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Transcriptional Induction and Expression of the Endoglucanase *celA* Gene from a Ruminal *Clostridium* sp. ("*C. longisporum*")

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Northern (RNA) blot analysis of RNA from *Clostridium* sp. revealed induction of transcription of the *celA* gene when barley β-glucan was used as carbon source, while no *celA* mRNA was detected after growth on cellobiose. Western blots (immunoblots), prepared by using a rabbit antiserum raised against CelA protein purified from *Escherichia coli*, revealed the extracellular location of CelA in *Clostridium* sp. Despite the absence of detectable *celA* mRNA, significant quantities of CelA were detected in the culture supernatant during growth on cellobiose. This finding indicated a low constitutive expression of *celA*. A 6.7-fold increase in the total β-glucanase specific activity in the extracellular fraction was observed during growth on β-glucan. The transcriptional start site of *celA* was mapped by extension and was found to be the same in *Clostridium* sp. and in *E. coli* expressing the cloned *celA* gene. A consensus *E. coli* −10 promoter region (AATAAT), but not a −35 promoter region, could be identified. Two direct repeats (TATTGAATTTAT) separated by 15 nucleotides flank the region where the consensus −35 promoter regions would have been. The size of the *celA* mRNA transcript corresponded with the size of the open reading frame. A potential stem-loop structure was found 18 nucleotides downstream of the 3′ stop codon, which could be responsible for termination of transcription.

The regulation of cellulase production in the competitive and fluctuating rumen environment is important for the survival of the ruminal microflora. A large number of cellulolytic and hemicellulolytic enzymes are produced by ruminal microorganisms. Many of these enzyme systems have been found to be regulated, and induction by their substrates, or degradation products thereof, has been found in *Butyrivibrio fibrisolvens* (29), *Ruminococcus flavefaciens* (5, 7, 28), a *Piromyces*-like ruminal fungus (21), *Neocallimastix frontalis* (2), and *Neocallimastix patriciarum* (30, 31). Catabolite repression by more readily fermentable substrates is used to down-regulate the enzyme levels (3, 13). Constitutive expression of total endoglucanase activity and of selected enzymes has been reported for *Fibrobacter succinogenes* (15, 18), although individual enzymes seem to be regulated. A number of cellulase genes from ruminal microorganisms have been cloned, but their transcriptional regulation in the original hosts has been investigated in only a few cases, e.g., *celA*, *celB*, *celC*, and *celD* from *R. flavefaciens* (5, 28) and *celA*, *celB*, *celC*, and *celD* from *N. patriciarum* (30, 31).

The gene encoding endoglucanase CelA from the ruminal bacterium *Clostridium* sp. ("*C. longisporum*") has been cloned and sequenced, and the product has been characterized in *Escherichia coli* (20). Here we report the transcriptional induction by β-glucan of *celA* in *Clostridium* sp.

Transcriptional induction of the *celA* gene in *Clostridium* sp. *Clostridium* sp. was cultured in an anaerobic cabinet on nonrumen fluid medium containing either 0.1% (wt/vol) cellobiose or 0.1% (wt/vol) barley β-glucan (Sigma) as a carbon source (20). A third culture was grown on cellobiose supplemented with sophorose (1 mg/ml), which has been reported to act as an inducer of cellulases in *Cellulomonas fimi* (25) and *Trichoderma viride* (17). For the preparation of total RNA, cultures were harvested at late log phase; mid-log-phase cultures were not a feasible option because of low cell densities and concomitant low RNA yields. *E. coli* BL21 (F^- *hsdS gal*) (26) was used to prepare RNA for various controls in the Northern (RNA) blot and the primer extension experiments. The cloning of the *celA* gene and the construction of plasmids pCMC, pCML, and pCMJII (Fig. 1) are described elsewhere (20).

The Northern blot clearly indicated that transcription of the *celA* gene was induced when β-glucan was used as a carbon source (Fig. 2, lane 2) but not when cellobiose was provided as carbon source (lane 4) or when sophorose was present (lane 3). The faint bands visible in lane 1 could be due to nonspecific hybridization of the probe to certain RNAs present in the standards. At least two bands are visible in lane 2: a dark band corresponding to full-length *celA* mRNA transcripts (1,650 bases) and more rapidly migrating, fainter bands which might be due to premature transcription termination or mRNA degradation. The transcriptional induction of the *celA* gene when *C. longisporum* was cultured on β-glucan medium is not unexpected since CelA was found to be an endo-β-glucanase with a high substrate specificity for barley β-glucan (20).

Fifteen bases downstream of the stop codon of the *celA* gene, a stem-loop structure with a minimum free energy of −19.8 kcal (ca. −82.8 kJ)/mol (9) which probably constitutes a transcription termination signal was identified. Judging from the size of the full-length *celA* transcripts on the Northern blot, it appears that *celA* forms a monocistronic transcription unit since the *celA* open reading frame is 1,548 bp long. Most cellulase genes have been found to be transcribed in monocistronic units (3), and stem-loop structures have been identified at the 3′ ends of many cellulase genes (4, 11, 22).

Identification of the transcriptional start site. Figure 3a shows the results of two separate primer extension and termination reactions separated by a sequencing ladder and flanked by ddA and ddT termination ladders in lanes 2 and 11, respectively. Lane 1 constitutes a negative control, in which RNA isolated from *E. coli* BL21 was hybridized with pCMP single-

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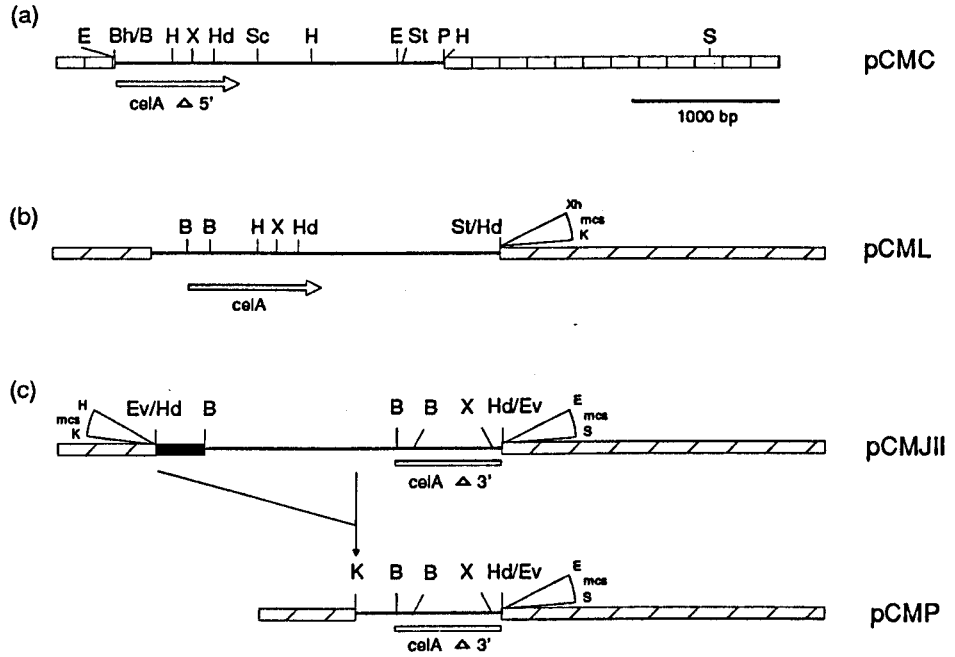

FIG. 1. Restriction maps of pCMC (a), pCML (b), and pCMJII and pCMP (c). The construction of the first three plasmids has been described previously (20). pCMP was obtained by exonuclease III shortening of pCMJII. The position of the *celA* gene is indicated by the large hollow arrow, and truncations at the 5′ and 3′ ends of the gene are shown. Restriction sites of selected endonucleases: B, *Bgl*II; Bh, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hd, *Hin*dII; K, *Kpn*I; P, *Pst*I; S, *Sac*I; Sc, *Sca*I; St, *Stu*I; X, *Xba*I; Xh, *Xho*I. mcs, multiple cloning site containing other unique restriction sites as indicated by the wedge-shaped segment. Bh/B, Hd/Ev, and St/Hd are the *Bam*HI-*Bgl*II, *Hin*dII-*Eco*RV, and the *Stu*I-*Hin*dII cloning junctions, respectively.

stranded DNA (ssDNA) and the end-labelled primer. Lanes 3 and 10 show termination bands formed in the vicinity of the 5′ end of the *celA* open reading frame by the T4 DNA polymerase on a pCMP ssDNA template annealed with *E. coli* BL21 (pCML) RNA. The termination band in lane 10 as indicated by the arrow is very faint because very little pCMP ssDNA was present in the reaction. Lanes 4 and 9 show termination bands formed by the T4 DNA polymerase on the pCMP ssDNA template annealed with *Clostridium* sp. RNA isolated from cells grown on barley β-glucan to induce the *celA* gene. The major termination bands in lanes 3, 4, 9, and 10 clearly correspond with the T bands in lanes 8 and 11. Minor termination bands were ignored, as they did not constitute definite signals compared. As the major bands indicate the length of the extension products which terminated one base upstream of the first nucleotide of the *celA* mRNA molecules hybridized to the ssDNA template, the C nucleotide 27 bases upstream of the ATG start codon of the *celA* gene was identified as the first nucleotide of the *celA* mRNA (Fig. 3b). Another important finding was that the mRNA transcripts in *Clostridium* sp. and in *E. coli* started at the same base.

It was not determined whether *C. longisporum* utilized separate transcription initiation sites for the induction of *celA* during growth on β-glucan and for the low constitutive expression of *celA* during growth on cellobiose. The main reason for this was the inability to detect *celA* mRNA on Northern blots during growth on cellobiose. In the *Ruminococcus albus* EgI gene, transcription is initiated 59 bp upstream of the ATG codon in both the parent strain and *E. coli* (23), but *celA* from *Prevotella ruminicola* utilizes different transcription initiation sites (27). *Clostridium thermocellum celA* and *celD* (4, 19) and *Cellulomonas fimi cenA* and *cenB* (11, 12) were shown to make use of two different promoters, depending on the host or on the culture conditions.

Localization of CelA in *Clostridium* sp. The Western blot (immunoblot) (Fig. 4b) shows that CelA was predominantly present in the culture supernatant in *Clostridium* sp. This result was predicted by the presence of a signal sequence at the 5′ end of the *celA* gene (20). The positive control in lane 1 contains purified CelA protein and shows two bands due to proteolytic cleavage of the full-length CelA protein in the linker region separating the catalytic domain and the cellulose-binding domain (CBD) (20). The 55-kDa band represents full-length CelA, while the 44-kDa band represents the catalytic domain without the CBD. The molecular mass of mature CelA predicted from the sequence is 55.1 kDa. The full-length *E. coli*-derived CelA protein (lane 1) migrates more slowly than the material in culture supernatants from β-glucan-induced *Clostridium* sp. (lane 2). A simple explanation for this apparent difference in molecular mass is a difference in signal peptide cleavage of CelA in *Clostridium* sp. and in *E. coli*. The lower band in lane 2 (Fig. 4b) is smaller than the 44-kDa band in lane 1. It could be a proteolytic degradation derivative of CelA that is processed differently in *Clostridium* sp. than in *E. coli*, or it could be another protein which is antigenically related to CelA.

Induction of CelA as seen on the Western blot and the zymogram. Zymograms (activity gels) containing 0.1% (wt/vol) carboxymethyl cellulose were performed as described elsewhere (20). Both the Western blot and the zymogram (Fig. 4b and c, respectively) show that CelA and other carboxymethyl cellulases were present in both uninduced and induced *Clostridium* sp. cultures, although the quantities were considerably higher in the latter. In contrast, in the transcriptional induction study, no *celA* mRNA was detected in the uninduced culture. These data indicate that the low levels of constitutively expressed *celA* mRNA were not detectable, and it is possible that the nonradioactively labelled probe used on the Northern blot

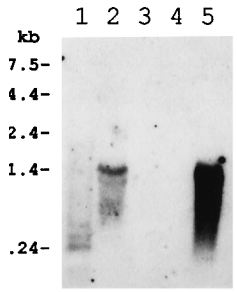

FIG. 2. Northern blot showing transcriptional induction of *celA*. Total RNA was prepared from *E. coli* BL21(pCML), which contains the *celA* gene on a high-copy-number vector for use as a positive control. RNA from *Clostridium* sp. and *E. coli* cultures was prepared by using a protocol slightly adapted from that of Aiba et al. (1). For the Northern blots, RNA preparations were mixed with RNA-denaturing tracking dye (8) and 1 μl of ethidium bromide (1 mg/ml) was added, after which samples were run on a 0.8% (wt/vol) agarose gel with Tris-borate-EDTA electrophoresis buffer. The RNA was transferred onto a Hybond-N+ charged nylon membrane (Amersham), using 0.05 M NaOH for the alkaline transfer in a capillary blot. The membrane was probed with a nonradioactive digoxigenin-labelled 1,930-bp *Eco*RI restriction fragment of pCMC (Fig. 1) as instructed by the manufacturer (Boehringer Mannheim). Probe bound to the membrane was detected with a chemiluminescence reaction utilizing AMPPD [3-(2′-spiroadamantane)4-methoxy-4-(3′-phosphoryloxy)-phenyl-1,2-dioxetane] (Boehringer Mannheim). The RNA molecular standards were obtained from Gibco BRL. Lanes: 1, RNA size markers; 2 to 4, *Clostridium* sp. RNA from cultures grown on β-glucan, cellobiose with sophorose, and cellobiose, respectively; 5, *E. coli* BL21(pCML) RNA. Lanes 2 to 5 contained 37, 20, 20, and 11 μg of RNA, respectively.

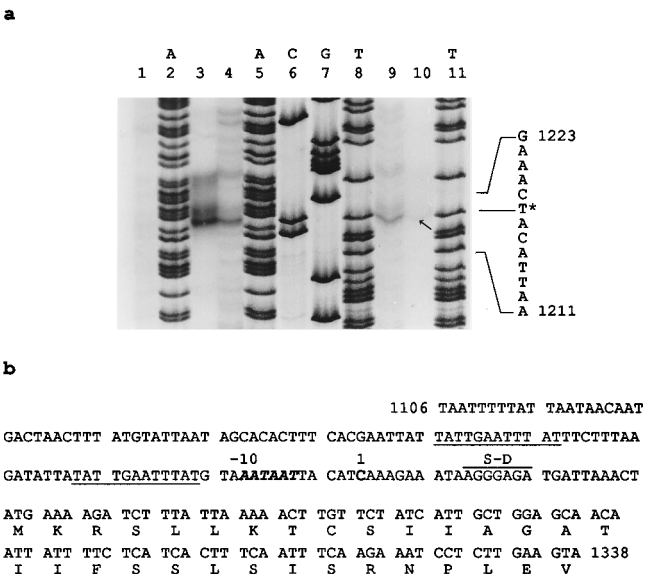

FIG. 3. Primer extension to map the transcriptional start site (a) and nucleotide sequence of the 5′ end of *celA* (b). pCMP (Fig. 1) contained the 3′ truncated *celA* gene (only the first 821 bp of the total 1,548-bp open reading frame were present) together with 231 bp of 5′ upstream sequence in the Bluescript vector pSK(M13+). pCMP was transformed into *E. coli* XL1-Blue, and ssDNA was obtained after infection with the helper phage VCS-M13 by using a standard protocol from the supplier (Stratagene). The primer extension protocol was adapted from that of Hu and Davidson (14). pCMP ssDNA (250 to 600 ng) was hybridized to total RNA from *Clostridium* sp. or *E. coli* (5 to 15 μg). γ-^{32}P-end-labelled T7 sequencing primer (5′ AATACGACTCACTATAG 3′) was annealed to the RNA-ssDNA pellets in T4 DNA polymerase buffer. The synthesis reaction was carried out by adding T4 DNA polymerase and deoxynucleoside triphosphates. The reaction was stopped after 10 min by the addition of sequencing stop buffer (Sequenase kit), and the samples were denatured at 85 to 90°C for 3 min. Aliquots of the reaction mixtures were loaded on a sequencing gel together with sequencing ladders obtained from the same phagemid construct (pCMP) and the same primer, using the Sequenase sequencing kit. Lanes 2, 5 to 8, and 11 show the sequencing ladder obtained from double-stranded DNA dideoxy termination sequencing; lanes 1, 3, 4, 9, and 10 show the primer extension and termination reactions which contained mRNA from *E. coli* or from *Clostridium* sp. Lane 1 shows the *E. coli* negative control; lanes 3 and 10 contained *E. coli* BL21(pCML) mRNA, and lanes 4 and 9 contained *Clostridium* sp. mRNA. The arrow indicates the position of a very faint band. The sequence of the region of interest is shown on the right; T* corresponds with the main termination band in lanes 3, 4, 9, and 10 and nucleotide sequence of the 5′ end of *celA*. In the nucleotide sequence (b), the direct repeats are underlined, and the −10 promoter binding region is in italic boldface. The transcription start site is in boldface and numbered 1. The putative Shine-Dalgarno site (S-D) is overlined. The translated amino acid sequence is shown. The sequence numbering corresponds with that of GenBank entry L02868.

was not sufficiently sensitive. The generally accepted model of the regulation of cellulase genes involves the induction of transcription by enzymatic degradation products of the large extracellular substrate, e.g., cellodextrins or derivatives thereof. Some low constitutive expression of extracellularly located cellulases would be required to produce these for uptake into the cells (3).

The specific β-glucanase activities of the extracellular fractions of both the induced and uninduced cultures were determined by using the dinitrosalicylic acid (DNS) method as recommended by the Commission on Biotechnology (10, 20), except that the substrate carboxymethyl cellulose was replaced by barley β-glucan. A 6.7-fold increase in the specific β-glucanase activity was observed in the induced culture, which agrees with the transcriptional induction of *celA* and the induction of CelA and other β-glucanases visible on zymograms. This increase is, however, an underestimation because of the presence of two highly induced proteins with molecular masses of about 80 to 90 kDa present in the supernatant of the induced culture (indicated with triangles in lane 2 of Fig. 3a), which show little or no carboxymethyl cellulase activity on the zymogram (Fig. 3c). The presence of these two proteins also leads to an underestimation of the induction of CelA as seen on the Western blot and zymogram, because equal quantities of protein were loaded in each lane. Quantitation of the increase in CelA detected on the Western blot and of the increase of β-glucanase activity on the zymogram was not done because the results would have been biased by the presence of these two proteins.

A conspicuous band of activity of ca. 35 kDa which shows no induction is visible on the zymogram. A combination of inducible and constitutively expressed cellulases has been found in other cellulolytic bacteria, namely, *R. flavefaciens* and *F. succinogenes* (5, 15), and in *Cellulomonas fimi* (11, 12) and *C. thermocellum* (19).

Identification of the −10 sequence, a direct repeat, and a ribosome binding site. The exact determination of the transcriptional start site enabled the tentative identification of an AATAAT −10 RNA polymerase recognition sequence which closely resembled the *E. coli* σ^{70} consensus sequence TAT AAT (Fig. 3b). No *E. coli* −35 region could be identified, but a 12-bp direct repeat was found flanking this region. The TAT TGAATTTAT direct repeats (underlined in Fig. 3b) are separated by 15 bases. Analysis of the 1,218 bp of *Clostridium* sp. DNA upstream of *celA* revealed that this is the only significant repeat present. Eleven bases downstream of the start of the mRNA transcript and 12 bases upstream of the ATG start codon, a possible Shine-Dalgarno ribosome binding site, AGGGAGA, was identified tentatively.

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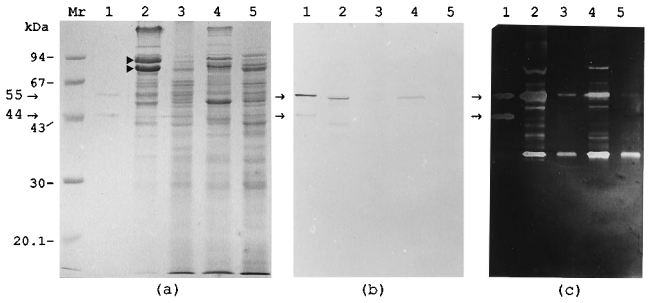

FIG. 4. Induction and localization of CelA in *Clostridium* sp. as shown by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (a), on a Western blot (b), and on a carboxymethyl cellulose zymogram (c). The SDS-polyacrylamide gel shows the molecular mass markers. CelA purified from *E. coli* was used as a positive control in lane 1. The 55- and 44-kDa bands indicated with the arrows show the full-length CelA and the proteolytically truncated CelAΔCBD (i.e., CelA from which the cellulose-binding domain had been deleted), respectively. The black triangles indicate two highly induced proteins. Lane 2 contains the extracellular proteins, which were concentrated by ultrafiltration, and lane 3 contains the cell-associated proteins of a *Clostridium* sp. culture grown on β-glucan. The extracellular proteins and the cell-associated proteins shown in lanes 4 and 5, respectively, were obtained from an uninduced culture grown on cellobiose. Lanes 1 of panels a to c were loaded with 2.3, 0.7, and 0.12 μg of purified CelA, respectively; 50 μg of protein was loaded in lanes 2 to 5 of panels a and b, and 5 μg was loaded in lanes 2 to 5 of panel c. Antiserum against CelA previously purified from *E. coli* BL21(pCML) (20) was prepared. Standard protocols were used for the Western blots (24). Protein samples were run on SDS–10% (wt/vol) polyacrylamide gels and electroblotted onto nitrocellulose membranes. These were probed with the CelA antiserum (1:7,500 dilution), and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma Immuno Chemicals) was used as the secondary antibody. Bound antibodies were detected calorimetrically by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

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