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Regulatory Mutations Affecting Cellulase Synthesis in *Bacillus subtilis* BS5

T.O. Femi-Ola

Department of Microbiology, University of Ado-Ekiti, P.M.B. 5363, Ado-Ekiti, Nigeria.

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Isolation and characterization of mutations in the regulatory system controlling cellulase synthesis in *Bacillus subtilis* BS5 isolated from the hindgut of wood-eating termites *Amitermes evuncifer* Silvestri was carried out. Mutation was chemically induced using ethyl methane sulphonate (EMS). Derepression of the cellulase gene at various levels was achieved in these mutants resulting in differential rate of the enzyme biosynthesis. Three classes of mutants were identified with respect to their cellulase induction rate and level of cellulase production in the presence of high concentration of glucose (2% w/v).

Key words: Cellulase, biosynthesis, *Bacillus*, mutation, termites.

Cellulases are the principal enzymes involved in the biodegradation of cellulose. They are generally assumed to be adaptive enzymes, which are subject to some kind of genetic regulation (Stewart and Leatherwood, 1976; Beguin, 1990). The biosynthesis of cellulase is regulated by in induction and repression. The inducer is usually the substrate for or some structurally related compound (Prescott *et al*., 1999). In most organisms, cellulase production is repressed in the presence of high concentration of readily metabolized carbon source (Coughlan, 1985). The rate of enzyme-mediated hydrolysis of the cellulose has been reported to be inhibited by products of hydrolysis and fermentation products, particularly when hydrolysis and fermentation are been carried out at the same time (Mosier *et al.*, 1999; Lynd *et al*., 2002). As a result of the presence of repressing substance and end product associated with the fermentation of waste cellulose, which have increased the cost of cellulose productions. Attempts have been made to get mutant organisms resistant to repression. It is believed that the use of organisms resistant to catabolite repression would circumvent the repression of cellulase synthesis and allow production of high yields of cellulase in direct microbial fermentation. The aim of the work reported here was to study the regulation of the synthesis of cellulase and to compare the mutants generated from a *Bacillus subtilis* strain BS5 isolated from the hindgut of the wood-eating termites *Amitermes evuncifer* Silvestri.

MATERIAL AND METHODS

Organism and growth condition

Bacillus subtilis BS5 was the parent strain; it was isolated from the hindgut of wood-eating termite *Amitermes evuncifer* Silvestri and maintained on nutrient agar slant. The organism was cultured in liquid basal medium containing the following (g/l) K_2HPO_4, 1.5; KH_2PO_4, 0.5; NaCl, 1.5; $MgSO_4{\cdot}7H_2O$, 0.05; $(NH_4)_2SO_4$, 1.0;

* To whom all correspondence should be addressed.
E-mail: titifemi2006@yahoo.com.

$CaCl_2 2H_2O$, 0.02; $FeSO_4 7H_2O$, 0.01; yeast extract, 0.5. Soluble carboxymethylcellulose High viscosity BDH, UK or any other carbon source was autoclaved separately and added to basal medium to give a final concentration of 1% Carbon source unless otherwise stated.

Isolation of *B. subtilis* mutants

The modified method of Shonukan and Nwafor (1989) was employed to mutagenize the cells of *B. subtilis* using ethyl methyl sulphonate (EMS) (Sigma, UK). A 25ml broth culture of the organism was incubated for 18hr at 35°C on a Gallenkamp rotary shaker at a speed of 120 rpm. The cells were recovered by centrifugation at 5000 rpm for 20min. The culture was washed twice with sterile normal saline and resuspended in minimal salt medium. A 0.08ml of EMS (1%) was then added to 2ml of the washed exponential phase nutrient broth culture of *Bacillus subtilis* BS5 at 1.0×10^9 cells per ml. The mixture of EMS and the cells was incubated for 1hr at 35°C. The cells were then washed twice with normal saline and diluted 1:10 in a minimal medium containing $MgSO_4$, 1.0mM; $Fe_2(SO_4)_3$, 1.25mM; $CaCl_2 2H_2O$, 50mm; $(NH_4)_2SO_4$, 15mM and 0.2% glucose. The culture was incubated at 35°C for 12h to allow for segregation of mutations. Then 0.2ml of the growing cell was plated out on a nutrient agar plate using a glass spreader. The plates were incubated at 35°C for 24h to give rise to individual mutant colonies. Distinct colonies were then replica plated on Nutrient agar plates containing CMC as sole carbon source 1% Congo red and incubated at 35°C for 48h. The selection of mutants for further studies was based on the diameter of clear zone surrounding the colonies. Selected mutants and wild type strains of *B. subtilis* were cultured in basal medium and incubated at 35°C for 30h. The cultures were harvested by centrifugation at 5000 rpm for 20minutes. Culture filtrates were assayed for cellulase production, while protein content was determined by the method of Lowry *et al*. (1951).

Effect of glucose and glycerol on cellulase production.

The wild type and mutant strain were grown in basal medium containing either glucose or glycerol with or without carboxymethyl cellulose as inducer. Cultures were incubated at 35°C for 30h and centrifuged at 5000 rpm for 20minutes. Cellulase activity and the protein content of the clear supernatant were determined.

Screening for catabolite repression

Selected mutants and wild type strain of *B. subtilis* were cultured in the basal medium containing high concentration of glucose (1% w/v). Cellulase synthesis was induced by adding 0.5% (w/v) carboxymethyl cellulose. Cultures were then incubated for 30h at 35°C.

Cellulase assay

Cellulase activity was determined by the method of Somogyi (1952). To a 0.5ml of 1% CMC in a 0.1M citrate phosphate buffer pH 6.8 was added a 0.5ml o f the enzyme preparation. The reaction mixture was incubated at 35°C for 1h in a water bath. The reaction was terminated by heating at 100°C for 10min. The control tube contained heat denatured enzyme to which the substrate was added. All assays were done in triplicates. One unit of cellulase activity was expressed as the amount of enzyme required to liberate 1µg glucose per minutes under the specified condition of the reaction.

Statistical analysis

Analyses of variance (ANOVA) and Duncan multiple comparison tests of data obtained were carried out using SPSS (Statistical Package for Social Sciences) version 11.0 software.

RESULTS

A total of nineteen mutant strains showing cellulolytic activities on carboxymethyl cellulose agar medium were isolated. Cellulase enzyme was synthesized by all the mutants at varying quantities (Table 1). Approximately 52.6% of the mutant strain showed higher cellulolytic activity than the wild type, while 47.4% of the mutants had lower cellulolytic activity when compared with the wild type. The presence of glucose and glycerol had varied influence on the cellulolytic activity of the mutant and wild type strain. Various derepressed levels of cellulase system were observed. Cellulase synthesis in the organism appeared repressed when glucose and carboxymethylcellulose were both present in the growth medium (Table 2). The highest value of induction was obtained for cellulase production when glycerol and CMC were placed together in the growth medium (Table 3).

Table 1. Cellulolytic activity of wild and mutant strains (MS1-MS19) of *Bacillus subtilis* BS5

Strain	Protein content (mg/ml)	Specific activity (UC/mg protein)	T-calculated
Wild type	0.18	0.49±0.01	-
MS1	0.23	0.74± 0.02	23.780
MS2	0.20	0.92±0.01	43.522
MS3	0.21	0.42± 0.03	2.962
MS4	0.17	0.93±0.01	31.298
MS5	0.28	0.33±0.02	16.405
MS6	0.30	0.05±0.01	44.957
MS7	0.16	0.46±0.01	3.624
MS8	0.28	0.08±0.01	36.892
MS9	0.20	0.80±0.01	28.597
MS10	0.16	1.06±0.06	45.523
MS11	0.23	0.11±0.01	43.325
MS12	0.13	0.34±0.05	5.347
MS13	0.21	1.00±0.02	54.611
MS14	0.20	0.27±0.01	22.064
MS15	0.13	0.70±0.02	6.318
MS16	0.15	0.37±0.01	10.166
MS17	0.11	0.65±0.03	8.959
MS18	0.15	1.37±0.02	42.584
MS19	0.12	0.79±0.02	5.032

T-table =2.776

Three classes of mutants were identified based on the degree of response to glucose repression (Table 4). The effect of high concentration of glucose (2% w/v) on cellulase production is shown in Table 5. Approximately 26.3% of the mutants completely lost the ability to produce the enzyme when subjected to 2% (w/v) glucose. However, 73.7% of the mutants could still express appreciable cellulase activity. Cellulase synthesis was also repressed in the wild type. Mutant strain MS18 had the highest level of cellulase production in the presence of high concentration of end product and was considered as catabolite repression insensitive mutant. The mutant was selected for further study to establish the cellulase regulation within *Bacillus subtilis* BS5.

DISCUSSION

Subjection of *Bacillus subtilis* strain BS5 to chemical mutagen, ethyl methane sulphonate (EMS) had an effect on the organism. A large percentage (52.6 %) of the mutant strains showed increased cellulolytic activity in comparison with the wild type. Cellulase synthesis was found to be

Table 2. Effect of glucose on cellulolytic activity of wild type and mutant strains (MS1-MS19) of *Bacillus subtilis* BS5

Strain	Cellulase activity (U/mg protein)		
	CMC only	CMC+Glucose	Glucose only
Wild type	0.49 ± 0.01	0.05 ± 0.01	0.03 ± 0.01
MS1	0.74 ± 0.02	0.08 ± 0.01	0.04 ± 0.01
MS2	0.92 ± 0.01	0.03 ± 0.01	0.02 ± 0.00
MS3	0.42 ± 0.03	0.03 ± 0.01	0.03 ± 0.01
MS4	0.93 ± 0.01	0.12 ± 0.02	0.07 ± 0.02
MS5	0.33 ± 0.02	0.05 ± 0.01	0.54 ± 0.01
MS6	0.05 ± 0.01	0.09 ± 0.03	0.02 ± 0.00
MS7	0.46 ± 0.01	0.13 ± 0.02	0.04 ± 0.01
MS8	0.08 ± 0.01	0.05 ± 0.02	0.06 ± 0.02
MS9	0.80 ± 0.01	0.15 ± 0.02	0.04 ± 0.01
MS10	1.06 ± 0.06	0.13 ± 0.02	0.04 ± 0.01
MS11	0.11 ± 0.01	0.18 ± 0.01	0.10 ± 0.01
MS12	0.34 ± 0.05	0.18 ± 0.02	0.08 ± 0.03
MS13	1.00 ± 0.02	0.03 ± 0.01	0.04 ± 0.01
MS14	0.27 ± 0.01	0.09 ± 0.00	0.09 ± 0.01
MS15	0.70 ± 0.02	0.22 ± 0.02	0.12 ± 0.02
MS16	0.37 ± 0.01	0.06 ± 0.02	0.04 ± 0.00
MS17	0.65 ± 0.03	0.22 ± 0.01	0.08 ± 0.01
MS18	1.37 ± 0.02	0.56 ± 0.02	0.17 ± 0.02
MS19	0.79 ± 0.02	0.09 ± 0.01	0.04 ± 0.01

Table 3. Effect of glycerol on cellulolytic activity of wild type and mutant strains (MS 1- MS 19) of *Bacillus subtilis* BS5

Strain	Cellulase activity (U/mg protein)		
	CM Conly	CMC+Glucose	Glucose Only
WT	0.49 ± 0.01	0.21 ± 0.03	0.10 ± 0.01
MS1	0.74 ± 0.02	0.13 ± 0.01	0.04 ± 0.01
MS2	0.92 ± 0.01	0.09 ± 0.02	0.04 ± 0.01
MS3	0.42 ± 0.03	0.65 ± 0.03	0.06 ± 0.02
MS4	0.93 ± 0.01	0.46 ± 0.02	0.06 ± 0.02
MS5	0.33 ± 0.02	0.06 ± 0.01	0.04 ± 0.01
MS6	0.05 ± 0.01	0.36 ± 0.02	0.06 ± 0.01
MS7	0.46 ± 0.01	0.60 ± 0.02	0.08 ± 0.02
MS8	0.08 ± 0.01	0.40 ± 0.01	0.04 ± 0.01
MS9	0.80 ± 0.01	0.36 ± 0.01	0.05 ± 0.01
MS10	1.06 ± 0.06	0.23 ± 0.02	0.09 ± 0.02
MS11	0.11 ± 0.01	0.06 ± 0.01	0.01 ± 0.00
MS12	0.34 ± 0.05	0.48 ± 0.02	0.09 ± 0.02
MS13	1.00 ± 0.02	0.06 ± 0.02	0.04 ± 0.01
MS14	0.27 ± 0.01	0.40 ± 0.01	0.03 ± 0.01
MS15	0.70 ± 0.02	0.19 ± 0.02	0.07 ± 0.01
MS16	0.37 ± 0.01	0.46 ± 0.01	0.06 ± 0.01
MS17	0.65 ± 0.03	0.04 ± 0.01	0.04 ± 0.01
MS18	1.37 ± 0.02	0.42 ± 0.02	0.08 ± 0.01
MS19	0.79 ± 0.02	0.35 ± 0.01	0.07 ± 0.02

Table 4. Classes of mutants

Class	Similar strains	Growth condition		Cellulase activity (U/mg) protein)	Induction
		C-Sourse*	Inducer		
Wild		Glycerol	None	0.10	2.1
type		Glycerol	CMC	0.21	
		Glucose	None	0.03	
		Glucose	CMC	0.05	
I (MS13)	MS1, MS2	Glycerol	None	0.04	1.5
	MS5, MS10	Glycerol	CMC	0.06	
	MS13, MS15	Glucose	None	0.04	
	MS17	Glucose	CMC	0.03	
II(MS18)	MS4,MS6,MS7	Glycerol	None	0.08	5.25
		Glycerol	CMC	0.42	
	MS9, MS11	Glucose	None	0.17	
	MS12, MS16	Glucose	CMC	0.56	
	MS18, MS19				
III (MS3)	MS3, MS8	Glycerol	None	0.06	
	MS14	Glycerol	CMC	0.65	
		Glucose	None	0.03	
		Glucose	CMC	0.03	10.83

* Carbon source

Table 5. Screening for catabolite repression insensitive mutants: effect of 2% (wt/vol) glucose on the cellulase production by wild type and mutant strains (MS1- MS19) of *Bacillus subtilis* BS5

Strain	Cellulase activity (U/mg protein)	T- calculated
Wild type	0.00	
MS1	0.00	0.00
MS2	0.00	0.00
MS3	0.00	0.00
MS4	0.12 ± 0.02	13.793
MS5	0.06 ± 0.01	10.200
MS6	0.10 ± 0.03	8.006
MS7	0.00	0.00
MS8	0.13 ± 0.03	13.393
MS9	0.05 ± 0.01	10.231
MS10	0.03 ± 0.01	5.167
MS11	0.03 ± 0.00	4.167
MS12	0.12 ± 0.04	5.272
MS13	0.18 ± 0.02	40.923
MS14	0.02 ± 0.00	3.728
MS15	0.09 ± 0.02	9.760
MS16	0.12 ± 0.02	20.941
MS17	0.00	0.00
MS18	0.21 ± 0.02	26.375
MS19	0.18 ± 0.04	13.286

T-table = 2.776

higher in the carboxymethylcellulose medium containing glucose. The production of cellulase in this organism was also sensitive to catabolite repression due to the presence of rapidly metabolized carbon source. Coughlan (1985) and Mosier *et al.* (1999) reported the repression of cellulase production in the presence of high concentration of readily metabolized carbon source and product of cellulose hydrolysis. Regulation of cellulase production by catabolite repression has been reported by several workers in different organisms. They include *Clostridium thermocellum* (Zhang and Lynd, 2005), *Bacillus subtilis* (Moreno *et al*., 2001) and *Thermobifida fusca* (Spiridonov and Wilson 1998; 2000) *Bacillus pumilus*. (Kotchoni and Shonukan, 2002; Kotchoni *et al*., 2003).

The result (Table 1) shows that some mutants of *B.subtilis* BS5 cellulase were synthesized constitutively on non inducing carbon sources. The *B. subtilis* BS5 used in this study is inducible for cellulase. Reduction in the observed activity of cellulase in the presence of glucose alone and when glucose and carboxymethylcellulose were both present in the growth medium suggest that the cellulase gene in *Bacillus subtilis* BS5 is sensitive to glucose. The repression of cellulase by high concentration of glucose also suggests that its production is regulated by both induction and repression as it has been reported in most other cellulolytic microorganism (Ali and Sayed, 1992; Zhang and Lynd, 2005). Insignificant cellulase activity was observed when the wild type and mutant strains of *Bacillus subtilis* BS5 were cultured on either glucose or glycerol as the sole carbon and energy sources. It has been reported that inducible enzyme are normally produced in small units in the absence of inducers because the repressor system will not function in an absolute block, thus allowing constitutive production of cellulase which could easily yield soluble hydrolysis product of natural cellulose (Beguin, 1990). The soluble products of hydrolysis could then enter the cell and then function as inducers because cellulase can not enter the cell and act as an inducer because of its large size and complexity (Beguin, 1990; Lynd *et al*., 2002). The isolated mutants were grouped according to their induction ratios using carboxymethylcellulose as the inducer and glycerol as the carbon and energy sources because of the inhibitory nature of glucose on the enzyme synthesis. The reduced secretion of cellulase observed in the Class 1 mutant may reflect probable alteration in the regulatory gene as suggested by Ishola and Shonukan (1997).

CONCLUSION

Cellulase biosynthesis in *Bacillus subtilis* BS5 was regulated by induction and repression mechanisms. Mutants producing cellulase in varying quantities under different growth medium were generated using ethyl methane sulphonate (EMS). Some mutants were resistant to catabolite repression. Even though the mechanism through which EMS triggered the catabolite repression in these mutants were not established in this study, this mutation brought out new strains that were cellulase hyper producers in the presence of glucose. Among the

mutant strains generated, MS18 has a potential for enhanced cellulase biosynthesis and greater resistance to catabolite resistance.

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