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Regulation of β-1,4-Endoglucanase Synthesis in *Thermomonospora fusca*

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In *Thermomonospora fusca* YX, endocellulase synthesis varies over a 100-fold range depending on the carbon source used. This study shows that the variation is caused by two regulatory mechanisms: an induction mechanism that increases the rate of endocellulase synthesis about 20-fold and a growth rate-dependent repression mechanism that changes the rate of synthesis over a 6-fold range in both induced and noninduced cells. In *T. fusca*, endocellulase synthesis can be induced by cellulose, cellobiose, or cellodextrin. Cellulase is involved in inducer generation from cellulose. Growth rate-dependent repression can be reversed by limiting cultures for carbon, nitrogen, or, to a lesser extent, phosphorus. Further evidence for two separate regulatory mechanisms is provided by the isolation of mutants (CC-1 and CC-2) whose endocellulases are synthesized constitutively but are still sensitive to growth rate-dependent repression. These conclusions about total endocellulase synthesis were extended to the individual endocellulases by showing that three *T. fusca* endocellulases are coordinately regulated.

There is evidence that the synthesis of cellulolytic enzymes is regulated by induction and repression, in both bacteria (2, 5, 11, 18–20) and fungi (4, 7, 8, 15, 17, 21), even though little is known about the molecular mechanisms of either regulatory system in either organism.

Induction usually causes the biggest change in endocellulase synthesis in bacteria, and the inducers are cellulose and its derivatives, cellobiose, and other oligosaccharides (2, 11, 18–20). The true inducer(s) in cultures grown on cellulose is probably the cellulolytic products or their derivatives. In addition, metabolizable carbon sources (e.g., glucose and cellobiose) repress endocellulase synthesis (2, 5, 11, 18–20). The effects of the nitrogen source on endocellulase synthesis are largely unknown, although some preliminary results suggested an inverse correlation between growth rate and enzyme synthesis in *Cellulomonas flavigena* (11). The highest endocellulase levels are usually achieved by growing the culture on cellulose. Because cellulose is degraded slowly by all cellulolytic microorganisms, they grow more slowly on cellulose than on cellobiose and are limited for carbon source.

Thermomonospora fusca YX is a thermophilic actinomycete producing extracellular β-1,4-endoglucanases (endocellulases) and xylanases (3, 10). Five immunologically distinct endocellulases have been purified and characterized from *T. fusca* culture supernatant (3; D. B. Wilson, Methods Enzymol., in press). All these enzymes digest both carboxylmethyl cellulose and filter paper, although the ratios of their activity on carboxylmethyl cellulose to filter paper vary greatly. These enzymes are thermostable (10) and have a low sensitivity to end product inhibition (6), properties that are valuable for practical applications. It has been shown that the endocellulase level in *T. fusca* increases when cellulose or cellobiose is present (16), but no systematic study of endocellulase regulation has been reported for this organism. In this report, the factors regulating endocellulase synthesis in *T. fusca* YX and two mutant strains were investigated under a range of growth conditions. In addition, the levels of three different *T. fusca* endocellulases were shown to be coordinately regulated by immunoinhibition experiments.

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MATERIALS AND METHODS

Culture conditions. *T. fusca* YX was maintained on LB glucose agar and used to inoculate LB broth containing 0.2% glucose. The liquid medium used in most experiments was modified Hägerdal medium (9): NaCl, 1.5 g; $(NH_4)_2SO_4$, 3.1 g; Na_2HPO_4, 9.1 g; KH_2PO_4, 0.9 g; disodium EDTA, 50 mg; $MgSO_4$–H_2O, 200 mg; $FeSO_4$–$7H_2O$, 20 mg; $CuCl_2$, 20 mg; $MnSO_4$–H_2O, 15 mg; $ZnSO_4$–$7H_2O$, 9 mg; thiamine hydrochloride, 1 mg; D-biotin, 1 mg (all measurements expressed per liter), supplemented with 0.2% carbon source. The cellulose used for growth was Sigmacell type 100 (Sigma Chemical Co.). The growth temperature was 55°C, and the shaking speed was 200 rpm on a Gyrotory shaker (New Brunswick Scientific Co., Inc.). To inoculate cultures, mycelia from a stationary-phase glucose culture were centrifuged at 3,000 × *g* for 5 min, washed with minimal salt medium, and transferred to 20 times the original volume of fresh medium.

Mutagenesis of *T. fusca* YX. *T. fusca* YX was grown on minimal glucose plates at 55°C for 2 days. Spores were eluted from each plate with 5 ml of sterile water and filtered through glass wool. The spore suspension was diluted into 1 ml of minimal salt solution containing 0.33 M ethyl methyl sulfate and incubated at 37°C for 3 h. The amount of spores used was adjusted to give 100 to 200 colonies on each LB-glucose plate (the survival rate under the above conditions is 1%). The suspension was centrifuged at 4,500 × *g* for 5 min, and the pellet was washed twice with 3 ml of minimal salt solution, suspended in the solution, plated on LB–0.2% glucose plates (20 per experiment), and incubated at 55°C for 2 days. Each plate was replicated into two minimal plates; one containing 0.2% glucose, the other containing 0.2%

TABLE 1. Protein and β-1,4-endoglucanase levels in *T. fusca* batch cultures

Strain	Carbon source	Growth rate[a]	Total culture protein (mg/ml)[b]	β-1,4-Endoglucanase (U/mg of protein)
YX	Glucose	2.1	0.14	0.33
	Xylose	2.5	0.14	0.55
	Sucrose	3.4	0.17	0.40
	Xylan	3.5	0.13	1.0
	Maltose	3.8	0.07	0.89
	Ribose[c]	(>10)	0.04	1.2
	Galactose[c]	(>10)	0.03	1.9
	Cellobiose	2.3	0.13	8.5
	Cellodextrin[d]	(2.0–2.5)	0.21	6.2
	Cellulose	3	0.10	75
CC-1	Glucose	(1.9–2.3)	0.16	1.3
	Xylose	(2.0–2.4)	0.17	1.9
	Cellobiose	(2.3–2.5)	0.11	13
	Cellulose	3	0.10	68
CC-2	Glucose	(2.2–2.5)	0.12	4.5
	Xylose	(2.2–2.5)	0.12	10
	Cellobiose	(2.2–2.5)	0.12	7.7
	Cellulose	3	0.11	50

[a] Growth rate was defined as the minimum doubling time for culture protein in early log phase. The figures in parentheses are less accurate estimates. The ratio of intracellular protein to cell dry weight is about 0.11.

[b] In the cellulose-grown cultures, about 30% of the total protein was secreted; in all the other cultures, only 5 to 10% of the total protein was secreted.

[c] Data measured after 48 h of growth.

[d] Cellodextrin was prepared as described in reference 14.

cellobiose. After 2 days of growth at 55°C, all plates were overlaid wih 5 ml of 1% agarose containing 0.05% carboxylmethyl cellulose and 10 mM Tris (pH 6.5). After the overlay solidified, the plates were incubated at 55°C for 7 to 8 min, transferred onto a cool metal surface, stained with Congo Red dye (1 mg/ml) for 10 min, and destained with 1 M NaCl for 15 min. The colonies surrounded by a yellow ring on both the glucose plate and the cellobiose plate were isolated and purified for further characterization (wild-type colonies give a yellow ring only on the cellobiose plate). Two mutants were found among the approximately 5,000 colonies that were screened.

Protein assay. Samples were centrifuged at 3,000 × *g* for 5 min, and the supernatants were assayed for extracellular protein. The pellets were washed with and suspended in 0.05 M potassium phosphate (pH 6.5) and lysed with a French press at 4°C. The cell lysates were diluted to the volume of the original culture, mixed, and centrifuged at 4,000 × *g* for 5 min. The lysate supernatants and the culture supernatants were assayed for protein by the Bradford method (1) by using bovine serum albumin fraction V (Sigma) as a standard.

Endocellulase assay. The culture supernatant obtained as described above was assayed for endocellulase by a procedure modified from that described in reference 10. All assays were done at 55°C in a total volume of 400 μl of potassium phosphate (50 mM, pH 6.5) containing 0.5% carboxylmethyl cellulose as the substrate. Each sample was diluted to give 30 to 80 μg of reducing sugar in a reaction time of 15 to 30 min. At the end of the incubation, dinitrosalicylic acid reagent was added to determine the amount of reducing sugar (13). One unit of endocellulase is defined as the amount of enzyme required to produce the reducing power equivalent to 1 μmol of glucose per min under the reaction conditions.

Xylanase assay. Xylanase was assayed as described for endocellulase except that the substrate used was 0.5% oat xylan. One unit of xylanase is the amount of enzyme producing reducing power equivalent to 1 μmol of xylose per min.

Immunoinhibition experiment. Endocellulase samples were incubated with 10 μl of rabbit antisera prepared against each of three purified *T. fusca* cellulases (3) at 37°C for 1 h and at 0°C for 1 h and then assayed for endocellulase as described above. This level of antiserum gave complete inhibition of the activity of the corresponding immunogenic endocellulase. The reducing sugar background caused by each antiserum was measured and subtracted from the total reducing sugar of the assay.

RESULTS

Endocellulose synthesis with different carbon sources. To study the effect of carbon sources on endocellulase synthesis, *T. fusca* YX was grown in minimal medium supplemented with 0.2% carbohydrate. The cultures were grown to early stationary phase (or for 48 h for poor carbon sources) and assayed for total endocellulase and total protein (Table 1). The growth rate was measured by a protein assay, since it is impossible to reproducibly measure the turbidity of a mycelial suspension. There was a significant variation of growth rate with different carbon sources and an even larger variation of endocellulase level. Of all the carbon sources tested, only cellulose, cellobiose, and cellodextrin gave a high level of endocellulase activity. Cellulose and its hydrolysis products seem to be inducers of endocellulase synthesis. In addition, the endocellulase level varied over a sixfold range with the noninducing carbon sources and showed an inverse correlation with the growth rate. A similar phenomenon was also observed with inducers; i.e., cells grown on cellulose, which gives slow growth, produced eight times as much endocellulase as those grown on cellobiose or cellodextrin. These variations in endocellulase level suggest that there is a second regulatory mechanism controlling endocellulase synthesis in addition to induction. This regulation appears to lower the rate of endocellulase synthesis when the growth rate increases. The concentration of reducing sugar may also be an important factor that is partially responsible for the high endocellulase level in cellulose-grown cultures. On this carbon source, the doubling time was somewhat longer than on cellobiose, whereas the level of free reducing sugar was always undetectable.

The time courses of mycelia growth and endocellulase synthesis were also determined for *T. fusca* YX growing on several different carbon sources. With all of the carbon sources except cellulose, the rate of endocellulase synthesis was nearly proportional to cell mass throughout the growth phase. With cellobiose as the carbon source, a typical growth curve and continuous endocellulase synthesis were observed (Fig. 1a). The specific activity of endocellulase reached its highest value by 3 h after the mycelium was transferred from glucose medium to cellobiose medium (Fig. 1b) and then decreased slightly. This result shows that endocellulase induction occurred before the rapid growth began. The exact time required for induction is not known, because the enzyme level was too low to be measured accurately before 3 h of incubation. When *T. fusca* was

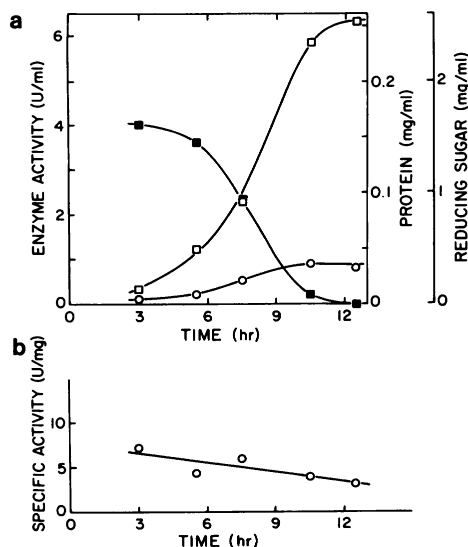

FIG. 1. Endocellulose synthesis by cellobiose-grown *T. fusca* YX. Symbols: (a) ■, reducing sugar; □, protein; ○, endocellulase; (b) ○, specific activity of endocellulase.

grown on cellulose (Fig. 2), growth began after a lag of several hours, and the growth phase was followed by a short period of protein decrease (always observed, even though not very significant in this figure) and then very slow growth until cellulose was depleted (Fig. 2a). The two growth phases may result from two types of cellulose being used, one of which is degraded faster than the other. The increase in endocellulase activity occurred only when the culture was in the growth phase (compare Fig. 2a and b), suggesting that cellulolytic products are the inducers of endocellulase.

Role of endocellulases in induction by cellulose. Cellulose is an insoluble compound that bacteria cannot take up. To explain its induction effect, it is widely believed that the basal level of cellulase generates a small amount of oligosaccharides which serves as a continuous, yet limited, supply of both the inducer and the carbon source. This is consistent with our observation that *T. fusca* endocellulase induction by cellulose has a long lag. This lag was significantly shortened by adding *T. fusca* endocellulases E_2 and E_4 to the culture along with the inoculum (Fig. 3). As predicted, the lag time decreased as the basal level of enzyme was increased by adding endocellulase. This result indicates that endocellulases in *T. fusca* are involved in the generation of inducers from cellulose.

Transient repression. To confirm the existence of a repression control, transient repression experiments were conducted. *T. fusca* YX was grown on 0.2% cellulose as before. When endocellulase activity reached 0.2 to 0.4 U/ml, the culture was divided, 0.2% of a second carbon source was added, and the cultures were grown for an additional 3 h. Under these conditions, both the new carbon source and the original one remained at a high level in all the experiments (data not shown). The difference between the endocellulase level at the beginning and end of the 3-h incubation was divided by the final culture protein and incubation time to calculate the absolute rate of endocellulase synthesis. The value for the repressed culture was divided by the value for the control culture to determine the relative synthesis rate. Because the absolute synthesis rate depends on the exact time when the repressor is added, the relative synthesis rate

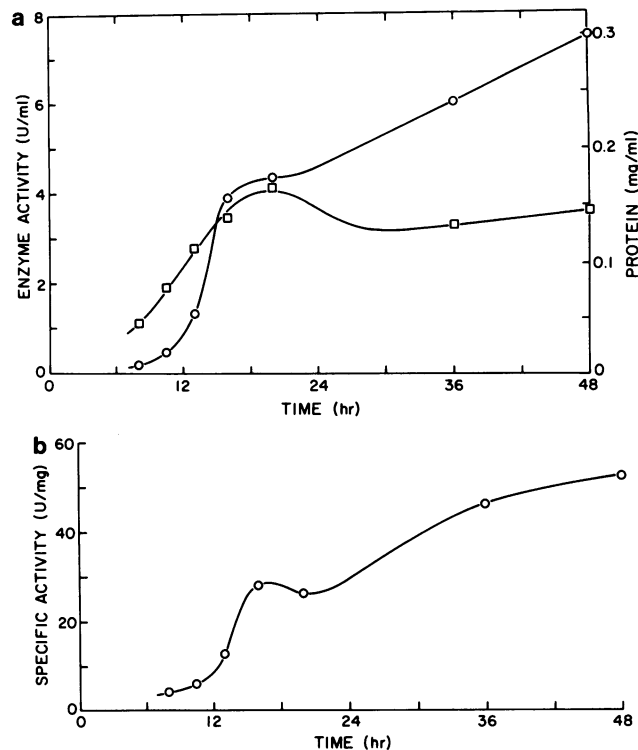

FIG. 2. Endocellulase synthesis by cellulose-grown *T. fusca* YX. No reducing sugar accumulated in the culture. Symbols: (a) □, protein; ○, endocellulase; (b) ○, specific activity of endocellulase.

is more reproducible. The values of the relative synthesis rates measured in several experiments were averaged and are given in Table 2. The transient repression effect was seen with all metabolizable carbon sources that allow fast growth, with the better carbon sources having a stronger effect. For example, cellobiose is a strong repressor, while succinate and α-methylglucoside, which are not utilized by *T. fusca*, have no repression effect. The repression effect can be

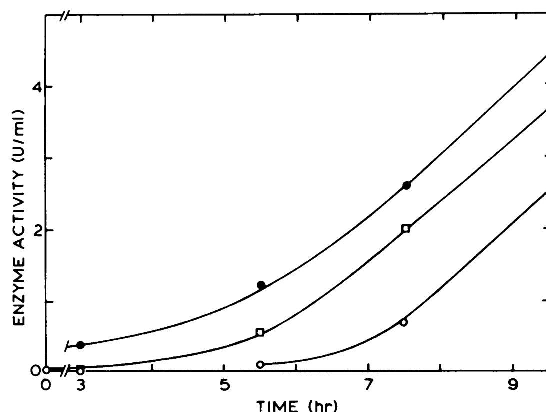

FIG. 3. Endocellulase induction in *T. fusca*. The culture was grown on 0.2% cellulose without enzyme addition (○) or with exogenous *T. fusca* endocellulase E_2 (●) or E_4 (□), both at 0.1 U of filter paper cellulase. The carboxymethyl cellulase activity of added enzymes has been subtracted from the final data.

TABLE 2. Transient repression of β-1,4-endoglucanase synthesis in *T. fusca* YX

Additions		β-1,4-Endoglucanase (% of control) with cellulose as inducer
Carbon source	Nucleotide	
		100
	cAMP	92
Glucose		31
Glucose	cAMP	51
Glucose	AMP	37
Cellobiose		36
Xylose		56
Maltose		69
Galactose		67
L-Alanine		72
Succinate		101
α-Methylglucoside		105

caused by both inducing and noninducing compounds. The repression effect was partially relieved when 5 mM cyclic AMP (cAMP) was added concomitantly with the repressing carbon source; however, cAMP never completely abolished repression. The absolute enzyme synthesis rates for the most important repression experiments are shown in Table 3. The enzyme synthesis rate in cellobiose medium was the same as the rate in medium containing cellulose and glucose ($1.4\ U \cdot mg^{-1} \cdot h^{-1}$). In both media, the enzyme level is regulated by both induction and repression. Since cellobiose is as strong a repressor as glucose, this result indicates that cellobiose is probably as good an inducer as cellulose.

Effects of nitrogen source and phosphorus source. It has been reported that limiting the nitrogen source relieves the repression of endocellulase synthesis in *C. flavigena* (11). In *T. fusca* YX, the effect of nitrogen source was tested by replacing $(NH_4)_2SO_4$ in the medium with other compounds, to give an equimolar amount of nitrogen. The cultures were grown as before for 30 h, harvested, and assayed. The results (Table 4) show that a better nitrogen source gave a lower endocellulase specific activity. It appears that the endocellulase activity of *T. fusca* YX increased when the culture was limited for nitrogen. The effects of different phosphorus sources were also tested (Table 4). The endocellulase activity increased when the culture was limited for phosphorus, even though the effect was less dramatic than the effect of limiting carbon or nitrogen sources.

Properties of mutants constitutive for endocellulase synthesis. The hypothesis of an induction-repression dual control of endocellulase synthesis in *T. fusca* YX predicts that it should be possible to isolate mutants that are constitutive for endocellulase synthesis and mutants that are resistant to repression but that each class of mutant should still retain the other type of regulation. To test this hypothesis, *T. fusca*

TABLE 3. Absolute synthesis rate of β-1,4-endoglucanase in *T. fusca* YX

Additional carbon source	Amt (U mg of protein^{-1} h^{-1}) of β-1,4-endoglucanase synthesized with the following original carbon source			
	Glucose	Xylose	Cellobiose	Cellulose
None	0.12	0.33	1.47	4.1
Glucose		ND[a]	0.44	1.4
Xylose	ND		ND	2.4

[a] ND, Not determined.

TABLE 4. Effect of nitrogen source on β-1,4-endoglucanase synthesis in *T. fusca* YX[a]

Source and limiting nutrient	Total culture protein (mg/ml)	Cellulase (U/mg of protein)
Nitrogen		
Casamino Acids[b]	0.33	2.1
$(NH_4)_2SO_4$	0.11	6.3
L-Histidine	0.026	7.6
$NaNO_3$	0.016	16
Phosphorus		
Potassium phosphate	0.15	2.7
Acetyl phosphate	0.06	2.1
Galactose-1-phosphate	0.055	3.3
Flavin mononucleotide	0.030	3.1
AMP	0.021	4.2
ADP	0.020	4.1
D-Fructose-6-phosphate	0.008	6

[a] Data were obtained after 30 h of growth (before cellobiose depletion) for all cultures except that the data for the best N or P sources were obtained upon the early stationary phase. *T. fusca* was grown in Hägerdal medium lacking $(NH_4)_2SO_4$ containing cellobiose as a carbon source and the indicated compound as a nitrogen source or phosphorus source at equal molar N or P concentrations. The cultures were grown and assayed as described in Materials and Methods.

[b] Difco Laboratories.

spores were mutagenized with ethyl methyl sulfate and screened for mutants with higher carboxymethyl cellulase activity on noninducing medium as described in Materials and Methods. Two strains, CC-1 and CC-2, were isolated and characterized from about 5×10^3 survivors. The endocellulase activity of both strains (Table 1) was higher than that of the wild-type strain when grown on noninducing carbon sources but about the same as that of the wild type on inducing carbon sources. CC-2 gave about the same endocellulase level for all the good carbon sources tested (glucose, xylose, and cellobiose), like a fully constitutive mutant, whereas CC-1 was only partially constitutive. The difference in the endocellulase level in CC-2 caused by different carbon sources is presumably determined solely by repression. The existence of repression was confirmed by transient-repression experiments conducted as described earlier. The endocellulase synthesis rate of strain CC-2 grown on cellulose was decreased by 0.2% glucose to 45% of the original level (55% inhibition), compared with a decrease to 31% (69% inhibition) for the wild-type strain. This indicates that CC-2 is nearly as sensitive to transient repression as the wild-type strain. Strain CC-2 was not constitutive for xylanase synthesis, as the specific activity of xylanase in glucose-grown cultures was 6 U/mg, slightly lower than the

TABLE 5. Activity of individual β-1,4-endoglucanases in *T. fusca* cultures

Strain	Carbon source	β-1,4-Endoglucanase activity [U/ml] (%)			
		Total	E_1[a]	E_2[a]	E_5[a]
YX	Glucose	0.057	0.031 (55)	0.002 (4)	0.007 (12)
	Cellobiose	0.73	0.45 (61)	0.08 (10)	0.10 (14)
	Cellulose	6.9	4.7 (69)	0.64 (9)	0.79 (12)
CC-2	Glucose	0.81	0.43 (54)	0.10 (13)	0.13 (16)
	Cellulose	5.6	3.9 (70)	0.65 (12)	1.06 (19)

[a] As defined by D. Wilson (Methods Enzymol., in press).

value of 10 U/mg found for the parent strain. The values for xylanase in cells grown on xylan were 120 U/mg for CC-2 and 180 U/mg for YX, showing that induction occurred in both strains.

Coordinate synthesis of the different endocellulases. Antisera prepared against each of three major endocellulases were used to measure the regulation of these individual enzymes by antibody inhibition experiments. The amounts of the different enzymes were determined in wild-type cells grown on glucose, cellobiose, or cellulose and in CC-2 cells grown on glucose or cellulose. The results (Table 5) show that in both strains all three enzymes were present at the same relative level under all growth conditions except for the lowest values, for which the error in the measurement is very large. Therefore, the *T. fusca* endocellulases appear to be coordinately regulated by both the induction and repression controls.

DISCUSSION

Endocellulase synthesis in *T. fusca* YX is subjected to dual control by both induction and repression, as has been found for most other cellulolytic bacteria. The induction system appears to be specific for endocellulases, because xylanase induction required a different inducer (xylan) and was not constitutive in strains CC-1 and CC-2. In contrast, the repression control appears to regulate both endocellulase and xylanase synthesis (data not shown).

Our study shows that *T. fusca* endocellulases are involved in generating the inducer(s) from cellulose. The inducer must be one (or some) of the oligosaccharide products of hydrolysis. Identification of the true inducer depends on the study of the induction mechanism, which is still under way; however, cellobiose is the most probable inducer for the following reasons. (i) Cellobiose is the major product of endocellulase hydrolysis. (ii) Cellobiose is a strong inducer, and no stronger inducer has been found. Cellulose hydrolysates (cellodextrin; Table 1) prepared with YX culture supernatant or HCl or cellotriose and cellotetraose isolated from the hydrolysates gave the same level of endocellulase activity as cellobiose did (for cellobiose, 8.5 U/mg; for cellotriose, 7.7 U/mg; for cellotetraose, 6.7 U/mg; for cellodextrin, 6.2 U/mg. (iii) Cellobiose is the smallest oligosaccharide which gives induction.

Derepression of endocellulase synthesis was achieved by limiting the source of either carbon, nitrogen, or phosphorus. The simplest explanation of all the derepressions is to propose a single repression mechanism that depends on the growth rate of the culture even though an additional mechanism (e.g., catabolite repression) may be involved. A similar observation has been made with another cellulolytic bacteria, *C. flavigena* (11). These results are quite different from those for catabolite repression in *Escherichia coli*, in which starvation for nitrogen or phosphorus increases repression (12). We cannot rule out the possibility that the increase of endocellulase level resulting from limiting the carbon or nitrogen source is also caused by independent mechanisms, especially because limiting the carbon or nitrogen source caused a greater derepression than limiting the phosphorus source. Fennington et al. (5) isolated a mutant of *Thermomonospora curvata* that was resistant to repression but did not present data on whether it was still inducible, as would be predicted by our model of two separate mechanisms controlling endocellulase synthesis. That glucose repressed endocellulase synthesis in the constitutive strain CC-2 grown on cellulose to nearly the same extent as it did with the wild-type strain proves that the transient repression effect is not caused by inducer exclusion.

One of the major obstacles to the study of the regulation of cellulase gene is the presence of multiple cellulases encoded by different genes. This problem was overcome for the *T. fusca* endocellulases by antibody inhibition experiments. The finding that these enzymes are coordinately regulated greatly simplifies future studies of *T. fusca* endocellulase gene regulation. Finally, this phenomenon may indicate that different endocellulases function together in degrading cellulose rather than each enzyme functioning to degrade different types of cellulose.

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