Analysis of a *Thermotoga maritima* DNA fragment encoding two similar thermostable cellulases, CelA and CelB, and characterization of the recombinant enzymes

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Recombinant *Escherichia coli* clones displaying thermostable β -glucanase activity were isolated from two different gene libraries of the hyperthermophilic bacterium *Thermotoga maritima* MSB8 (DSM 3109), and the nucleotide sequence of a 1,4- β -glucanase gene designated *celA* was determined. Amino-terminal sequencing of cellulase I previously detected in *T. maritima* cells indicated that the *celA* gene encodes this β -glucanase, which is now designated CelA. CelA, which has a calculated molecular mass of 29732 Da, was purified from a recombinant *E. coli* strain to apparent homogeneity as judged by SDS-PAGE with a 44% yield. The enzyme was most active against soluble substrates such as mixed-linkage β -glucan and CM-cellulose. CelA displayed remarkable thermostability, which was enhanced in the presence of high concentrations of salt. Downstream of the *celA* gene we found a second open reading frame, *celB*, whose nucleotide sequence was 58% identical to *celA*. Experimental proof that *celB* also encodes a β -glucanase was obtained by separation from *celA* and expression in *E. coli* under the control of an efficient host promoter. According to the deduced amino acid sequences, CelB, in contrast to CelA, contains a signal peptide at the amino terminus. CelB and CelA had similar substrate specificities and temperature optima, but differed in their pH optima. Also, the addition of salt had a less stabilizing effect on CelB than on CelA. Nine 30 bp direct repeats, each itself representing a sequence with imperfect dyad symmetry, were detected upstream of the *celA*–*celB* cellulase gene cluster.

Keywords: cellulase, β -glucanase, nucleotide sequence, purification, thermostability

INTRODUCTION

Two *Thermotoga* species, i.e. *Thermotoga maritima* and *Thermotoga neapolitana*, represent the most thermophilic organotrophic eubacteria described to date. They grow fermentatively at temperatures up to 90 °C (Huber & Stetter, 1992). *Thermotoga* species are able to utilize various carbohydrates for growth, including the polymers starch, xylan and cellulose (Huber & Stetter, 1992; own unpublished results). Enzymes investigated from these species so far have generally displayed high thermostability (e.g. Gabelsberger *et al.*, 1993a, b; Jaenicke, 1993; Liebl *et al.*, 1992; Ostendorp *et al.*, 1993; Rutter-

smith & Daniel, 1991; Wrba *et al.*, 1990), which makes them potentially interesting candidates for the investigation of protein stability as well as for biotechnological applications.

Cellulose, the major structural polysaccharide of plant cell walls, consists of D-glucose units linked together to linear chains via 1,4- β -glycosidic bonds. The breakdown of cellulose by cellulolytic micro-organisms is normally accomplished in two steps. First, cellulases act by hydrolysis of the polymer chains. Then, in a second step, the cello-oligosaccharides liberated by cellulase action are degraded further by β -glucosidases or cello-dextrin phosphorylases.

The cloning, sequence analysis and purification of a thermostable β -glucosidase which may be involved in the

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final step of the breakdown of cellulose or cellodextrins to glucose by *T. maritima* MSB8 has been reported (Gabelsberger *et al.*, 1993a, b; Liebl *et al.*, 1994). Ruttersmith & Daniel (1993) have characterized a β -glucosidase of *Thermotoga* sp. strain FjSS3-B.1. Only three reports about *Thermotoga* enzymes that hydrolyse 1,4- β -glycosidic bonds of polymeric β -glucans are available. One deals with an exoglucanase (1,4- β -D-glucan cellobiohydrolase; EC 3.2.1.91) from the culture supernatant of *Thermotoga* sp. strain FjSS3-B.1 (Ruttersmith & Daniel, 1991). Dakhova *et al.* (1993) have reported the cloning of *T. neapolitana* DNA fragments that confer β -glucanase production upon *E. coli* transformants. Finally, Bronnenmeier *et al.* (1995) demonstrated the presence of two cellulases, cellulase I and II, in the crude cellular extract of *T. maritima* strain MSB8. Both enzymes were able to cleave CM-cellulose, β -glucan and *p*-nitrophenyl β -D-cellobioside. Cellulase I was classified as an endoglucanase (1,4- β -D-glucan glucanohydrolase; EC 3.2.1.4), while cellulase II also attacked microcrystalline cellulose, yielding cellobiose and glucose, and therefore has exo-1,4- β -D-glucanase activity (Bronnenmeier *et al.*, 1995). Primary structures of cellulases from hyperthermophilic organisms have not been reported to date. We now describe the isolation and analysis of a *T. maritima* MSB8 DNA fragment encoding β -glucanase production and present the results of the characterization of an extremely thermostable cellulase purified from a recombinant *E. coli* strain.

METHODS

Strains, plasmids and substrates. The cloning vector/host strain combinations used for gene library constructions and expression studies were pSU1/*E. coli* M5219 (Schüller *et al.*, 1985; Remaut *et al.*, 1981), pUN121/*E. coli* JM83 (Nilsson *et al.*, 1983; Yanisch-Perron *et al.*, 1985), pT7-7/*E. coli* BL21 (USB; Studier & Moffatt, 1986) and pWLQ2/*E. coli* XL1-Blue (Liebl *et al.*, 1992; Stratagene). *E. coli* strains were routinely grown in Luria-Bertani medium (LB; 1%, w/v, peptone; 0.5% w/v, yeast extract; 0.5%, w/v, NaCl; pH 7.2) supplemented with 100 μ g ampicillin ml⁻¹ or 12 μ g oxytetracycline ml⁻¹ where appropriate. Cellulase enzyme substrates were purchased from Sigma (CM-cellulose, low viscosity; lichenan; *p*-nitrophenyl β -D-cellobioside), Serva (microcrystalline cellulose Avicel, 0.02 mm; cellulose MN300, 0.002–0.02 mm), Fluka (oat spelt xylan) and Megazyme (barley β -glucan). Acid-swollen Avicel was prepared by incubating Avicel in concentrated hydrochloric acid with continuous agitation for 2 h.

DNA methodology, sequencing and computer analysis. Plasmid DNA isolation, transformation of *E. coli*, DNA modifications and Southern hybridization analysis were done with standard techniques (Ausubel *et al.*, 1987). Enzymes for DNA modification were purchased from Boehringer Mannheim or Pharmacia. The nucleotide sequence was determined on both strands of the plasmid employing the DIG Taq DNA sequencing kit (Boehringer Mannheim). Computer analysis of the DNA sequences was done with programs of the University of Wisconsin Genetics Computer Group (UWGC) software package (Devereux *et al.*, 1984) for Unix version 7.3.

Protein analytical methods. Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as a standard. Protein molecular masses were determined via discontinuous SDS-PAGE as described by Laemmli (1970). Unless mentioned otherwise, samples were

mixed with 1 vol. sample buffer (15%, v/v, glycerol; 5%, v/v, β -mercaptoethanol; 2.3%, w/v, SDS; 62.5 mM Tris/HCl, pH 6.8; 0.05%, w/v, bromphenol blue) and boiled for 5 min prior to application.

Preparation of crude extracts. A lysozyme/freezing-thaw method was employed for the rapid preparation of crude extracts for the screening of the gene library and for initial enzyme characterization. For this purpose, the clones were grown in 50 ml LB broth with ampicillin selection. The cells were harvested, washed with 5 ml 50 mM Tris/HCl, pH 8, resuspended in 1.5 ml 50 mM Tris/HCl, pH 8.0, 10 mM EDTA, and treated with 100 μ g lysozyme ml⁻¹, 10 μ g DNaseI ml⁻¹ and 10 μ g RNaseA ml⁻¹ for 60 min at 20 °C. Lysis was enhanced by a threefold freezing (–20 °C)–thaw procedure. Supernatants of the lysates were obtained after a 40 min centrifugation step (40 000 g, 30 min, 4 °C). In order to obtain an enzyme preparation enriched in β -glucanase for further characterization, the crude extract was heated at 70 °C for 30 min, cooled on ice, and then cleared of precipitated host proteins by centrifugation.

Detection of β -glucanase activity on plates and after SDS-PAGE. Clones producing thermoactive endoglucanase were identified after growing them on CM-cellulose plates (10 g tryptone, 6 g yeast extract, 2.5 g NaCl, 2 g CM-cellulose medium viscosity, 6 g agar and 1000 ml H₂O, pH 7.2) at 37 °C. The plates were incubated at 60–70 °C for 3–4 h and subsequently stained with 0.1% (w/v) Congo red (15 min). Excess dye was removed with 1 M NaCl (10 min). Location of enzymic activity in protein bands after SDS-PAGE was accomplished after separation of enzyme samples in an SDS-polyacrylamide gel containing 0.1% (w/v) CM-cellulose. The gel was washed at ambient temperature first for 30 min in a mixture (4:1, v/v) of 100 mM succinate buffer, pH 6.0, and 2-propanol, then for another 30 min in 100 mM succinate buffer, pH 6.0. During the whole period, the wash solution was renewed every 15 min. The gel was submerged in 100 mM succinate buffer, pH 6.0, at 60 °C for 30–120 min. Subsequently, the gel was stained with 0.1% (w/v) Congo red for 20 min and excess dye was removed by washing with 1 M sodium chloride.

Assay of enzyme activity. The pH values of buffers were adjusted at the temperatures of use. Unless mentioned otherwise, enzyme assays were done in duplicate at 85 °C in 0.5 ml reaction mixtures containing 50 mM MES buffer, pH 6.0, and 4 mM *p*-nitrophenyl β -D-cellobioside, 0.5% (w/v) soluble polysaccharide substrates or 1% (w/v) non-soluble substrates. The enzymic liberation of reducing groups was determined with the dinitrosalicylic acid reagent (Bernfeld, 1955). One unit of enzyme activity was defined as the amount of enzyme needed to release 1 μ mol glucose-equivalent reducing groups min⁻¹. Non-enzymic hydrolysis of the substrates at elevated temperatures was corrected for with appropriate controls in every experiment. Stability at elevated temperatures was measured by determination of residual activity with the standard assay after preincubation of enzyme samples in 50 mM MES buffer, pH 6.0, at various temperatures and NaCl concentrations.

TLC of oligosaccharides. TLC of β -glucan degradation products was done by an ascending technique on 0.2 mm silica-gel-coated aluminum sheets (type 60; Merck). Separation of the cello-oligosaccharides was achieved by three consecutive ascents using a solvent system consisting of chloroform/acetic acid/water (5:7:1, by vol.). Carbohydrate spots were visualized by spraying the chromatogram with aniline-diphenylamine reagent as described previously (Liebl *et al.*, 1992).

Construction of pT7-7-celA. Two synthetic oligonucleotide primers with the nucleotide sequences 5'-GGCATTCCATA-

Table 1. Purification of recombinant CelA

Enzyme activities are expressed as CM-cellulose-hydrolysing activities measured in 30 min assays at pH 6.0 at 85 °C.

Step	Protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification (-fold)	Yield (%)
Cleared lysate	4002	11 090	2.76	1.0	100
Heat treatment (20 min at 75 °C)	228	8 740	40.4	14.6	79
After Q-Sepharose	56.4	6 470	71.9	26.5	58
After Phenyl-Sepharose	16.8	4 900	291	105	44

TGGTACTGATGACAAAACCG-3' and 5'-CGGAATTCA-TTCTCTCACCTCCAGATC-3', designated celA-V and celA-R, respectively, were constructed on the basis of the nucleotide sequence of the *celA* gene. These primers were used in PCR-aided amplification of the *celA*-coding region. The reaction mixture contained 200 ng *Eco*RI-linearized pTEGL-H as the template DNA, 50 pmol of each primer, deoxynucleotide triphosphates (200 µM each), 2.5 U *Pyrococcus furiosus* (Pfu) DNA polymerase and 10 µl 10 × Pfu reaction buffer (both Stratagene) in a total volume of 0.1 ml. The template strands were separated at 94 °C (3 min) before starting 40 reaction cycles (94 °C/30 s, 51 °C/2 min, 72 °C/4 min). The PCR primers celA-V and celA-R contained recognition sites for the restriction endonucleases *Nde*I and *Eco*RI, respectively, which were used for the in-frame fusion of the *celA* open reading frame to the start codon downstream of the P_{T7} promoter and ribosome-binding site of expression vector pT7-7 pretreated with the same restriction enzymes. The correct insertion of the PCR product into pT7-7 was verified by nucleotide sequence analysis. The resulting plasmid, designated pT7-7-celA, was transformed into an *E. coli* BL21 expression host strain with an IPTG-inducible chromosomal copy of the RNA polymerase gene of phage T7 (Studier & Moffatt, 1986).

Purification of recombinant CelA from *E. coli* BL21(pT7-7-celA) and cellulase I from *T. maritima* MSB8 cells. *E. coli* BL21(pT7-7-celA) was inoculated (1%, v/v) into 5 l baffled flasks containing 2.5 l 2 × LB medium supplemented with 100 µg ampicillin ml⁻¹. After growth for 14 h on a rotary shaker at 35 °C, the cells were harvested, washed and resuspended with 20 mM Tris/HCl, pH 7.0. The cells were lysed by passage of the suspension through a French pressure cell (American Instrument Company) at 6.9 MPa. Remaining whole cells and debris were removed by centrifugation (27 000 g, 30 min). The cleared lysate was incubated at 75 °C for 20 min and centrifuged again in order to precipitate the majority (about 94%) of heat-labile host cell proteins. The supernatant, which contained nearly 80% of the initial amount of thermostable cellulase, was adjusted to pH 8.0 and loaded onto a Q-Sepharose fast-flow column (Pharmacia). Elution was achieved with a 10 bed volume gradient of 0–0.5 M sodium chloride in 20 mM Tris/HCl, pH 8.0. The fractions with thermostable CM-cellulase activity were pooled, adjusted to 1.5 M ammonium chloride and 50 mM Tris/HCl, pH 8.0, and applied to a HiLoad Phenyl-Sepharose column (Pharmacia). The enzyme was eluted from the column with a 10 bed volume gradient of 1.5–0 M ammonium chloride in 50 mM Tris/HCl, pH 8.0. Finally, 16.8 mg purified recombinant CelA was obtained with a yield of 44% (see Table 1). Cellulase I from cells of *T. maritima* for

comparison with recombinant CelA was prepared as described previously (Bronnenmeier *et al.*, 1995). Its amino-terminal amino acid sequence was determined by Edman degradation using a gas-phase amino acid Sequenator model 477A (Applied Biosystems).

RESULTS AND DISCUSSION

Isolation of *T. maritima* MSB8 genes encoding thermostable β-glucanase activity

Two previously constructed libraries of *T. maritima* genomic DNA (Gabelsberger *et al.*, 1993a; Ostendorp *et al.*, 1993) were screened for clones producing thermostable CM-cellulose-hydrolysing activity. The first, pSU1-based, library was screened after preparing crude lysates of pools, consisting of 12 clones each, with the lysozyme/freeze-thaw method described above. The lysates were tested for thermostable β-glucan- and CM-cellulose-hydrolysing activity. Positive pools were investigated further by checking the single clones for activity in the same way. Upon screening several thousand pools by this procedure, eight independent clones were finally obtained which displayed thermostable hydrolytic activity against barley β-glucan and CM-cellulose. The recombinant plasmids of these clones were named pTG1, 2, 5, 6, 7, 8, 9 and 10. The other, pUN121-based, library was screened with the plate assay described in the Methods after replicating clones onto agar plates containing 0.2% CM-cellulose. Five different recombinant plasmids, designated pTEGL-A, -G, -H, -P and -R2, were isolated from positive clones of this screen. Restriction analysis of the β-glucanase plasmids revealed that the insert fragments of all of the pTG plasmids and at least three of the pTEGL plasmids (pTEGL-A, -G and -H) overlapped (data not shown). The DNA segment common to all of these plasmids had a length of about 0.9 kb, representing a polypeptide coding capacity of up to 300 residues or about 35 kDa. These numbers are consistent with the molecular masses determined via SDS-PAGE for the known *T. maritima* cellulases I and II (about 27 kDa and 29 kDa, respectively; Bronnenmeier *et al.*, 1995). pTEGL-P and pTEGL-R2, which may contain additional CM-cellulase genes, were not investigated further in this study.

Fig. 1. Genetic map of an approximately 5 kb large segment of the *T. maritima* MSB8 genome. Segments thereof cloned in various recombinant plasmids (see text) are drawn as solid lines.

Nucleotide sequence analysis

Primary structures of cellulases or their genes from hyperthermophilic organisms have not been reported to date. Therefore, it was of interest to sequence the cloned cellulase-encoding DNA fragment of *T. maritima*. An *Eco*RI-*Sau*I fragment of pTG7 containing the complete 1.7 kb *Sau*3A/partial *T. maritima* chromosomal DNA insert of this plasmid was cloned into the multiple cloning site of the sequencing vector pBluescript KS(+) (Stratagene), yielding pBS-EGL7 (Fig. 1). After constructing various deletion derivatives, the nucleotide sequence of both strands of the insert was determined. The *T. maritima* chromosomal DNA fragment present in pBS-EGL7 and in pTG7 represents the sequence between the *Sau*3A sites at positions 469 and 2131 which contains one complete open reading frame (residues 1107–1880), termed *celA*.

The amino-terminal amino acid sequence of cellulase I purified from cells of *T. maritima* MSB8 as described previously (Bronnenmeier *et al.*, 1995) was determined in order to find out if this enzyme is encoded by *celA*. Indeed, the sequence obtained, MVLMTKPGTSDFF, supports this view since it is found at positions 1 through 12 of the CelA primary structure deduced from the nucleotide sequence of the gene.

The nucleotide sequence analysis of the pBS-EGL7 insert revealed several 30 bp direct repeats upstream of *celA* and an incomplete reading frame downstream of *celA*. Therefore, the sequence was extended in both directions via primer walking using the β -glucanase plasmids pTEGL-A, -G and -H, whose inserts overlap with the pBS-EGL7 insert (Fig. 1), as templates. In this way, the contiguous 2.8 kb sequence of the *T. maritima* MSB8 genome shown in Fig. 2 was obtained. The now completed second open reading frame was named *celB*. It is presently not known if *celB* encodes the second cellulase purified from cells of *T. maritima*, i.e. cellulase II (Bronnenmeier *et al.*, 1995), because an amino-terminal sequence of this enzyme is not available. The coding regions of *celA* and *celB* were 58% identical at the nucleotide sequence level. Using sequencing primers constructed to anneal to sequences of the vector pUN121 flanking the inserts, we also obtained single-strand nucleotide sequences of the ends of the inserts of pTEGL-

Fig. 2. Nucleotide sequence of the *T. maritima* cellulase gene region and deduced primary structures of CelA and CelB. The putative ribosome-binding sites are underlined. The putative catalytic glutamate residues are circled. Nine 30 bp repeats are shown as black boxes with white letters. An inverted repeat which could represent a transcription termination structure is indicated by arrows.

A, -G and -H. Based on the analysis of these data, we were able to assemble a gene map of an approximately 5 kb region of the *T. maritima* genome (Fig. 1). Two open reading frames, both in the opposite orientation to *celA*-*celB*, were found whose putative translation products shared significant amino acid sequence similarity with known protein sequences. A truncated open reading frame upstream of *celA*-*celB* could be part of the *T. maritima* aspartate-semialdehyde dehydrogenase (Asd) gene. Asd is an enzyme involved in the biosynthesis of L-lysine, L-methionine, L-threonine and L-isoleucine from aspartate. The amino acid sequence of the postulated *T. maritima* Asd fragment was 49% identical to residues 1–277 of the *Vibrio cholerae* Asd and shared more than 40% identity with the corresponding parts of various other known Asds (not shown). The deduced amino acid sequence of a small open reading frame downstream of *celA*-*celB* displayed low-level similarity with a 141 residue, hypothetical protein (YjeB) of unknown function in the *purA*-*vacB* intergenic region of *E. coli*.

The DNA region upstream of *celA* contains nine 30 bp direct repeats (Figs 1–3), seven of which are 100% identical. The repeats are separated by non-similar se-

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340  TGGAGGTCRAAAAACATCTTGCCTTCAGAGTGTGTTTGTGGTATAATTTCTTTGAACT
400  CAACGGTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
471  TCTTCCCTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
537  TTGTCCCTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
602  CATTGAGTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
668  TTCTAAATTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
734  TCATACCTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
800  TTCTGCTTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
866  CCGACATTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
933  GAGCCATTTTCAATAATTCCTTAGAGTATGGGAATTCAGTTCCTCCCTGCTTCTGGATTGTTAAGCGAGGA
999  TTAATACCCCTGTTTCTTTTATTTTGGGTGATTAGTTCTATTTCGGAATTTTCAGATTTTGAATGTAA
1069 ACATTTCATAATAAGATGTTTCTGAGGTGATTAATGGTGGTACTGATGACAAAACCGGGAACATCGGAT

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M V L M T K F G T S D

Consensus: **GTTTCAATACTTCCTTAGAGGTATGGAAAC**

Fig. 3. Alignment of nine 30 bp sequence repeats found upstream of *celA*. Repeats 1 through 7 are 100% identical, while repeats 8 and 9 have one and nine mismatches, respectively, when compared to the consensus. Each repeat can itself be considered to be a region with dyad symmetry, and is indicated by converging arrows drawn below the consensus.

Fig. 4. Design of recombinant plasmids for determining whether *celB* represents a functional cellulase-encoding cistron. A restriction map of the 2.8 kb DNA fragment whose nucleotide sequence is shown in Fig. 3 is given. A deletion was introduced into pTEGL-H (Fig. 1) by *Bam*HI digestion and religation, yielding pTEGL-H-Bam-del, which lacks most of *celA* but carries the complete *celB* open reading frame. For the construction of pWLQ-celB, the *celB* gene was isolated as a *Bam*HI-*Ssp*I fragment and inserted in expression vector pWLQ2 downstream of the strong P_{tac} promoter.

quences of 34–41 bp. Interestingly, each repeat itself represents a sequence with imperfect dyad symmetry (Fig. 3). Although the function of the DNA sequence repeats is unknown, their vicinity to the 5'-end of the *celA*-*celB* gene cluster makes it tempting to speculate that they may be involved in the regulation of cellulase expression.

Separation from *celA* and functional heterologous expression of *celB*

Sequence analysis of the open reading frame *celB* immediately downstream of *celA* revealed that its translation product was nearly 50% identical to CelA, making it seem likely that it may encode another cellulase. This assumption was proven by expressing this gene after separation from *celA*. For this purpose, we first tried to delete the DNA region upstream of the *Bam*HI site (within the *celA* gene at position 1664 of Fig. 2) in plasmid

pTEGL-H, which contains *celA* and *celB*. However, this deletion completely abolished thermostable cellulase production in the corresponding *E. coli* transformant. On the other hand, placement of *celB* (as a 1.1 kb *Bam*HI-*Ssp*I fragment of pTEGL-H; corresponds to residues 1664–2777 of Fig. 2) under the transcriptional control of the P_{tac} promoter of expression vector pWLQ2 (Liebl *et al.*, 1992) and transformation of the resulting plasmid pWLQ-celB (Fig. 4) into *E. coli* XL1-Blue (Stratagene) resulted in a recombinant strain which produced thermostable CM-cellulose-hydrolysing activity upon induction with IPTG (data not shown). This experiment clearly demonstrates that *celB* represents an intact cellulase gene. The genetic organization of the analysed cellulase gene region suggests that *celB* is co-transcribed with *celA*. Downstream of *celB* a potential rho-independent terminator structure was detected (Fig. 2). As the ATG start codon of *celB* overlaps with the last glutamate codon and the stop codon of *celA*,

Fig. 5. (a) Alignment of the amino acid sequences of CelA and CelB. Identical residues in both primary structures are indicated. (b) Local amino acid sequence similarity between *T. maritima* CelA (Tm_CelA), *T. maritima* CelB (Tm_CelB), *Erwinia carotovora* CelS (Eca_Cel) and an *Aspergillus aculeatus* cellulase (Aac_Cel). The *T. maritima* enzymes share only very low overall similarity with the other two cellulases. Therefore, only short stretches of sequences around two conserved glutamate residues which may be involved in the catalytic mechanism of the enzymes (see Saarihahti *et al.*, 1990; Törrönen *et al.*, 1993) are shown here. The numbering of Eca_Cel and Aac_Cel is according to sequences retrieved from the SWISS-PROT database, accession numbers P16630 and P22669, respectively.

translational coupling might also occur. Interestingly, the putative ribosome-binding sites of *celA* and *celB* appear to be identical, contained within an 11 bp sequence (TCTGGAGGTGA) found 5 bp and 4 bp, respectively, upstream of each of the corresponding start codons (Fig. 2).

CelA and CelB have similar primary structures, but only CelB has an amino-terminal signal peptide

The primary structures of CelA (257 residues; 29 732 Da) and CelB (274 residues; 31 732 Da) deduced from the nucleotide sequences of their genes are highly similar. The amino acid sequences can be aligned (Fig. 5a) with the introduction of merely one single-residue gap and share 49.8% identical residues (68.8% similarity, if conservative exchanges are considered). Due to the high level of relatedness on the protein primary structure level as well as the nucleotide sequence level (58% identical), it seems likely that the *celA-celB* gene cluster evolved via gene duplication from an ancestral cellulase gene. CelB, in contrast to CelA, carries a hydrophobic amino-terminal extension with all the features of typical signal peptides (von Heijne, 1985). The predicted signal peptidase cleavage site lies between serine residues 17 and 18 of CelB (see Fig. 2 or 5a). The putative mature polypeptide would

therefore have a size of 257 amino acid residues with a calculated molecular mass of 29 767 Da, which is almost the same as CelA (257 residues; 29 732 Da). Thus it may be that the two products of the *T. maritima celA-celB* cellulase gene cluster are targeted to different cellular compartments. CelA (cellulase I) was originally isolated from a crude cellular extract of washed *T. maritima* MSB8 cells (Bronnenmeier *et al.*, 1995), but detailed studies to elucidate the precise subcellular localization have yet to be performed. Due to its signal peptide, CelB is expected to be either a periplasmic or an extracellular enzyme. If CelA turns out to be a cytoplasmic β -glucanase, its physiological role could be the breakdown of cello-oligomers internalized by the cell.

Amino acid sequence similarity between CelA and CelB and other proteins

We attempted to find polypeptides related to CelA or CelB by searching through the available protein sequence databases (PIR, SWISS-PROT) using the standard search algorithms (programs FASTA and BLASTP). Only one enzyme, an endoglucanase of *Erwinia carotovora*, exhibited low but significant amino acid sequence similarity with CelA (25.2% identity over the entire length of the polypeptide chains). This *E. carotovora* enzyme belongs to glycosyl hydrolase family 12 (Henrissat, 1991) and has only one known 'relative', a β -glucanase (FI-CMCase) of *Aspergillus aculeatus*. The amino acid sequence identities between *T. maritima* CelA and the latter enzyme, as well as between *T. maritima* CelB and the *Erwinia* and *Aspergillus* β -glucanases, are on the border of detection (20–22%). However, all four enzymes share the two short peptide sequences shown in Fig. 5(b). Also, hydrophobic cluster analysis revealed significant similarity between the enzymes (data not shown). Therefore, we propose that CelA and CelB of *T. maritima* MSB8 belong to glycosyl hydrolase family 12 (corresponds to β -glucanase family H; Gilkes *et al.*, 1991; Henrissat, 1991), which doubles the number of known members of this small cellulase family.

It has been proposed by Törrönen *et al.*, (1993) on the basis of hydrophobic cluster analysis that the low-molecular-mass endo-xylanases comprising glycosyl hydrolase family 11 (corresponds to β -glucanase family G) and the two members of family 12 cellulases (family H) known at that time shared weak but significant similarity. Members of both these enzyme families hydrolyse glycosidic bonds with retention of the anomeric configuration (Gebler *et al.*, 1992; Schou *et al.*, 1993). For one family 11 enzyme, the *Bacillus pumilus* xylanase, the elucidation of the three-dimensional structure and site-directed mutagenesis studies have led to the identification of two glutamate residues essential for the general acid catalysis mechanism of this enzyme (Ko *et al.*, 1992). By analysing similarity, Törrönen *et al.* (1993) predicted that the corresponding glutamate residues of the family 12 cellulases (Glu₁₅₈ and Glu₂₄₆ for *E. carotovora* CelS, Glu₁₃₄ and Glu₂₁₈ for *A. aculeatus* FI-CMCase) are essential for the catalytic activity of these enzymes. The fact that both *T. maritima* cellulases also have glutamate residues at the

Fig. 6. Thermostability of purified CelA at 85 °C (a) and 95 °C (b) at various NaCl concentrations (○, none; □, 1 M; △, 2.5 M; ▽, 5 M). The data represent the means of duplicate assays. The highest activity at each salt concentration was defined as 100%. Note the different time scales in the graphs.

corresponding positions (Glu₁₃₄ and Glu₂₃₁ for CelA, Glu₁₅₂ and Glu₂₅₀ for CelB; see Fig. 5b) strengthens the hypothesis that these are indeed the catalytically essential acidic residues of family 12 glycosyl hydrolases (family H β -glucanases).

Comparison of the amino acid compositions of CelA and CelB with the less thermostable cellulases of family 12 revealed some differences, for example an increased percentage of the charged residue glutamate (1.8 mol% for *A. aculeatus* FI-CMCase and 3.4 mol% for *E. carotovora* CelS versus 10.9 mol% for *T. maritima* CelA and 8.2 mol% for *T. maritima* CelB), and a slight increase in proline (3.2 mol% and 2.6 mol%, versus 5.5 mol% and 4.7 mol%, respectively). However, it is not possible to tell if these differences could account for the extreme thermostability of CelA and CelB because the set of data is too small for a statistically significant analysis, and the degree of relatedness between the *Thermotoga* cellulases and their mesophile counterparts is too low to even construct reliable full-length pairwise alignments. Therefore, these observations must be interpreted with caution until more primary structures, or preferably high-resolution three-dimensional structures, of family 12 cellulases of mesophilic and thermophilic origin become available.

Purification and characterization of the recombinant β -glucanase CelA

In order to increase the recombinant enzyme level, the *celA*-coding region was amplified from pTEGL-H via PCR and inserted downstream of the transcription and translation initiation signals of expression vector pT7-7, yielding the plasmid pT7-7-*celA* (see Methods). pT7-7-*celA* was introduced into the expression host *E. coli* BL21, which carries an IPTG-inducible copy of the phage T7 RNA polymerase gene. Unexpectedly, induction of P_{T7}-driven expression by addition of 0.4 mM IPTG in the exponential growth phase of *E. coli* BL21(pT7-7-*celA*) resulted in severe growth impairment of the host strain. However, the basal expression level without induction was high enough to allow the purification of the recombinant cellulase. A preliminary enzyme localization experiment with a different strain (*E. coli* M5219/pTg1) had revealed that the majority of the thermostable β -

glucanase was cell-associated, and only about 9% of the activity was released by cold osmotic shock (not shown). Therefore, the β -glucanase was purified from a whole-cell lysate of *E. coli* BL21(pT7-7-*celA*) by heat precipitation, anion-exchange chromatography and hydrophobic interaction chromatography as described in Methods. Starting with about 4 g total protein, about 17 mg pure CelA was obtained (Table 1). The specific CM-cellulose-hydrolysing activity of this preparation was 291 U (mg protein)⁻¹. Amino-terminal sequencing of the purified recombinant cellulase yielded the sequence MVLMTKP-GTSDVFWNGIPLS for the first 20 residues, which corresponds precisely to the result expected on the basis of the nucleotide sequence of the gene (see Fig. 2) and contains the 12 residue sequence also obtained by sequencing of the authentic cellulase purified from *T. maritima* cells (see above). Recombinant CelA production in *E. coli* led to an enzyme preparation which was amino-terminally heterogeneous. In addition to the expected residue, each sequencing cycle yielded a second amino acid derivative corresponding to the next residue of the sequence, meaning that a significant proportion of the CelA molecules lacked the amino-terminal methionine residue.

The recombinantly produced CelA had an apparent molecular mass of about 30 kDa as determined by SDS-PAGE analysis (not shown), which corresponds well with the value expected from the nucleotide sequence of the gene (29732 Da) and the size of the authentic enzyme from *T. maritima* cells (cellulase I; Bronnenmeier *et al.*, 1995). Using the substrates β -glucan, *p*-nitrophenyl β -D-cellobioside and xylan, CelA displayed a broad range of near-maximum (> 75%) activity between pH 6.0 and 8.5 (data not shown). With CM-cellulose as the substrate, the pH optimum was shifted to more acidic conditions (pH 4.5–6.5). A substrate-dependent shift in the pH-activity profile has been reported before for a β -glucanase (Hitomi *et al.*, 1994) and has also been described for mammalian α -amylases (Ishikawa *et al.*, 1990, 1993). This phenomenon may be caused by pH-dependent differences in the binding affinities of substrate-binding subsites of these enzymes for different substrates. The 'temperature optimum' of CelA, which was determined in 30 min assays in 50 mM MES buffer, pH 6.0, with 1% CM-cellulose, was about 90 °C.

Table 2. Substrate specificity of the *T. maritima* β -glucanases CelA and CelB

Assays were performed at substrate concentrations of 4 mM *p*-nitrophenyl β -D-cellobioside, 1% (w/v) non-soluble polymeric substrates or 0.5% (w/v) soluble polymers in 50 mM MES buffer, pH 6.0, at 85 °C using an enzyme concentration of 81 ng ml⁻¹ (purified CelA) or 3 μ g ml⁻¹ (crude CelB). Incubation was carried out for 10–60 min for β -glucan, CM-cellulose and *p*-nitrophenyl β -D-cellobioside, 24 h for xylan and acid-swollen Avicel, and up to 48 h for Avicel and cellulose MN300. Activity on barley β -glucan was defined as 100%.

Substrate	CelA		CelB	
	Specific activity (U mg ⁻¹)	Relative activity (%)	Specific activity (mU mg ⁻¹)	Relative activity (%)
Barley β -glucan	1785	100	2905	100
CM-cellulose	302	17	890	30.6
<i>p</i> -Nitrophenyl β -D-cellobioside	7.4	0.42	50	1.70
Acid-swollen Avicel	8.0	0.48	26	0.89
Avicel	0	0	0	0
Cellulose MN300	0	0	0	0
Oat speltis xylan	2.5	0.14	8	0.28

Thermostability of recombinant CelA

Purified CelA was incubated at a concentration of 1.6 μ g ml⁻¹ in 50 mM MES, pH 6.0, containing 0, 1, 2.5 or 5 M NaCl at 70, 85 or 95 °C. After various periods of time, the remaining CM-cellulose-hydrolysing activity was determined under standard assay conditions (pH 6.0, 85 °C). At 70 °C, the enzyme was stable over a period of at least 56 h at all NaCl concentrations tested (data not shown). At 85 °C in the absence of NaCl or at 1 M NaCl, CelA still displayed remarkable thermostability, showing more than 40% of its initial activity after a 48 h incubation, whereas at 95 °C under otherwise identical conditions the enzyme was inactivated within about 30 min (Fig. 6). The addition of high salt concentrations led to a dramatic increase in thermostability. At 5 M NaCl, the activity of CelA was not significantly reduced after 6 h at 95 °C (Fig. 6). The protein-stabilizing effect brought about by the addition of certain salting-out co-solvents such as MgSO₄ or NaCl was recently reviewed by Timasheff (1993).

Substrate specificity and hydrolysis products

The relative rates of hydrolysis of a variety of substrates are summarized in Table 2. Like many other β -glucanases, *T. maritima* CelA displayed much higher activity with barley β -glucan, a 1,4- β -/1,3- β -mixed linkage substrate, than with the modified cellulose model substrate CM-cellulose. α -Glucans like soluble starch or pullulan were not attacked. The products formed during hydrolysis of barley β -glucan were analysed by TLC (not shown). The initial products resolved with this technique were a mixture of oligosaccharides with a degree of polymerization (DP) of 3 and larger. The major products after extended incubation were tri- and tetrasaccharides, but a substantial amount of DP 2 oligosaccharides and

some glucose were also formed. Based on these data, we conclude that CelA probably represents an endo- β -glucanase (1,4- β -D-glucan glucanohydrolase; EC 3.2.1.4). Hydrolysis of *p*-nitrophenyl β -D-cellobioside, which is often used as a model substrate for exo- β -glucanases, was also observed. However, the differentiation of exo- and endocellulases on the basis of their specificities using small soluble substrates does not appear to be a very useful method (see Claeysens & Henrissat, 1992) and therefore does not contradict the classification of CelA as an endoglucanase. The enzyme apparently is not strictly specific for 1,4- β -D-glucosidic bonds because oat speltis xylan, a polymer consisting predominantly of 1,4- β -linked xylose units, was also hydrolysed, although with low catalytic efficiency (Table 2). Xylan hydrolysis has also been observed with *E. carotovora* CelS (Saarilahti *et al.*, 1990), a distantly related endoglucanase belonging to the same enzyme family as CelA (see above).

Comparison of CelA and CelB properties

Some properties of recombinant CelB were determined in order to compare this enzyme with the structurally similar enzyme CelA. For this purpose, cells of *E. coli* XL1-Blue(pWLQ-celB) (see above and Fig. 4) were grown to the exponential growth phase and induced with IPTG. CelB was prepared from crude lysate by heat treatment at 70 °C for 20 min and removal of precipitated proteins (typically 90–95% of the host proteins) by centrifugation. The specific CM-cellulose-hydrolysing activity of this crude CelB preparation was 0.89 U (mg protein)⁻¹ (determined in 50 mM MES, pH 6.0, at 85 °C). The 'temperature optimum' (85–90 °C) and substrate specificity (Table 2) of CelB were similar to CelA. However, CelA and CelB differed significantly in their pH-activity profiles

Fig. 7. Effect of pH on the hydrolysis of CM-cellulose by CelA (a) and CelB (b). The buffers used were succinate/NaOH (○; pH 3.0–6.0), MES/NaOH (□; pH 4.5–7.0), Tris/HCl (△; pH 7.0–10.0) and glycine/NaOH (▽; pH 10.5–11.0). The assays were performed at 80 °C.

Fig. 8. Long-term thermostability of CelA (○, □) and CelB (△, ▽) at 90 °C. After preincubation of enzyme (1.6 µg pure CelA ml⁻¹ or 4.5 µg crude CelB ml⁻¹) for various periods of time in the presence of 2.5 M NaCl (□, ▽) or without the addition of salt (○, △), residual activities were determined under standard assay conditions. The highest activity at each salt concentration was defined as 100%.

(Fig. 7) and long-term thermostability (Fig. 8). Also CelB was less well stabilized by salt addition than CelA (Fig. 8). Since microcrystalline cellulose was not significantly hydrolysed by CelB (Table 2), this enzyme apparently does not represent cellulase II isolated previously (Bronnenmeier *et al.*, 1995) from *T. maritima* MSB8 cells.

It seems quite obvious to us that the highly similar, adjacent cellulase genes *celA* and *celB* of *T. maritima* MSB8 arose from a single predecessor gene, possibly by a recent gene duplication event. Depending on the starting scenario, one of the genes subsequently must have either lost or acquired an in-frame signal-peptide-coding region. It would be interesting to obtain more information about the precise localization of the structurally similar enzymes CelA and CelB in *T. maritima* cells in order to obtain clues about possible differences in their physiological roles. Also, the function of the unusual multiple sequence repeats upstream of the *celA*–*celB* gene cluster has yet to be solved.

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REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: John Wiley and Sons.
- Bernfeld, P. (1955). Amylases α and β . *Methods Enzymol* **1**, 149–158.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Bronnenmeier, K., Kern, A., Liebl, W. & Staudenbauer, W. (1995). Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. *Appl Environ Microbiol* **61**, 1399–1407.
- Claeysens, M. & Henrissat, B. (1992). Specificity mapping of cellulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. *Protein Sci* **1**, 1293–1297.
- Dakhova, O. N., Kurepina, N. E., Zverlov, V. V., Svetlichnyi, V. A. & Velikodvorskaya, G. A. (1993). Cloning and expression in *Escherichia coli* of *Thermotoga neapolitana* genes coding for enzymes of carbohydrate substrate utilization. *Biochem Biophys Res Commun* **194**, 1359–1364.
- Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.
- Gabelsberger, J., Liebl, W. & Schleifer, K. H. (1993a). Cloning and characterization of β -galactoside and β -glucoside hydrolysing enzymes of *Thermotoga maritima*. *FEMS Microbiol Lett* **109**, 131–138.
- Gabelsberger, J., Liebl, W. & Schleifer, K. H. (1993b). Purification and properties of recombinant β -glucosidase of the hyperthermophilic bacterium. *Thermotoga maritima*. *Appl Microbiol Biotechnol* **40**, 44–52.
- Gebler, J., Gilkes, N. R., Claeysens, M., Wilson, D. B., Béguin, P., Wakarchuk, W. W., Kilburn, D. G., Miller, R. C., Jr, Warren, R. A. J. & Withers, S. G. (1992). Stereoselective hydrolysis catalyzed by related β -1,4-glucanases and β -1,4-xylanases. *J Biol Chem* **267**, 12559–12561.
- Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr & Warren, R. A. J. (1991). Domains in microbial β -1,4-glycanases:

- sequence conservation, function, and enzyme families. *Microbiol Rev* **55**, 303–315.
- von Heijne, G. (1985). Signal sequences. The limits of variation. *J Mol Biol* **184**, 99–105.
- Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **280**, 309–316.
- Hitomi, J., Park, J.-S., Nishiyama, M., Horinouchi, S. & Beppu, T. (1994). Substrate-dependent change in the pH-activity profile of alkaline endo-1,4- β -glucanase from an alkaline *Bacillus* sp. *J Biochem* **116**, 554–559.
- Huber, R. & Stetter, K. O. (1992). The Order Thermotogales. In *The Prokaryotes. A Handbook on the Biology of Bacteria, Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. II, pp. 3809–3815. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer-Verlag.
- Ishikawa, K., Matsui, I. & Honda, K. (1990). Substrate-dependent shift of optimum pH in porcine pancreatic α -amylase-catalyzed reactions. *Biochemistry* **29**, 7119–7123.
- Ishikawa, K., Matsui, I., Kobayashi, S., Nakatani, H. & Honda, K. (1993). Substrate recognition at the binding site in mammalian pancreatic α -amylases. *Biochemistry* **32**, 6259–6265.
- Jaenicke, R. (1993). Structure-function relationship of hyperthermophilic enzymes. In *Biocatalyst Design for Stability and Specificity*, pp. 53–67. Edited by M. E. Himmel & G. Geogios. ACS Symposium Series No. 516. American Chemical Society.
- Ko, E. P., Akatsuka, H., Moriyama, H., Shinmyo, A., Hata, Y., Katsube, Y., Urabe, I. & Okada, H. (1992). Site-directed mutagenesis of aspartate and glutamate residues of xylanase from *Bacillus pumilus*. *Biochem J* **288**, 117–121.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Liebl, W., Feil, R., Gabelsberger, J., Kellermann, J. & Schleifer, K. H. (1992). Purification and characterization of a novel thermostable 4- α -glucanotransferase of *Thermotoga maritima* cloned in *Escherichia coli*. *Eur J Biochem* **207**, 81–88.
- Liebl, W., Gabelsberger, J. & Schleifer, K. H. (1994). Comparative amino acid sequence analysis of *Thermotoga maritima* β -glucosidase (BglA) deduced from the nucleotide sequence of the gene indicates distant relationship between β -glucosidases of the BGA family and other families of β -1,4-glycosyl hydrolases. *Mol Gen Genet* **242**, 111–115.
- Nilsson, B., Uhlen, M., Josephson, S., Gatenbeck, S. & Philipson, L. (1983). An improved positive selection plasmid vector constructed by oligonucleotide mediated mutagenesis. *Nucleic Acids Res* **11**, 8019–8030.
- Ostendorp, R., Liebl, W., Schurig, H. & Jaenicke, R. (1993). The L-lactate dehydrogenase gene of the hyperthermophilic bacterium *Thermotoga maritima* cloned by complementation in *Escherichia coli*. *Eur J Biochem* **216**, 709–715.
- Remaut, E., Stanssens, P. & Fiers, W. (1981). Plasmid vectors for high-efficiency expression controlled by the P_L promoter of coliphage lambda. *Gene* **15**, 81–93.
- Ruttersmith, L. D. & Daniel, R. M. (1991). Thermostable cellobiohydrolase from the thermophilic eubacterium *Thermotoga* sp. strain FjSS3-B.1. *Biochem J* **277**, 887–890.
- Ruttersmith, L. D. & Daniel, R. M. (1993). Thermostable β -glucosidase and β -xylosidase from *Thermotoga* sp. strain FjSS3-B.1. *Biochim Biophys Acta* **1156**, 167–172.
- Saarilahti, H. T., Henrissat, B. & Palva, E. T. (1990). CelS: a novel endoglucanase identified from *Erwinia carotovora* subsp. *carotovora*. *Gene* **90**, 9–14.
- Shou, C., Rasmussen, G., Kaltoft, M.-B., Henrissat, B. & Schülein, M. (1993). Stereochemistry, specificity and kinetics of the hydrolysis of reduced celloextrins by nine cellulases. *Eur J Biochem* **217**, 947–953.
- Schüller, A., Harkness, R. E., Rüther, U. & Lubitz, W. (1985). Deletion of C-terminal amino acid codons of PhiX174 gene E: effect on its lysis inducing properties. *Nucleic Acids Res* **13**, 4143–4152.
- Studier, F. W. & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**, 113–130.
- Timasheff, S. N. (1993). The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Annu Rev Biophys Biomol Struct* **22**, 67–97.
- Törrönen, A., Kubicek, C. P. & Henrissat, B. (1993). Amino acid sequence similarities between low molecular weight endo-1,4- β -xylanases and family F cellulases revealed by clustering analysis. *FEBS Lett* **321**, 135–139.
- Wrba, A., Jaenicke, R., Huber, R. & Stetter, K. O. (1990). Lactate dehydrogenase from the extreme thermophile *Thermotoga maritima*. *Eur J Biochem* **188**, 195–201.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

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