

Regulation of Major Cellulosomal Endoglucanases of *Clostridium thermocellum* Differs from That of a Prominent Cellulosomal Xylanase

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The expression of scaffoldin-anchoring genes and one of the major processive endoglucanases (CelS) from the cellulosome of *Clostridium thermocellum* has been shown to be dependent on the growth rate. For the present work, we studied the gene regulation of selected cellulosomal endoglucanases and a major xylanase in order to examine the previously observed substrate-linked alterations in cellulosome composition. For this purpose, the transcript levels of genes encoding endoglucanases CelB, CelG, and CelD and the family 10 xylanase XynC were determined in batch cultures, grown on either cellobiose or cellulose, and in carbon-limited continuous cultures at different dilution rates. Under all conditions tested, the transcript levels of *celB* and *celG* were at least 10-fold higher than that of *celD*. Like the major processive endoglucanase CelS, the transcript levels of these endoglucanase genes were also dependent on the growth rate. Thus, at a rate of 0.04 h^{-1} , the levels of *celB*, *celG*, and *celD* were threefold higher than those obtained in cultures grown at maximal rates (0.35 h^{-1}) on cellobiose. In contrast, no clear correlation was observed between the transcript level of *xynC* and the growth rate—the levels remained relatively high, fluctuating between 30 and 50 transcripts per cell. The results suggest that the regulation of *C. thermocellum* endoglucanases is similar to that of the processive endoglucanase *celS* but differs from that of a major cellulosomal xylanase in that expression of the latter enzyme is independent of the growth rate.

The cellulosome complex of the thermophilic anaerobe *Clostridium thermocellum* is a highly active extracellular cellulolytic system (5, 8, 14, 15, 18, 28, 29, 31, 33, 38, 39). Individual cellulosomes consist of up to nine catalytic subunits attached to a noncatalytic scaffolding protein, or scaffoldin (1, 3, 12, 13, 20, 21, 37), originally termed CipA (cellulosome-integrating protein A). To date, >20 cellulosomal enzymes are known to be produced by *C. thermocellum* (5, 6, 38, 39, 40). These catalytic components include mostly endoglucanases, exoglucanases, xylanases, and other hemicellulases.

Evidence from various sources has indicated that the expression of cellulosomal components from different cellulosome-producing bacteria is generally regulated by growth conditions rather than being constitutive. These changes in cellulosome composition have been shown to influence the activity of the entire complex (4, 9–11, 16, 19, 22–24, 26, 29, 35, 36, 41). Cellulosomes prepared from cells grown on cellulose exhibited higher activities on crystalline cellulose than cellulosomes prepared from cells grown on cellobiose (16). The increase in cellulosome activity was found to be accompanied by an increase in the transcript level of the major cellulosomal processive endoglucanase, CelS (Cel48A). More insights into the factors that influence the transcript level of *celS* were obtained from studies involving continuous cultures operated at differ-

ent dilution rates. With this approach, it was determined that the expression level of *celS* increased with decreasing growth rates. The influence of the growth rate was not exclusively limited to the transcription of the *celS* gene, as transcription of the scaffoldin gene, *cipA*, and of other *cipA*-related genes was also affected (17).

In order to study the alteration of the expression patterns of cellulosomal genes due to changes in physiological conditions, we determined their transcript levels. Among the genes selected as targets for this study, we chose two that encode major cellulosomal endoglucanases, CelB (Cel5A) and CelG (Cel5D), from glycoside hydrolase family 5 (7, 32), and another that encodes a family 9 endoglucanase, CelD (Cel9A) (27). In addition, the regulation of expression of the family 10 xylanase XynC (25) was also investigated in order to compare whether gene regulation of a hemicellulase subunit is similar to that of the endoglucanases. Our results established that the patterns of transcription are similar for the various endoglucanase genes studied in this work, which are, in turn, similar to that observed for previously studied cellulosomal genes, i.e., the major processive endoglucanase *celS* and the primary scaffoldin *cipA* (16, 17). In contrast, the expression of *xynC* represented a distinctive regulation pattern.

MATERIALS AND METHODS

Organism, substrates, and culture conditions. *C. thermocellum* YS was originally isolated from soil samples obtained at the hot springs of Yellowstone National Park (2, 29, 30). Cells were grown in batch culture at 60°C in Duran anaerobic bottles (Schott Corporation, Mainz, Germany) in a medium containing the following reagents (per liter): 0.65 g of $\text{K}_2\text{HPO}_3 \cdot 3\text{H}_2\text{O}$, 0.5 g of KH_2PO_4 ,

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1.3 g of $(\text{NH}_4)_2\text{SO}_4$, 42 g of morpholinepropanesulfonic acid (MOPS), 5 g of yeast extract, 1 g of cysteine, 0.5 g of MgCl_2 , and 2 mg of resazurin. The medium included 1.0% of the desired carbon source, either cellobiose from Acros Organics (Geel, Belgium) or microcrystalline cellulose (Avicel), obtained from Teva-Pharmaceutical Industries (Kfar Sava, Israel). Before the turbidity was measured, cellulose-grown cultures were vortexed vigorously and centrifuged at $100 \times g$ for 1 min to remove the insoluble substrate. Continuous cultures were performed in a BIOFLO 3000 fermentor (New Brunswick Scientific, Edison, N.J.) in a 1.5-liter working volume at 60°C . The pH was maintained at 7.2 by the automatic addition of 5 N NaOH. Agitation was kept constant at 100 rpm. For the maintenance of anaerobic conditions, the headspace of the bioreactor contained 99.99% CO_2 for initial growth and then changed to 99.99% N_2 while the continuous process started. Continuous cultures were operated under conditions of cellobiose limitation (2 g/liter), whereby *C. thermocellum* adapted to different growth rates. The residual cellobiose concentration at the steady state was assessed by a reducing sugar assay using the dinitrosalicylic acid reagent (34). The cellobiose concentration correlated, as expected, to the dilution rate. Typical values were 0.22, 0.17, and 0.14 g/liter for dilution rates of 0.23, 0.19, and 0.15 h^{-1} , respectively. Note that *C. thermocellum* YS cannot utilize yeast extract as a carbon source. The establishment of steady-state conditions was assumed when the culture had been growing for a period of at least three generations and the cell density (monitored spectrophotometrically) had remained unchanged for at least one generation.

RNA extraction. Precipitated cells, derived from 10 ml of culture medium, were washed twice with 10 mM Tris-HCl, pH 7.5, and the pellets were snap-frozen in liquid nitrogen and stored at -80°C . The total RNA was extracted from cells by use of an RNeasy kit (QIAGEN GmbH, Hilden, Germany), with minor modifications as described earlier (16). The RNA concentration was estimated by measuring the A_{260} , and the resultant RNA preparations were stored in aliquots at -80°C .

RPA. RNase protection assays (RPAs) were performed by use of an RPAII kit (Ambion Inc., Austin, Tex.). Different amounts of RNA were hybridized to ^{32}P -labeled antisense probes, and the protected RNAs were placed directly in a scintillation counter for quantification. For visualization, the protected RNAs were separated in 5% polyacrylamide gels containing 7 M urea and visualized by use of a phosphorimager system. Probes were labeled by use of a Maxiscript in vitro transcription kit (Ambion) and T7 RNA polymerase. The appropriate fragments were cloned into pGEM (Promega, Madison, Wis.). The different probes included the following DNA fragments (positions are relative to the initial ATG): *celG*, from -77 to $+283$; *celB*, from -101 to $+119$; *celD*, from -88 to $+499$; *celF*, from -211 to $+338$; and *xynC*, from -174 to $+419$.

Determination of amount of RNA per cell. The amount of RNA was established for each of the cultures wherein the transcript levels of the genes were determined. A pellet derived from 30 ml of culture was washed with 10 mM Tris-Cl, pH 7.6, and resuspended in 10 ml of 10% cold trichloroacetic acid (TCA). The suspension was kept on ice for 30 min. Supernatant fluids were discarded after 10 min of centrifugation at $10,000 \times g$. The cells were then resuspended in 5% TCA and centrifuged, and the pellet was dissolved gently in 1.5 ml of 0.1 N NaOH by use of a sealed Pasteur pipette. The samples were incubated overnight at 37°C to allow for the complete hydrolysis of RNAs. The solution was neutralized with 1 ml of 10% TCA, incubated for 15 min on ice, and then centrifuged. For determination of the RNA concentration in the sample, 1 volume of appropriately diluted supernatant fluid was mixed with 1 volume of orcinol reagent (1% orcinol dissolved in 0.1% FeCl_3 in concentrated HCl), and the solution was boiled for 15 min. After the tubes were cooled under running tap water, 2 volumes of distilled water was added. The absorbance was recorded at two wavelengths, namely, 600 nm (background) and 660 nm (background plus green complex). For determination of the RNA or nucleotide concentration, a deoxyadenosine solution at 50 $\mu\text{g}/\text{ml}$ was used as a standard. Since the results were obtained as purine riboside equivalents of RNA, the values were doubled to obtain nucleotide equivalents (purines plus pyrimidines) and multiplied by the average nucleotide molecular weight. For determination of the amount of total RNA per cell, cells were counted under a microscope by use of a Petroff-Hausser counter chamber. The number of transcripts of each gene per cell was then calculated from the resultant values of counts per minute per microgram of total RNA, the specific activity of the probe, and the average amount of total RNA determined per cell.

RESULTS

In previous studies of *C. thermocellum* (4, 16), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

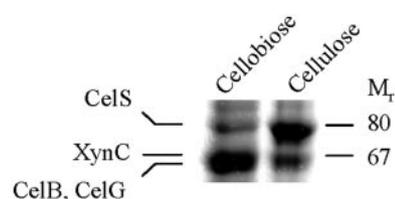


FIG. 1. SDS-PAGE analysis of cellulosome preparations purified from cellobiose- and cellulose-grown cells. Cellulosomes, derived from the late stationary phase of *C. thermocellum* grown either on microcrystalline cellulose or on cellobiose, were isolated from the culture medium by affinity chromatography with cellulose. The preparations were separated by SDS-6% PAGE. The cellulosomal subunits XynC, CelB, and CelG were identified previously from polyvinylidene difluoride blots of the gels by amino acid sequence analysis (16).

analyses revealed that the compositions of cellulosome subunits derived from cellulose- versus cellobiose-grown stationary-phase cultures differed greatly. The most prominent difference was a reduction in the family 48 CelS band in cellobiose-grown cells compared to cellulose-grown cells (Fig. 1). The regulation of *celS* was investigated in detail and was described in an earlier work (16). Another major difference revealed by SDS-PAGE analysis was an enhancement of a 67-kDa band upon growth on cellobiose (4). An analysis of the N-terminal amino acid sequence of this 67-kDa band indicated that it actually represented three separate cellulosomal subunits, namely, the two endoglucanases CelB and CelG and the xylanase XynC (16). For further study of the factors responsible for the regulation of these proteins, cultures of *C. thermocellum* were grown under more defined growth conditions.

Due to the difficulty in distinguishing among these cellulosomal subunits by SDS-PAGE, an alternative strategy was required for estimating the level of each enzyme. Another problem with studying the regulation of cellulosomal genes at the protein level is that the cellulosome complex is present in both cell-free and cell-bound forms. In this context, most studies have focused on the cell-free fraction, due to the relatively facile methods for its isolation, and the cell-bound fraction (e.g., from exponential-phase cells) has often been ignored. For the circumvention of such problems, the expression level of each gene was determined at the transcriptional level by RPAs. RPA was the method of choice because of its capacity to provide quantitative results whereby the expression levels of genes can be obtained as numbers of transcripts per cell.

Effect of carbon source on transcript levels of selected cellulosomal genes. As mentioned above, our initial results indicated that the overall combined level of expression of three cellulosomal enzymes (the endoglucanases CelB and CelG and the xylanase XynC, represented by a common 67-kDa band) was higher in late-stationary-phase cellobiose-grown cells than in cellulose-grown cells. In order to determine the effect of the carbon source on the expression of these genes, we assessed the mRNA level of each in cultures grown on the relevant substrate. Cells were harvested during the exponential and late-exponential phases, when culture turbidities (A_{660}) reached 0.85 and 1.8, respectively, for cellobiose-grown cells, and 0.65 and 2.0, respectively, for cellulose-grown cells. An RPA was used to assess the level of mRNA for each gene. To ensure that the amount of the probe in the hybridization re-

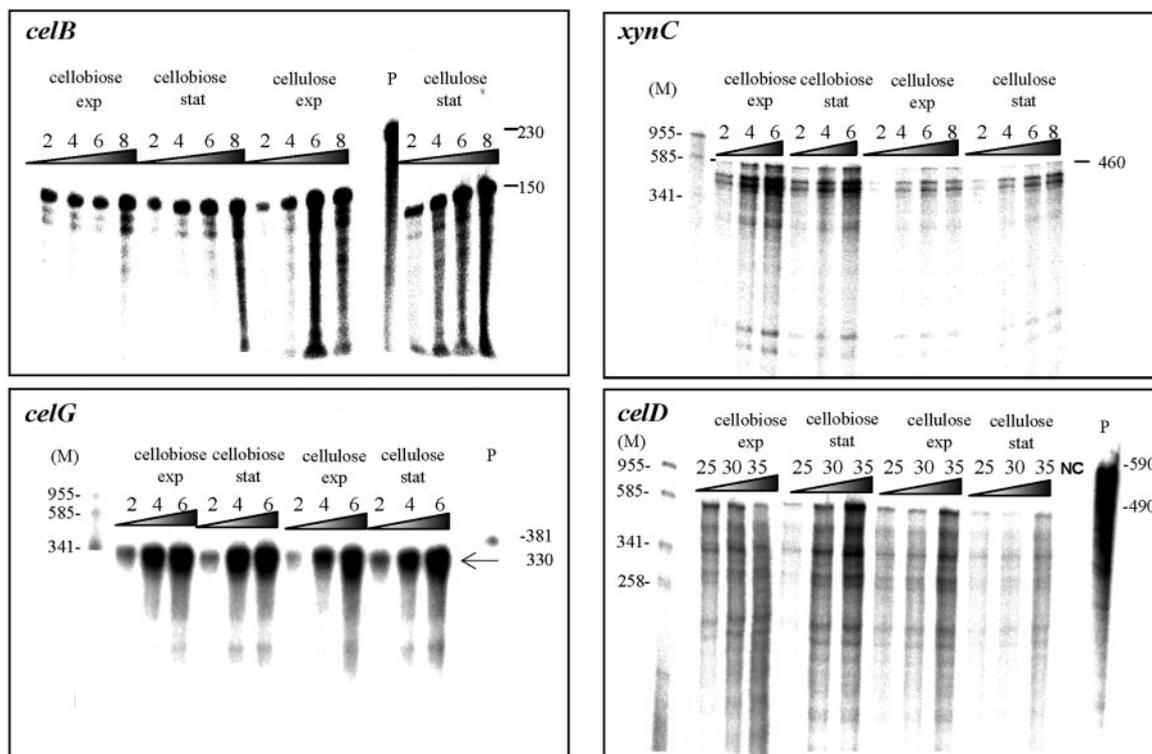


FIG. 2. Transcript levels of endoglucanase genes *celB*, *celG*, and *celD* and the xylanase gene *xynC*. RPAs were performed with a relevant ³²P-labeled antisense probe as described earlier (16), using RNAs from exponential (exp)- or stationary (stat)-phase cultures grown on either cellobiose or crystalline cellulose, as designated. The different amount (in micrograms) of RNA used to hybridize with the probe is indicated at the top of each lane. Autoradiographs of the respective RPA products were visualized with a phosphorimager system. Negative control lanes (NC) contained RNAs from yeast. Lanes M, size markers (in base pairs). The full-length probe is shown in lanes P. Values at the right indicate the size of the undigested full-length antisense probe and the estimated sizes of the protected products, in bases.

action was not limiting, we hybridized different amounts of the isolated RNAs with each ³²P-labeled antisense probe. After digestion with RNases, the RPA product was precipitated, the supernatant fluids were removed, and the radioactivity was assessed in a scintillation counter. The protected product was separated in a polyacrylamide gel for visualization by a phosphorimager system (Fig. 2). A linear correlation was obtained in all cases examined between the amount of total RNA used in the assay and the radioactivity of the protected product. The

calculated number of transcripts of each gene is presented in Table 1.

The results indicated that among the three 67-kDa-band-related genes, only the transcript level of *xynC* was higher in cellobiose-grown cells than in cellulose-grown cells. During exponential growth on cellobiose, the expression of *xynC* was 54 transcripts per cell, and the value increased to 80 upon entering the stationary phase of growth (Fig. 2). The *xynC* mRNA level in cellulose-grown cells was only about 30 transcripts per cell. In contrast to the case for *xynC*, the transcript level of *celG* was only slightly affected by the carbon source and was about 20 transcripts per cell during exponential growth on both substrates. The transcript level of the endoglucanase gene *celB* was twice as high in cellulose-grown cells but was still two- to threefold lower than that obtained for *celG* (6 to 12 versus 20 transcripts per cell). These two endoglucanase genes were unaffected by the phase of growth, since no increase was observed in the transcript level when cells entered stationary phase. Nevertheless, the overall combined transcript level of the two endoglucanases, together with that of the xylanase, during the late exponential phase of cellobiose-grown cells proved to be higher than that observed for cellulose-grown cells in the corresponding phase of growth (110 compared to 66 transcripts per cell). This finding is in accord with the SDS-PAGE results described above, wherein the intensity of the 67-kDa band in cellobiose-grown cells was higher than that

TABLE 1. Transcript levels of selected endoglucanase genes and a xylanase gene in *C. thermocellum* batch cultures grown on either cellobiose or cellulose

Gene	No. of transcripts per cell on indicated substrate ^a			
	Cellobiose		Crystalline cellulose	
	Exponential phase	Late exponential phase	Exponential phase	Late exponential phase
<i>celB</i>	6	8	11	12
<i>celG</i>	22	22	20	24
<i>celD</i>	0.7	0.9	1	0.8
<i>celF</i>	1	1	2	1
<i>xynC</i>	54	80	28	30

^a Values represent the averages ± 15% standard error of at least three determinations of mRNA levels.

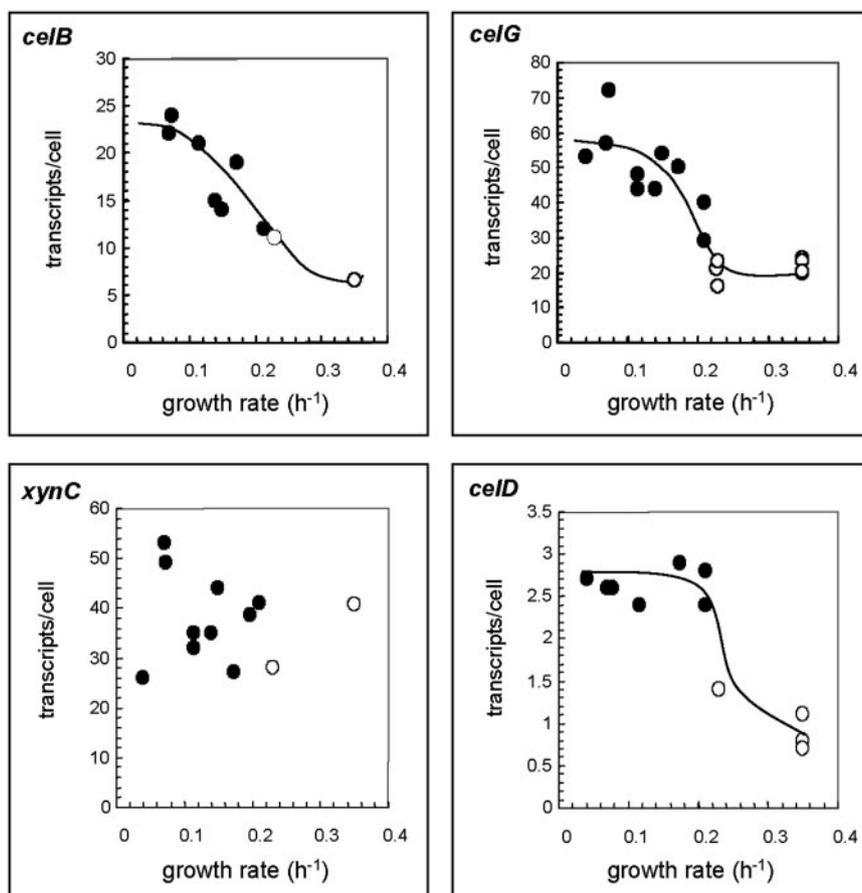


FIG. 3. Transcript levels of the indicated cellulosomal genes as a function of the growth rate. The amounts of the respective mRNAs were determined by RPAs and converted to the numbers of transcripts per cell, based on the average measured amount of total RNA in a single cell (0.18 pg/cell). Values represent the averages of several (at least three) measurements, with accuracies of $\pm 25\%$. Continuous cultures (filled circles) were operated with carbon (cellobiose) limitation at dilution rates from 0.04 to 0.21 h^{-1} . Batch cultures (open circles) were grown to exponential phase on either cellulose or cellobiose at rates of 0.23 and 0.35 h^{-1} , respectively. Note the differences in the scales of the different graphs.

observed in cellulose-grown cells. These results suggest that this difference mainly reflects an increase in the expression of the xylanase gene *xynC* upon entry into the stationary phase of growth. To compare the transcription patterns of *celB* and *celG* to those of other cellulosomal endoglucanases, we also determined the expression levels of two different family 9 genes, *celD* and *celF*. The regulation of the last two genes was previously characterized (35), and thus they were used as a reference for the present work. The transcript levels of *celD* and *celF* were previously shown to undergo an increase at the end of the exponential phase of growth, while during the early phase the levels of both genes were undetectable. In agreement with these earlier findings, the transcript levels obtained for both genes in the present work were only about one transcript per cell under all conditions studied (Table 1). The low transcript levels of these genes were consistent with the fact that it was not possible to detect these proteins by SDS-PAGE and N-terminal sequencing of candidate bands.

One possible factor that has been shown to play a role in the expression of the endoglucanases studied here is the rate of cell growth, as demonstrated previously for other cellulosome-related genes (16, 17). The difference in maximal growth rates of cellobiose- versus cellulose-grown cultures probably re-

sulted from the difference in the immediate availability of the two substrates. The growth rates achieved on cellobiose and cellulose were 0.35 and 0.23 h^{-1} (i.e., doubling times of 2 and 3 h), respectively. In this context, it seems that the growth rate either has a contrary effect on the transcription of *xynC* or that the transcription of this gene is influenced by another factor(s). To address these issues, we used chemostat cultures.

Transcript levels of selected cellulosomal genes during growth in continuous culture with limited cellobiose. Chemostat studies were conducted to investigate the influence of the growth rate (i.e., the cellobiose concentration) on the transcription of the desired cellulosomal genes of *C. thermocellum*. This system allowed the study of lower growth rates than those achieved with cellulose-grown batch cultures. Moreover, the chemostat enabled us to study the regulation of the desired genes under defined steady-state conditions. Continuous cultures were operated with limiting concentrations of cellobiose. The ability to control the growth rate is achieved by controlling the flow rate of the medium (dilution rate) introduced into the fermentor. The dilution rates established ranged from 0.21 to 0.04 h^{-1} , and the resultant cell turbidities (A_{660}) were within the range of 0.5 to 0.7.

As shown in Fig. 3, the levels of transcripts obtained for the

endoglucanase genes *celB* and *celG* were only slightly higher at a dilution rate of 0.21 h^{-1} (13 and 29 transcripts per cell, respectively) than those achieved in cultures grown on cellulose at a growth rate of 0.23 h^{-1} (11 and 24 transcripts per cell, respectively). In contrast, the transcript level of *celD* increased up to threefold when the rate of growth decreased from 0.23 to 0.21 h^{-1} . A further decrease in the dilution rate to 0.04 h^{-1} failed to influence the transcript level of *celD*, and the level stabilized at about two to three transcripts per cell. In the cases of *celB* and *celG*, a decrease in the dilution rate was accompanied by a gradual increase in the transcript level. Thus, at a dilution rate of 0.04 h^{-1} , the transcript levels of these genes were two- to threefold higher than those obtained in cellulose-grown cells (25 and 55 transcripts per cell for *celB* and *celG*, respectively). Interestingly, no clear trend was observed for the transcript level of *xynC* upon altering the dilution rate. The level of *xynC* mRNA fluctuated from 30 to 55 transcripts per cell, irrespective of the growth rate.

DISCUSSION

The most important conclusion to be drawn from the results of the present study is that there appears to be a common trend in the regulation of the different cellulosomal genes. In this context, the expression of *celG* and *celB* was found to be growth rate dependent, as observed earlier for the genes encoding the major processive endoglucanase and the primary scaffoldin (*celS* and *cipA*, respectively). As observed in our earlier studies on the expression of cellulosomal components (16, 17), upon the transition from conditions of rapid exponential growth to very low growth rates, the transcript levels of *celB*, *celG*, and *celD* increased about threefold. In contrast, transcription of the xylanase gene *xynC* appeared to follow a different pattern of regulation, and its expression remained at a relatively high level, irrespective of the growth rate.

In general, the influence of the growth phase on the expression of the genes studied in this work was relatively minor, although the transcript level of *xynC* exhibited a defined increase during stationary-phase growth on cellobiose. Interestingly, this finding is in contrast to the response observed earlier for the transcript levels of *celS* and *cipA*, both of which decreased upon entry into the stationary phase of growth (16, 17). The observed difference in the transcript levels of *xynC* between the stationary- and exponential-growth phases may in fact denote a physiological function essential to the fundamental lifestyle of the bacterial cell. In this context, *C. thermocellum* specializes in the hydrolysis and utilization of cellulose and its degradation products alone. Although it readily hydrolyzes xylan and other hemicelluloses, it cannot assimilate their components.

In the native ecosystem, the plant cell wall is structured such that the cellulose microfibrils are embedded in a colloidal matrix of lignin and hemicellulose. To commence effective biodegradation of the growth-relevant cellulose substrate, the cells must first penetrate the hemicellulose barrier. Therefore, relatively high levels of xylanases, such as XynC, would serve as a primary means for gaining access to the main substrate. In the next stage, the cells attach to and begin to degrade the cellulosic substrate. Under these conditions, the growth rate is relatively low and the production of cellulases would be stim-

ulated in order to support further growth. The growth rate would then increase as a function of the accumulation of soluble degradation products; further production of cellulases would be less critical, and their transcript levels would decrease, as supported by the results of this work.

To date, more than two dozen *C. thermocellum* enzymes have been sequenced by conventional means, the majority of which appear to be dockerin-containing cellulosomal enzymes. About half are endoglucanases and/or processive cellulases, and the other half are xylanases and other hemicellulases. Moreover, the *C. thermocellum* draft genome sequence is now available at the Joint Genome Institute website (<http://www.jgi.doe.gov>). The genome sequence has ensured the identification of additional enzymes and other cellulosome-related genes. It will thus be interesting to determine in future research whether the trends observed in the present work reflect a more general feature of this bacterium, particularly with respect to the regulation of cellulases versus hemicellulases. In this context, Doi and colleagues (23, 24) have recently examined the regulation of expression of cellulosomal enzymes in a mesophilic cellulosome-producing bacterium, *Clostridium cellulovorans*, and the coordinated expression of cellulases and hemicellulases was demonstrated.

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