APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1984, p. 201–204
0099-2240/84/010201-04$02.00/0

Vol. 47, No. 1

Cellulase Biosynthesis in a Catabolite Repression-Resistant Mutant of *Thermomonospora curvata*

GEORGE FENNINGTON, DEBBIE NEUBAUER, AND FRED STUTZENBERGER*
Department of Microbiology, Clemson University, Clemson, South Carolina 29631

Received 13 June 1983/Accepted 10 October 1983

A catabolite repression-resistant mutant of the thermophilic actinomycete *Thermomonospora curvata* was obtained by treatment with ethyl methanesulfonate and UV light. Cellulase biosynthesis was undiminished by glucose, 2-deoxyglucose, or alpha-methyl glucoside, which are potent repressors in the wild type. Intracellular cyclic AMP levels were higher in the mutant in both the absence and the presence of repressors.

Cellulase biosynthesis is subject to induction and catabolite repression in both bacteria and fungi (16, 19, 21). Although the exact mechanism regulating cellulase biosynthesis is unknown, cellulose, cellobiose, and sophorose are considered inducers (12, 19), whereas glucose and glycerol are effective repressors (11). Repression of cellulase biosynthesis severely limits the ability of wild-type cultures to produce cellulase on a commercial scale and hampers its application in biomass conversion processes. Therefore, methods have been developed to obtain catabolite repression-resistant mutants (8, 14, 20). These methods involve culture mutagenesis, followed by plating of survivors on cellulosic media containing one or more cellulase repressors. 2-Deoxyglucose (2DG) is particularly valuable as a repressor since it acts as an antimetabolite in colonies unable to secrete cellulase and therefore is selective for catabolite repression-resistant mutants (13). This report describes a mutant of *Thermomonospora curvata* which appears resistant to the action of 2DG and other cellulase repressors. To our knowledge, this is the first report of a catabolite repression-resistant thermophilic actinomycete.

Medium used for the maintenance and growth of *T. curvata* cultures has been described in earlier reports (22, 24). This cellulose-mineral salts (CMS) medium contains ground cotton fibers as the sole carbon and energy source. The plating agar used for mutant screening contained about 1% microcrystalline cellulose (Avicel; FMC Corp.) instead of cotton fibers. Mutants were obtained by adding 0.1 ml of ethyl methanesulfonate to 5-ml samples of exponential-phase cells. To disperse these cells before ethyl methanesulfonate addition, they were sonicated for two 15-s periods (separated by a 30-s cooling period) with the sterilized microtip of a Bronwill Biosonik sonicator set at maximum output. After 24 h of exposure at 55°C, survivors (about 0.00174% of control CFU) were plated on cellulose agar containing 2 mM 2DG and 0.01% yeast extract. After 5 days of incubation at 55°C, colonies were streaked for confluent growth on cellulose agar containing 10 mM 2DG and UV irradiated at 40 ergs per mm^2 per min for 3 min. The rate of survival from the UV treatment was calculated to be 2.2×10^7. After another 5-day incubation period, any survivor producing a colony surrounded by a zone of cellulose clearing was transferred to 100 ml of CMS supplemented with 0.01% yeast extract. Cultures exceeding 0.25 filter paper units per ml during daily testing for 5 days at 53°C in shake culture were serially transferred in the same system to test for stability. The filter paper assay was performed according to the standardized procedure of Mandels et al. (10); pH and temperature of reaction were 6.2 and 65°C, respectively.

The mutant described here has been designated G-11; it has been the most stable of the ca. 30 mutants we have selected from over 1,200 colonies arising on 2DG-containing CMS agar. In our comparison of mutant G-11 with the wild type, we measured dry cell weight accumulation (determined as insoluble nitrogen as previously described) (22), soluble protein by the Bradford dye-binding method (3), endoglucanase (EG) activity on carboxymethyl cellulose (23), filter paper activity, and soluble sugar in culture fluid by the Bernfeld method (1). Table 1 summarizes the maximum values of these products observed for the wild type and mutant G-11 during a 3-day shake culture. In the absence of added repressors in liquid culture, cellulase production of G-11 was only about 15 to 40% better than that of the wild type on cotton fibers or on filter paper powder (a cellulosic substrate less resistant to degradation) (8, 25). With the exception of increased cellulase activity, values for the G-11 mutant and the wild type were quite similar. However, when the cultures were cultured in the presence of repressors added to CMS, the similarity ended. When either 2DG or alpha-methyl glucoside (αMG), another potent cellulase repressor (11), was present at the time of inoculation, cellulase production of the wild type was reduced by ca. 60 to 80% during 3 days in shake culture at 53°C. Since growth is dependent on cellulase activity in CMS, a decrease in cellulase biosynthesis was also reflected in decreased growth of the wild type in the presence of the cellulase repressors. In contrast, cellulase production by the G-11 mutant appeared to be stimulated by the presence of low concentrations of 2DG (1 to 2 mM) and only slightly inhibited by the presence of 5 mM αMG (Table 2). G-11 growth also appeared unaffected. It should be noted that in the absence of repressors, G-11 cultures lagged noticeably behind wild-type cultures in every respect.

We did some kinetic studies to determine the time required for diminution of cellulase secretion rates by repressors in wild-type *T. curvata* growing in CMS. In these studies we measured only EG activity, since addition of soluble sugars to the cultures increased the color in the filter paper assay blank above acceptable levels. In CMS, cellulase biosynthesis was maximally induced and accumulated in the culture fluid at approximately linear rates during early

* Corresponding author.

TABLE 1. Comparison of wild type and mutant G-11 during growth on cotton fibers and filter paper powder[a]

Strain	Substrate	Dry cell wt (mg/ml)	Soluble protein (μg/ml)	Endoglucanase activity (U/ml)	Filter paper activity (U/ml)	Soluble sugar (μg/ml)[b]
Wild type	Cotton fibers	0.65	208	24.0	0.26	38
	Filter paper	0.68	238	25.8	0.26	41
G-11	Cotton fibers	0.74	195	27.8	0.29	36
	Filter paper	0.67	238	36.7	0.30	35

[a] All data are the maximum values observed during 72 h of incubation at 53°C in shake culture. The average standard deviation between triplicate flasks for each of the above analyses was as follows: dry cell weight, ±24.6%; soluble protein, ±7.5%; endoglucanase activity, ±14.4%; filter paper activity, ±6.2%; and soluble sugar, ±3.0%.

[b] Expressed as glucose equivalents.

exponential growth. If production ceased, cellulase activity decreased at the rate of ca. 6%/h in shaken culture fluid at 53°C (data not shown). When a non-metabolizable repressor such as 5 mM αMG was added to a wild-type culture, the inhibitory effect on both cellulase activity and extracellular protein was apparent within 2 to 3 h after addition; within 20 h, a complete cessation of cellulase biosynthesis occurred

TABLE 2. Comparison of wild type and mutant G-11 in the presence of 2DG or αMG[a]

Strain	Repressor[b]	Culture time (days)	Dry cell wt (mg/ml)	Soluble protein (μg/ml)	Endoglucanase activity (U/ml)	Filter paper activity (U/ml)
Wild type	None	1	0.47	90	8.8	0.16
		2	0.62	195	20.2	0.27
		3	0.66	197	24.4	0.24
G-11		1	0.36	15	3.4	0.03
		2	0.71	130	20.7	0.23
		3	0.78	207	32.2	0.29
Wild type	1 mM 2DG	1	0.37	20	3.5	0.05
		2	0.37	38	4.6	0.07
		3	0.42	60	4.8	0.09
G-11		1	0.46	25	5.6	0.09
		2	0.71	190	31.8	0.32
		3	0.80	213	38.9	0.32
Wild type	2 mM 2DG	1	0.32	19	3.5	0.04
		2	0.34	33	4.1	0.06
		3	0.34	50	4.8	0.09
G-11		1	0.41	18	4.8	0.08
		2	0.62	138	27.8	0.29
		3	0.67	185	37.4	0.29
Wild type	5 mM αMG	1	0.08	10	0	0.03
		2	0.09	23	3.3	0.04
		3	0.09	32	3.7	0.03
G-11		1	0.43	70	3.9	0.05
		2	0.55	160	18.1	0.25
		3	0.71	181	23.8	0.22

[a] All values are averages of duplicate determinations.

[b] 2DG, 2-Deoxyglucose; αMG, alpha-methyl glucoside.

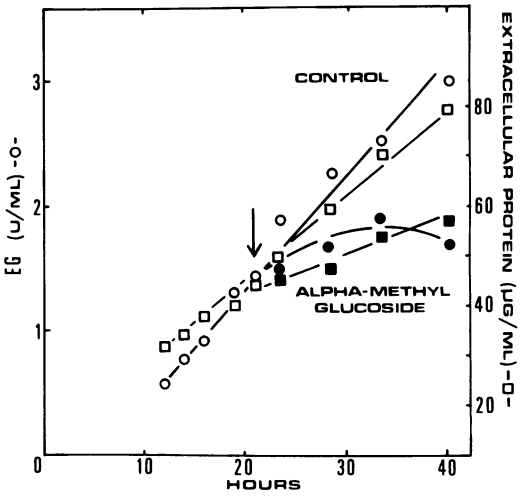

FIG. 1. Inhibition of EG production and protein secretion in *T. curvata* after addition of 5 mM αMG to the CMS liquid medium. Arrow indicates time of addition. Open symbols represent control values, and closed symbols represent values obtained after addition of αMG.

(Fig. 1). When a readily metabolizable cellulase repressor such as 10 mM glucose was added, a doubling of growth rate was observed (data not shown). However, cellulase secretion stopped until the glucose was depleted in the medium, and then the rate resumed at a lower level (Fig. 2). In G-11 cultures, addition of 10 mM glucose stimulated growth, whereas addition of 2DG had no apparent effect on the slow growth rate in CMS (Fig. 3a). However, in contrast to the wild-type response, both sugars caused an immediate stimulation of cellulase secretion (Fig. 3b). Stimulation by glucose became less pronounced toward the end of the experiment, and the EG units per milligram of cells were slightly decreased (17 in control to 13 in glucose culture). 2DG caused an unusual effect, an initial stimulation similar to that seen with glucose, followed by a decline to the levels of the control and then a recovery to a level comparable with the glucose-stimulated culture. We have no explanation for this effect at present. 2DG apparently is non-metabolizable in *T. curvata* since it cannot serve as the sole carbon and energy source, nor did we see any significant increase in cell mass

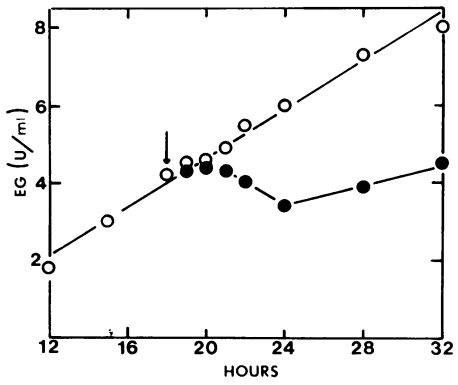

FIG. 2. Inhibition of EG production in *T. curvata* after addition of 10 mM glucose to the CMS liquid medium. Arrow indicates time of addition. Symbols: ○, control; ●, glucose.

TABLE 3. cAMP levels in wild type and mutant G-11 under conditions of cellulase induction and repression in CMS[a]

Strain	Soluble sugar added	pmol of cAMP per mg of dry cells	
		Range	Avg
Wild type	None	20–28	24
	10 mM glucose	3–9	7
G-11	None	21–36	31
	10 mM glucose	15–24	22

[a] Values are averages and ranges for hourly samples taken within 5 h after glucose addition. The average standard deviation was ±13.9% for triplicate assays on a given sample. For each sample taken, the assay value which fell outside the standard deviation range was discarded, and the other two were averaged.

due to its addition to G-11 cultures growing in CMS. If 2DG were metabolizable in G-11, the effect seen in Fig. 3b might be explained as a sequence of effects correlated to shifting levels of 2DG-derived products in the cells.

The repression resistance of mutant G-11 to metabolizable glucose repressors may be due in part to its ability to maintain its cyclic AMP (cAMP) levels. The evidence supporting the currently accepted model for the mechanism of enzyme biosynthesis control by cAMP in procaryotes has been recently reviewed (2); cAMP acts through a cAMP receptor protein (CRP). The CRP is a dimer having two identical subunits each capable of binding one molecule of cAMP. The CRP has two distinct domains. The N-terminal portion binds to cAMP, and the C-terminal portion binds to DNA. In the presence of cAMP, the CRP undergoes an allosteric configuration change which results in preferential binding to specific portions of DNA near the promoter regions of cAMP-dependent operons. This binding enables RNA polymerase to bind and initiate transcription at a second distinct site 30 to 50 nucleotides distal to the CRP binding region. Rapidly metabolizable substrates such as glucose lead to increased ATP levels and lowered cAMP levels, a shift which represses cAMP-dependent operons.

To test the relationship of cellular cAMP levels to rates of cellulase biosynthesis, we extracted cAMP from 5-ml samples of both wild-type and G-11 cells before and after glucose addition to cellulose-grown cultures. Our extraction procedure was essentially that described by Hylemon and Phibbs (9). Since *T. curvata* produces the clumping growth characteristic of many actinomycetes, we sonicated cells for 10 s during the extraction procedure by using the microtip of an Ultrasonics Inc. Model W-220F to provide a confluent suspension. The extracted cAMP was measured by the method of Gilman and Murad (7). The clumping growth of *T. curvata* and the presence of insoluble cellulose make precise intracellular cAMP measurements especially difficult. Therefore, we took at least four samples during a 4- to 6-h period before and after glucose addition. When we measured cAMP levels (expressed as picomoles of cAMP per milligram of dry cell weight) in the experiment shown in Fig. 2 and 3, we obtained the data shown in Table 3. The range and average of cAMP levels in mutant G-11 appeared to be slightly higher than those in wild-type cells when measured under conditions of cellulase induction. The addition of 10 mM glucose to wild-type cultures reduced the intracellular cAMP concentrations levels by ca. 70%, whereas in mutant G-11, the glucose reduced cAMP concentrations to levels comparable to those of the wild-type in the induced state.

The mechanism of induction and repression of cellulase biosynthesis remains unknown. The roles of glucose, cellobiose, sophorose, and other soluble sugars as repressors or inducers of cellulase biosynthesis vary from organism to organism. For example, sophorose is a good inducer of endoglucanase in *Trichoderma reesei* (18), whereas in *Acetivibrio cellulolyticus*, it represses endoglucanase production as efficiently as glucose (17). Cellobiose is an excellent inducer in *Neurospora crassa* (4) and *Sporotrichum pulverulentum* (5), but is a relatively poor inducer in *Trichoderma* spp. when compared with sophorose (5). In *T. curvata*, cellobiose induces only ca. 30% as much endoglucanase on cellobiose than does a resistant cellulose substrate (22), whereas in another *Thermomonospora* species, cellobiose appears to be as good an inducer as cellulose (15). Clearly, studies at the molecular level are needed to resolve these inconsistencies and to determine whether cyclic nucleotides are the major regulatory effectors in cellulase biosynthesis.

The potential for G-11 in industrial applications is as yet undetermined; it grows more slowly than wild-type *T. curvata*, it requires additional growth factors in the form of low concentrations of yeast extract in CMS, and it produces only ca. 15 to 40% more cellulase than the wild type in the

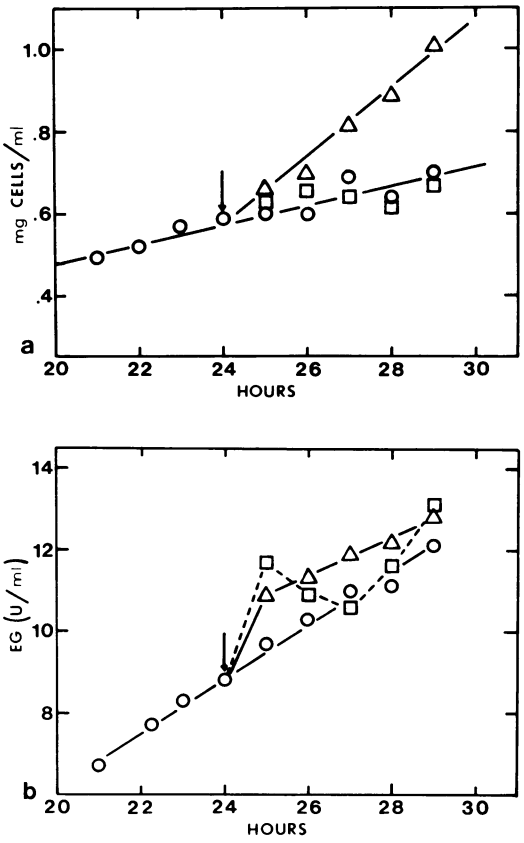

FIG. 3. (a) Addition of soluble sugars to *T. curvata* mutant G-11 cultures growing in CMS. Increase in cell mass, estimated by insoluble nitrogen, was measured before and after addition of 10 mM glucose (△) or 10 mM 2DG (□) compared with control receiving no sugar (○). Arrow indicates time of addition. (b) Stimulation of cellulase biosynthesis in *T. curvata* mutant G-11 by addition of 10 mM glucose (△) or 10 mM 2DG (□) compared with control (○) receiving no soluble sugar in CMS liquid medium. Arrow indicates time of sugar addition.

absence of the repressors. Its major merit may be its usefulness as a model for the study of cellulase induction and repression compared with the wild type. In this regard, we are currently comparing phosphodiesterase and adenyl cyclase levels in mutant G-11 to those of the wild type. We are also testing the effects of 2DG on rates of cAMP synthesis and excretion to explore the mechanism by which it represses cellulase biosynthesis.

We thank Lyndon Larcom and Ellis Kline for helpful discussions during preparation of this manuscript.

This work was supported by U.S. Army Research Office contract DAAG-29-81-K-0026.

LITERATURE CITED

1. **Bernfeld, P.** 1955. Amylases, alpha and beta. Methods Enzymol. **1**:149–154.
2. **Botsford, J. L.** 1981. Cyclic nucleotides in procaryotes. Microbiol. Rev. **45**:620–642.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Eberhart, B. M., R. S. Beck, and K. M. Goolsby.** 1977. Cellulase of *Neurospora crassa*. J. Bacteriol. **130**:181–186.
5. **Eriksson, K. E., and S. G. Hamp.** 1978. Regulation of endo-1,4-beta-glucanase production in *Sporotrichum pulverulentum*. Eur. J. Biochem. **90**:183–190.
6. **Ghosh, V. K., T. K. Ghosh, and K. S. Gopalkrishnan.** 1982. Improvement of *T. reesei* strain through mutation and selective screening techniques. Biotechnol. Bioeng. **24**:241–243.
7. **Gilman, A. G., and F. Murad.** 1974. Assay of cyclic nucleotides by receptor protein binding displacement. Methods Enzymol. **38**:49–62.
8. **Hofsten, B. V., and B. Berg.** 1972. Cellulase formation in *Cellvibrio fulvus*, p. 731–734. *In* G. Terui (ed.), Proceedings of the IV International Fermentation Symposium: Fermentation Technology Today. Society for Fermentation Technology, Kyoto, Japan.
9. **Hylemon, P. B., and P. V. Phibbs.** 1974. Evidence against the presence of cyclic AMP and related enzymes in selected strains of *Bacteroides fragilis*. Biochem. Biophys. Res. Commun. **60**:88–95.
10. **Mandels, M., R. Andreotti, and C. Roche.** 1976. Measurement of saccharifying cellulase, p. 21–33. *In* E. L. Gaden, Jr., and M. H. Mandels (ed.), Enzymatic conversion of cellulosic materials: technology and applications. Biotechnology Bioengineering Symposium No. 6. John Wiley & Sons, Inc., New York.
11. **Mandels, M., and E. T. Reese.** 1957. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J. Bacteriol. **73**:269–278.
12. **Mandels, M., and E. T. Reese.** 1975. Growth and cellulase production by *Trichoderma*, p. 81–109. *In* M. Bailey, T. M. Enari, and M. Linko (ed.), Symposium on Enzymatic Hydrolysis of Cellulose. The Finnish National Fund for Research and Development, Helsinki, Finland.
13. **Montenecourt, B. S., and D. E. Eveleigh.** 1979. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. Adv. Chem. Ser. **181**:289–301.
14. **Montenecourt, B. S., and D. E. Eveleigh.** 1979. Production and characterization of high yielding cellulase mutants of *Trichoderma reesei*, p. 101–108. *In* TAPPI Annual Meeting Proceedings. Technical Association of the Pulp and Paper Industry, Atlanta, Ga.
15. **Moreira, A. R., J. A. Phillips, and A. E. Humphrey.** 1981. Production of cellulases by *Thermomonospora* sp. Biotechnol. Bioeng. **23**:1338–1347.
16. **Nisizawa, T., H. Suzuki, and K. Nisizawa.** 1972. Catabolite repression of cellulase formation in *Trichoderma viride*. J. Biochem. **71**:999–1007.
17. **Saddler, J. N., A. W. Khan, and S. M. Martin.** 1980. Regulation of cellulase synthesis in *Acetivibrio cellulolyticus*. Microbios **28**:97–106.
18. **Sternberg, D., and G. R. Mandels.** 1979. Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose. J. Bacteriol. **139**:761–769.
19. **Sternberg, D., and G. R. Mandels.** 1980. Regulation of the cellulolytic system in *Trichoderma reesei* by sophorose: induction of cellulase and repression of β-glucosidase. J. Bacteriol. **144**:1197–1199.
20. **Stewart, B. J., and J. M. Leatherwood.** 1976. Derepressed synthesis of cellulase by *Cellulomonas*. J. Bacteriol. **128**:609–615.
21. **Stoppok, W., P. Rapp, and F. Wagner.** 1982. Formation, location, and regulation of endo-1,4-β-glucanases and β-glucosidases from *Cellulomonas uda*. Appl. Environ. Microbiol. **44**:44–53.
22. **Stutzenberger, F. J.** 1972. Cellulolytic activity of *Thermomonospora curvata*: nutritional requirements for cellulase production. Appl. Microbiol. **24**:77–82.
23. **Stutzenberger, F. J.** 1972. Cellulolytic activity of *Thermomonospora curvata*: optimal assay conditions, partial purification, and product of the cellulase. Appl. Microbiol. **24**:83–90.
24. **Stutzenberger, F. J.** 1979. Degradation of cellulosic substances by *Thermomonospora curvata*. Biotechnol. Bioeng. **21**:909–913.
25. **Szakacs, G., K. Reczey, P. Hernadi, and M. Dobozi.** 1981. *Penicillium verruculosum* WA 30: a new source of cellulase. Eur. J. Appl. Microbiol. Biotechnol. **11**:120–124.