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A gene encoding a novel extremely thermostable 1,4-β-xylanase isolated directly from an environmental DNA sample

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Abstract Small-subunit (SSU) rRNA genes (rDNA) were amplified by PCR from a hot pool environmental DNA sample using *Bacteria*- or *Archaea*-specific rDNA primers. Unique rDNA types were identified by restriction fragment length polymorphism (RFLP) analysis and representative sequences were determined. Family 10 glycoside hydrolase consensus PCR primers were used to explore the occurrence and diversity of xylanase genes in the hot pool environmental DNA sample. Partial sequences for three different xylanases were obtained and genomic walking PCR (GWPCR), in combination with nested primer pairs, was used to obtained a unique 1,741-bp nucleotide sequence. Analysis of this sequence identified a putative XynA protein encoded by the *xynA* open reading frame. The single module novel xylanase shared sequence similarity to the family 10 glycoside hydrolases. The purified recombinant enzyme, XynA expressed in *E. coli* exhibited optimum activity at 100°C and pH 6.0, and was extremely thermostable at 90°C. The enzyme showed high specificity toward different xylans and xylooligosaccharides.

Keywords 16S rDNA · β-xylanase · Environmental DNA · Genomic walking PCR (GWPCR) · Thermostability

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Introduction

Xylans are heterogeneous polysaccharides consisting of a main chain of 1,4-linked β-D-xylopyranosyl residues that often carry acetyl, arabinosyl, and glucuronosyl substituents. The action of the main xylanolytic enzyme, β-endoxylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is to convert polymeric xylan to xylooligosaccharides (Biely 1985). Xylanases are classified into two families, 10 and 11, according to the similarity of amino-acid sequences of their catalytic domain in hydrophobic cluster analyses (Henrissat 1991).

The classical approach to obtaining new xylanases has been based on the screening and isolation of xylanolytic microorganisms. However, typically only a small fraction (< 1%) of naturally occurring microorganisms can be cultivated using standard techniques (Amann et al. 1995). Several approaches have been developed recently to overcome this limitation. One is based on the use of environmental DNA for the construction of DNA libraries and direct screening for functional gene products (Cottrell et al. 1999; Henne et al. 2000). An alternative method is to use PCR primers based on conserved regions of known genes to amplify new genes from DNA extracted from noncultured biomass (Seow et al. 1997; Cottrell et al. 2000). In this study we used a modified genomic walking PCR (GWPCR) technique to retrieve xylanase genes from a hot pool environmental DNA sample. We report here the cloning, sequencing, and expression of a novel xylanase, XynA, along with some biochemical properties of the purified enzyme.

Materials and methods

Bacterial strains and genomic DNA

Escherichia coli INVαF′ One-Shot cells (Invitrogen, Carlsbad, CA, USA) were used as the bacterial host for all DNA cloning and expression studies. Genomic DNA was prepared as described previously (Morris et al. 1995) from environmental samples from

the hot pool "KP" in Kuirau Park, Rotorua, New Zealand (for a description of the pool see Saul et al. 1999).

PCR amplification, cloning, and sequencing of 16S rDNA

PCR was performed with AmpliTaq Gold polymerase kit (Perkin-Elmer, Foster City, CA, USA) and a pair of *Bacteria*- and *Archaea*-specific primers (Table 1) for the SSU 16S rRNA gene (rDNA) sequences. The *Bacteria*-specific primers were designed to obtain the almost complete 16S rRNA genes (Saul et al. 1993) while amplification with the *Archaea*-specific primers resulted in a 1,000-bp fragment. The PCR was performed in a GeneAmp PCR System 2400 (Perkin-Elmer) for 40 cycles with denaturation at 95°C for 30 s, annealing at 50°C and primer extension at 72°C. Amplified DNAs were purified, ligated into the pCR2.1 vector (Invitrogen), and transformed into *E. coli* INVαF′ according to the manufacturer's instructions. Plasmids containing inserts were prepared for restriction fragment length polymorphism (RFLP) analysis using the enzyme *Bsp*143I (MBI Fermentas, Amherst, NY, USA). Restriction fragments were separated by agarose gel electrophoresis, and plasmids showing different restriction patterns were analyzed again with a combination of the enzymes *Hin*fI, and *Msp*I (MBI Fermentas). Representative 16S SSU isolates were sequenced in both strands using a combination of 16S SSU sequencing primers (Table 1).

Consensus and GWPCR

A consensus PCR fragment from glycoside hydrolase family 10 xylanase was amplified from the environmental DNA sample using the XYNFA–XYNFR and XYNFB–XYNFR consensus primer pairs (Table 1). The fragments were ligated into the pCR2.1 vector and transformed into *E. coli* and analyzed by RFLP using the restriction enzymes *Tsp*509I and *Hae*III (New England Biolabs, Beverly, MA, USA). Plasmids having identical restriction patterns were grouped together into families, representatives of which were fully sequenced on both strands. Linker assembly and linker library construction were performed as described by Morris et al. (1995, 1998). GWPCR was performed as described previously (Morris et al. 1998) but using nested pairs of specific primers. Basically, reactions were performed in standard 50-µl PCR mixtures by using 12 pmol of linker primer (Morris et al. 1995), 12 pmol of the genomic walking primer (Table 1) and 1 µl of the appropriate environmental DNA restriction fragment linker library. The first PCR was performed with the external genomic walking primer in combination with its linker primer for 20 cycles. The products obtained were diluted as necessary and used as template for the nested PCR using an internal genomic walking primer in combination with its respective linker primer. Nested PCR was performed for 35 cycles as described above. The forward and reverse genomic walking primers used in this study are shown in Table 1. The GWPCR fragments obtained were purified from the reaction mixtures and ligated into the pCR2.1 vector and transformed into *E. coli* INVαF′.

Construction of a recombinant plasmid containing *xynA* and purification of XynA

The *xynA* specific primers UNCULTXYNAF and UNCULTXYNAR (Table 1) were synthesized (based on the nucleotide sequence of *xynA* obtained from GWPCR) to allow PCR amplification of DNA coding for the mature *xynA* gene directly from the environmental DNA sample. 3′ A-overhangs were added post-amplification following the manufacturer's instructions for the pCR2.1 cloning kit, and the product was ligated into the expression plasmid pCR2.1 to give an in-frame fusion with *lacZ* open reading frame; this resulted in recombinant plasmid pSUNXYNA. Xylanase-positive transformants were identified on plates containing xylan using Congo Red staining (Teather and Wood 1982). Both strands of the recombinant plasmid encoding the XynA xylanase were sequenced in order to confirm that there were no PCR-derived base changes in the DNA. Recombinant cells were grown, harvested, and purified as previously described (Morris et al. 1998). The final extract was desalted and purified by anion-exchange chromatography on a HiTrapQ column (Amersham Pharmacia Biotech) as described previously (Sunna et al. 2000a). The purified XynA was concentrated, desalted, and stored in 50 mM sodium phosphate buffer (pH 6.0) at 4°C.

Enzyme assay, protein determination and electrophoresis

Xylanase activity was determined by the dinitrosalicylic acid method (Bernfeld 1955) as described previously (Sunna et al. 2000a) but with a standard incubation temperature of 100°C (15 min). The standard assay reaction mixture consisted of 0.5% (w/v) xylan supplemented with 120 mM universal buffer (Britton and Robinson 1931), pH 6·0, and enzyme (66.2 mXU) to give a final volume of 0.1 ml. All reactions were performed in 1.5-ml vials

Table 1 Oligonucleotides used in this study

Bacteria-specific16S rDNA primers[a]	
PB36 (11–30)	5′ AGRGTTTGATCMTGGCTCAG 3′
16S. 1 (369–379)	5′ ACTCCTACGGGAGGCAGCAG 3′
16SF1 (539–560)	5′ TGCCAGCAGCCGCGGTAATACG 3′
16SR1 (538–559)	5′ GTATTACCGCGGCTGCTGGCAC 3′
16S. 3 (808–829)	5′ GGATTAGATACCCKGGTAGTCC 3′
16SF2 (1125–1146)	5′ GGTTAAGTCCCGCAACGAGCGC 3′
16SR2 (1125–1146)	5′ GCGCTCGTTGCGGGACTTAACC 3′
PB38 (1534–1551)	5′ GMTACCTTGTTACGACTT 3′
Archaea-specific 16S rDNA primers [a]	
ARCH 358 (338–358)	5′ GGCCCTAYGGGGYGCASCAGG 3′
ARCH 1378 (1378–1399)	5′ GTGTGTGCAAGGAGCAGGGAC 3′
Family 10 xylanase consensus primers	
XYNFA	5′ CACACKCTKGTKTGGCA 3′
XYNFB	5′ CATACKTTKGTTTGGCA 3′
XYNFR	5′ TMGTTKACMACRTCCCA 3′
Genomic walking primers	
UN2GW1F	5′ CCAGTAAGTAAAGAGGTACTTTTGCAG 3′
UN2GW2F	5′ CAACAGGTGCCTGCTTGGGTTTTTAGG 3′
UN2GW1R	5′ CTGCAAAAGTACCTCTTTACTTACTGG 3′
UN2GW2R	5′ CCTAAAAACCCAAGCAGGCACCTGTTG 3′
Noncultured *xynA*-specific primers	
UNCULTXYNAF	5′ GGAGGTTGCCATGGGTAGTGAGATTCCTTC 3′
UNCULTXYNAR	5′ GGGAGGAGCTTAAGTATTAAAGTTTACAAT 3′

[a]Primer binding positions relative to *E. coli* 16S rDNA (Brosius et al. 1978). Internal *Bacteria*- specific16S rDNA primers used for DNA sequencing are underlined

with O-ring screw caps. One xylanase unit (XU) is defined as the amount of enzyme required to liberate 1 μmol of xylose per minute at the assay temperature. Protein concentrations were determined using the BCA protein quantification kit (Pierce, Rockford, IL, USA). SDS-PAGE was performed by the method of Laemmli (1970) as previously described (Sunna et al. 2000a).

Effects of temperature, pH, and thermostability on xylanase activity

The effects of temperature, pH, and thermostability on the activity of XynA were determined as described by Sunna et al. (2000b). For experiments above 100°C, vials were incubated fully immersed in an oil bath. Universal buffer (Britton and Robinson 1931) was used for determination of optimal pH for activity. The purified enzyme was incubated in the absence of substrate for thermostability experiments. Samples were removed at increasing time intervals and the residual xylanase activity was measured under standard conditions with an assay time of 5 min.

Substrate specificity and mode of action of XynA

The substrate specificity of XynA was determined by incubating the purified enzyme with different polymeric substrates under standard assay conditions. The hydrolysis products arising after the action of XynA on birchwood xylan (Sigma) and xylooligosaccharides (Megazyme International, Bray, Ireland) at 85°C were analyzed by thin-layer chromatography as previously described (Sunna et al. 2000a, b).

DNA sequencing

DNA sequencing and analysis was carried out as previously described (Morris et al. 1998). The DNA sequence of the recombinant xylanase reported here has been submitted to the GenBank database and has been assigned the accession number AY048712. The 16S rDNA sequences reported here have also been submitted to GenBank.

Results

Microbial diversity of the hot pool environmental DNA

A total of 86 positive transformants carrying the amplified bacterial or archaeal 16S rDNA fragments were screened by RFLP to identify unique types for sequence determination. From a total of 40 plasmids carrying the bacterial 16S rDNA, 14 RFLP types were distinguished visually on agarose gels, whereas from 46 plasmids carrying the archaeal 16S rDNA, only six RFLP types were distinguished. Fragments representing unique RFLP types were sequenced on both strands. Complete 16S rDNA nucleotide sequences of RFLP selected plasmids revealed similarity to cultured and noncultured bacterial and archaeal representatives. Table 2 lists the percentage similarities (nearest neighbor) between 16S rDNA PCR fragments generated from DNA of the hot pool "KP" in Kuirau Park and the sequence of described species or deposited environmental clone sequences after analysis using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi). A homology search using BLAST showed that the inserts could be classified into three major phylotypes: the *Aquificale*s, the *Thermus*/*Deinococcus* group, and *Proteobacteria*. Most of the bacterial genes (60%) represented known cultured thermophile sequences, whereas the rest of the bacterial genes represent sequences sharing similarities to environmental sample sequences obtained from different hot springs. Interestingly, most of the archaeal SSU rDNA sequences (41 sequences) were represented by the noncultured archaeon clone A10 whose gene sequence (GenBank accession no. AF325184) was obtained from a microbial community in an arsenite oxidizing, acidic, hot spring. The only cultured representative sequence was that of *Thermococcus waimanguensis*.

Analysis of consensus and GWPCR

Two 160-bp fragments were amplified from the environmental DNA sample with the XYNFA–XYNFR and the XYNFB–XYNFR consensus PCR primer pairs (Table 1). From a total of 48 transformants, three RFLP types were distinguished and 16 recombinant plasmids representing the unique RFLP types were sequenced on both strands. Nucleotide sequencing of RFLP selected plasmids revealed similarity to three different bacterial xylanases belonging to the glycoside hydrolase family 10 (Henrissat 1991). Inserts AS231 and AS255 (containing the 160-bp fragment amplified with XYNFA–XYNFR) exhibited amino acid sequence similarity to *Caldicellulosiruptor* sp. Tok7B.1 XynA (Gibbs et al. 2000; 100% sequence similarity) and to *C. saccharolyticus* XynI (V. S. J. Te'o, unpublished data; 98% sequence similarity), respectively. Insert AS233 (containing the 160-bp fragment amplified with XYNFB–XYNFR) exhibited low amino-acid sequence similarity to *Caldibacillus cellulovorans* XynA (Sunna et al 2000a; 54% sequence similarity). Accordingly, this fragment was chosen for amplification of the full xylanase gene, *xynA*. The amplification of the DNA fragments upstream and downstream of the *xynA* consensus fragment region was achieved using genomic walking primers (see Table 1) and GWPCR as described in the Materials and methods section. This procedure differs from that described earlier (Morris et al. 1995, 1998) in that it was necessary to use nested genomic walking primers to get specific amplification of the xylanase sequence. The nucleotide sequence data generated from the upstream and downstream *xynA* genomic walking were combined to generate a 1,741-bp consensus sequence, which encompassed a complete 1,083-bp *xynA* open reading frame (Fig. 1).

Analysis of the consensus nucleotide sequence revealed the presence of a 1,083-bp open reading frame, *xynA*, coding for a β-xylanase, XynA, with a putative size of 360 amino acids. Similarity searches were carried out between the deduced amino acid sequence of XynA against entries in the GenBank and SWISSPROT databases. The N-terminal sequence contained a putative signal peptide sequence with a predicted cleavage site between position 26 (Val) and position 27 (Met). Removal of the signal peptide yielded a mature protein

Table 2 Phylogenetic affiliation of bacterial and archaeal SSU rDNA nucleotide sequences

RFLP representative 16S rDNAs and accession no.	Taxonomic group	Nearest phylogenetic neighbor	GenBank accession no.	Similarity to nearest neighbor (%)	Number of 16S rDNAs in RFLP Group
BACTERIA					
p1, AF402971	*Thermus*/*Deinococcus*	*Thermus* W28A.1	L10068	97–98	9
p2, AF402972	*δ-Proteobacteria*	*Thermodesulforhabdus* sp.	AF170420	92	1
p9, AF402973	Unclassified bacteria	Unidentified bacterium strain NKB19[a]	AB013271	90	1
p14, AF402974	*Proteobacteria*	Unidentified proteobacterium strain BD1–5[a]	AB015518	92–93	3
p16, AF402975	*β-Proteobacteria*	*Hydrogenophilus thermoluteolus*	AB009828	95–96	7
p17, AF402976	Unclassified bacteria	Uncultured eubacterium AA26[a]	AF275921	89	1
P24, AF402977	*Cytophaga-Flavobacteria-Bacteroides* (CFB)/Green sulfur bacteria group	Unidentified Cytophagales/ green sulfur bacterium OPB56[a]	AF027009	97	2
P25, AF402978	Unclassified bacteria	Uncultured bacterium TA07[a]	AF229780	85	1
P28, AF402979	Unclassified *Aquificales*	*Aquificales* strain SRI-40	AF255598	94	4
P35, AF402980	Unclassified clone	–	–	–	2
P39, AF402981	*Nitrospora* group	*Nitrospira* sp. SRI-9	AF255603	97	1
P43, AF402982	*δ-Proteobacteria*	*Desulfurella kamchatkensis*	Y16941	97	3
P48, AF402983	Unclassified bacteria	Uncultured eubacterium SRang2.5[a]	AF047624	95	1
P49, AF402984	Unclassified bacteria	Candidate division OP8 clone OPB95[a]	AF027060	97	4
ARCHAEA					
P1, AF402985	Unclassified archaea	Uncultured archaeon clone A10[a]	AF325184	93	41
P3, AF402986	Unclassified clone	–	–	–	1
P11, AF402987	Unclassified *Crenarchaeota*	Crenarchaeotal sp. clone pJP 41[a]	L25301	93	1
P14, AF402988	Unclassified clone	–	–	–	1
P24, AF402989	*Thermococcales*	*Thermococcus waimanguensis*	AF098975	99	1
P28, AF402990	Unclassified clone	–	–	–	1

[a]Sequence from environmental sample

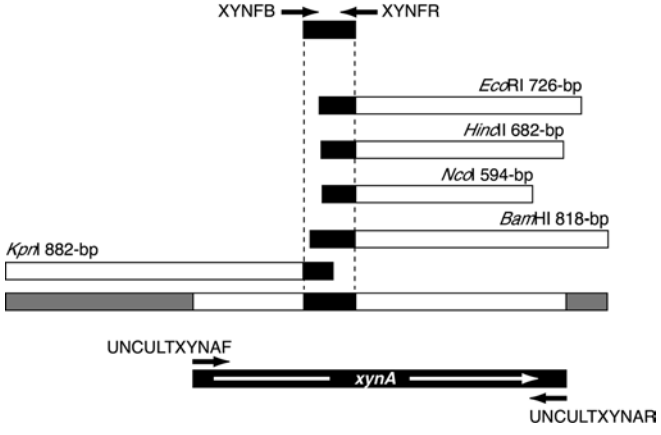

Fig. 1 Diagrammatic overview of upstream and downstream GWPCR products obtained with genomic walking primers. The family 10 xylanase consensus fragment amplified with by the XYNFB and XYNFR consensus PCR primer pair is indicated by a black box. The 1,083-bp *xynA* open reading frame is indicated by the *white arrow* and the position of the UNCULTXYNAF and UNCULTXYNAR primer pair used for amplification of *xynA* during construction of recombinant pSUNXYNA expression plasmid is also shown

that had a predicted M_r of 39.2×10^3. The mature XynA peptide was identified as a β-1,4-xylanase belonging to glycoside hydrolase family 10. Figure 2 shows an alignment of members of glycoside hydrolase family 10 xylanases and the amino acid sequence of the noncultured mature XynA. The noncultured xylanase shares 69% sequence similarity with the xylanase XynA from *Dictyoglomus thermophilum* Rt46B.1 (Gibbs et al. 1995), and 48% sequence similarity with the exo-xylanase XynX from *Aeromonas caviae* ME-1 (Usui et al. 1999).

Biochemical properties of the purified noncultured recombinant XynA enzyme

A recombinant plasmid containing the *xynA* amplified directly from the environmental DNA sample was obtained using the *xynA* specific primers UNCULTXYNAF and UNCULTXYNAR (Table 1). The recombinant XynA fusion protein contained at its N-terminus the first 25 amino acid residues of the pCR2.1 vector *lacZ* open reading frame. The recombinant XynA xylanase was purified to electrophoretic homogeneity with a total yield of 1.4 mg per liter recombinant *E. coli* culture. The final specific activity was 31.1 XU/mg. The apparent M_r of the purified enzyme was estimated to be 43×10^3 by SDS-PAGE (data not shown), consistent with the 42×10^3 M_r deduced from the translated amino acid sequence of the in-frame fusion *lacZ* open reading frame – XynA in the expression plasmid pSUNXYNA.

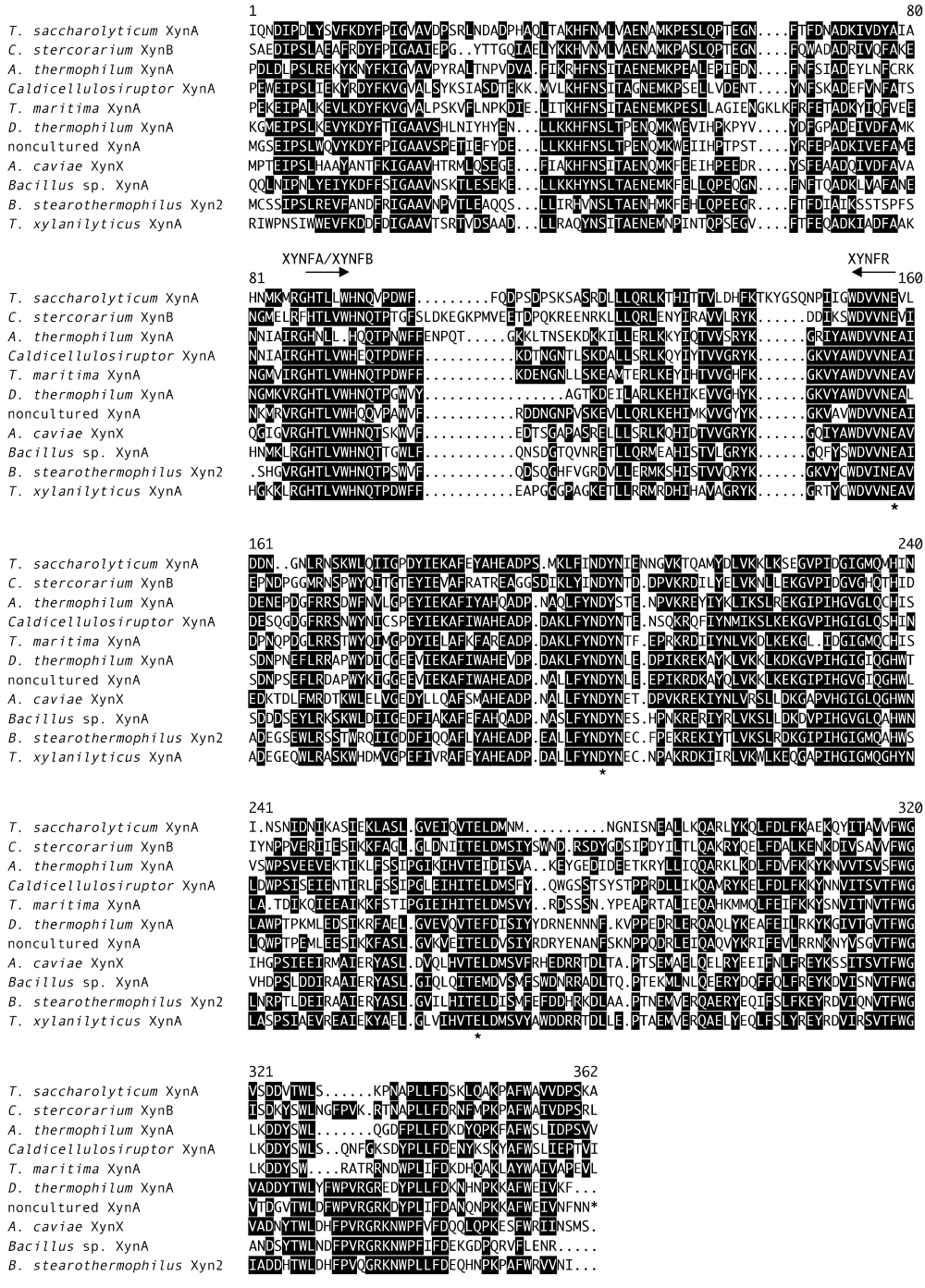

Fig. 2 Amino acid similarity alignment of xylanases belonging to family 10 glycoside hydrolase and the recombinant noncultured xylanase XynA. Conserved residues are *highlighted*. The sequences used were sequences of *Thermoanaerobacterium saccharolyticum* XynA (GenBank access no. M97882), *Clostridium stercorarium* XynB (GenBank access no. D12504), *Anaerocellum thermophilum* XynA (GenBank access no. Z69782), *Caldicellulosiruptor* sp. Tok7B.1 XynA (GenBank access no. AF078737), *Thermotoga maritima* XynA (GenBank access no. Z46264), *Dictyoglomus thermophilum* XynA (GenBank access no. L39866), noncultured XynA (GenBank access no. AY048712, this study), *Aeromonas caviae* XynX (GenBank access no. AB015980), *Bacillus* sp. N137 XynA (GenBank access no. Z35497), *Bacillus stearothermophilus* Xyn2 (GenBank access no. D28121), and *Thermobacillus xylanilyticus* XynA (GenBank access no. Y16849). *Horizontal arrows* indicate positions of family 10 xylanase consensus primers. The amino acid residues important for the catalytic activity of family 10 xylanases are indicated by *asterisks*

The recombinant XynA displayed an optimal temperature for activity under the conditions used at 100°C (Fig. 3A). At 125°C, 27% of the initial activity was still detected. Thermostability experiments indicated that in the absence of substrate, the enzyme displayed half-lives at 100° and 90°C of 2.5 min and 10 h, respectively, while at 85°C the enzyme retained 90% of its initial activity after 66 h incubation (data not shown). When assayed at 100°C, the purified xylanase was active at pH values between 5.0 and 10 (Fig. 3B). The optimal pH for activity was 6.0, with 68% and 28% of the xylanase initial activity remaining at pH 8.0 and 9.0, respectively.

Substrate specificity and mode of action

The xylanase hydrolyzed birchwood xylan, wheat arabinoxylan, oat spelts xylan, beechwood xylan, and larchwood xylan, liberating different amounts of reducing sugars (data not shown). Barley β-glucan and lichenan were poorly hydrolyzed (25% and 19%

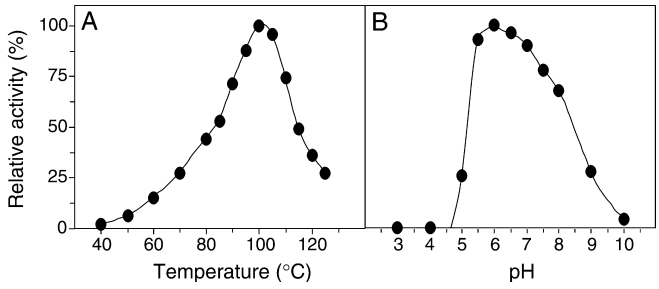

Fig. 3 Effect of temperature (**A**) and pH (**B**) on the enzyme activity of the purified recombinant noncultured XynA. Assays were performed as described in the Materials and Methods section for 15 min at the indicated temperature and pH

reducing sugars liberated compared with birchwood xylan), indicating the low specificity of the enzyme for mixed β-1,3-/β-1,4-linked D-glucose polysaccharides. No detectable reducing sugars were released from laminarin, hydroxyethyl-, or carboxymethyl-cellulose.

The products released by the action of the purified xylanase on partially soluble birchwood xylan and xylooligosaccharides at 85°C were analyzed qualitatively by thin-layer chromatography (data not shown). At the early stages of the hydrolysis (15–30 min), a mixture of larger products and shorter xylooligosaccharides was liberated. Xylobiose was the major end-product of the hydrolysis of xylan after 4 h incubation, with some larger products starting to accumulate after the first 30 min of hydrolysis. After 4 h incubation at 85°C, the purified recombinant β-xylanase completely hydrolyzed xylohexaose, xyloopentaose, xylootetraose, and xylotriose, yielding xylobiose and xylose. Xylobiose was not hydrolyzed, indicating that the enzyme is not a β-xylosidase. Furthermore, no transferase activity was observed after 4 h incubation with xylose.

Discussion

The analysis of microbial diversity of the "KP" hot pool environmental DNA studied here indicates the presence of three major phylotypes: the *Aquificale*s, the *Thermus*/*Deinococcus* group, and *Proteobacteria*. The presence of mostly thermophile cultured representatives is not surprising when considering the high temperature (78°C) of the pool (Saul et al. 1999). The restricted diversity of amplified archaeal rDNA is consistent with a previous study by Hugenholtz et al. (1998) carried out in the Obsidian Pool, a 75°–95°C hot spring, in Yellowstone Park. In their study, a culture-independent molecular phylogenetic survey indicated that bacterial rRNA genes dominate the PCR-amplified community of rDNA by at least 75:1. In general, it is agreed that *Bacteria* communities dominate in most hydrothermal environments with moderate temperatures (50°–90°C), whereas *Archaea* may only dominate in environments at above 90°C (Reysenbach and Shock 2002).

Although many types of cultured microorganisms are known to produce xylanases (Sunna and Antranikian 1997; Sunna et al. 1997; Kulkarni et al. 1999), there have been no reports on xylanases from noncultured microorganisms. Direct phylogenetic comparisons of 16S rDNA from environmental samples have indicated no correlation between cultured strains and their corresponding 16S rDNA libraries (Suzuki et al. 1997; Felske et al. 1999; Brambilla et al. 2001). It could be expected that the same correlation would apply for genes from cultured and noncultured organisms from the same environmental source. Thus, the classical approach clearly represents a limit to the number of genes than can be isolated when screening only for culturable organisms is undertaken.

Several approaches have recently been developed to overcome this limitation. One is based on the use of environmental DNA for the construction of DNA libraries and direct cloning of functional genes (Cottrell et al. 1999; Henne et al. 2000). An alternative method is to use PCR primers based on conserved regions of known genes to identify new genes from noncultured microorganisms (Seow et al. 1997; Cottrell et al. 2000). The most significant new approach is the determination and analysis of entire DNA sequence of an organism. However, the putative function of most of the genomic genes is identified by sequence similarity and their function needs to be confirmed by characterization of the expressed gene products (Hough and Danson 1999).

The consensus PCR approach we use relies on sequence similarities associated with the active site region of the xylanase. Previously, we have used GWPCR successfully to amplify glycoside hydrolase genes from different cultured thermophilic microorganisms (Morris et al. 1995, 1998; Sunna et al. 2000a, b) but this technique was unsuccessful for the direct amplification of genes from environmental DNA. The use of the modified GWPCR technique described here, with the addition of nested primers, allowed us to amplify a novel glycoside hydrolase family 10 xylanase directly from an environmental DNA sample. This culture-independent molecular screening technique simplifies the time-consuming task of screening microorganisms, their isolation and obtaining them in pure cultures. In addition, the amino acid sequence of the noncultured xylanase, XynA, showed low similarity to other reported xylanases, indicating the potential of this method for isolating genes that differ greatly from those of cultured bacteria. However, the procedure is limited to genes that define family 10 and 11 enzymes and a completely novel xylanase without sequence similarity to the consensus regions would not be recovered.

Most xylanases described hydrolyze xylan by an endo-type mechanism to produce a mixture of xylooligosaccharides (Sunna and Antranikian 1997). The noncultured recombinant XynA hydrolyzes xylan to liberate mainly xylobiose and traces of larger sugars. Similarly, xylanase V from *Aeromonas caviae* ME-1 has been reported to hydrolyze birchwood xylan to produce

exclusively xylobiose, with the accumulation of larger products as the hydrolysis proceeded (Kubata et al. 1994). In both cases, the formation and accumulation of larger products may indicate the presence of side-chain residues that sterically hinder the action of both xylanases. Accordingly, due to its hydrolysis pattern, xylanase V from *A. caviae* was classified as an exo-xylanase rather than an endoxylanase. However, the noncultured XynA seems to hydrolyze birchwood xylan by an endo-mechanism to produce a range of larger hydrolysis products in the early stages (15–30 min) of hydrolysis. This finding and the ability of XynA to hydrolyze xylooligosaccharides to mainly xylobiose and xylose suggest that xylanase XynA is an endo-xylanase.

The most striking characteristic of the purified noncultured recombinant XynA expressed in *E. coli* is its extreme optimum temperature for activity and its thermostability in the absence of substrate. The noncultured XynA has a higher optimal temperature (100°C) for activity than that reported for most other xylanases. This is comparable only to the thermostable xylanases described from the genus *Thermotoga*, with optimal temperatures for activity under the assay conditions used between 80° and 105°C (Sunna et al. 1997). All *Thermotoga* xylanases are either single or modular family 10 glycoside hydrolases. The single module XynA purified from *Thermotoga* sp. strain FjSS3-B.1 has the highest optimum temperature for activity reported so far, at 105°C (Simpson et al. 1991), but no members of the *Thermotogales* were identified in the environmental DNA from 16S sequences. Archaea growing at high temperatures seem to be unable to utilize xylan as a carbon source or to produce thermostable xylanases (Sunna and Antranikian 1997). Recently, however, it was shown that the extreme thermophilic archaeon *Thermococcus zilligii* was capable of producing a novel thermostable xylanase (Uhl and Daniel 1999). This xylanase, however, could not be amplified using consensus primers designed to either family 10 or 11 xylanases (M.D. Gibbs, unpublished data). Although there is no data on the optimum temperature for activity of the purified archaeal xylanase; the enzyme displayed a half-life of 8 min when incubated at 100°C. The pH profile of the noncultured XynA described is also remarkable; the purified enzyme retains 68% and 28% of its initial activity after 100°C incubation at pH 8.0 and 9.0, respectively.

Substantial interest has been focused on thermostable xylanases due to their potential application in the development of environmentally friendly technologies in the paper and pulp industry. The use of xylanases in the manufacture of kraft pulp has been shown to promote pulp bleaching (Viikari et al. 1991, 1994). One obstacle, however, remains the need to use xylanases capable of enduring harsh conditions of alkaline pH and temperatures above 80°C. The biochemical characteristics of the noncultured recombinant xylanase, XynA, described here meets the basic requirements for such biotechnological applications, namely thermostability and activity at pH values above 7.0. However, direct application tests on kraft pulp will ultimately determine the bleaching-aid potential of this enzyme.

Exploiting the natural biodiversity of environmental DNA may result in a dramatic increase in the number of novel genes encoding thermostable xylanases being discovered. Amplifying functional genes directly from environmental DNA and their subsequent expression in mesophilic hosts avoids the problems arising from the need to grow extremophiles, which may or may not be readily culturable. This procedure may increase the range of applications for thermostable enzymes in biotechnological processes with high-temperature environments.

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