Cloning, sequencing and analysis of expression of a *Butyrivibrio fibrisolvens* gene encoding a β -glucosidase

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The cloning, expression and nucleotide sequence of a 3.74 kb DNA segment on pLS215 containing a β -glucosidase gene (*bglA*) from *Butyrivibrio fibrisolvens* H17c was investigated. The *B. fibrisolvens bglA* open reading frame (ORF) of 2490 bp encoded a β -glucosidase of 830 amino acid residues with a calculated M_r of 91800. In *Escherichia coli* C600(pLS215) cells the β -glucosidase was localized in the cytoplasm and these cells produced an additional protein with an apparent M_r of approximately 94000. The *bglA* gene was expressed from its own regulatory region in *E. coli* and a single mRNA initiation point was identified upstream of the *bglA* ORF and adjacent to a promoter consensus sequence. The primary structure of the β -glucosidase showed >40% similarity with a domain of 237 amino acids present in the β -glucosidases of *Kluyveromyces fragilis* and *Clostridium thermocellum*. The *B. fibrisolvens* β -glucosidase hydrolysed cellobiose to a limited extent, cellotriose to cellobiose and glucose, and cellotetraose and cellopentaose to predominantly glucose.

Introduction

The importance of *Butyrivibrio fibrisolvens* in the digestion of cellulose and hemicellulose in the rumen has been reported by Shane *et al.* (1969), Margherita & Hungate (1963), Orpin *et al.* (1985) and Hespell *et al.* (1987). These reports have shown that *B. fibrisolvens* is the predominant bacterium in the rumen of animals surviving under adverse conditions, e.g. the high-arctic Svalbard reindeer and the semi-starved Zebu cattle in Kenya. They therefore suggest that this species may be more resilient than other rumen bacteria. Recombinant DNA technology was used to investigate the cellulolytic enzymes of *B. fibrisolvens*.

The hydrolysis of cellulose to glucose requires at least three groups of enzymes: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β -glucosidases or cellobiases (EC 3.2.1.21) (Coughlan, 1985). Berger *et al.* (1989) reported the cloning, nucleotide sequence and characterization of an endoglucanase (*endI*) gene from the cellulolytic *B. fibrisolvens* strain H17c that showed regions of similarity with the *Clostridium thermocellum celE* endoglucanase. Analysis of β -glucosidase activity is

essential as these enzymes can be rate-limiting in cellulose hydrolysis (Coughlan, 1985). We have constructed a gene bank of *B. fibrisolvens* H17c DNA in *E. coli* and report the molecular analysis of a β -glucosidase (*bglA*) gene and the activity of the encoded enzyme.

Methods

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The cloning vector pEB1 is an *E. coli*/*Bacillus subtilis* shuttle vector with positive selection for inserted DNA fragments in *E. coli*. pEB1 was constructed by replacing the pBR322 fragment of pLP1202 (Robson & Chambliss, 1986; Ostroff & Pène, 1983) with pEcoR252, which is a derivative of pEcoR251 lacking the *Pst*I site in the β -lactamase gene (P. Janssen, personal communication). The structure of pEcoR251 (a gift from M. Zabeau, Plant Genetic Systems, Gent, Belgium) is similar to other plasmids utilizing inactivation of the *Eco*RI endonuclease gene as a selective marker (e.g. Cheng & Modrich, 1983) and has been described previously (Zappe *et al.*, 1986). *B. fibrisolvens* H17c was grown in M10 medium (Caldwell & Bryant, 1966). The growth conditions were described previously by Strydom *et al.* (1986). *E. coli* strains were grown in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982) and ampicillin (100 μ g ml⁻¹) was used in selecting for transformants.

Preparation of DNA. Chromosomal DNA from *B. fibrisolvens* was obtained by the method of Berger *et al.* (1989). Plasmid DNA was prepared by an alkaline lysis procedure (Ish-Horowitz & Burke, 1981). The DNA was purified by CsCl-ethidium bromide density gradient centrifugation, n-butanol extraction and 2-propanol precipitation (Maniatis *et al.*, 1982).

Abbreviations: MUC, 4-methylumbelliferyl β -D-cellobioside; MUG, 4-methylumbelliferyl β -D-glucoside; ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M31120.

Table 1. *Bacterial strains and vectors*

	Relevant characteristics	Reference
<i>Strain</i>		
<i>B. fibrisolvens</i> H17c	Wild-type	Dehority (1966)
<i>E. coli</i> C600	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21 supE44 λ⁻</i>	Wood (1966)
<i>E. coli</i> K514	<i>r_K⁻ m_K⁺</i> derivative of <i>E. coli</i> C600	
<i>E. coli</i> LK111	<i>lacI lacZ Δ</i> M15 derivative of <i>E. coli</i> K514	Zabeau & Stanley (1982)
<i>E. coli</i> JM103	<i>Δ(lac-pro) thi strA supE endA sbcB hsdR⁻ F' traD36 proAB lacI^q Z Δ</i> M15	Messing <i>et al.</i> (1981)
<i>Vector</i>		
pEB1	Cm ^r Ap ^r	This paper
pLS215	Cm ^r Ap ^r	
Bluescript [®]	Ap ^r	

Construction and screening of a *B. fibrisolvens* H17c genomic library. *B. fibrisolvens* chromosomal DNA was partially digested by *Sau*3A endonuclease and fractionated on a sucrose density gradient (Maniatis *et al.*, 1982). The DNA fragments ranging from 4–10 kb in length were pooled and ligated with pEB1 which had been digested with *Bgl*II endonuclease. The ligated DNA was used to transform competent *E. coli* LK111 cells (Dagert & Ehrlich, 1979). Transformants were selected on LB agar containing ampicillin (100 µg ml⁻¹). Colonies were lifted off the plates on Whatman no. 1 filter paper discs and saturated with 1 ml 4-methylumbelliferyl β-D-cellobioside (MUC; Sigma) at a concentration of 0.02% (w/v) in 0.1 M-sodium acetate buffer, pH 5.0. The discs were incubated for 10 min at 37 °C and examined for fluorescent halos under UV light (254 nm). Plasmids were analysed by standard procedures (Maniatis *et al.*, 1982).

Southern blot analysis. *B. fibrisolvens* chromosomal DNA and pLS215 were digested to completion with *Eco*RV, fractionated by electrophoresis in 0.8% (w/v) agarose gels in 40 mM-Tris/acetate, 2 mM-EDTA (pH 8.0) buffer, and transferred to Hybond N (Amersham) (Smith & Summers, 1980). pLS215, nick-translated with [α -³²P]dCTP (Rigby *et al.*, 1977) was used as a hybridization probe.

Nucleotide sequencing. DNA fragments were subcloned in Bluescript vectors (Stratagene) and exonuclease III was used to generate two sets of overlapping deletions of opposite polarity (Henikoff, 1984). Sequencing was done by the chain termination method of Sanger *et al.* (1977) using a Sequenase kit (United States Biochemical Corp.). The nucleotide and deduced amino acid sequences were analysed using an IBM XT computer and DNA tools or Genepro (version 3.1) programs.

Primer extension. mRNA from *E. coli* C600(pLS215) was extracted by the method of Aiba *et al.* (1981). A synthetic 17-oligomer (5'-GCTGTAACCCTTTCACC-3') complementary to nucleotide positions 235 to 251 (Fig. 2) was labelled at the 5'-end with [γ -³²P]ATP [2000 Ci mmol⁻¹ (74 TBq mmol⁻¹)] using T4 polynucleotide kinase. The labelled primer (1 pmol) was hybridized to 5 µg of mRNA isolated from *E. coli* C600 (pLS215) in TE buffer (10 mM Tris/HCl, pH 8.3, 0.35 mM-EDTA). After ethanol precipitation, the samples were incubated in 25 µl 50 mM-Tris/HCl, pH 8.3, 8 mM-MgCl₂, 2 mM-DTT, 1 mM-dNTPs and 34 U of reverse transcriptase at 42 °C for 60 min. A DNA sequencing reaction was carried out using the same oligonucleotide as a primer and electrophoresed alongside the RNA-primer-extended products on a 6% polyacrylamide/7 M-urea sequencing gel.

In vitro transcription and translation. A prokaryotic-DNA-directed *in vitro* transcription and translation kit (no. N380; Amersham) was used

as specified by the manufacturers. The resulting proteins were separated by SDS-PAGE (Laemmli, 1970). Pharmacia low-molecular-mass standards were used as markers.

Cell fractionation. Periplasmic extracts were prepared from early stationary phase *E. coli* C600(pLS215) cultures (200 ml) by the osmotic shock procedure of Willis *et al.* (1974). Cytoplasmic fractions were prepared from osmotically shocked cells by sonication on ice (10 s bursts for a total of 100 s). The preparation was clarified by centrifugation for 15 min at 27000 g at 4 °C and samples were stored at -20 °C.

Enzyme assays. β-Glucosidase activity was assayed by the method of Deshpande *et al.* (1984) with the following modifications. The assay mixture contained 0.25 ml of diluted cell extract and 0.25 ml of 3.4 mM-*p*-nitrophenyl β-D-glucoside in 0.05 M-phosphate/citrate buffer (pH 5.0). The reaction mixtures were incubated at 42 °C for 30 min, and the reaction was stopped by the addition of 0.5 ml 14% (w/v) Na₂CO₃; after dilution with 1 ml H₂O the absorbance at 400 nm was measured. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol min⁻¹.

Activities of β-galactosidase and β-lactamase in cell extracts were assayed by the methods of Pardee *et al.* (1959) and Sykes & Nordstrom (1972), respectively.

Hydrolysis of cellobiose, cellotriose, cellotetraose and cellopentaose (obtained from AECI) was determined by incubating the cell extracts (20 µl) with 100 µl of the dextrin (10 mg ml⁻¹ in phosphate/citrate buffer) at 42 °C. Samples were taken at various times and analysed in an HPLC system equipped with a model 156 refractive index detector (Beckman). Separation was achieved on a C18 column (Waters). The column was held at room temperature and filtered water was used as an eluant at a flow rate of 1.5 ml min⁻¹.

Protein concentrations were determined by the dye-binding method of Bradford (1976) using BSA as a standard.

Results and Discussion

Cloning and expression of a β-glucosidase gene from B. fibrisolvens in E. coli

A MUC-positive *E. coli* LK111 transformant was isolated on LB agar plates containing ampicillin. The *E. coli* cells contained a recombinant plasmid which was

Fig. 1. Restriction map and sequencing strategy of plasmid pLS215. Single and bold lines and open boxes represent vector DNA, *B. fibrisolvens* insert DNA and antibiotic resistance genes, respectively. The circle and arrow beneath denote the position and direction of the λ rightward promoter. The extent of the *bglA* transcript is indicated by the solid line below the restriction map. The broken line indicates the extent of the nucleotide sequence shown in Fig. 2. The arrows denote the direction and extent of sequence determination.

designated pLS215. *E. coli* C600(pLS215) cells also showed activity towards 4-methylumbelliferyl β -D-glucoside (MUG) but no carboxymethylcellulase activity. pLS215 contained a 3.7 kb DNA insert which was characterized by restriction analysis (Fig. 1). Deletion of the λ rightward promoter on the 0.9 kb *EcoRI* fragment did not affect expression of the β -glucosidase gene (*bglA*) in *E. coli*, and it was concluded that the *bglA* gene was expressed from a regulatory region present in the insert.

The origin of the cloned fragment on pLS215 was determined by Southern blotting with 32 P-labelled pLS215 as a probe against *B. fibrisolvens* chromosomal DNA digested with *EcoRV* endonuclease. The insert DNA on pLS215 contained four *EcoRV* restriction sites (Fig. 1) and the plasmid hybridized to five bands of *EcoRV*-digested chromosomal DNA. The three which

represented the internal fragments of pLS215 had the predicted sizes of 0.6, 1.1 and 1.4 kb (results not shown).

Nucleotide sequencing

The nucleotide sequence of the 3.74 kb DNA fragment contained an open reading frame (ORF) encoding 830 amino acids with a calculated M_r of 91800 (Fig. 2).

The G + C content of the nucleotides within the ORF was 46.1 mol% which corresponds well with the average G + C content of the *B. fibrisolvens* genome of 42 mol% (Mannarelli, 1988).

The region upstream of the ORF, between nucleotides 55 and 84, contained a promoter sequence which consisted of a TTCAA – 35 region and TTTTAT – 10 region separated by 17 bp (Fig. 2). This promoter sequence resembled the consensus sequence of the σ^{70} RNA polymerase recognition site of *E. coli* (Hawley & McClure, 1983; Cowing *et al.*, 1985). Primer extension experiments indicated that this was a functional promoter sequence in *E. coli* and a transcriptional initiation point was detected 7 bp downstream of the – 10 region (Fig. 3). No other transcriptional initiation point could be detected in the region upstream of the *bglA* gene.

A possible ribosome-binding site (AACGAGG) was situated 8 bp upstream of the putative ATG initiation codon. The stop codon of the *bglA* gene is followed by an inverted repeat sequence and a region encoding a stretch of 5 U residues (Fig. 2). The potential stem/loop structure that could be formed in the resultant mRNA message had a $\Delta G = -1.21$ kcal mol $^{-1}$ (-5.06 kJ mol $^{-1}$) (calculated according to Salser, 1977) and consisted mainly of A and U residues. It is therefore unlikely that the structure would function as a ρ -independent terminator in *E. coli*.

Table 2. Distribution of β -glucosidase in *E. coli* C600(pLS215) cells

Values are means of three experiments; standard errors of means were less than 10% of the reported values.

Fraction	Total activity (units)*		
	β -Glucosidase	β -Galactosidase	β -Lactamase
Supernatant	1 (4)	79 (<1)	110 (30)
Periplasmic	<1 (<1)	47 (<1)	231 (63)
Cytoplasmic	25 (96)	34000 (99)	26 (7)

* Activity units: β -glucosidase, μ mol *p*-nitrophenol released min $^{-1}$; β -galactosidase, nmol *o*-nitrophenol released min $^{-1}$; β -lactamase, μ mol ampicillin hydrolysed min $^{-1}$. Numbers in parentheses represent the percentage of total activity for the respective enzyme.

AACAAACAGAATTGTTGGAACTTTTTTATATGCAATATTACTTTAACACAGAATGTCTG	60
GTGATGGTATATGTTTTTCTTCCTGAGTTATATATTTCAAATCTTAGATGTGCTATGCT	120
TTTATGGCAGAAACATGTTATATACAAGGTAAAGATAAGTAGGAT <u>AACGAGG</u> TATTTATA	180
ATGGAGAAATGGGCAAGAATCAAATATACACCAAATCTTCCGCTTGGAGAGAATGGTGAA	240
M E K W A R I K Y T P N L P L G E N G E	
AGGGTTACAGCGAGTCAGAAGCACATTGAGCTTTCATGCGAGGCAGCATGTGAGGGAATG	300
R V T A S Q K H I E L S C E A A C E G M	
GTACTTCTCAAGAATGACAGAAACGTTCTTCCTATCAGAAAGGGCACAAGAGTAGCCCTC	360
V L L K N D R N V L P I R K G T R V A L	
TTTGGAAAGGGAGTATTTGACTATGTAAAAGGCGGCGGTGGTAGCGGAGATGTAAACAGTT	420
F G K G V F D Y V K G G G G S G D V T V	
CCTTACATCAGAAACCTCTACGAAGGCCTTTCTCAGTACACATCAGACATTTCAATTTAC	480
P Y I R N L Y E G L S Q Y T S D I S I Y	
GACAAATCTGTCAGATTCTATCAGGAATATGTAGCAGACCAGTACAGACTTGAATTGCA	540
D K S V R F Y Q E Y V A D Q Y R L G I A	
CCAGGCATGATCAAAGAGCCGGCTCTTCCGGAAGATATTCTTGCAGATGCAGCAGCCTAT	600
P G M I K E P A L P E D I L A D A A A Y	
GCAGATACTGCAATCATCGCAATCAGCAGATTCTCCGGAAGGCTGGGACAGAAAGGTT	660
A D T A I I A I S R F S G E G W D R K V	
GCAGGCGTTGACAGAGAAATCAAGTGCGAAGCCAAGGACCTCGTAGAGCAGGGCAACAAG	720
A G V D R E I K C E A K D L V E Q G N K	
ATATTTGATCATGGTGATTTCTACCTCACAAATGCTGAGAAGAAGATGGTCAAGATGGTA	780
I F D H G D F Y L T N A E K K M V K M V	
AAAGAGAACTTCTCAAGCGTCATTGTAGTCATGAATGTCGGAGGAGTCGTAGACACAACA	840
K E N F S S V I V V M N V G G V V D T T	
TGGTTTAAAAAGGATGACCAGATTTTCATCAGTCCTCATGGCATGGCAGGGTGGAATTGAA	900
W F K K D D Q I S S V L M A W Q G G I E	
GGCGGACTTGCCGCAGCCAGGATCCTTCTTGGCAAGGTTAATCCTTCAGGTAAGCTCTCA	960
G G L A A A R I L L G K V N P S G K L S	
GATACATTCGCAGCAAGGCTTGAAGACTATCCTTCAACAGAGGGCTTCCACGAAGATGAT	1020
D T F A A R L E D Y P S T E G F H E D D	
GACTACGTGGATTACACAGAAGATATCTACGTTGGCTATAGATATTTTCGAGACCATTTCC	1080
D Y V D Y T E D I Y V G Y R Y F E T I P	
GGGGCAAAAGAGAAAGTTAACTACCCCTTTGGCTATGGCCTTTCCTATACAACTTTCCTG	1140
G A K E K V N Y P F G Y G L S Y T T F L	
CTTGAAGACTATAAGGCAGAGCCTTTTGTGGCTTCAGCAGCAGACGAGGTCGGTAAATCT	1200
L E D Y K A E P F V A S A A D E V G K S	

GATAGCGACCTTGCAGATGCAATCGTAGCCTCAGTTACAGTCACAAACATTGGCAAGATT 1260
 D S D L A D A I V A S V T V T N I G K I
 CCGGGCAAAGAGGTTGTTTCAGCTCTACTACAGCGCTCCTCAGGGCAAGCTCGGTAAGCCT 1320
 P G K E V V Q L Y Y S A P Q G K L G K P
 GCTAAAGTCCTTGGCGGCTATGCCAAGACAAGGCTACTGCAGCCGGGAGAGAGCCAGAGA 1380
 A K V L G G Y A K T R L L Q P G E S Q R
 GTGACAATTGCTCTTTATATGGAGGATATGGCATCTTACGACGACCTTGGCAAGGTTAAA 1440
 V T I A L Y M E D M A S Y D D L G K V K
 AAGGCTGCCTGGCTCCTTGAAAAAGGTGAATATCATTTCTTCCTTGGAACATCAGTAAGA 1500
 K A A W L L E K G E Y H F F L G T S V R
 GACACAAGGCTTCTTGATTACACCTATGAACCTTCTAAGAACATAATAGTTGAACAGGTC 1560
 D T R L L D Y T Y E L S K N I I V E Q V
 TCAAACAAGCTCGTTCCAACATCTCTTCCCAAGAGAATGCTTGCTGATGGCACATATGAG 1620
 S N K L V P T S L P K R M L A D G T Y E
 GAACTTCCTCAGACAGAACCTGTAGATACTTATGCAACAATCTTCCCAAGACCTAAGAAC 1680
 E L P Q T E P V D T Y A T I F P R P K N
 TGGAAAGAAACAATTGAGCACGACGTATTAAAGACTCCTGTAGTTTCGTCCACAGGACAGA 1740
 W K E T I E H D V L K T P V V R P Q D R
 TTCCAGCTCTTTTTGCCACCTAAGGAAGGTGACCCTAAGAAATTTATCGAAGTTGCAGAA 1800
 F Q L F L P P K E G D P K K F I E V A E
 TGCAAGGTGACACTTGAAGACTTTATTGCACAGCTATCTAACGAGCAGCTTGCAAGCCTT 1860
 C K V T K E D F I A Q L S N E Q L A S L
 CTTGGAGGACAGCCAAATGTCGGAATGGCTAACACCTTTGGATACGGCAACCTTCCTGAG 1920
 L G G Q P N V G M A N T F G Y G N L P E
 GTTGGAGTTCCTAATGCCCAGACCTGTGATGGTCCTGCAGGTGTCCGTATTGCACCGGAA 1980
 V G V P N A Q T C D G P A G V R I A P E
 GTTGGTGTGTGACTACAGCATTTCCCATGTTCAACCCTTCTTGCATGCACATGGAATGAA 2040
 V G V V T T A F P C S T L L A C T W N E
 GATATCTGCTACGAAGTCGGAGTTGCAGGCGGAGAAGAGGCCAAGGAGTGCAATTTTGGT 2100
 D I C Y E V G V A G G E E A K E C N F G
 GCATGGCTTACTCCTGCTGTTAACATCCATAGAAGCCCTCTTTGCGGCAGAACTTTGAG 2160
 A W L T P A V N I H R S P L C G R N F E
 TACTACTCCGAAGATCCATTCTTGCAGGTAAACAGGCAGCAGCTATGGTTCGTGGTATC 2220
 Y Y S E D P F L A G K Q A A A M V R G I
 CAGAGCAACAACATAATTGCTACACCTAAACATTTTGCCCTCAACAACAAGGAATCCAAT 2280
 Q S N N I I A T P K H F A L N N K E S N
 AGAAAAGGCAGCGATTACGTGCTTCTGAGCGTGCATCAGAGAAATATATTTAAAGGCC 2340
 R K G S D S R A S E R A I R E I Y L K A

TTTGAAATCATTGTTAAAGAGCAGAGCCCTGGAGCATCATGTCTTCAATACAATATAGTT	2400
F E I I V K E Q S P G A S C L Q Y N I V	
AACGGTCAGAGATCATCCGAATCTCACGACCTCCTCACAGGAATCCTCCGCGATGAGTGG	2460
N G Q R S S E S H D L L T G I L R D E W	
GGCTTTGAAGGTGTTGTAGTCAGCGACTGGTGGGGCTTTGGTGAGCATTACAAGGAAGTC	2520
G F E G V V V S D W W G F G E H Y K E V	
CTTGCAGGCAACGATATCAAGATGGGCTGTGGCTATACAGAACAGCTCCTTGAAGCAATT	2580
L A G N D I K M G C G Y T E Q L L E A I	
GATAAGAAAGCTCTTAAGAGAAAAGATTTGGAAAAGAGGCAGAGCGAGTCCTCAAGATGC	2640
D K K A L K R K D L E K R Q S E S S R C	
TTCTCAAACCTCGACTAAGCTCAAAGCCGCTTAGAATAAACGTTTAAGTCATAAAAAAGTA	2700
F S N S T K L K A A *	
ATGTATTGTTTCATAAACTATCAATATTTTTCTGACAATTTGTCGTATAATATAAATATGA	2760
TATAGTTGTTGGGAATATCTTCTGAGAATTGTATCAGGAGAAAGTTGTAGAACTACAAC	2820
CTCGGAGGTATGTCTATGAACAAAAAGCTATTGTTGGTATTTTTATGTCCATTTTGATG	2880
<u>M N K K A I V G I F M S I L M</u>	
GCAGGGCTCGTTGGATGTGCCGGTAGCAGTGATGCCCAGGCAGGAGATGACCTCAAGCCG	2940
<u>A G L V G C A G S S D A Q A G D D L K P</u>	
GTTATTTATCTTTATCCACAGGAAGATAATACCGAGATTTTCAGTAAGCCTTGATTATAAC	3000
V I Y L Y P Q E D N T E I S V S L D Y N	
GGAAATCTGGTTGACCTGATTCCTGAGTTTAATGCAGATAAGACATGGAATGTTACAGCT	3060
G N L V D L I P E F N A D K T W N V T A	
AACAAAGATGGCAAGATTACCTTTGAAGGACAGACTTATGACTATCTGTTTTGGGAAGGC	3120
N K D G K I T F E G Q T Y D Y L F W E G	
GATC	3124
D	

Fig. 2. Nucleotide sequence of the *B. fibrisolvens* *bglA* structural gene and flanking regions. Open circles indicate transcriptional start points for the *bglA* gene and the associated arrow indicates the direction of transcription. Consensus -10 and -35 regions of the *bglA* promoter are underlined and the ribosome-binding sequence (RBS) is boxed. Inverted repeat sequences are shown by converging arrows. The signal peptide of the downstream ORF is underlined.

Fig. 3. Primer extension analysis of the 5'-end of *bglA* mRNA. An oligonucleotide complementary to nucleotide positions 235–251 (Fig. 2) was hybridized to RNA from *E. coli* C600 (pLS215) cells (lane 2) and a control without RNA (lane 1). A sequencing reaction was done using the oligonucleotide as a primer with DNA from pLS215. The stars mark two possible transcription initiation sites.

Amino acid sequence, localization and *in vitro* transcription-translation

The deduced amino acid sequence of the *B. fibrisolvens* β -glucosidase was compared with the sequences of the β -glucosidases from the fungi *Kluyveromyces fragilis* (Raynal *et al.*, 1987) and *Candida pelliculosa* (Kohchi & Toh-e, 1985), and the bacterium *Clostridium thermocellum* (Gräbnitz *et al.*, 1989), and showed 13, 13 and 12% similarity respectively. In contrast to the thermophilic bacterial β -glucosidase from *Clostridium thermocellum*, which showed extensive homology with the fungal β -glucosidases (Gräbnitz *et al.*, 1989), the *B. fibrisolvens* β -glucosidase is not related to the fungal enzymes. The peptide sequence implicated in the catalytic activity of the fungal (Bause & Legler, 1980) and *Clostridium thermocellum* (Gräbnitz *et al.*, 1989) β -glucosidases was not present in the *B. fibrisolvens* β -glucosidase. Although there was low overall similarity between the *B. fibrisolvens* β -glucosidase and the fungal-type β -glucosidases, a sequence of significant similarity between the *B. fibrisolvens* enzyme (residues 207–444), and the β -glucosidases of *K. fragilis* (residues 618–830, 42%

similarity) and *Clostridium thermocellum* (residues 448–661, 41% similarity) was detected (Fig. 4). This region of homology was absent in the *Candida pelliculosa* β -glucosidase (Gräbnitz *et al.*, 1989).

The homology between the amino acid sequence of the *B. fibrisolvens* β -glucosidase and the bacterial β -glucosidases from *E. coli* (Schnetz *et al.*, 1987), *Caldocellum saccharolyticum* (Love *et al.*, 1988) and an *Agrobacterium* spp. (Wakarchuk *et al.*, 1988) was determined. The *B. fibrisolvens* enzyme showed <15% similarity with these bacterial β -glucosidases which also show insignificant similarity when compared with one another.

Experiments on the localization of the *B. fibrisolvens* β -glucosidase in *E. coli* cells showed that 95.8% of the enzyme activity was present in cytoplasmic fractions and only low levels of activity were detected in periplasmic fractions and the culture supernatant (Table 2). β -Galactosidase, a cytoplasmic enzyme and β -lactamase, a periplasmic enzyme, were used as controls to confirm the correct fractionation of the cell compartments.

SDS-PAGE analysis of *in vitro* translation products of pLS215 showed the presence of, amongst others, a protein with an apparent M_r of approximately 94000 (Fig. 5a, lane 2). The apparent M_r of this protein is in agreement with a predicted M_r of 91800 encoded by the *bglA* gene. The proteins with lower apparent M_r values are likely to include proteins and breakdown products encoded by both the *bglA* gene and the second ORF shown in Fig. 2. The vector pEB1 did not encode any proteins with apparent M_r values >30000 (Fig. 5a, lane 1).

The cytoplasmic fraction of *E. coli* C600(pLS215) cells (Fig. 5b, lane 4) also contained a protein with an apparent M_r of 94000. This protein was absent in the corresponding periplasmic fraction and in the periplasmic and cytoplasmic fractions of *E. coli* C600(pEB1) cells (Fig. 5b, lanes 2, 1 and 3 respectively). The distribution of this protein between the periplasmic and cytoplasmic fractions of *E. coli* C600(pLS215) (compare Fig. 5b, lanes 2 and 4) reflects the distribution of β -glucosidase activity in the respective cell fractions shown in Table 2.

Enzyme activity

The *B. fibrisolvens* β -glucosidase cloned in *E. coli* had a pH optimum of 5.0 and activity decreased rapidly at pH values above and below pH 5.0. The optimum temperature for activity of the β -glucosidase was 42 °C.

The activity of the cloned β -glucosidase against various cellodextrins was examined by HPLC (Fig. 6). The β -glucosidase was able to hydrolyse cellobiose to a limited extent. The enzyme hydrolysed cellotriose to cellobiose and glucose. Cellotetraose and cellopentaose were hydrolysed predominantly to glucose.

Bf	207	VIVVMNGGVVDTTWFKKDDQISSVLMWQGGIEGGLAAARILLGKVNPSG
Kf	618	VIV-NSG--TPVEFPWLEEANALV-QAWYGGNELGNIAADVLYGDVVPNG
Ct	448	V-VVLNG--SPVEMPWIDKVK-SVLEAYLGGQALGGRWRMC-YSVKSIVG
Cd	557	TIVIVTGQQIDLEPFIDNENVTAIVIYSSYLGQDFGTVLAKVLFGDENPSG
Bf	257	KLSDTFAARLEDY-PSTEGFHEDDDYVDYTEDIVVGYRYFETIPGAKEKV
Kf	664	KLSLSWPFLQDN-PSFLNFKTEFGRVVYGEDIFVGYRYYEKLQR---KV
Ct	493	KLAETFPVKLSHN-PSYLNFPGEDDRVEYKEGLFVGYRYYDTKGI---EP
Cd	607	KLPFTIAKDVNDYIPVIEKVDVDPVDKFTESIYVDYRYFDKY--HK-PV
Bf	301	NYPFGYGLSYTTFFLEDYKAEPFVASAADEVGKSDSDLADAIVASVTVTN
Kf	710	AFPFYGLSYTTFFEL-DIS--DFK-----VK--D-DKIDISV-DVKNT-
Ct	539	LFPFHGGLSYTKFEYSDISV-DKK----D-V---K-SIINVSV---KNV-
Cd	654	RYEFGYGLSYSNFSLSDIEIQTLQFFSENAEPAANYSETYQYKOSNMDPS
Bf	356	IGKIPGKEVVQLYYSAPQGKLGKPAKVLGGYAKTRLLQPGESQRTIALY
Kf	746	GDKFAGSEVVQVYFSALNSKVVRPVKELKGFEEKVH-LEPGEKKTVNIELE
Ct	575	-GKMAGKEIVQLYVKDVKSSVRHPEKELKGFEEKVF-LNPGEKKTVTFTLD
Cd	704	EYTVPEGFKELANYTVPIYHDASSIKANSSYDYPEGY-STEQLDGPKSLA
Bf	401	MEDMASYDDLGVKVKAAWLLKGEYHFFLGTSVRDTRL
Kf	795	LKDAISYFNEELGK---WHVEAGEYLVSVGTS-SDDIL
Ct	623	-KRAFAYYNTQI-KD--WHVESGEYLILIGRS-SRDIV
Cd	748	AGGLGGNHTCGMLVTLSSL--KSQIKVLMVLVGLHLNCM

Fig. 4. Homologous regions of the β -glucosidases of *B. fibrisolvens* (Bf), *Kluyveromyces fragilis* (Kf), *Clostridium thermocellum* (Ct) and *Candida pelliculosa* (Cp). Homology was maximized by introducing gaps denoted by a dash (-). Numbers on the left refer to the first amino acid in each line.

Fig. 5. SDS-PAGE analysis of proteins encoded by pLS215 and pEB1 *in vitro* (a) and *in vivo* (b). (a) Lane 1, pEB1; lane 2, pLS215. (b) Lanes 1 and 2, periplasmic fractions of *E. coli* K514(pEB1) and K514(pLS215), respectively; lanes 3 and 4, cytoplasmic fractions of *E. coli* K514(pEB1) and K514(pLS215), respectively; lane 5, M_r standards: 94000, rabbit muscle phosphorylase b; 67000, BSA; 43000, ovalbumin 30000, bovine carbonic anhydrase. The arrow heads indicate the position of the *bglA* gene product.

To our knowledge this is the first report of a β -glucosidase whose major role in the degradation of cellulose is the cleavage of cellooligosaccharides rather than cellobiose. As *B. fibrisolvens* H17c is able to grow on glucose (Strydom *et al.*, 1986), its genome is likely to encode an enzyme with greater cellobiase activity than the BglA enzyme. We are continuing to screen our gene library for such an enzyme.

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Fig. 6. HPLC analysis of the hydrolysis products of cellooligosaccharides by the cloned β -glucosidase. A, standards. B and C, cell extracts incubated at 42 °C for 60 min (B) and 90 min (C) with 200 μ g of cellooligosaccharides in 0.05 M-phosphate/citrate buffer (pH 5.0). Substrates used were: panel 1, cellobiose; panel 2, cellotriose; panel 3, cellotetraose; panel 4, cellopentaose. Peaks represent: a, glucose; b, cellobiose; c, cellotriose; d, cellotetraose; e, cellopentaose.

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