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Regulation of Cellulase Formation in *Clostridium thermocellum*

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True cellulase activity (i.e. degradation of crystalline cellulose) was markedly derepressed when cellobiose-grown cells were transferred to fructose or sorbitol, especially while the cells were adapting over many hours to these carbon sources. On the other hand, the long lag phase on glucose was not accompanied by derepression. Transfer to cellobiose resulted in low cellulase production. Growth on crystalline cellulose derepressed cellulase production. Addition of cellobiose (the preferred carbon source for growth) to cells adapting to fructose caused a rapid burst of growth and cessation of cellulase synthesis. After cells had adapted to growth on fructose or sorbitol, repression set in and, after a number of transfers, growth on these carbon sources yielded cellulase at a level even lower than with cellobiose or glucose. It appears that rapid growth on a soluble sugar such as cellobiose causes carbon source repression which is relieved during slow growth on crystalline cellulose or during the growth lag on fructose or sorbitol. The reason for the lack of cellulase derepression during the growth lag on glucose is unexplained.

INTRODUCTION

The anaerobic degradation of cellulose by bacteria is limited by the accumulation of inhibitory fermentation products and by the low titre of extracellular cellulase (Avgerinos & Wang, 1981; Mandels, 1981; Wolin & Miller, 1983). Toxic concentrations of fermentation end-products can be avoided by growing the primary hydrolytic decomposer in the presence of a secondary organism, such as a methanogen, resulting in an increase in the extent of cellulose utilization (Weimer & Zeikus, 1977; Wolin & Miller, 1983; Zeikus, 1983). The rate of cellulose decomposition does not increase appreciably in co-culture, since the quantity of extracellular cellulases secreted by anaerobes is still very low. The limited synthesis of extracellular proteins under anaerobic conditions imposes a selective pressure for the secretion of enzymes of high specific activity. This is supported by our finding that the *Clostridium thermocellum* cellulase in the presence of dithiothreitol (DTT) and calcium has a high 'true' cellulase activity (degradation of crystalline cellulose) compared to the enzyme from *Trichoderma reesei* (Johnson & Demain, 1984; Johnson *et al.*, 1982). The unusual requirements of the clostridial cellulase for DTT and calcium and its sensitivity to oxygen were not recognized by earlier investigators, and the conclusions that clostridial cellulase synthesis is constitutive and not subject to carbon source repression (Garcia-Martinez *et al.*, 1980; Hammerstrom *et al.*, 1955; Lee & Blackburn, 1975) were based on short term cellulolytic assays or on the determination of carboxymethylcellulase activity (degradation of soluble cellulose derivatives, e.g. carboxymethylcellulose). In the present study we have used an improved cellulase assay to reassess the effects of carbon nutrition on the formation of cellulase in *C. thermocellum*.

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METHODS

Materials. Hungate tubes (18 × 150 mm) and butyl rubber stoppers for bacterial culture were obtained from Bellco Glass. An anaerobic chamber (Coy Laboratories) was inflated with a gas mix (90% N_2, 5% CO_2, 5% H_2) prepared by Matheson. Phosphate salts, glucose and urea were from Fisher Scientific. Cysteine.HCl, MOPS, cellobiose, pyridoxamine.HCl, biotin and DTT were purchased from Sigma. Cyanocobalamin was obtained from Merck, and *p*-aminobenzoic acid and D-fructose were obtained from J. T. Baker Chemical Co. Sodium citrate and succinic acid were from Mallinckrodt. Avicel (type PH105, 20 μm particles) was obtained from FMC. Cellotriose was a gift from M. Ladisch, Purdue University, USA, cellodextrins were kindly supplied by H. Cellard and C. L. Cooney, M.I.T., and thiocellobiose was a gift of H. Driguez, Pulp and Paper Research Institute of Canada.

Bacterial strain. A wild-type strain of *Clostridium thermocellum* (ATCC 27405) was used throughout this study. It was plated periodically in an anaerobic glove box on solid MJ medium (Johnson *et al.*, 1981) containing 0·8% Avicel as the carbon source. A cellulolytic colony was recovered with a toothpick, grown in cellobiose broth, and re-isolated on an Avicel plate. This culture was grown in cellobiose medium and stored at 4 °C. Stock cultures were also maintained by lyophilization in complex medium (Johnson *et al.*, 1981) and at −80 °C in 50% (v/v) glycerol.

Preparation of media and cultivation of bacteria. A minimal, defined medium (MJ; Johnson *et al.*, 1981) was used throughout. It was prepared by mixing the basal medium (1·5 g KH_2PO_4, 2·9 g K_2HPO_4, 2·9 g urea, 10·0 g MOPS, 1·0 mg resazurin and 3·0 g sodium citrate in 850 ml H_2O). This was boiled to remove O_2, 1·0 g cysteine.HCl was added, and the mixture was transferred to the anaerobic chamber. The medium was dispensed and capped inside the chamber, and then sterilized for 15 min at 121 °C. A 10-fold concentrated trace salts solution (10·0 g $MgCl_2.6H_2O$, 1·5 g $CaCl_2.2H_2O$ and 12·5 mg $FeSO_4.7H_2O$ per litre) was autoclaved separately and combined with a filter-sterilized, 1000-fold concentrated vitamin solution (20 mg biotin, 200 mg pyridoxamine.HCl, 40 mg *p*-aminobenzoic acid and 20 mg cyanocobalamin per litre) which was then added by syringe. Soluble carbon sources were added at a concentration of 5 g l^{-1}.

Cells were grown at 60 °C and growth was measured by monitoring the optical density at 600 nm in a Turner model 330 spectrophotometer: 1 mg cell dry weight ml^{-1} corresponds to 1·8 optical density units. Growth on insoluble cellulose was determined by microscopic counts in a Petroff Hausser chamber: 1 mg cell dry weight is equivalent to 9 × 10^8 cells. Sampling of Hungate tubes during experiments was usually done in the anaerobic glove box to prevent leakage of air and changes in the environment of *C. thermocellum*.

Cellulase assay. A turbidimetric assay of cellulase activity using Avicel as the substrate was carried out as previously described (Johnson & Demain, 1984; Johnson *et al.*, 1982). This procedure measures the degradation of insoluble, microcrystalline cellulose to cellobiose (the principal product of cellulase activity); it represents the composite activity of the multienzyme cellulase complex. The decrease in optical density at 660 nm is correlated with loss in dry weight of cellulose. Since crystalline cellulose is a heterogeneous substrate, and is degraded more slowly as the reaction proceeds, we felt that it was important to measure its solubilization over a long time period (24 h) that allowed total saccharification. Over this time period, the decrease in optical density is first order and is proportional to a protein concentration up to approx. 25 μg protein ml^{-1}. One cellulase unit is defined as the amount of cellulase that gives a first order rate measured over 24 h and completely degrades Avicel producing 1 nmol cellobiose min^{-1}.

Protein assay. Extracellular protein was measured with Coomassie blue reagent by the method of Bradford (1976).

RESULTS

C. thermocellum can utilize cellulose, cellobiose, glucose, fructose and sorbitol as carbon sources. Cellobiose is the principal product of cellulase activity and is also the carbon source preferred by *C. thermocellum*. To investigate the effect of carbon substrate on cellulase synthesis, a cellulolytic colony was grown in cellobiose broth and then inoculated to minimal medium containing fructose, sorbitol or glucose (Table 1). *C. thermocellum* grew very poorly on these carbon sources from the cellobiose inoculum; the poor growth in fructose or sorbitol (but not the poor growth in glucose) was accompanied by a five-to-sixfold increase in the specific production of cellulase. It appeared that synthesis of cellulase and not activation of the enzyme caused the increased titre since no activation of cellulase activity occurred when fructose and cellobiose broths were mixed.

The kinetics of growth and cellulase formation were examined in fructose and cellobiose (Fig. 1). Growth was rapid in cellobiose and the volumetric titre of cellulase paralleled growth. Cells inoculated to fructose medium from an inoculum grown on cellobiose had a 60–70 h growth lag, during which cellulase accumulated in the medium. The accumulation of cellulase as a function of increase in cell dry weight was 50 times higher during adaptation to fructose than during

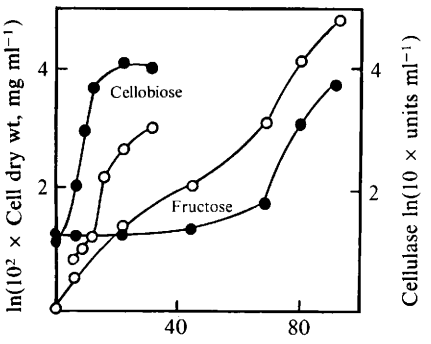

Fig. 1

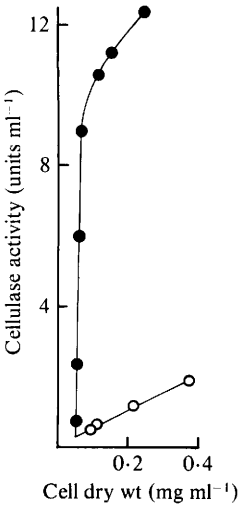

Fig. 2

Fig. 1. Growth of *C. thermocellum* (●) and cellulase formation (○) in cellobiose or fructose defined medium. Carbon sources were at a concentration of 10 g l^{-1}. The inoculum (1%, v/v) was a culture growing exponentially in cellobiose.

Fig. 2. Cellulase activity as a function of cell dry weight during the growth phase in fructose (●) or during the exponential growth phase in cellobiose (○).

Table 1. *Cellulase synthesis by cells previously grown in cellobiose when transferred as a small inoculum (1%, v/v) to different carbon sources*

Cultures were assayed after reaching stationary phase.

Medium	Lag (h)	Cell dry wt (mg ml^{-1})	Cellulase activity (units ml^{-1})	Cellulase activity [units (mg cell dry wt)$^{-1}$]
No carbon source (starved)	0	0·08	0·3	4
Cellobiose	0	0·48	7·2	15
Glucose	120	0·26	5·4	21
Fructose	110	0·13	11·8	90
Sorbitol	84	0·17	12·8	75

growth on cellobiose (Fig. 2). When the cells started to grow on fructose, this differential rate dropped but was still 5–6 times higher than on cellobiose (Fig. 2).

The addition of cellobiose to a culture in the lag phase on fructose (Fig. 3) caused a rapid burst of growth and cessation of cellulase synthesis. The extent of growth and decrease in cellulase synthesis depended on the dose of cellobiose. When the added cellobiose was exhausted, the growth lag on fructose was re-established and formation of cellulase again occurred at a high rate. Therefore, the addition of a desired carbon source to cells in a lag phase on fructose promoted rapid growth and a sharp decline in cellulase synthesis.

The derepression of cellulase formation during adaptation to fructose suggests that rapid catabolism of cellobiose (the principal product of cellulolysis and the preferred energy source of *C. thermocellum*) leads to carbon source repression of cellulase synthesis. This concept was supported by limitation of catabolism by growth on insoluble crystalline cellulose. Under this condition, growth is limited by the supply of soluble carbon source (cellobiose) (Weimer & Zeikus, 1977). We observed a significant increase in cellulase titre with cellobiose limitation (Table 2).

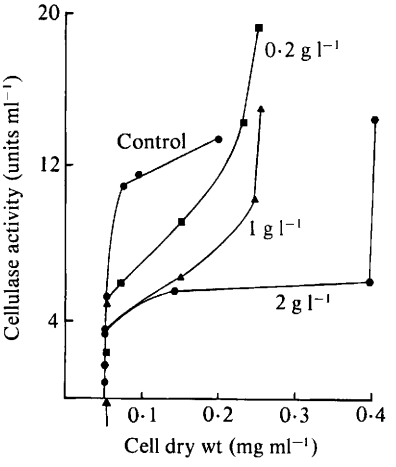

Fig. 3.

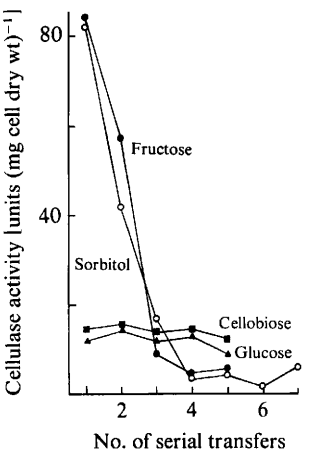

Fig. 4.

Fig. 3. Effect of the addition of cellobiose at various concentrations on cellulase formation during the growth lag on fructose. The arrow indicates the time of addition.

Fig. 4. Effect of serial transfer on the specific titre of cellulase in culture broths of *C. thermocellum*. Cultures were grown in cellobiose and then inoculated into the various carbon sources. Each transfer corresponds to five mass doublings.

Table 2. *Cellulase synthesis by C. thermocellum grown on cellobiose and inoculated (1%, v/v) to cellobiose or Avicel media*

Cultures were grown for 60 h before assays were done.

Carbon source*	Cell dry wt (mg ml⁻¹)	Cellulase activity (units ml⁻¹)	Cellulase activity [units (mg cell dry wt)⁻¹]	Extracellular protein (μg ml⁻¹)
Cellobiose	0·33	2·8	8·6	62·5
Avicel	0·22	12·8	58	65·0
Cellobiose in increments†	0·19	5·8	31	38·0
Avicel in increments†	0·14	10·2	73	65·0

* Final concentration was 2 g l⁻¹.
† Added in three doses of 0·5 g l⁻¹ after limitation of growth at each stage. The initial charge was 0·5 g l⁻¹.

The increase in cellulase titre during growth on fructose or sorbitol was not limited to prior growth in cellobiose; cells previously adapted to glucose and inoculated into fructose or sorbitol also substantially increased their specific titre of cellulase (Table 3). This high specific production dropped when growth was established, indicating that the catabolism of the sugars was responsible for the repression.

Although the levels of cellulase were high in cultures adapting to fructose or sorbitol, continued growth on these sugars resulted in a severe drop in the titre (Fig. 4). Eventually, a specific activity was obtained which was less than that produced in cellobiose medium, even though growth was faster in cellobiose. The severe drop in cellulase titre on serial transfer in fructose was not caused by the selection of non-cellulase producing mutants, as equal numbers of fructose-adapted cells grew on cellulose or fructose agar. Furthermore, when individual cells from the Avicel agar plates were isolated, grown in cellobiose, and transferred back to fructose, they underwent another cycle of increased cellulase synthesis followed in later transfers by a very low specific titre. Thus no genetic change had occurred in the cells.

The drop in cellulase titre during adaptation to fructose or sorbitol was not caused by the depletion of a hypothetical inducer derived from cellulose, such as cellobiose or cellotriose. This was shown by the observation that fructose- or sorbitol-adapted cultures were not 'induced' for

Table 3. *Growth and cellulase formation by cells adapted to different carbon sources*

Inocula were allowed to adapt for at least eight serial transfers. Cellulase assays were done when maximum cell dry weight was reached. ND, Exponential growth did not occur.

Adapted inoculum	Carbon source (0·5%, w/v)	Lag (h)	Growth rate [μ (h^{-1})]	Max. cell dry wt (mg ml^{-1})	Cellulase activity (units ml^{-1})	Cellulase activity [units (mg cell dry wt)$^{-1}$]
Cellobiose	Sorbitol	110	ND	0·12	13	94
	Fructose	110	ND	0·13	12	92
	Cellobiose	0	0·35	0·38	6·4	17
	Glucose	125	ND	0·26	4·0	16
	None	—	—	0·08	0·24	3·0
Glucose	Sorbitol	70	ND	0·14	15	106
	Fructose	30	ND	0·11	10	94
	Cellobiose	0	0·21	0·44	6·6	15
	Glucose	30	0·18	0·41	3·2	8·0
	None	—	—	0·04	0	0
Fructose	Sorbitol	5	0·20	0·31	1·2	4·0
	Fructose	7	0·30	0·43	1·0	2·2
	Cellobiose	3	0·45	0·56	1·0	1·8
	Glucose	12	0·14	0·47	0·2	0·4
Sorbitol	Sorbitol	15	0·20	0·31	1·6	5·0
	Fructose	10	0·25	0·39	1·9	4·4
	Cellobiose	0	0·25	0·40	2·0	4·6
	Glucose	15	0·25	0·38	0·6	1·4
	None	—	—	0·05	0	0

cellulase synthesis when transferred back to cellobiose, even after five mass doublings (Table 3). Transferring the cells three times in cellobiose gradually brought the cellulase titre up to the level observed in the original inoculum developed in cellobiose. The use of a medium pre-conditioned with cellobiose-adapted cells, or the addition (0·1 g l^{-1}) of possible cellulase inducers (including glucose-1-phosphate, cellotriose, cellotetraose or a mixture of soluble cellulose dextrins) to fructose or cellobiose medium failed to boost the production of cellulase from a fructose inoculum in a single transfer (about five mass doublings). Cellobiose analogues including lactose, salicin and thiocellobiose were also inactive. Taken together, the above experiments argue against the requirement for a cellulose-derived inducer in cellulase synthesis.

DISCUSSION

Previous studies with *C. thermocellum* (Garcia-Martinez *et al.*, 1980; Hammerstrom *et al.*, 1955) and other cellulolytic bacteria (Fusee & Leatherwood, 1972; Lee & Blackburn, 1975) demonstrated that carboxymethylcellulase is not strongly repressed by catabolism of soluble carbon sources. This was surprising since it would be advantageous for *C. thermocellum* to repress its level of cellulase if a soluble carbon source is available in the medium. We have found in this study that the cellulase component(s) responsible for the degradation of microcrystalline Avicel is repressed during rapid growth on cellobiose and after adaptation to fructose, sorbitol and glucose. We have not determined the specific enzyme component(s) which is repressed, but it is possible that it is an exoglucanase that is not involved in the breakdown of amorphous cellulose. It is interesting that the cellulase component(s) active on Avicel but not on carboxymethylcellulose is sensitive to thiol reagents and oxidation (Johnson & Demain, 1984; this may be a property of a key exoglucanase or other material (Ljungdahl *et al.*, 1983) involved in the breakdown of crystalline cellulose.

The results presented in this paper suggest that cellulase production may be brought on by a low energy level in the cell. This would be expected to be the case in cells growing slowly on crystalline cellulose or during adaptation to growth on fructose or sorbitol; in these cases, cellulase production was high. However, once the cells were induced and could grow rapidly on fructose or sorbitol, the energy level would be expected to be high, correlating with low cellulase production. On cellobiose, the favoured carbon source for growth, the energy level would be

expected to be high and we have shown cellulase production to be low. It is not clear why there is poor cellulase production during adaptation to glucose (since the energy level should be low in this case). Further work will be required to answer this question.

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