Cloning and sequencing of the *celA* gene encoding endoglucanase A of *Butyrivibrio fibrisolvens* strain A46

GEOFFREY P. HAZLEWOOD,^{1*} KEITH DAVIDSON,¹ JUDITH I. LAURIE,¹ MAREK P. M. ROMANIEC² and HARRY J. GILBERT³

¹Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK

²Fermentation Microbiology Laboratory, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

³Department of Agricultural Biochemistry and Nutrition, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, UK

(Received 5 April 1990; revised 3 June 1990; accepted 4 July 1990)

Genomic DNA from *Butyrivibrio fibrisolvens* strain A46 was digested with *EcoRI* and ligated into λ gt11. Two recombinant phages isolated from the gene bank hydrolysed carboxymethylcellulose and were shown to contain the same 2.3 kb *EcoRI* restriction fragment, which was cloned into pUC12 to generate pBA46. *Escherichia coli* JM83 harbouring pBA46 expressed an endoglucanase (EGA) which hydrolysed a range of other substrates including barley β -glucan, Avicel, filter paper and *p*-nitrophenyl β -D-cellobioside. Nucleotide sequencing of the *B. fibrisolvens* strain A46 DNA cloned in pBA46 revealed a single open reading frame (ORF) of 1296 bp, encoding a protein of 48863 Da. Confirmation that the ORF coded for EGA was obtained by comparing the N-terminal sequence of the purified endoglucanase with that deduced from the nucleotide sequence. EGA contains a typical prokaryotic signal peptide at its N-terminus and shows some homology with the *Bacillus* family of cellulases. The enzyme does not contain distinct functional domains, which are prevalent in cellulases from *Pseudomonas fluorescens* subsp. *cellulosa* and *Cellulomonas fimi*.

Introduction

Butyrivibrio fibrisolvens plays an important role in the ruminal breakdown of plant structural polysaccharides. In ruminants such as the high-arctic Svalbard reindeer, *B. fibrisolvens* is the major culturable cellulolytic bacterium in both summer and winter, representing 66% and 55% of the cellulolytic population respectively (Orpin *et al.*, 1985). In view of the well-documented involvement of members of this genus in a variety of rumen processes, it is surprising that their cellulase system has received so little attention. Kopecny (1986) reported that the cellulase of *B. fibrisolvens* UC142 comprised extracellular and cell-associated enzymes, including an endo-1,4- β -glucanase (EC 3.2.1.4) and three β -glucosidases (EC 3.2.1.21). More recently, an endoglucanase gene (*end-1*)

from *B. fibrisolvens* H17c was cloned and sequenced (Berger *et al.*, 1989); the encoded protein was homologous with an endoglucanase (EGE) from *Clostridium thermocellum*.

Cellulase genes from a range of micro-organisms have now been sequenced, revealing considerable diversity in the molecular structure of these proteins (see Béguin, 1990, for a review). Some bacteria synthesize enzymes whose structure is highly conserved, while others express cellulases which show little homology at the amino acid level. Cellulases produced by *Pseudomonas fluorescens* subsp. *cellulosa* (Gilbert *et al.*, 1990), *Cellulomonas fimi* (Ong *et al.*, 1989) and *Bacteroides succinogenes* (McGavin & Forsberg, 1989) typically contain distinct structural regions responsible for cellulose binding and catalytic activity. In contrast, endoglucanases from *Bacillus* spp., *Clostridium* spp. (Béguin, 1990) and *Ruminococcus albus* (Poole *et al.*, 1990) in general do not appear to contain substrate-binding domains distinct from the active site. Similarities in molecular architecture, and the conserva-

Abbreviations: EGA, endoglucanase A; ORF, open reading frame.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M37031.

tion of sequence between cellulases expressed by *B. fibrisolvens* remain to be elucidated.

A detailed knowledge of cellulases will contribute greatly to our understanding of fibre digestion in the rumen, and may also facilitate commercial exploitation of this important group of enzymes. With these objectives in mind, we describe here the cloning and sequencing of the *celA* gene from *B. fibrisolvens* strain A46, encoding endoglucanase A (EGA).

Methods

Bacterial strains, plasmids and culture conditions. *B. fibrisolvens* strain A46 was originally isolated from the rumen of the high-arctic Svalbard reindeer (Orpin *et al.*, 1985), and was cultured statically at 39 °C in rumen-fluid-containing medium (Hobson, 1969; medium 2) with cellobiose (0.25%, w/v) and carboxymethylcellulose (CMC; sodium salt, low viscosity, DS > 0.4; 0.25%, w/v) as carbon sources. *Escherichia coli* strains HB101 (Boyer & Roulland-Dussoix, 1969) and JM83 (Norrander *et al.*, 1983) were cultured at 37 °C in Luria broth (LB) adjusted to pH 7.5 with NaOH. Agar (1.5%, w/v), and filter-sterilized ampicillin (100 µg ml⁻¹) and tetracycline (12.5 µg ml⁻¹), were added as required. Vectors used for subcloning and sequencing the endoglucanase gene were pUC12, pUC18, pUC19, M13mp18 and M13mp19 (Norrander *et al.*, 1983). Plasmid pNM52, containing *lacP*, was described by Gilbert *et al.* (1986).

Isolation of DNA. Total DNA from *B. fibrisolvens* strain A46 was extracted and purified as described by Romaniec *et al.* (1987). Large-scale preparations of plasmid DNA were made from *E. coli* by alkaline lysis and purified by caesium chloride density-gradient centrifugation (Maniatis *et al.*, 1982). The methods of Birnboim & Doly (1979) and Holmes & Quigley (1981) were used for small-scale plasmid DNA extraction and rapid screening of plasmid-containing clones. Large-scale preparations of λ phage DNA were made from liquid lysates essentially as described by Maniatis *et al.* (1982). *E. coli* Y1090 (Young & Davis, 1983), infected with recombinant λ phage which had been recovered in agar plugs from CMCase⁺ plaques, was cultured in NZB medium until lysis occurred. NZB medium contained (g l⁻¹): tryptone, 10; yeast extract, 5; maltose, 2; NaCl, 5; MgCl₂·7H₂O, 2.

Construction and screening of the genomic library. Genomic DNA from *B. fibrisolvens* strain A46 was digested with *Eco*RI and fragments 2–9 kbp in size were recovered by electroelution and ligated into *Eco*RI-digested λgt11 DNA (Young & Davis, 1983) (Protophone λgt11 system, Promega Biotec), using conditions specified in the manufacturer's technical bulletin. *In vitro* packaging of the λ DNA was done using protocol II of Maniatis *et al.* (1982). Recombinant phage (LacZ⁻) were enumerated by plating dilutions of phage suspension, mixed with *E. coli* Y1090, on NZB soft agarose medium supplemented with isopropyl β-D-thiogalactoside (IPTG; 100 µg ml⁻¹) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 250 µg ml⁻¹). For screening the gene bank, λ recombinants (20000 in total) were cultured on lawns of *E. coli* Y1090 to a plaque density of 10 cm⁻². Each plate was overlaid with medium containing CMC (0.5%, w/v) and agarose (1.5%, w/v) in PC buffer (50 mM-K₂HPO₄, 12 mM-citric acid, pH 6.5), and incubated at 39 °C for 16 h. Plaques producing CMCase activity were identified by staining with Congo Red (1%, w/v) and destaining with 1 M-sodium chloride (Teather & Wood, 1982). CMCase⁺ plaques were removed in plugs of agar and phage contained therein were allowed to diffuse into 1 ml SM buffer [10 mM Tris/HCl, pH 7.8, containing 100 mM-NaCl, 20 mM-MgCl₂ and 0.05% (w/v) gelatin].

General recombinant DNA procedures. Agarose gel electrophoresis, transformation of *E. coli* and the modification of DNA using restriction enzymes and T4 DNA ligase were done essentially as described by Gilbert *et al.* (1987). Southern blot hybridization was performed according to Romaniec *et al.* (1987).

Nucleotide sequencing. The 2.3 kb *Eco*RI fragment carrying the *B. fibrisolvens* strain A46 *celA* gene in pBA46 (Fig. 1) was cleaved with *Pvu*II and the two fragments generated (0.8 and 1.5 kb) were each cloned into pUC18 and pUC19 which had been digested with *Eco*RI and *Pvu*II. Sequential digestion of each insert from either end, with *Bal*31 exonuclease, was used to generate sets of nested deletions; the deleted fragments were cloned into M13mp18 and M13mp19 for nucleotide sequencing. An internal *Hind*III fragment of 0.14 kb (Fig. 1) was separately cloned into M13 for determining the sequence over the region of the *Pvu*II site. Nucleotide sequence was determined using the dideoxy chain-termination method of Sanger *et al.* (1980), with the exception that T7 DNA polymerase (Tabor & Richardson, 1987) was substituted for the Klenow fragment of DNA polymerase I. Sequences were compiled and ordered using the computer programs described by Staden (1980). The entire sequence of *celA* was determined in both strands.

Bal31 deletions. To delete the 5' region of *celA*, pUC18 containing full-length *celA* on a 1.4 kb *Sca*I–*Cla*I fragment (Fig. 1) was linearized with *Eco*RI, digested with *Bal*31, and rendered blunt-ended with T4 DNA polymerase (Hall *et al.*, 1988). The truncated fragment was excised by digesting with *Sph*I, and was ligated into pUC18 which had been cleaved with *Sma*I and *Sph*I, such that the coding sequence of *celA* was in frame with the N-terminus of *lacZ*. The 3' end of *celA* was deleted by linearizing pUC18 containing *celA* on the same *Sca*I–*Cla*I fragment with *Sph*I, before digesting with *Bal*31 and filling in with T4 DNA polymerase. The deleted fragment was excised with *Eco*RI and ligated into pUC18 previously digested with *Eco*RI and *Sma*I.

Assays. Cellulase-related enzyme activities were assayed at 37 °C in PC buffer, pH 6.5, with substrate concentration in the range 2.5 to 10 mg ml⁻¹. Hydrolysis of acid-swollen cellulose (Sigmacell type 50 treated according to Wood, 1971), Avicel (PH105, FMC Corporation), barley β-glucan (Biocon, UK), CMC (sodium salt, Sigma), filter paper (Whatman no. 1), laminarin (Sigma), lichenan (Sigma) and the soluble fraction of oat spelt xylan (Sigma) was measured by following the release of reducing sugar colorimetrically using 3,5-dinitrosalicylic acid (Miller, 1959). Hydrolysis of the chromogenic substrates 4-methylumbelliferyl β-D-cellobioside and p-nitrophenyl β-D-cellobioside was estimated colorimetrically. One unit (U) of enzyme activity was defined as that which resulted in the liberation of 1 µmol product per min⁻¹. Qualitative measurement of endoglucanase activity was done either by incorporating CMC into LB agar or by overlaying LB plates of *E. coli* clones with PC buffer containing 0.5% (w/v) CMC and 1% (w/v) molten agar. After incubating at 37 °C, activity was revealed by staining with Congo Red. Protein was measured by dye binding (Sedmak & Grossberg, 1977) or by the Lowry method, with bovine serum albumin as standard.

Fractionation of *E. coli*. Periplasmic and cytoplasmic fractions of *E. coli* clones harbouring *celA* were prepared as described previously (Hazlewood *et al.*, 1990).

Purification of endoglucanase A (EGA). *E. coli* JM83 harbouring full-length *celA* cloned in pUC18 was cultured for 16 h at 37 °C in LB containing ampicillin (100 µg ml⁻¹). Cells from 3 litres of culture were collected by centrifugation and were resuspended in 50 mM Tris/HCl buffer, pH 8, to approximately 1/30 of their original volume. Cell-free extract prepared by ultrasonication was loaded onto an anion-exchange column (2.6 × 21 cm; DEAE-Trisacryl M; IBF Biotechnics) which was eluted with a 0–500 mM-NaCl linear gradient in 50 mM-Tris/HCl

buffer, pH 8. Fractions containing endoglucanase activity were pooled, dialysed against 10 mM-Tris/HCl buffer, pH 8, and applied to a second column containing DEAE-Trisacryl M, which was eluted with a 100–400 mM-NaCl linear gradient. Active fractions were dialysed as above and concentrated by vacuum evaporation.

Sequencing of EGA. EGA, partially purified by anion-exchange chromatography, was fractionated by SDS-PAGE (10% acrylamide, 0.1% SDS) (Laemmli, 1970) and transferred to Immobilon PVDF (Millipore) by electroblotting. Putative EGA (47 kDa), stained with Page Blue G90, was excised from the transfer membrane and applied directly to a 470A gas-phase sequencer equipped with a 120A on-line PTH analyser (Applied Biosystems; Hunkapillar *et al.*, 1983).

Results and Discussion

Isolation and characterization of CMCase⁺ clones

The genomic library constructed by ligating *Eco*RI fragments of *B. fibrisolvens* strain A46 DNA into λ gt11 had an insertion rate of the order of 70%. Twenty-thousand λ gt11 recombinants were screened and two CMCase⁺ plaques were found. DNA was isolated from phage recovered from each of these plaques and the *B. fibrisolvens* *Eco*RI restriction fragments were excised and ligated into pUC12. Restriction enzyme analysis showed that both plasmids contained the same 2.3 kb *Eco*RI fragment, which could be cleaved with *Hind*III or *Pvu*II, but was devoid of *Bam*HI, *Bgl*II, *Pst*I, *Sal*I or *Sma*I sites (Fig. 1). In Southern blot hybridizations, a ³²P-labelled probe prepared by nick-translation of pUC12 containing the cloned fragment (pBA46) hybridized with a single 2.4 kb *Eco*RI fragment of *B. fibrisolvens* strain A46 genomic DNA. The same probe also hybridized with genomic DNA digested with *Hind*III and *Pvu*II in a manner which confirmed the physical map and indicated that the fragment had been cloned without deletion or rearrangement (data not shown); pUC12 alone was not homologous with *B. fibrisolvens* strain A46 genomic DNA.

Subcloning and expression of *celA* in *E. coli* JM83

The 2.3 kb *Eco*RI fragment carrying the *celA* gene was subcloned from pBA46 into pUC18 and pUC19 and transformed into *E. coli* JM83. In both orientations *celA* directed the synthesis of functional EGA, which suggests that the *celA* promoter is operative in *E. coli*. A 1.4 kb *Sca*I–*Cla*I restriction fragment (Fig. 1) directed the synthesis of an active endoglucanase when cloned into pUC18, but not when inserted in the opposite orientation into pUC19; this finding indicates that the *Sca*I–*Cla*I fragment contains the protein-coding region of *celA*, but not the regulatory sequences of the gene, and confirms that *celA* is transcribed in the direction indicated in Fig. 1.

Fig. 1. Restriction enzyme map of *B. fibrisolvens* strain A46 genomic DNA in the vicinity of the *celA* gene. The positions of the cleavage sites for *Bcl*I (Bc), *Cla*I (Cl), *Eco*RI (EI), *Hind*III (Hi), *Pvu*II (Pv) and *Sca*I (Sc) are shown. The extent and orientation of *celA* are indicated by the solid bar and arrow respectively.

Table 1. Substrate specificity of EGA expressed in cell-free extract from *E. coli* JM83 harbouring full-length *celA*

The assays were repeated several times with different preparations. Typical results are shown.

Substrate	Specific activity [U (mg protein) ⁻¹]
Acid-swollen cellulose	0.009
Avicel	0.006
Barley β -glucan	1.4
CMC*	0.2
Cotton	0.003
Filter paper	0.0006
Laminarin	0.004
Lichenan	0.2
4-Methylumbelliferyl β -D-cellobioside	0.02
4-Methylumbelliferyl β -D-glucoside	0
p-Nitrophenyl β -D-cellobioside	0.07
p-Nitrophenyl β -D-glucoside	0
Xylan	0.006

* There was no significant difference between the activity displayed against high-, medium- and low-viscosity CMC.

When the *Sca*I–*Cla*I fragment cloned in pUC18 was transformed into *E. coli* JM83 already harbouring pNM52, a multicopy plasmid carrying the *lac*^a gene, transcription of the *celA* gene was brought under the control of *lacZp*, and high-level synthesis was induced by the addition of IPTG (0.1 mM) to an exponential-phase culture; about 25% of the EGA synthesized under these conditions was secreted into the periplasm, and the total yield of EGA was considerably (6 to 10 times) higher than for *E. coli* JM83 harbouring the 2.3 kb *Eco*RI fragment cloned into pUC18.

Substrate specificity of EGA

With respect to substrate specificity, EGA from *B. fibrisolvens* strain A46 was quite similar to End1 of *B. fibrisolvens* H17c (Berger *et al.*, 1989). Enzyme contained in cell-free extracts made from *E. coli* harbouring *celA* was most active in hydrolysing barley β -glucan (70–75% β -1,4-; 25–30% β -1,3-linkages) (Table 1). Lichenan, a

-200

ACAATATTAGAAATAAAAGAGTATGTTAATTCTCTTAGCAACGAGGAATTAGATAAGTTTATCGACGAGG

-100

TAGATTTTGCAGATTATGACTTCGATTTAGAGGCTCTTGATTGCTTACAAGCTCACGTCTTTACGACTA

+1

CATTCAGTACTCACACAATGGAGAGGCTACTATATCATTTGACTAATTGGGAGGAATTATAATGGTTAGTA

M V S

AGAAGCAGAAGTTCTTAACAGTCATACTTGTAATAGTTCTTGCTATAGTTATTGTTGGCGGAGTTTTTGG
 K K Q K F L T V I L V I V L A I V I V G G V F G

100

AATCAGCTTTGTTAAGGGCAGGGTTACTTTCCCTTGGCAGCTTCAAATAGCGAAGCTAAACTGAACAG
 I S F V K G R V T F P W Q L Q N S E A K T E Q

200

GTGAAGGAGCCTGCAAAGGAAGAGCCTAAGCTTGTAATAAAGGAAAAGAAACAGGACGAGTCCGCAAAAA
 V K E P A K E E P K L V I K E K K Q D E S A K

AGGAACAAGAGCTTAAGAAAGCCAAAGAAGAGGCAGAGGCAGCTGTAGAAAAAGAAACAGAAAAAACTGA
 K E Q E L K K A K E E A E A A V E K E T E K T E

300

AGAAGAGCCTGTTGATAATCTTCTTAACGATATGAAGCTTAAATATTATGGCAAGCTTGCTGTTGAGGGT
 E E P V D N L L N D M K L K Y Y G K L A V E G

400

TCTCATCTTGATGATGCAGATGGCCATGAGGTTCTTCTTATGGGCGTCAGCACTCATGGAATTAAGTGGT
 S H L V D A D G H E V L L M G V S T H G I N W

ATCCAGAATATGCTTCAGCAGAGACTATCAAATCCTTAAGAGATACCTGGGGCATCAATGTCATCAGACT
 Y P E Y A S A E T I K S L R D T W G I N V I R L

500

TGCTATGTACACAAGCGACTATAATGGTTATTGCGTGGCAGGTAAGGAAAACAGGAAAAGCTTAAGGAT
 A M Y T S D Y N G Y C V A G K E N Q E K L K D

600

ATTATTGATGATGCAGTAGAAGCTGCAACTGATAACGATATGTATGTCATTATTGACTGGCACACATTAA
 I I D D A V E A A T D N D M Y V I I D W H T L

700

ATGATGCAGACCCTAACGAATATAAGGCTGATGCTATTGAGTTCTTCGGTGAAATGGTGCGCAAATACAA
 N D A D P N E Y K A D A I Q F F G E M V R K Y K

GGATAACGAGAATGTTATCTACGAAATTTGTAACGAACCAAATGGAGATACCACTTGGAATGACGTTAGA
 D N E N V I Y E I C N E P N G D T T W N D V R

800

AGATACGCTAACGAGGTTATTCCGGTCATCAGAAATGTTGATGCTATCATTTTAGTGGAACACCTAAGT
 R Y A N E V I P V I R N V D A I I L V G T P K

900

GGGCTACCGATTAGATTTCAGTTTTAGATAAACCACTTGATTTTGATAATATCATGTACACATATCATTT
W A T D L D S V L D K P L D F D N I M Y T Y H F

TTATGCAGGCACTCATCATAAGGCTGAAAGAAACGCTCTTAGAGATGCGTTGGATGAAGGTCTTCCAGTA
Y A G T H H K A E R N A L R D A L D E G L P V

1000

TTCATTTTCAGAGTATGGTTTGGTAGATGCTGATGGAGATGGAACTTGAATGAGAAAGAGGCTGATTATT
F I S E Y G L V D A D G D G N L N E K E A D Y

1100

GGTATGATATGATTCGCAAGGAATATGGTGTATCTTCTTGCATGTGGAATCTTTCAAACAAGGATGAAGG
W Y D M I R K E Y G V S S C M W N L S N K D E G

TTCTGCCATGATTAATGCTGATTGTGATAAGCTTTTCAGATTTTACAGAAGAAGACCTTTCTGAATCAGCA
S A M I N A D C D K L S D F T E E D L S E S A

1200

ATGTGGTTGATTGATCAAATTTTCACAGCTTAAGCACAGTGATTTAGAACAAGGTGTGGATTGGATCACAC
M W L I D Q I S Q L K H S D L E Q G V D W I T

1300

CTGAGAATAATAATAGATAGGAGATATTTAGCAATGACAACTAATGAGAAAATCGCAGC
P E N N N R

Fig. 2. Nucleotide sequence of the *celA* gene from *B. fibrisolvens* strain A46. The derived amino acid sequence of EGA is given in one-letter code. The signal peptide is indicated by a solid underline, and the broken underline shows the extent of the experimentally-determined N-terminal sequence of mature EGA. The putative Shine-Dalgarno sequence is boxed.

similar mixed-linkage polysaccharide from *Cetraria islandica* was degraded to about the same extent as CMC. The enzyme also released reducing sugar during prolonged incubation with recalcitrant forms of cellulose (acid-swollen cellulose, Avicel, filter paper and cotton), but the activity against these substrates amounted to only 0.1% to 4% of the activity against CMC. A low level of activity was also observed against the soluble fraction from oat spelt xylan and against the β -1,3-linked polysaccharide laminarin (Table 1). Release of reducing sugar from CMC was accompanied by a rapid decline in substrate viscosity, characteristic of endo- β -1,4-glucanase activity. Cleavage of the aglycone bond in *p*-nitrophenyl β -D-cellobioside and 4-methylumbelliferyl β -D-cellobioside suggests that EGA also has some endoglucanase activity, but numerous other prokaryotic exoglucanases and xylanases also show activity against these substrates, so some caution is advisable in interpreting these results. No activity was detected against the aryl-glucosides *p*-nitrophenyl β -D-glucoside and 4-methylumbelliferyl β -D-glucoside.

Nucleotide sequence of *celA*

Translation of the nucleotide sequence of the 2.3 kb *EcoRI* fragment cloned from *B. fibrisolvens* strain A46 revealed a single open reading frame (ORF) of 1296 bp coding for a polypeptide of 432 amino acid residues, with a molecular mass of 48863 Da (Fig. 2). The putative translation initiation codon was immediately preceded by a potential ribosome-binding site (GGGAGGA), and the N-terminus of the polypeptide had the general characteristics of a prokaryotic signal peptide, being composed of a sequence containing 19 hydrophobic amino acids, preceded by a shorter sequence of positively-charged residues. Confirmation that the ORF codes for EGA was obtained experimentally by determining the molecular size and N-terminal sequence of EGA synthesized in *E. coli* JM83 harbouring full-length *celA*. Endoglucanase activity from cell-free extract, partially purified by anion-exchange chromatography and fractionated by SDS-PAGE, contained a major band corresponding to a molecular mass of 47 kDa (data not

Table 2. Codon usage of *B. fibrisolvens* strain A46 *celA* gene and *B. fibrisolvens* H17c *end-1* gene

Amino acid	Codon	<i>celA</i>	<i>end-1</i>	Amino acid	Codon	<i>celA</i>	<i>end-1</i>
F	TTT	5	12	S	TCT	5	12
F	TTC	5	8	S	TCC	2	6
L	TTA	7	3	S	TCA	7	16
L	TTG	4	1	S	TCG	0	0
L	CTT	21	14	P	CCT	7	17
L	CTC	0	2	P	CCC	0	2
L	CTA	0	0	P	CCA	4	10
L	CTG	0	2	P	CCG	1	6
I	ATT	15	13	T	ACT	8	10
I	ATC	9	15	T	ACC	3	1
I	ATA	4	7	T	ACA	8	23
M	ATG	11	13	T	ACG	0	1
V	GTT	15	24	A	GCT	17	14
V	GTC	5	1	A	GCC	2	5
V	GTA	8	20	A	GCA	12	15
V	GTG	5	2	A	GCG	1	2
Y	TAT	14	19	C	TGT	2	2
Y	TAC	5	9	C	TGC	2	6
Ter	TAA	0	1	Ter	TGA	0	0
Ter	TAG	1	0	W	TGG	10	14
H	CAT	6	5	R	CGT	0	3
H	CAC	2	3	R	CGC	2	0
Q	CAA	4	0	R	CGA	0	0
Q	CAG	7	13	R	CGG	0	0
N	AAT	16	26	S	AGT	2	3
N	AAC	11	17	S	AGC	4	9
K	AAA	12	6	R	AGA	8	13
K	AAG	24	19	R	AGG	1	4
D	GAT	34	32	G	GGT	9	9
D	GAC	6	12	G	GGC	7	10
E	GAA	27	12	G	GGA	7	13
E	GAG	16	10	G	GGG	0	1

shown). The N-terminal sequence of this protein (VTFP-QLQNSEAKTEQ) agreed with the sequence deduced for residues 35 to 50 of EGA (Fig. 2). The G+C content of *celA* was 38.9 mol%; this compares with figures of 42 and 41.3 mol% reported for the G+C ratio of the *B. fibrisolvens* H17c genome (Mannarelli, 1988) and the *end-1* gene (Berger *et al.*, 1989) respectively. Codons having G or C in the third position occurred in *celA* at frequencies of 18.6% and 15.2% respectively, indicating some bias towards A and T in the 'wobble' position. Synonymous codons were not utilized with equivalent frequencies in *celA*, but a comparison between this gene and *end-1* of *B. fibrisolvens* H17c revealed that the two genes were quite similar in their codon preferences (Table 2). With two exceptions (AGA, GGA), both *celA* and *end-1* make little use of those codons corresponding to the minor tRNAs which in *E. coli* are presumed to act as modulators (Minton *et al.*, 1986).

Sequence homology

Comparison of sequences revealed that residues 151 to 283 of EGA had approximately 50% homology with N-terminal regions of endoglucanases from *Bacillus* sp. (Fukumori *et al.*, 1989), *Clostridium acetobutylicum* (Zappe *et al.*, 1988) and *Erwinia chrysanthemi* (Guisseppi *et al.*, 1988) (Fig. 3). No homology was found with End-1 from *B. fibrisolvens* H17c. A classification based on hydrophobic cluster analysis has recently been employed to assign the cellulases of prokaryotic and lower eukaryotic organisms to six distinct families, some of which can be subdivided into subfamilies (Henrissat *et al.*, 1989; Béguin, 1990). The subfamily A2 contains endoglucanases from *Bacillus* spp. and from *C. acetobutylicum* and *Er. chrysanthemi*. These endoglucanases show extensive homology over the entire length of their N-terminal catalytic domain, which comprises 300 to 400

EGA	151	ETIKS	L	RDT	W	GIN	V	I	RLAM	YTS	DYN	GY	CVA
CEL-A	73	DSLKW	L	RDD	W	GIT	V	F	RAAM	YTSS	G	GY	IE
CEL-B	71	ESMKW	L	RDD	W	GIT	V	F	RAAM	YTSS	G	GY	IE
Bs1	77	DSLKW	L	RDD	W	GIT	V	F	RAAM	YTAD	G	GY	I
Bs2	76	DSLKW	L	RDD	W	GIT	V	F	RAAM	YTAD	G	GY	ID
EGZ	72	DTVAS	L	LKD	W	KSS	I	V	RAAM	GVQESG	GY	LQ	
Ca1	82	DSMKF	L	RDK	W	GMN	V	I	RAAM	YTNE	G	GY	ISN
CEL-C	114	NAFAA	L	AND	W	GSN	V	I	RLAL	YIGE	N	GY	PR
EGA	181	GKENQEK	L	K	DI	I	DDA	VE	A	A	T	D	ND
CEL-A	101	D PS V	K	NK	V	KEA	VE	A	A	I	D	LG	IYVII
CEL-B	99	D PS V	K	EK	V	KEA	VE	A	A	I	D	LG	IYVII
Bs1	104	DNPS V	K	NK	V	KEA	VE	A	A	K	E	LG	IYVII
Bs2	104	NPSVKNK	K	KV		KEA	VE	A	A	K	E	LG	IYVII
EGZ	101	D PAGNKA	K	V		ERV	VD	A				ND	MYAII
Ca1	111	PSSQKEKI	K	KI	V	QDA	ID			L	N	MYVII	D W
CEL-C	142	N PLIE	K		V	YAG	IE	I	A	K	E	ND	MYVII
EGA	211	H TLNDAD	PN	EY				KAD	A	IQ	FF		
CEL-A	127	H ILSDND	PN	IY				KEE	A	DE	FF		
CEL-B	125	H ILSDND	PN	IY				KEE	A	KD	FF		
Bs1	137	H ILNDGN	PN	QN				KEK	A	KE	FF		
Bs2	132	H ILNDGN	PN	QN				KEK	A	KE	FF		
EGZ	126	H S HSAE	N	N				RSE	A	IR	FF		
Ca1	137	H ILSDNN	PN	TY				KEQ	A	KS	FF		
CEL-C	168	H VHRPGD	PN	ADY	QGGVN	EDGE	EYLG	A	KD	FF			
EGA	230	GEMVRK	Y	KDNEN	VIYEI	C	NEP	NG	DTT	W	N		
CEL-A	145	DEMSAL	Y	GDYPN	VIYEI	A	NEP	TG	HNV	RW	D		
CEL-B	143	DEMSEL	Y	GDYPN	VIYEI	A	NEP	NGSDVT	WDN				
Bs1	151	KEMSSL	Y	GNTPN	VIYEI	A	NEP	NG	DVN	WKR			
Bs2	150	KEMSSL	Y	GNTPN	VIY I	A	NEP	NG	DVN	WKR			
EGZ	141	QEMARK	Y	GNKPN	VIYEI	Y	NEP	L	QVS	W	S		
Ca1	155	QEMAE	Y	GKYSN	VIYEI	C	NEP	NG	GTN	WAN			
CEL-C	198	LHIAEK	Y	PNDPH	LIYEL	A	NEP	SSNSSG	G	P			
EGA	258	D VRRYANEV	IPV	IR	N							VD	
CEL-A	173	SHIKPYAEEV	IPV	IR	A							ND	
CEL-B	172	Q IKPYAEEV	IPV	IR	N							ND	
Bs1	179	D IKPYAEEV	ISV	IR	K							ND	
Bs2	177	D IKPYAEEV	ISV	IR	K							ND	
EGZ	167	NTIKPYAE	AVIS	IR	A							ID	
Ca1	183	D VRPYANYI	IPA	IR	A							ID	
CEL-C	226	G I TNDEDG	WEA	VR	EYAQPIVD	ALRDS	GNA						
EGA	275	A IILVGT	PK										
CEL-A	189	PNN IVIVGT	A										
CEL-B	188	PNN IIVVGT	GT										
Bs1	195	PDN IIVVGT	GTW										
Bs2	193	PDN IIVVGT	GT										
EGZ	183	PDN LIIVGT	PSW										
Ca1	198	PNN IIVVGT	S										
CEL-C	255	EDN IIVVGS	PNW										

Fig. 3. Amino acid sequence alignments for endoglucanases from *B. fibrisolvens* strain A46 (EGA; this study), *Bacillus* sp. strain N-4 (CEL-A, CEL-B and CEL-C; Fukumori *et al.*, 1989), *Bacillus subtilis* PAP115 (Bs1; Zappe *et al.*, 1988), *B. subtilis* DLG (Bs2; Robson & Chambliss, 1987), *Erwinia chrysanthemi* (EGZ; Guiseppe *et al.*, 1988) and *Clostridium acetobutylicum* (Ca1; Zappe *et al.*, 1988). Residues which are identical or similar in structure in all sequences compared are enclosed within a box.

amino acid residues. Our finding that EGA displays significant homology with these enzymes suggests that the *celA* gene of *B. fibrisolvens* strain A46, like the other genes in the A2 subfamily, evolved from a common ancestral gene. Alternatively, conservation of sequence in cellulases from such a variety of species may have resulted from the convergent evolution of structures having a common function.

To date, the primary structures have been determined for five endoglucanases produced by anaerobic rumen bacteria. Cellulases from *Ruminococcus albus* are highly conserved (Poole *et al.*, 1990), suggesting a common ancestral gene and strong selection pressure for the retention of conserved sequences. In contrast, the endoglucanases (EGA and End1) produced by two strains of *B. fibrisolvens* clearly belong to different cellulase subfamilies. This lack of homology between *B. fibrisolvens* cellulases could reflect the apparent ease with which genetic exchange occurs between members of this genus and other bacteria (Teather, 1985), thus providing a mechanism for the acquisition of cellulase genes from diverse sources. Alternatively, it may be further evidence of the inadequacy of the classification applied to rumen bacteria. If, as seems likely, rumen butyrivibrios comprise a genetically heterogeneous group consisting of two or more genera and numerous species (Hazlewood & Teather, 1988) it would not be particularly surprising to find that their cellulase enzymes derive from more than one evolutionary source.

Structure of EGA in relation to function

There is now clear evidence that the catalytic function of some prokaryotic cellulases is vested in a clearly defined independent catalytic domain which may account for less than half of the total gene (Béguin, 1990; Gilbert *et al.*, 1990). In such cases, a substantial non-essential portion of the gene may be deleted without affecting the catalytic function of the encoded protein. To determine whether this is the case for EGA from *B. fibrisolvens* strain A46, truncated derivatives of *celA* were generated by digesting either the 5' or the 3' end of the gene with *Bal31* exonuclease. Removal of 240 bp from the 5' end or 190 bp from the 3' end of *celA* resulted in complete inactivation of EGA. These results indicate that virtually the whole polypeptide is required to maintain the functional integrity of EGA. The apparent absence of a cellulose-binding domain distinct from the active site is supported by cellulose-binding studies (data not shown) which revealed that recombinant EGA produced by *E. coli* harbouring full-length *celA* did not bind strongly to Avicel in the manner typical of endoglucanases having distinct cellulose-binding domains (Gilbert *et al.*, 1990; Ong *et al.*, 1989).

Aspects of the molecular architecture of cellulases from the rumen species *B. fibrisolvens*, *R. albus* and *Bacteroides succinogenes* have now been reported. To date, only the latter organism has been shown to produce an endoglucanase containing a well-defined cellulose binding domain which is distinct from the catalytic centre (McGavin & Forsberg, 1989). From an evolutionary viewpoint, the prospect of there being major differences between the cellulase systems of rumen bacteria is surprising, since all organisms evolving in the rumen have presumably been subjected to similar environmental influences. All three of these species are widely distributed in ruminants and are effective in degrading dietary cellulose. *In vivo* each is found colonizing plant tissue, but unlike *Bac. succinogenes*, *R. albus* and *B. fibrisolvens* produce cellulases which have not as yet been shown to contain functional cellulose-binding domains distinct from the active site, and form only a loose association with plant cell walls (Chesson & Forsberg, 1988).

Future work by this group will focus on the mechanisms by which different bacterial cellulase systems hydrolyse plant cell wall polysaccharides in the rumen.

We wish to thank the AFRC (Grant no. LRG 138) for supporting this work.

References

- BÉGUIN, P. (1990). Molecular biology of cellulose degradation. *Annual Review of Microbiology* **44** (in the Press).
- BERGER, E., JONES, W. A., JONES, D. T. & WOODS, D. R. (1989). Cloning and sequencing of an endoglucanase (*end1*) gene from *Butyrivibrio fibrisolvens* H17c. *Molecular and General Genetics* **219**, 193–198.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- BOYER, H. W. & ROULLAND-DUSOIX, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *Journal of Molecular Biology* **41**, 459–472.
- CHESSON, A. & FORSBERG, C. W. (1988). Polysaccharide degradation by rumen microorganisms. In *The Rumen Microbial Ecosystem*, pp. 251–284. Edited by P. N. Hobson. London & New York: Elsevier Applied Science.
- FUKUMORI, F., KUDO, T., SASHIHARA, N., NAGATA, Y., ITO, K. & HORIKOSHI, K. (1989). The third cellulase of alkalophilic *Bacillus* sp. strain N-4: evolutionary relationships within the *cel* gene family. *Gene* **76**, 289–298.
- GILBERT, H. J., BLAZEK, R., BULLMAN, H. M. S. & MINTON, N. P. (1986). Cloning and expression of the *Erwinia chrysanthemi* asparaginase gene in *Escherichia coli* and *Erwinia caratovora*. *Journal of General Microbiology* **132**, 151–160.
- GILBERT, H. J., JENKINS, G., SULLIVAN, D. A. & HALL, J. (1987). Evidence for multiple carboxymethylcellulase genes in *Pseudomonas fluorescens* subsp. *cellulosa*. *Molecular and General Genetics* **210**, 551–556.
- GILBERT, H. J., HALL, J., HAZLEWOOD, G. P. & FERREIRA, L. M. A. (1990). The N-terminal region of an endoglucanase from *Pseudomonas fluorescens* subspecies *cellulosa* constitutes a cellulose-binding domain that is distinct from the catalytic centre. *Molecular Microbiology* **4**, 759–767.

- GUISEPPI, A., CAMI, B., AYMERIC, J.-L., BALL, G. & CREUZET, N. (1988). Homology between endoglucanase Z of *Erwinia chrysanthemi* and endoglucanases of *Bacillus subtilis* and alkalophilic *Bacillus*. *Molecular Microbiology* **2**, 159–164.
- HALL, J., HAZLEWOOD, G. P., BARKER, P. J. & GILBERT, H. J. (1988). Conserved reiterated domains in *Clostridium thermocellum* endoglucanases are not essential for catalytic activity. *Gene* **69**, 29–38.
- HAZLEWOOD, G. P. & TEATHER, R. M. (1988). The genetics of rumen bacteria. In *The Rumen Microbial Ecosystem*, pp. 323–342. Edited by P. N. Hobson. London & New York: Elsevier Applied Science.
- HAZLEWOOD, G. P., DAVIDSON, K., CLARKE, J. H., DURRANT, A. J., HALL, J. & GILBERT, H. J. (1990). Endoglucanase E, produced at high level in *Escherichia coli* as a *lacZ* fusion protein, is part of the *Clostridium thermocellum* cellulosome. *Enzyme and Microbial Technology* (in the Press).
- HENRISSAT, B., CLAEYSSSENS, M., TOMME, P., LEMESLE, L. & MORNON, J.-P. (1989). Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81**, 83–95.
- HOBSON, P. N. (1969). Rumen bacteria. *Methods in Microbiology* **3B**, 133–149.
- HOLMES, D. S. & QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**, 193–197.
- HUNKAPILLAR, M. W., HEWICK, R. E., DREYER, W. J. & HOOD, L. E. (1983). High sensitivity sequencing with gas-phase sequencer. *Methods in Enzymology* **91**, 399–413.
- KOPECNY, J. (1986). Cellulolytic enzymes of *Butyrivibrio fibrisolvens*. *XIV International Congress of Microbiology*, Abstract P.B17-25, p. 104.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MANNARELLI, B. M. (1988). Deoxyribonucleic acid relatedness among strains of the species *Butyrivibrio fibrisolvens*. *International Journal of Systematic Bacteriology* **38**, 340–347.
- MCGAVIN, M. & FORSBERG, C. W. (1989). Catalytic and substrate binding domains of endoglucanase 2 from *Bacteroides succinogenes*. *Journal of Bacteriology* **171**, 3310–3315.
- MILLER, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* **31**, 426–428.
- MINTON, N. P., BULLMAN, H. M. S., SCAWEN, M. D., ATKINSON, T. & GILBERT, H. J. (1986). Nucleotide sequence of the *Erwinia chrysanthemi* NCPPB1066 L-asparaginase gene. *Gene* **46**, 25–33.
- NORRANDER, J., KEMP, T. & MESSING, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101–116.
- ONG, E., GREENWOOD, J. M., GILKES, N. R., KILBURN, D. G., MILLER, R. C. & WARREN, R. A. J. (1989). The cellulose-binding domains of cellulases: tools for biotechnology. *Trends in Biotechnology* **7**, 239–243.
- ORPIN, C. G., MATHIESEN, S. D., GREENWOOD, Y. & BLIX, A. S. (1985). Seasonal changes in the ruminal microflora of the high-arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*). *Applied and Environmental Microbiology* **50**, 144–151.
- POOLE, D. H., HAZLEWOOD, G. P., LAURIE, J. I., BARKER, P. J. & GILBERT, H. J. (1990). Nucleotide sequence of the *Ruminococcus albus* SY3 endoglucanase genes *celA* and *celB*. *Molecular and General Genetics* (in the Press).
- ROBSON, L. M. & CHAMBLISS, G. H. (1987). Endo- β -1,4-glucanase gene of *Bacillus subtilis* DLG. *Journal of Bacteriology* **169**, 2017–2025.
- ROMANIEC, M. P. M., CLARKE, N. G. & HAZLEWOOD, G. P. (1987). Molecular cloning of *Clostridium thermocellum* DNA and the expression of further novel endo- β -1,4-glucanase genes in *Escherichia coli*. *Journal of General Microbiology* **133**, 1297–1307.
- SANGER, F., COULSON, A. R., BARRELL, B. G., SMITH, A. J. H. & ROE, B. A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *Journal of Molecular Biology* **143**, 161–178.
- SEDMAN, J. J. & GROSSBERG, S. E. (1977). A rapid, sensitive and versatile assay for protein using Coomassie Brilliant Blue G250. *Analytical Biochemistry* **79**, 544–552.
- STADEN, R. (1980). A new computer method for the storage and manipulation of DNA gel reading data. *Nucleic Acids Research* **16**, 3673–3694.
- TABOR, S. & RICHARDSON, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 polymerase. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 4767–4771.
- TEATHER, R. M. (1985). Application of gene manipulation to rumen microflora. *Canadian Journal of Animal Science* **65**, 563–574.
- TEATHER, R. M. & WOOD, P. J. (1982). Use of Congo Red polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology* **43**, 777–780.
- WOOD, T. M. (1971). The cellulase of *Fusarium solani*. Purification and specificity of the β -(1 $\rightarrow$ 4)-glucanase and the β -D-glucanase components. *Biochemical Journal* **121**, 353–362.
- YOUNG, R. A. & DAVIS, R. W. (1983). Efficient isolation of genes by using antibody probes. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 1194–1198.
- ZAPPE, H., JONES, W. A., JONES, D. T. & WOODS, D. R. (1988). Structure of an endo- β -1,4-glucanase gene from *Clostridium acetobutylicum* P262 showing homology with endoglucanase genes from *Bacillus* spp. *Applied and Environmental Microbiology* **54**, 1289–1292.