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Sequence and Expression of a Xylanase Gene from the Hyperthermophile *Thermotoga* sp. Strain FjSS3-B.1 and Characterization of the Recombinant Enzyme and Its Activity on Kraft Pulp

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A gene expressing xylanase activity was isolated from a genomic library of *Thermotoga* sp. strain FjSS3-B.1. The sequence of the gene shows that it encodes a single domain, family 10 xylanase. The recombinant enzyme has extremely high thermal stability, activity over a relatively broad pH range, and activity on *Pinus radiata* kraft pulp.

Biotechnology is receiving increasing attention from the pulp and paper industry because of its potential for use in primary-pulp manufacture. Interest has been focused on a number of areas within the industry, including pulp modification, waste treatment, and by-product conversion. Kraft pulping, a process widely used in paper manufacture, removes about 95% of the lignin by alkaline sulfate cooking. The remaining lignin gives the pulp a brown color which is removed in a multistage bleaching process with a variety of agents. Currently, there is concern about the environmental impact of some of the compounds used in the process. This is particularly the case with chlorine and chlorine dioxide. Enzymes, including xylanases (endo-1,4-β-xylanase, EC 3.2.1.8), have been shown to reduce the amount of chlorine required to achieve comparable levels of paper brightness (12, 16, 23, 24). However, the mesophilic enzymes currently in use have limitations because high temperatures are used in bleaching.

Thermotoga sp. strain FjSS3-B.1 (hereafter referred to as FjSS) was enriched from an intertidal hot spring on Savu-Savu beach in Fiji (11) and has been shown to produce a highly thermostable xylanase (21). Below we report the isolation of a xylanase gene from this organism, its overexpression in *Escherichia coli*, and preliminary analysis of the properties of the recombinant enzyme.

FjSS was provided as a cell pellet by Hugh Morgan of the University of Waikato, Hamilton, New Zealand. Chromosomal DNA was isolated and partially digested with *Sau*3AI. Fragments of 8 to 10 kb were inserted into λZapII (Stratagene, San Diego, Calif.) and packaged with Gigapack XL (Stratagene) as described by Gibbs et al. (7). A total of 1,500 plaques from the library were initially screened. This number ensured a 99% probability of representation for calculations based on estimates of genome sizes for other ancient bacterial genomes (3). The plaques were tested for the expression of xylanase activity by the Congo red assay of Teather and Wood (22), with incubations carried out at 70°C. Replicate plates were used because the assay procedure is lethal to the phage. Only a single plaque, designated TX1, which showed xylanase activity was found. It is usual for xylanolytic organisms to produce more than one active xylanase, and so a further 3,000 plaques were screened; no further xylanase-positive recombinants were observed. It is possible that *Thermotoga* genes are poorly expressed in *E. coli* and that the xylanase expression of TX1 was a fortuitous juxtaposition of the gene with vector sequences. Two other xylanase genes (*xynB* and *xynC*) have subsequently been found in this organism by PCR and genomic walking, but these genes were not detected by expression in the library (unpublished data). A full description of *xynB* and *xynC* will be made available on completion of the work.

TX1 was converted to a Bluescript SK− plasmid by the standard excision procedure described by Stratagene, and the resulting plasmid was found to contain an 11-kb genomic insert. The xylanase activity was confined to a 2.3-kb *Kpn*I-*Sma*I fragment at one end of the insert, and the pBS plasmid containing this fragment was designated pNZ2824. The fragment was transferred to M13 mp10 and was sequenced in full with an Applied Biosystems (Foster City, Calif.) model 373A DNA sequencer and a Catalyst 800 robotic workstation. A combination of double-stranded plasmid sequencing and single-stranded M13 sequencing was performed by both dye-primer and dye-terminator methods, and the fragment was sequenced fully on both strands.

Computer analysis of sequence data was carried out with the Genetics Computer Group software package (5) on a Silicon Graphics Indigo2 workstation. Percentage similarities of peptides were calculated with the comparison table of Risler et al. (17). The sequence data showed the fragment to possess a single, complete open reading frame which was designated *xynA*. Comparison of this open reading frame with other genes showed that *xynA* encoded a xylanase belonging to the glycosyl hydrolase family 10 (corresponding to β-glycanase family F [8]). The *xynA* gene translates to a peptide of 346 amino acids with a calculated molecular mass of 40.5 kDa. XynA is most closely related to XynZ from *Clostridium thermocellum* (44% identity and 88% similarity [9]) and XynA from the thermophilic anaerobe Rt8B.4 (42% identity and 86% similarity [6]).

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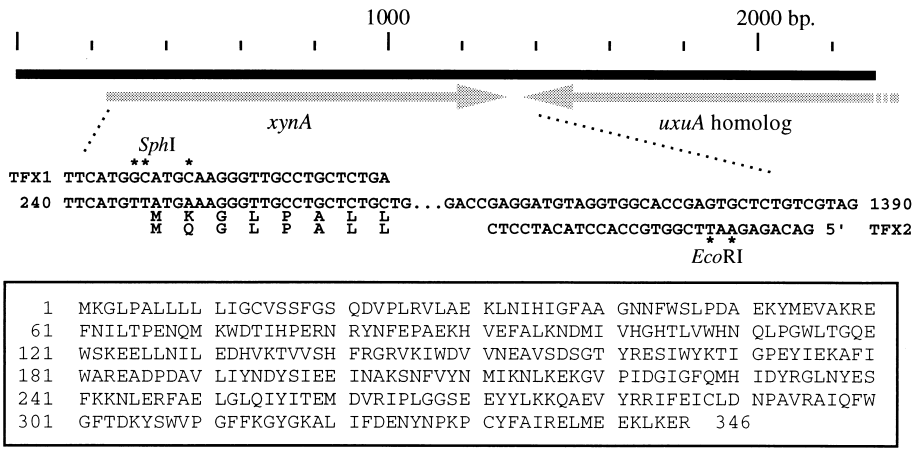

FIG. 1. At the top is a diagram showing the locations of the two open reading frames on the insert of pNZ2824. Below the diagram is a primer design for expression of FjSS *xynA* in the plasmid vector pJLA602. Coordinates refer to the base numbers in the sequence, which is available from GenBank. Restriction enzyme sites introduced and the bases changed to create them are shown. The upper amino acid sequence is the deduced N terminus of the native XynA, and the lower sequence is the sequence of the expressed recombinant enzyme. Asterisks highlight the mismatches in primer TFX1 which introduce the *Sph* I site. At the bottom is the deduced peptide sequence of FjSS XynA.

The *Thermotoga maritima xynA* gene (25) has only moderate homology with FjSS *xynA*. Furthermore, the *T. maritima* sequence is unlike FjSS *xynA* in that it encodes a multidomain enzyme. Preliminary sequence data suggest that the FjSS genes *xynB* and *xynC* have a structure similar to that of *T. maritima xynA* (see above). It is likely that the 40-kDa xylanase seen in zymograms by Winterhalter et al. is the *T. maritima* homolog of FjSS XynA.

The sequence upstream of *xynA* showed no detectable homology with any genes in the GenBank or EMBL database (releases 87 and 41, respectively). Downstream of *xynA* is a second open reading frame on the opposite strand of the DNA. The 5′ end of the gene is missing from the sequence data. The peptide sequence derived from this truncated reading frame has 45% identity and 85% similarity with UxuA of *E. coli*, which is a D-mannonate hydrolase involved in the glucuronate pathway (1, 18). A diagrammatic representation of the fragment is shown in Fig. 1.

The plasmid expression vector pJLA602 (19) was used for overexpression of *xynA* in *E. coli*. An expression plasmid was constructed as follows. Oligonucleotide primers were designed on the basis of the coding sequences for the amino and carboxy termini of XynA to allow PCR amplification of the *xynA* gene from FjSS genomic DNA. *Sph*I and *Eco*RI restriction endonuclease sites were incorporated into the forward and reverse primers, respectively (TFX1 and TFX2) to give a 1,141-bp PCR product with cleavable restriction sites at each end (Fig. 1). These sites allowed directional ligation of the PCR product into the same sites of pJLA602. The *Sph*I site in the multiple cloning site of the vector contains an in-frame ATG start codon. Ligation of the *Sph*I-digested vector and PCR product ensures that the start codon of *xynA* is optimally placed downstream of the vector promoter sequences. One codon change was made with the introduction of the 5′ restriction site. The *Sph*I site changes the second residue of XynA from lysine to glutamine. The introduction of an *Eco*RI site in the reverse primer changes only the DNA sequence downstream of the *xynA* gene and as a consequence does not introduce any amino acids changes into the expressed peptide. *E. coli* DH5α (10) was transformed by the recombinant pJLA602/*xynA* plasmid to ampicillin resistance. XynA enzyme was purified by the method of Gibbs et al. (7) except that the heat precipitation of host proteins was performed at 85°C. The enzyme produced was sufficiently pure (approximately 95%) for subsequent enzyme assays.

Xylanase activity was quantified by measuring reducing sugar release from oat spelt xylan or *Pinus radiata* kraft pulp with *p*-hydroxybenzoic acid hydrazide (13). Care was taken to use enzyme and substrate concentrations which did not lead to substrate limitation during the assay procedure. Enzyme activity is expressed in nanokatals. One katal is defined as the amount of enzyme required to release from xylan 1 mol of xylose reducing sugar equivalent per s (2).

pH profile. Buffers used were sodium acetate, 1-3-bis[Tris (hydroxymethyl)-methylamino]propane (bis Tris propane), or 3-[cyclohexylamino]-1-propanesulfonic acid. The pH was measured at 85°C and buffers were used at a concentration of 25 mM. Oat spelt xylan was added a final concentration of 0.22% (wt/vol), and 100 nkat of XynA was used in 500 μl of buffer. Measurements of enzyme activity were made in triplicate. The pH optimum for the enzyme at 85°C is 6.3, with 50% of maximum activity being retained between pH 5.1 and pH 8.1 (data not shown).

Temperature stabilities. Temperature stabilities were evaluated by measuring the residual activity of XynA after various incubation times in 500 μl of 25 mM bis Tris propane (pH 6.3). A total of 2,000 nkat of XynA (measured at 85°C) was added to each tube. Separate 600-μl PCR tubes were used for each time point to maintain large relative volumes, thus minimizing evaporation. At a variety of times, the tubes were removed and placed on ice. The residual activity in 50 μl of the sample was measured for each time point, and the results from five replicate experiments were combined. Incubations were performed in the absence of substrate. Values were normalized so that the activity of the zero time point equalled 100% (Fig. 2). The half-lives for XynA are 12 h at 95°C and 22 h at 90°C. No measurable loss of activity was seen when the enzyme was incubated at 85°C. Others have shown that the thermal stability of a similar xylanase from the same organism is further enhanced in the presence of a substrate (21).

Activity on *P. radiata* kraft pulp. One gram (dry weight) of *P. radiata* kraft pulp was presoaked overnight in 50 ml of 25 mM bis Tris propane (pH 6.3). A total of 1,000 nkat of XynA (measured at 85°C) was added, and the mixture was incubated

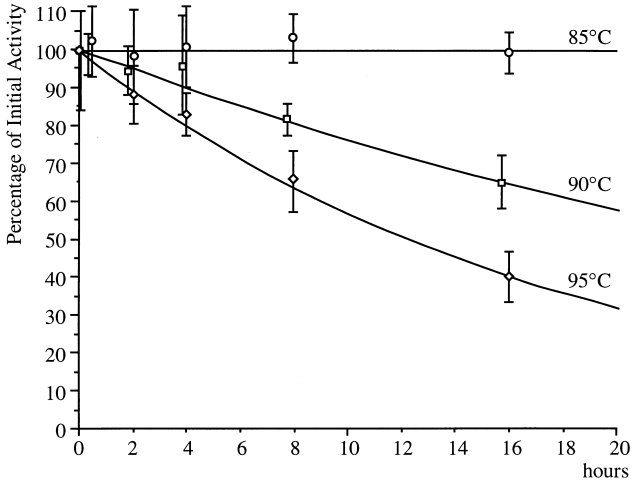

FIG. 2. Loss of activity of FjSS XynA at 85, 90, and 95°C when incubated in the absence of substrate in 25 mM bis Tris propane (pH 6.3). Activities are expressed as a percentage of the reducing sugar released by the untreated (time-zero) sample. Error bars represent two standard deviations calculated from five replicates.

with vigorous shaking at 95°C. One-milliliter samples were taken at a variety of time points and passed through a 0.2-μm-pore-size filter to remove fibers. A prefilter of cotton wool was used to prevent clogging of the membrane. The concentration of reducing sugars in the supernatant was measured as described above. The enzyme catalyzed a maximum release of approximately 15 μmol of reducing sugars or oligosaccharide reducing ends after 30 min (Fig. 3). A slight rise was seen over the next 4.5 h, but this also occurred in the control sample and so it is unlikely to have been the result of further hydrolysis by XynA. The cause of the loss of xylanase activity after 30 min is as yet uncertain. Similar results have been seen with another hemicellulase, β-mannanase, with which further activity was obtained by the addition of more enzyme (15). This result

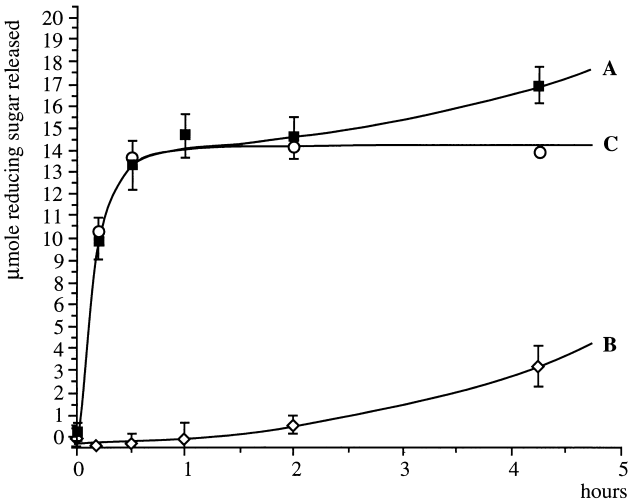

FIG. 3. Micromoles of reducing sugar released from 1 g (dry weight) of *P. radiata* kraft pulp. (A) reducing sugar released by 1,000 nkat (measured at 85°C) of FjSS XynA; (B) reducing sugar released by cooking with no enzyme; (C) release by 1,000 nkat of XynA, with the values corrected by subtraction of the "no enzyme" values (A − B). Hydrolysis was performed at 95°C in 50 ml of 25 mM bis Tris propane (pH 6.3). Error bars represent two standard deviations calculated from five replicates.

confirms that the decline in activity is not a result of substrate limitation. Senior et al. (20) have suggested that the loss of activity is the result of both inhibition by lignocellulosics and nonspecific adsorption to the pulp fibers. However, current work by others has shown that although enzyme activity is rapidly lost in the supernatant, hydrolysis of the pulp fiber continues for some time (14). This suggests that inhibition is not a major cause of enzyme inactivation. The implication of these observations is that if enzyme could be recycled by being released from the fiber, then less would be required in pulp processing.

The pH optimum and half-lives of XynA differ from those reported previously for a xylanase from FjSS (21). Furthermore, the N-terminal sequence of the xylanase reported by Simpson et al. does not match that predicted for XynA, although the sequences of some internal peptide fragments do match (4). From these data, we conclude that there may be more than one 40-kDa xylanase produced by FjSS and that XynA is similar but not identical to the enzyme previously reported.

The closer an enzyme's optimal environment is to the requirements of an industrial process, the more likely it is that the enzyme will offer an economically viable alternative to existing methods. *Thermotoga* sp. strain FjSS XynA has the combined characteristics of high thermal stability, broad pH profile, and activity on fiber-bound xylan. The gene has been expressed at levels which now make it possible for sufficient enzyme to be made for use in pulp bleaching trials.

Nucleotide sequence accession number. The sequence for fragment pNZ2824 has been assigned GenBank accession number U33060.

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