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Cloning, Sequencing, and Expression of a Xylanase Gene from the Extreme Thermophile *Dictyoglomus thermophilum* Rt46B.1 and Activity of the Enzyme on Fiber-Bound Substrate

MORELAND D. GIBBS,[1] ROSALIND A. REEVES,[1] AND PETER L. BERGQUIST[1,2]*

Centre for Gene Technology, University of Auckland, Auckland, New Zealand,[1] and School of Biological Sciences, Macquarie University, North Ryde, New South Wales 2109, Australia[2]

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A genomic library of the *Dictyoglomus* sp. strain Rt46B.1 was constructed in the phage vector λZapII and screened for xylanase activity. A plaque expressing xylanase activity, designated B6-77, was isolated and shown to contain a genomic insert of 5.3 kb. Subcloning revealed that the xylanase activity was restricted to a internal 1,507-bp *Pst*I-*Hin*dIII fragment which was subsequently sequenced and shown to contain a single complete open reading frame coding for a single-domain xylanase, XynA, with a putative length of 352 amino acids. Homology comparisons show that XynA is related to the family F group of xylanases. The temperature and pH optima of the recombinant enzyme were determined to be 85°C and pH 6.5, respectively. However, the enzyme was active across a broad pH range, with over 50% activity between pH 5.5 and 9.5. XynA was shown to be a true endo-acting xylanase, being capable of hydrolyzing xylan to xylotriose and xylobiose, but it could not hydrolyze xylobiose to monomeric xylose. XynA was also shown to hydrolyze xylan present in *Pinus radiata* kraft pulp, indicating that it may be of use as an aid in pulp bleaching. The equivalent xylanase gene was also isolated from the related bacterium *Dictyoglomus thermophilum*, and DNA sequencing showed these genes to be identical, which, together with the 16S small-subunit rRNA gene sequencing data, indicates that Rt46B.1 and *D. thermophilum* are very closely related.

Recently, xylanases (EC 3.2.1.8) have been shown to have potential use as aids in the bleaching of wood pulp used in paper production, effectively reducing the amount of chlorine required to achieve comparable levels of brightness (9, 22, 31, 32). Xylan is a heteropolymer of the pentose sugar xylose, which accounts for a major proportion of the 20 to 30% hemicellulose found in both hardwood and softwood plant species. Hardwood xylan is mainly glucuronoxylan, while softwoods contain arabinoglucuronoxylan. Kantelinen et al. (9) have suggested that during enzymatic prebleaching of pulp, xylanases primarily hydrolyze xylan which has reprecipitated onto the surface of pulp fibers. The removal of precipitated xylan is thought to enhance the accessibility of bleaching agents to residual lignin within the fibers. Xylanases may also act to solubilize residual lignin cross-linked to xylan.

A member of the genus *Dictyoglomus* has been isolated from a hot spring in Russia (the organism is tentatively called *Dictyoglomus turgidus* [29]), and recently, several isolates of *Dictyoglomus* have been reported. One strain, B1, isolated from a pulp mill cooling tank, could utilize only xylans as a carbon source (16). Secreted xylanases from *Dictyoglomus* sp. isolate B1 and from B4a, a *Dictyoglomus* isolate from an Icelandic thermal pool, were characterized along with xylanase from *Dictyoglomus thermophilum*. All three xylanase preparations were shown to have very similar activities across a broad pH range, with good activity from pH 5.5 to 9.0. All had temperature optima of around 80°C (17). Xylanase prepared from strain B1 has been shown to increase the brightness of pine kraft pulp after treatment for 2 h at 80°C, at both pH 6 and pH 8 (22).

The thermophilic bacterium Rt46B.1 was isolated from a New Zealand hot spring and was reported as being a strain of *D. thermophilum* on the basis of similarities in morphology, fermentation end products, and DNA base composition (20). Comparison of the small-subunit (SSU) 16S rRNA gene sequence of *D. thermophilum* and partial SSU 16S rRNA gene sequence of Rt46B.1 has shown that they are closely related. Phylogenetic analysis has placed the genus *Dictyoglomus* near the base of the phylum defined by members of the order *Thermatogales* (13).

We report here the cloning, sequencing, expression, and characterization of a xylanase gene from *Dictyoglomus* sp. strain Rt46B.1 and examine the potential of the recombinant xylanase as an aid to pulp bleaching, as demonstrated by the release of reducing sugar from fiber-bound substrate.

MATERIALS AND METHODS

Bacteria and culture conditions. *Escherichia coli* C600 (*thi-1 leuB6 lacY1 supE44 tonA* F^-) was used as a host for pJLA602 plasmid constructs. XL1-Blue {*endA1 hsdR17* [r_K^- m_K^+] *supE44 thi-1* λ^- *recA1 gyrA96 relA*1 [Lac^-] [F′ *proAB lacI*^{q} ZΔM15 Tn*10*(Tet^r)]} was used in λZapII genomic library construction. *Dictyoglomus* strains were cultured as described by Patel et al. (19).

Genomic library construction and screening. Chromosomal DNA from Rt46B.1 was isolated and partially digested with *Sau*3AI. Fragments (5 to 8 kb) were purified and partially backfilled with nucleotides A and G. λZapII vector (Stratagene, San Diego, Calif.) was digested with *Xho*I and partially backfilled with the nucleotides C and T to give a 2-bp 5′ overhang complementary to the partially backfilled genomic DNA. Partially backfilled vector and genomic DNA were ligated and packaged with Gigapack XL packaging extracts (Stratagene).

Construction of plasmids. All recombinant plasmids described below were derived from pNZ2906, an 8.3-kb pBluescript SK^- (pBS SK^-) plasmid excised from the λZapII library isolate B6-77. The plasmids pNZ2922, pNZ2923, and pNZ2924 were constructed by simple restriction enzyme digestion-recircularization of pNZ2906 with the restriction enzymes *Eco*RV, *Hin*dIII, and *Hin*cII, respectively. Plasmids pNZ2930, pNZ2931, and pNZ2932 were constructed by

* Corresponding author. Mailing address: Research Office, Macquarie University, North Ryde, New South Wales 2109, Australia. Phone: 61 2 850 8614. Fax: 61 2 850 8799. Electronic mail address: Peter.Bergquist@mq.edu.au.

digestion of pNZ2924 with *Hin*dIII in combination with either *Bgl*II, *Pst*I, or *Sac*I. DNA fragments of 1,700, 1,500, and 800 bp, respectively, were isolated and ligated in pBS KS$^+$ which had been digested previously with the same enzyme combinations. Nested deletions for the sequencing of pNZ2931 were constructed by the exonuclease-mung bean nuclease method as described in the Bluescript manual by Stratagene. pNZ2931 was digested with *Bst*XI and *Xba*I to give the appropriate 5′ and 3′ overhangs required for directed exonuclease III digestion. The plasmid pNZ2900 contains the complete *xynA* gene isolated by the PCR and ligated into pJLA602 (26) digested with *Sph*I and *Eco*RI (see Results).

Primer synthesis and PCR. Two DNA oligonucleotide primers (DictXynF [5′-ATGGTAGCATGCTTAACCAAAGGTTTTCTATC-3′] and DictXynR [5′-CCTATAGAATTCAAAACTTTACAATCTCCC-3′]) were designed to allow the PCR amplification of the entire *xynA* gene from Rt46B.1 genomic DNA. The primers DictXynF and DictXynR were designed to incorporate the restriction enzyme sites *Sph*I and *Eco*RI, respectively, at their 5′ ends, allowing the directional cloning of PCR product into the plasmid expression vector pJLA602 (see Fig. 4). PCR amplification of *xynA* with DictXynF and DictXynR was as follows: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, for a total of 30 cycles. Approximately 5 ng of pNZ2931 was used as the PCR template. All DNA oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA synthesizer.

Enzyme preparation. Purified XynA enzyme was produced as follows. One hundred to 500 μl of an overnight culture of C600 carrying pNZ2900 (grown at 30°C in L broth containing 60 mg of ampicillin per ml) was used to inoculate a fresh 600-ml culture which was grown to an A_{600} of 1.0 and then transferred to 42°C for 2 h to induce XynA production. Cells were pelleted by centrifugation at 3,000 × *g* for 5 min, resuspended in 50 ml of ice-cold TES buffer (0.05 M Tris [pH 8.0], 0.05 M NaCl, 0.005 M EDTA), and centrifuged again at 3,000 × *g* for 5 min, and the cell pellet was resuspended in 20 ml of TES buffer. The cells were then ruptured in a French pressure cell, and the resulting lysate was heated to 80°C for 30 min. After being heated, the cell lysate was centrifuged at 20,000 × *g* for 30 min to pellet denatured mesophilic protein and cell debris to give a supernatant containing relatively pure XynA enzyme. This purified enzyme was used for all subsequent enzyme assays. Enzyme activity was defined in XUs (1). One XU is defined as the amount of enzyme required to release 1 μmol of xylose-reducing sugar equivalent per min from xylan.

Plate assays for xylanase activity. λZapII plaques containing genomic inserts were screened for the expression of xylanase activity by the Congo red assay of Teather and Wood (30). Plaques were overlaid with 0.4% oat spelt xylan dissolved in 0.4% agarose and then incubated at 70°C for at least 5 h. Following incubation, plates were stained with 1% Congo red and destained with 1 M NaCl. Positive plaques were identified by a zone of clearing around xylanase-expressing plaques. Xylanase-positive plaques were converted to Bluescript plasmid recombinants by the excision procedure described by Stratagene.

Enzyme assays. Xylanase activity was assayed quantitatively by measuring reducing sugar release from oat spelt xylan or *Pinus radiata* kraft pulp with *p*-hydroxybenzoic acid hydrazide, as described by Lever (12), with colorimetric changes measured at A_{405}. Enzyme was used at concentrations determined from preliminary assays not to be substrate limiting over the period of the reaction. For pH profiles, appropriate amounts of enzyme (approximately 2.6×10^{-4} XU) were mixed with the pH-adjusted buffers, sodium acetate, 1,3-bis[tris(hydroxymethyl)-methylamino]propane, or 3-[cyclo hexylamino]-1-propanesulfonic acid. All buffers were pH adjusted at the appropriate assay temperature. Each tube included oat spelt xylan to a final concentration of 0.22% and a final buffer concentration of 50 mM. Assay times for pH measurements were 10 min. However, no difference in profile was seen when the incubation times were extended. The composition of xylose oligomers solubilized from 0.25% oat spelt xylan was determined by paper chromatography methods with butanol-acetic acid-H_2O (12:3:5) as the running solvent (7). Purified xylobiose was obtained from Megazyme Pty., Sydney, Australia.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cellular proteins (approximately 100 μg) were separated on Laemmli gels (10). The method of Beguin (2) was used to renature protein in SDS-polyacrylamide gels for the identification of enzyme activity. The gels were then soaked in a 0.2% solution of oat spelt xylan for 2 h at 70°C, removed, wrapped in cling-film (Glad Wrap; Glad Production, New South Wales, Australia), and incubated for a further 12 h at 70°C. Following incubation, the gels were stained in 1% Congo red for 1 h and destained for 4 to 5 h in 1 M NaCl. Clear regions in the gels indicated the presence of xylanase activity.

Protein purification. All protein purifications were carried out at room temperature. A two-step procedure was used to purify xylanase for N-terminal sequencing. Heat-treated cellular extract containing xylanase (10 ml) was passed through a PD-10 Sephadex G-25 column (Pharmacia, Uppsala, Sweden) to remove salt and smaller molecules. A HiLoad Q-Sepharose column (Pharmacia) equilibrated with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5) was used to purify XynA. Activity was eluted at 70 mM NaCl with a linear 0 to 150 mM salt gradient. The enzyme was estimated to be more than 95% pure on the basis of SDS-PAGE analysis after purification (data not shown).

DNA sequence analysis. The DNA sequence of pNZ2931 (1,507 bp) was determined by dideoxynucleotide sequencing (23) on an Applied Biosystems 373A DNA sequencer. Both dye-primer and dye-terminator methods were used to sequence pBS(+) and pBS(−) plasmid constructs and M13mp10 constructs. SSU rRNA sequence was obtained by the method described by Saul et al. (24). Computer analysis of sequence data was carried out with the Genetics Computer Group software package (5) on a Silicon Graphics 4D/30 workstation.

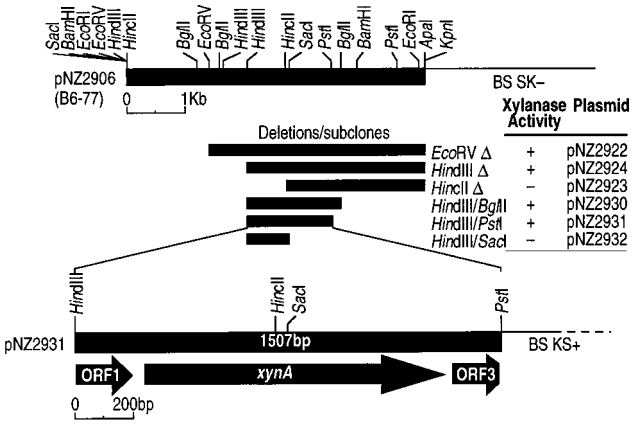

FIG. 1. A restriction map of pNZ2906 showing plasmid derivatives and their activity on oat spelt xylan. The main map is an enlargement of the sequenced insert of pNZ2931, showing the presence of *xynA* flanked by two partial open reading frames of unknown function.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this article have been assigned the accession numbers L39875 and L39866.

RESULTS

Library screening and genetic analysis. A total of 1,600 λZapII plaques containing genomic inserts were screened for xylanase activity by the substrate overlay method. This number ensured a full representation of the genome based on estimates of genome sizes for other ancient bacterial genomes (4). In total, six plaques expressing xylanase activity were detected. One plaque, designated B6-77, which exhibited the strongest xylanase activity, based on the size of the zone of clearing, was converted into a pBS SK$^-$ plasmid (pNZ2906) (see Fig. 1) by the ExAssist excision procedure (Stratagene). Restriction mapping followed by the construction of simple deletions and subcloning allowed the xylanase activity to be confined to a 1,500-bp *Hin*dIII-*Pst*I fragment derived from pNZ2906 (Fig. 1). The insert within the plasmid pNZ2931 was subsequently sequenced in full, and the sequence is presented in Fig. 2.

Analysis of the sequence of the genomic DNA fragment present in pNZ2931 revealed the presence of a single complete open reading frame, *xynA* (bp 244 to 1302), coding for a protein with a putative size of 352 amino acids (Fig. 1 and 2). The *xynA* gene is flanked by two incomplete open reading frames, designated ORF1 and ORF3. The possible functions of these two genes is unknown, and they have no homology with any sequence available in GenBank release 82.0.

Comparison of the amino acid sequence of XynA with those of other xylanases present in the GenBank database reveals that the enzyme is a single-domain family F xylanase as defined by Gilkes et al. (8). XynA had the highest homology with other thermophilic family F xylanases, and on the basis of percentage identity, XynA had the highest homology with the carboxy-terminal domain of XynA from Rt8B.4 (51.6%), a thermophilic anaerobic bacterium closely related to *Caldicellulosiruptor saccharolyticus* (formerly *Caldocellum saccharolyticum*). It had 50.1% homology with the amino-terminal xylanase domain of CelB from *C. saccharolyticus* and 47% homology with XynA from the same organism (6, 15, 25). At the DNA level, *xynA* also had the highest homology with these three genes.

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        10                  30                  50
AAGCTTGAGGAATTGTTGTTGGGTGATTTGAAGAAAGAAGGTTATGATGTATTTTACCAA
K  L  E  E  L  L  L  G  D  L  K  K  E  G  Y  D  V  F  Y  Q

        70                  90                  110
TATGTCTATATTAAAAGGAATTTTAGATTTGCGGAAAGAATAAAGAGCCTAATGAAAGAT
Y  V  Y  I  K  R  N  F  R  F  A  E  R  I  K  S  L  M  K  D

        130                 150                 170
TACAAATCTATATTTGTGGTAATAGGTGCAGGTCATTTAATAGGTGAAGAAAACGTGGTA
Y  K  S  I  F  V  V  I  G  A  G  H  L  I  G  E  E  N  V  V

        190                 210                 230
AAATTATTACAAAATTAGGGGTTAAAGTAAGTAAAATTTTAATGGAAAGGGGATGATGGT
K  L  L  Q  N  *

        250                 270                 290
AAAATGATTAACCAAAGGTTTTCTATCCTTGTGCTTCTTCTAATTTTGCTCACTTTCTCA
   M  I  N  Q  R  F  S  I  L  V  L  L  L  I  L  L  T  F  S

        310                 330                 350
TTAGGATTTTTAAAGGAGGAGGCTAAAGGTATGGAGATTCCATCACTGAAAGAAGTTTAT
L  G  F  L  K  E  E  A  K  G  M  E  I  P  S  L  K  E  V  Y

        370                 390                 410
AAAGATTACTTTACTATTGGAGCGGCAGTTAGTCACTTAAATATTTATCATTATGAGAAT
K  D  Y  F  T  I  G  A  A  V  S  H  L  N  I  Y  H  Y  E  N

        430                 450                 470
CTTTTGAAGAAGCATTTTAATAGTTTAACTCCTGAAAATCAAATGAAATGGGAAGTTATT
L  L  K  K  H  F  N  S  L  T  P  E  N  Q  M  K  W  E  V  I

        490                 510                 530
CATCCTAAACCATATGTTTATGATTTTGGACCTGCCGATGAGATCGTAGATTTTGCAATG
H  P  K  P  Y  V  Y  D  F  G  P  A  D  E  I  V  D  F  A  M

        550                 570                 590
AAGAATGGTATGAAGGTAAGAGGGCATACTTTGGTTTGGCATAATCAGACTCCAGGATGG
K  N  G  M  K  V  R  G  H  T  L  V  W  H  N  Q  T  P  G  W

        610                 630                 650
GTTTATGCAGGTACAAAGGATGAAATTCTTGCAAGATTAAAGGAACACATTAAAGAGGTA
V  Y  A  G  T  K  D  E  I  L  A  R  L  K  E  H  I  K  E  V

        670                 690                 710
GTAGGACATTATAAAGGTAAAGTTTATGCTTGGGATGTGGTTAACGAGGCCTTATCGGAT
V  G  H  Y  K  G  K  V  Y  A  W  D  V  V  N  E  A  L  S  D

        730                 750                 770
AATCCTAATGAATTTTTGAGAAGAGCTCCTTGGTATGATATTTGTGGTGAAGAAGTTATT
N  P  N  E  F  L  R  R  A  P  W  Y  D  I  C  G  E  E  V  I

        790                 810                 830
GAAAAGGCATTTATTTGGGCTCATGAGGTAGATCCTGATGCTAAATTATTTTATAACGAT
E  K  A  F  I  W  A  H  E  V  D  P  D  A  K  L  F  Y  N  D

        850                 870                 890
TACAATTTAGAAGATCCTATAAAGAGAGAAAAGGCTTATAAATTAGTTAAGAAGCTCAAG
Y  N  L  E  D  P  I  K  R  E  K  A  Y  K  L  V  K  K  L  K

        910                 930                 950
GATAAAGGAGTTCCTATTCATGGTATTGGAATACAGGGACATTGGACATTAGCATGGCCA
D  K  G  V  P  I  H  G  I  G  I  Q  G  H  W  T  L  A  W  P

        970                 990                 1010
ACTCCAAAGATGCTTGAGGATTCCATAAAGAGATTTGCAGAGCTTGGAGTTGAAGTACAA
T  P  K  M  L  E  D  S  I  K  R  F  A  E  L  G  V  E  V  Q

        1030                1050                1070
GTTACAGAGTTTGATATTTCTATTTACTACGATAGAAATGAAAATAATAATTTTAAGGTT
V  T  E  F  D  I  S  I  Y  Y  D  R  N  E  N  N  N  F  K  V

        1090                1110                1130
CCGCCTGAAGATAGGCTTGAAAGACAAGCTCAGCTTTATAAGGAGGCTTTTGAAATCTTA
P  P  E  D  R  L  E  R  Q  A  Q  L  Y  K  E  A  F  E  I  L

        1150                1170                1190
AGAAAATACAAAGGAATAGTTACTGGTGTTACTTTCTGGGGTGTAGCAGATGACTATACT
R  K  Y  K  G  I  V  T  G  V  T  F  W  G  V  A  D  D  Y  T

        1210                1230                1250
TGGCTCTATTTCTGGCCTGTTAGAGGAAGAGAAGATTATCCATTACTCTTTGATAAGAAT
W  L  Y  F  W  P  V  R  G  R  E  D  Y  P  L  L  F  D  K  N

        1270                1290                1310
CACAATCCTAAGAAGGCTTTCTGGGAGATTGTAAAGTTTTAGGTTGTATAGGAGGTTTAA
H  N  P  K  K  A  F  W  E  I  V  K  F  *

        1330                1350                1370
ATGAGAAAATTATTAAGGTTTTTGATTTTTTCTCTATTAGTTTTAGGACTTTCTTTCAAT
M  R  K  L  L  R  F  L  I  F  S  L  L  V  L  G  L  S  F  N

        1390                1410                1430
ATTATTTTAGCTGAGACTGAGAAATATGTGGATAAGAATTTAGGTTTTTCTATTGTGCCT
I  I  L  A  E  T  E  K  Y  V  D  K  N  L  G  F  S  I  V  P

        1450                1470                1490
CCTGAAGGGTGGGAGGTAAAGGATGGAAAGCCTTATAACTTGGCTGTAATTTTTGTTGGC
P  E  G  W  E  V  K  D  G  K  P  Y  N  L  A  V  I  F  V  G

CCTGCAG
P  A
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FIG. 2. Nucleotide sequence of the Rt46B.1 *xynA* gene and flanking regions. Coordinates in base pairs are shown above each line of sequence. The open reading frame encoding XynA has the coordinates of bp 244 to 1302. XynA is flanked by two partial open reading frames, ORF1 (positions 1 to 198) and ORF3 (positions 1321 to 1507). The putative amino acid sequence of each open reading frame is positioned below the DNA sequence.

Previous analysis of the GC nucleotide content of *D. thermophilum* by thermal denaturation techniques gave a value of 29.5% (20). To date, four genes have been sequenced from this organism, three amylase genes, *amyA*, *amyB*, and *amyC*, and the SSU rRNA gene (13). The GC content is 32.6% over the total 8,407 bp of sequence encompassing the three amylase genes and flanking noncoding DNA. For the 1,507-bp Rt46B.1 DNA of pNZ2931, the GC content is 33.9%, agreeing most closely with the high-performance liquid chromatography (HPLC)-derived value of 34% for *Dictyoglomus* sp. strain B1 (17).

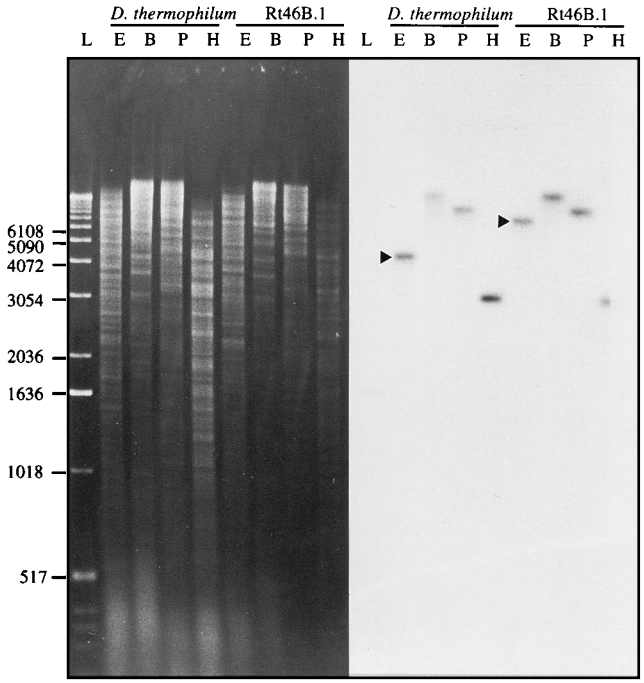

FIG. 3. Southern blot analysis of genomic DNA from Rt46B.1 and *D. thermophilum*. The *xynA* gene from *C. saccharolyticus* (15) was used as a probe against genomic DNA from each organism digested with the restriction enzymes *Eco*RV (lanes E), *Bam*HI (lanes B), *Pst*I (lanes P), and *Hin*dIII (lanes H). Arrowheads indicate a restriction fragment length polymorphism between *Eco*RV-digested DNA from each organism. Molecular mass marker sizes (in base pairs) are indicated on the left (lanes L).

Analysis of library isolates with *xynA* PCR primers. The six xylanase-positive plaques isolated from the Rt46B.1 genomic library were screened with the primers designed to amplify the XynA gene (3) to determine whether the XynA gene was also present in these recombinants. Only the library isolate B6-77 gave a PCR product, indicating the presence of *xynA*. This result suggested that Rt46B.1 possesses at least one other xylanase gene. Preliminary experiments using consensus PCR primer techniques have identified an additional xylanase gene present on these library isolates which has no homology with XynA, but it has homology with family G type xylanases (8) (data not shown).

Sequence comparison with *D. thermophilum*. Partial sequence of the SSU rRNA gene from Rt46B.1 has been reported previously by Love et al. (13) along with the complete SSU rRNA sequence of *D. thermophilum*. Phylogenetic analysis places both organisms in the order *Thermatogales*. The complete SSU rRNA sequence of Rt46B.1 was obtained with SSU rRNA gene-specific sequencing primers (24) and was found to be 100% identical to that of *D. thermophilum*, indicating that these two organisms are very closely related or identical. Southern blotting experiments using the *C. saccharolyticus xynA* gene as a probe against *Eco*RV-digested genomic DNA showed a difference in the restriction fragment length polymorphism patterns derived from these two organisms, which indicates that their genomes are not identical (Fig. 3). With the primers DictXynF and DictXynR, the *xynA* gene

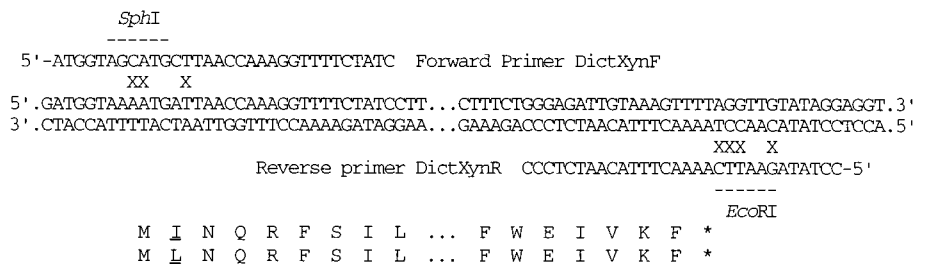

FIG. 4. Primer design strategy used to isolate the full-length *xynA* gene. This figure shows an alignment of oligonucleotide primers DictXynF and DictXynR with the amino- and carboxyl-terminal coding sequences of *xynA* which allowed the PCR amplification of the *xynA* open reading frame. X shows the positions where nucleotide changes occur with the introduction of restriction sites into the PCR primers.

was isolated from *D. thermophilum* by use of the PCR. The amplified product was ligated into M13mp10 and sequenced in full on one DNA strand. Comparison of DNA sequences shows that the *xynA* genes from Rt46B.1 and *D. thermophilum* are identical, giving further evidence that Rt46B.1 and *D. thermophilum* are very closely related. The full SSU rRNA sequence is not repeated here and has been submitted to GenBank under the accession number L39875.

N-terminal sequencing of recombinant XynA. Amino-terminal sequencing of XynA expressed from pNZ2931 was in perfect agreement with the predicted amino acid sequence of an internal portion of XynA, the first six residues of the purified enzyme being MEIPSL. This sequence starts 30 residues in from the predicted N-terminal methionine of XynA. It is unclear whether the first 30 amino acids were being cleaved from XynA by a signal peptidase or whether translation was occurring from the internal methionine. Residues 1 to 29 of the predicted full-length XynA are in perfect accordance with the general rules for signal sequence structure and suggest that XynA is being processed in *E. coli*. A charged residue (arginine) is present at the immediate N terminus, which is followed by an 18-residue hydrophobic core region containing 14 hydrophobic residues and no charged residues. The C terminus of the predicted signal sequence conforms to the AXB rule defined for *E. coli* signal sequences by Oliver (18), where the residue −3 from the cleavage site A is generally alanine, glycine, or serine, while the residue −1 from the cleavage site may include these amino acids as well as leucine, valine, or isoleucine. In the case of XynA, the last three residues of the putative signal sequence are alanine-lysine-glycine.

Overexpression of XynA in pJLA602. By using the primers DictXynF and DictXynR as described in Materials and Methods, the XynA gene was isolated by the PCR and ligated into the controlled expression vector pJLA602 (Fig. 4). *Sph*I and *Eco*RI restriction sites were introduced into the respective forward and reverse primers to allow the PCR product to be ligated in frame into the multiple cloning site of the controlled expression vector pJLA602. Three nucleotide changes were made in the amino-terminal primer to introduce an *Sph*I site into the PCR product. The *Sph*I site in the multiple cloning site of pJLA602 contains an in-frame ATG start codon. Ligation of *Sph*I-digested vector and PCR product ensured that the start codon of *xynA* was optimally placed downstream of the vector translation promoter sequences. Two codon changes are made with the introduction of the amino-terminal *Sph*I restriction site and the carboxy-terminal *Eco*RI site. The *Sph*I site changed codon two of XynA from leucine to isoleucine. The introduction of an *Eco*RI site at the carboxy terminus of the reverse primer converted the TAG stop codon of *xynA* to a TGA stop codon but did not introduce an amino acid change into the recombinant *xynA* gene product. Enzyme was expressed and purified as described in Materials and Methods. A 600-ml culture of *E. coli* C600 containing pNZ2900 produced a total of 165.2 XU of XynA. Approximately 56% (72.6 XU) of total XynA was found to be released into the medium, with 44% (92.6 XU) retained in the cell pellet. XynA purified from the cell pellet was used for all characterization assays.

Enzyme characterization. Zymogram analysis of XynA expressed from pNZ2931 revealed a single protein band approximately 33 kDa in size, which corresponds to the predicted size of XynA based on the *xynA* gene sequence (data not shown). Enzyme characteristics were determined with heat-purified extracts from pNZ2900. XynA showed optimal activity at 85°C, with 50% activity at 70 and 95°C. The pH optimum was found to be 6.5. However, the enzyme showed substantial activity across a broad pH range, with over 50% of maximum activity from pH 5.5 to 9.5. Thermostability at the temperature optimum was measured by incubation without substrate at pH 6.8 and 9.3, giving an enzyme half-life under these conditions of over 24 and 6.5 h, respectively. XynA was tested for its ability to release reducing sugars from unbleached *P. radiata* kraft pulp along with its ability to release lignin as measured by an increase of A_{280}. Release of reducing sugars was seen to match closely the ΔA_{280} of supernatants, suggesting either that there was a close association between the xylan and lignin released or that free xylan-oligomers produced the ΔA_{280}.

Breakdown products of xylan. Paper chromatography analysis of the hydrolysis products produced by the action of XynA on oat spelt xylan shows that it is broken down into oligomers, the major components being xylotriose and xylobiose. Small amounts of xylose were also released from the hydrolysis of oat spelt xylan. However, XynA showed no detectable activity on pure xylobiose and hence has no β-xylosidase activity. XynA showed significant activity on *p*-nitrophenyl-β-D-xylopyranoside, a substrate used to detect the presence of xylobiohydrolase activity. XynA was also shown to release reducing sugars from unbleached *P. radiata* kraft pulp, with a concomitant increase in A_{280} indicating the release of soluble lignocellulose compounds (7).

DISCUSSION

The xylanase gene described here from the extremely thermophilic anaerobe *Dictyoglomus* sp. strain Rt46B.1 codes for a family F single-domain xylanase of approximately 33 kDa which is active across a broad pH range. XynA had highest homology with xylanases from organisms classified as being members of the clostridial thermophilic anaerobic cellulolytics (21), namely *C. saccharolyticus* and its close relative Rt8B.4. The xylanase gene from *Dictyoglomus* sp. strain Rt46B.1 is not part of a cluster of genes coding for xylanases or related xylose-metabolizing enzymes as we have found in other thermophilic

cellulolytic anaerobes (6, 15), nor is it associated with a gene coding for multidomain cellulase-xylanase enzymes as seen with *celB* of *C. saccharolyticus* (3). The adjoining genes are unrelated to hemicellulose degradation, and their function is unknown. Moreover, unlike the thermophilic bacteria referred to above, Rt46B.1 appears to possess only two xylanase genes, one of family F and one of family G. Although five λZapII isolates were identified as carrying family G genes, all carried the same-sized fragment of DNA and are presumed to represent the same portion of the genome, although the possibility of a tandem repeat structure has not been ruled out. The genus *Dictyoglomus* is on a deeper branching of the phylogenetic tree than the genus *Caldicellulosiruptor* or its relations, and this observation plus the different organization of the xylanase genes sets Rt46B.1 and *C. saccharolyticus* and its relatives apart from one another.

Comparisons of the SSU rRNA genes of Rt46B.1 and XynA genes of Rt46B.1 and *D. thermophilum* show that these organisms are very closely related, and in each case, the *xynA* genes and the SSU rRNA genes of these organisms were identical. A single genetic difference based on restriction fragment length polymorphism patterns was detected. These results support the proposition of Patel et al. (20) that Rt46B.1 should be regarded as a strain of *D. thermophilum.*

The temperature optimum of XynA of 85°C is the highest reported for a recombinant xylanase to date. A recombinant xylanase from the thermophilic anaerobe *Thermoanaerobacter saccharolyticum* has been reported with a temperature optimum of 75°C (11), while recombinant XynA from *C. saccharolyticus* has a temperature optimum of 70°C (14). Xylanase prepared from the *Thermotoga* sp. strain FjSS3B.1 with a higher temperature optimum has been reported (28); however, the genes and gene types have yet to be identified. XynA retains 50% of its maximum activity at pH 9.3 and has a very broad activity profile at its optimum reaction temperature of 85°C. However, its temperature stability (half-life) is lower at alkaline pH, but the observed value of 7.5 h is more than adequate for batchwise processing of pulp. We have yet to test the possibility that the half-life under conditions of alkaline pH could be increased by lowering the reaction temperature. Mathrani et al. (17) have reported that xylanases produced by *Dictyoglomus* spp. produce enzymes with different thermostabilities depending on the temperature at which the organisms were cultured. This result could be due to the organisms possessing several xylanase genes which are expressed at different levels depending on the growth temperature.

XynA was seen to hydrolyze oat spelt xylan primarily to xylotriose and xylobiose, with the release of some xylose. However, XynA showed no detectable activity on pure xylobiose. These results are consistent with XynA acting in an endo fashion since most xylanases produce predominantly xylobiose and xylotriose from xylan (27). XynA also showed activity on *p*-nitrophenyl-β-D-xylopyranoside, and a similar result has been observed by Schofield et al. (27) for the family F xylanase XynA from *C. saccharolyticus*. They suggest that this result may be due to an initial transferase activity rather than endoxylanase activity.

The broad pH profile of XynA coupled with its thermal stability makes the enzyme an attractive candidate for bleaching application in the pulp and paper industry. Indeed, we have shown that it is able to release both reducing sugars and lignin from commercial *P. radiata* kraft pulp, but its utility depends on its ability to increase brightness at low chlorine charges.

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