resulting solution was mixed with an equal volume of 100% glycerol and kept at -20°C.

We attached the $(His)_6$-Tag to *Xor*II in hopes of purifying it in a single step, but unfortunately the eluted fraction from the HiTrap Chelating HP column always contained a non-negligible amount of contaminating proteins. We therefore employed cation exchange chromatography as a filtering step before performing affinity chromatography in order to achieve higher purity. The cation (HiTrap SP HP) and anion (HiTrap Q HP) exchange resins were tested to see if they could provide any useful information. The fraction that passed through the HiTrap SP HP column produced the best result (Fig. 3). It was surprising that in order to obtain a higher purity, it was better to choose the flow-through fraction from the cation exchange column rather than the bound fraction from the anion exchange column. One contaminant (the major band in lane 6, Fig. 3) was very hard to remove, even with the affinity column. However, it was effectively trapped inside the cation exchange column, and we confirmed that the trapped fraction had much lower enzymatic activity than the unbound fraction, which indicated that the band did not represent (functional) *Xor*II (data not shown). The flow-through fraction was directly loaded on to the HiTrap Chelating HP column. The two washing steps (glycerol and ethanol washing) proved to be more effective in removing contaminants than gradually

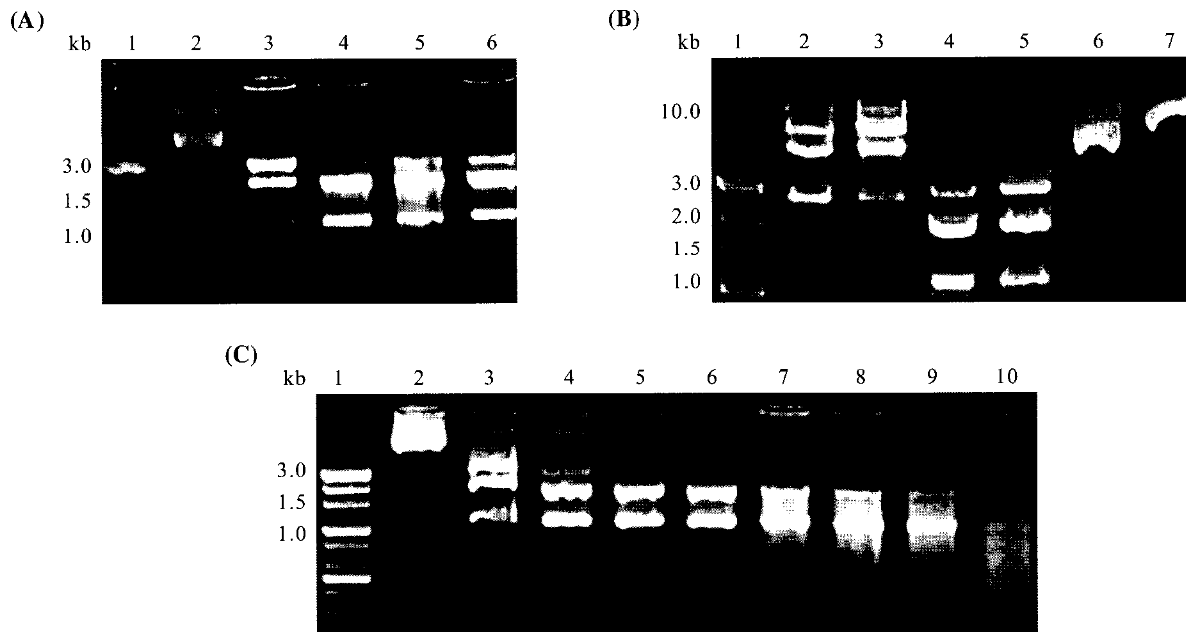

Fig. 4. (A) *Xor*II activity test with different buffers. Lane 1, size marker; lane 2, intact vector; lane 3-6, fragments from vector digestion in NEBuffers 1-4, respectively. (B) Comparison of the cleavage patterns of *Pvu*I and *Xor*II. Lane 1, size marker; lanes 2 and 3, YEp24 cleavage by *Pvu*I and *Xor*II, respectively; lanes 4 and 5, pBluescript KSII(+) cleavage by *Pvu*I and *Xor*II, respectively; lanes 6 and 7, ϕX174 cleavage by *Pvu*I and *Xor*II, respectively. (C) *Xor*II activity test at 25 and 42°C. Lane 1, size marker; lane 2, intact vector; lane 3-6, fragments from vector digestion at 25°C for 1, 2, 3, and 4 h, respectively; lane 7-10, fragments from vector digestion at 42°C for 1, 2, 3, and 4 h, respectively.

increasing the imidazole concentration. The wash containing 40% Buffer B successfully removed some portion of *Xor*II as well as the other contaminants (Fig. 3, lane 9); however, we regarded this as a sustainable compromise to get a pure protein (Fig. 3, lane 10). We also tried using gradient elution, but the 40% washing was faster and more effective in removing other contaminants. In fact, the speed of the purification was deemed the most important factor in the present study since it was imperative to minimize the loss of enzymatic activity. The amount of *Xor*II purified from one liter of LB medium was around 2.5 mg, as calculated from the absorbance at 280 nm using the theoretical extinction coefficient ε_{280} = 28880 M/cm or 0.860 ml/mg/cm. The final yield seems low; however, this result is due to the fact that the culture did not fully grow and a considerable portion of the produced *Xor*II formed an inclusion body (Fig. 3, lane 4). The concentration of the final product was adjusted to 0.25 mg/ml.

The pBluescript SK II(+) (Stratagene, USA) vector was used as a substrate in the enzyme activity test. The pBluescript SK II(+) vector contained two PvuI sites, and the expected fragments were 1045 and 1916 bp long. Two micrograms of the substrate DNA and 0.25 μg of *Xor*II were used in a 50 μl reaction volume. The buffers used to find the optimal chemical condition were NEBuffer 1, NEBuffer 2, NEBuffer 3, and NEBuffer 4 (New England Biolab, USA), which were readily available in our laboratory. We believe that those buffers represented a reasonable variation of buffer conditions. *Xor*II showed higher activity in NEBuffer 2, as shown in Fig. 4A. We might have explored more buffer conditions had the activity in any buffer not been high enough, but fortunately, NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol; pH 7.9 at 25°C) was found to be a match for *Xor*II. We also tested buffers in the presence of BSA, but did not observe any noticeable differences (data not shown). We reconfirmed that *Xor*II was, in fact, the isoschizomer of *Pvu*I as previously reported (Wang *et al.*, 1980). As shown in Fig. 4B, the cleavage pattern of Yep24, pBluescript KSII(+), or ϕX174 RF1 DNA by *Xor*II was the same as that by *Pvu*I; however, the cleavage reaction was not carried out to completion. Both enzymes can cut YEp24 and pBluescript twice, but neither can cut ϕX174 RF1 DNA. The enzymatic activities of both endonucleases seemed to be mostly directed toward the supercoiled DNA because the single-cut band persistently remained. We did not attempt to use more enzymes or incubate for a longer time for the following reason. The enzyme activity was first assayed at 37°C as described in Wang *et al.* (1980), but the digested band showed a smearing pattern in the agarose gel. As shown in Fig. 4C, at 42°C the smearing first began within an hour of incubation, although the reaction was faster than at 25°C. We suspected that this was due to nuclease contamination, but we obtained the same result from several attempts at purification. In addition, *Xor*II was tagged with hexahistidine, so the purity of the final product was very high, and we assume that *Xor*II has a native exonuclease activity at suboptimal conditions. A detailed study on this unusual activity is currently underway.

In conclusion, we successfully cloned the *XorII* gene, produced the enzyme in *E. coli*, and demonstrated that it was functional and an isoschizomer of *Pvu*I. A detailed biophysical and biochemical study of *Xor*II is currently underway.

Acknowledgements

The authors thank Prof. Kyung Nam Kim for providing helpful advice and discussion of the manuscript, as well as Prof. David Hindman for proofreading this manuscript. This work was supported by the RDA Biogreen21 Fund (grant 20050401-034-743-176-06-00).

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