JOURNAL OF BACTERIOLOGY, Dec. 1985, p. 1146–1152
0021-9193/85/121146-07$02.00/0

Vol. 164, No. 3

Regulation and Genetic Enhancement of Glucoamylase and Pullulanase Production in *Clostridium thermohydrosulfuricum*

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Received 3 May 1985/Accepted 4 September 1985

We studied the general mechanism for regulation of glucoamylase and pullulanase synthesis in *Clostridium thermohydrosulfuricum*. These amylases were expressed only when the organism was grown on maltose or other carbohydrates containing maltose units. Amylase synthesis was more severely repressed by glucose than by xylose. Catabolite repression-resistant mutants were isolated by using nitrosoguanidine treatment, enrichment on 2-deoxyglucose, and selection of colonies with large clear zones on iodine-stained glucose-starch agar plates. Amylases were produced in both wild-type and mutant strains when starch was added to cells growing on xylose but not when starch was added to cells growing on glucose. In both wild-type and mutant strains, glucoamylase and pullulanase were produced at high levels in starch-limited chemostats but not in glucose- or xylose-limited chemostats. Therefore, we concluded that amylase synthesis in *C. thermohydrosulfuricum* was inducible and subject to catabolite repression. The mutants produced about twofold more glucoamylase and pullulanase, and they were catabolite repression resistant for production of glucose isomerase, lactase, and isomaltase. The mutants displayed improved starch metabolism features in terms of enhanced rates of growth, ethanol production, and starch consumption.

Bacterial and fungal amylases, such as α-amylase, glycoamylase, and pullulanase, have been widely used in starch-processing industries (2, 3, 5, 7, 18, 19). High value is placed on extreme thermostability and thermoactivity of amylases used in the bioprocessing of starch (2, 3, 18). Nonetheless, most industrial amylases are produced by mesophilic microorganisms (5, 18, 19). However, thermophiles often possess thermostable enzymes (21, 27), and their amylases may have important features for the starch-processing industry.

Thermoanaerobic fermentations have been a focal point because of their potential for production of thermostable enzymes as well as ethanol (25, 27, 28). However, little is known about the starch fermentation metabolism of thermoanaerobes. We previously found that *Clostridium thermohydrosulfuricum* (11) produces an extremely thermostable cell-bound pullulanase (EC 3.2.1.41) and glucoamylase (EC 3.2.1.3), which are stable and optimally active at 85 and 75°C, respectively, and active over a wide range of pH values (3.5 to 10.5), whereas *Clostridium thermosulfurogenes* (10) produces an extracellular and thermostable β-amylase (EC 3.2.1.2) which is stable and optimally active at 80 and 75°C, respectively.

Previously, considerable work has dealt with elucidation of the regulation mechanisms involved in aerobic mesophilic bacterial and fungal α-amylase synthesis (7, 16, 20, 22, 23, 26), but there has not been work done on amylase synthesis in anaerobes or extreme thermophiles. However, little is known about the regulation mechanisms for glucoamylase or pullulanase synthesis in microorganisms. A few studies have shown that glucoamylase (1, 4) or pullulanase synthesis (9) is inducible; an exception is *Clostridium acetobutylicum* glucoamylase (6) which is produced on glucose during the logarithmic growth phase.

The purposes of this study were to determine the physiological mechanisms for regulation of glucoamylase and pullulanase synthesis in *C. thermohydrosulfuricum*, to obtain regulatory mutants in order to support the conclusions derived from physiological studies, and to assess the metabolic and physiological properties of mutant strains that are catabolite repression resistant for amylase synthesis.

MATERIALS AND METHODS

Unless indicated below, the materials and methods used were those described previously and in the accompanying papers (10–14). The techniques used for mutagenesis, enrichment, and isolation of mutants have been described

TABLE 1. Effect of carbon sources on production of amylases by *C. thermohydrosulfuricum*[a]

Growth substrate	Final cell concn (OD_{660})[b]	Amylase activity (U/mg of protein)	
		Pullulanase	Glucoamylase
Glucose	1.15	0.00	0.000
Xylose	0.98	0.00	0.000
Mannose	0.48	0.00	0.000
Fructose	0.63	0.00	0.000
Cellobiose	0.75	0.00	0.000
Lactose	0.56	0.00	0.000
Maltose	0.52	0.40	0.040
Maltotriose	0.59	0.36	0.041
Amylopectin	0.62	0.27	0.040
Soluble starch	0.80	0.27	0.041
Insoluble starch	0.46	0.18	0.030
Glycogen	0.66	0.37	0.040
Pullulan	0.24	0.51	0.030

[a] Cells were grown at 65°C for 24 h in serum bottles containing 50 ml of TYE medium supplemented with substrate at a concentration of 0.5%. Cultures grown in media containing individual carbon sources were used as inocula. Activities were measured in cell extracts.

[b] OD_{660}, Optical density at 660 nm.

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TABLE 2. Comparison of amylolytic enzyme activities in relation to the saccharide composition of growth medium for *C. thermohydrosulfuricum*[a]

Substrate(s)	Growth (OD$_{660}$)[b]	Amylase activity (U/ml)	
		Pullulanase	Glucoamylase
Maltose	0.57	0.048	0.0104
Glucose	1.60	0.000	0.0001
Glucose + maltose	1.60	0.000	0.0005
Xylose	1.08	0.001	0.0000
Xylose + maltose	1.45	0.002	0.0049
Fructose	1.60	0.000	0.0000
Fructose + maltose	1.60	0.000	0.0001
Mannose	1.58	0.000	0.0001
Mannose + maltose	1.48	0.000	0.0001
Cellobiose	1.49	0.000	0.0000
Cellobiose + maltose	1.45	0.001	0.0009
Lactose	1.40	0.000	0.0001
Lactose + maltose	1.40	0.002	0.0035

[a] Cultures pregrown on TYEG medium were washed three times with reduced LPBM medium and used as inocula. Cultures were grown without shaking in serum bottles containing 50 ml of TYE medium at 65°C for 30 h and then were washed with water and assayed for amylolytic enzymes. The concentrations of maltose and other saccharides were 0.5 and 1.5%, respectively.

[b] OD$_{660}$, Optical density at 660 nm.

TABLE 3. Effect of 2-deoxyglucose concentration on the growth of *C. thermohydrosulfuricum* when glucose or starch was used as a carbon source[a]

Carbon source(s)	Growth (OD$_{660}$) after:[b]	
	20 h	30 h
Controls		
No carbon source	0.05	0.05
0.5% 2-Deoxyglucose	0.10	0.07
Starch-containing media		
0.5% Starch	0.56	0.60
0.5% Starch + 0.5% 2-deoxyglucose	0.11	0.11
0.5% Starch + 0.05% 2-deoxyglucose	0.11	0.19
0.5% Starch + 0.005% 2-deoxyglucose	0.09	0.12
0.5% Starch + 0.0005% 2-deoxyglucose	0.52	0.59
Glucose-containing media		
0.5% Glucose	0.67	0.70
0.5% Glucose + 0.5% 2-deoxyglucose	0.36	0.44
0.5% Glucose + 0.05% 2-deoxyglucose	0.37	0.45
0.5% Glucose + 0.005% 2-deoxyglucose	0.46	0.54
0.5% Glucose + 0.0005% 2-deoxyglucose	0.63	0.68

[a] Cells were cultivated in pressure tubes containing LPBM medium supplemented with 0.3% yeast extract, 0.5% glucose or 0.5% starch, and various concentrations of 2-deoxyglucose without shaking at 65°C.

[b] OD$_{660}$, Optical density at 660 nm.

previously from *C. thermosulfurogenes* (14), except that cells were treated with 400 μg of nitro-*N*-nitrosoguanidine per ml at 65°C for 1 h. *C. thermohydrosulfuricum* wild-type strain ATCC 33223 and mutant strain Z21-109 have been deposited with the American Type Culture Collection, Rockville, Md.

RESULTS

Relationship of glucoamylase and pullulanase production to carbon sources. Table 1 shows that glucoamylase and pullulanase were detected only when cells were grown on stimulatory substrates, such as maltose or other carbohy-

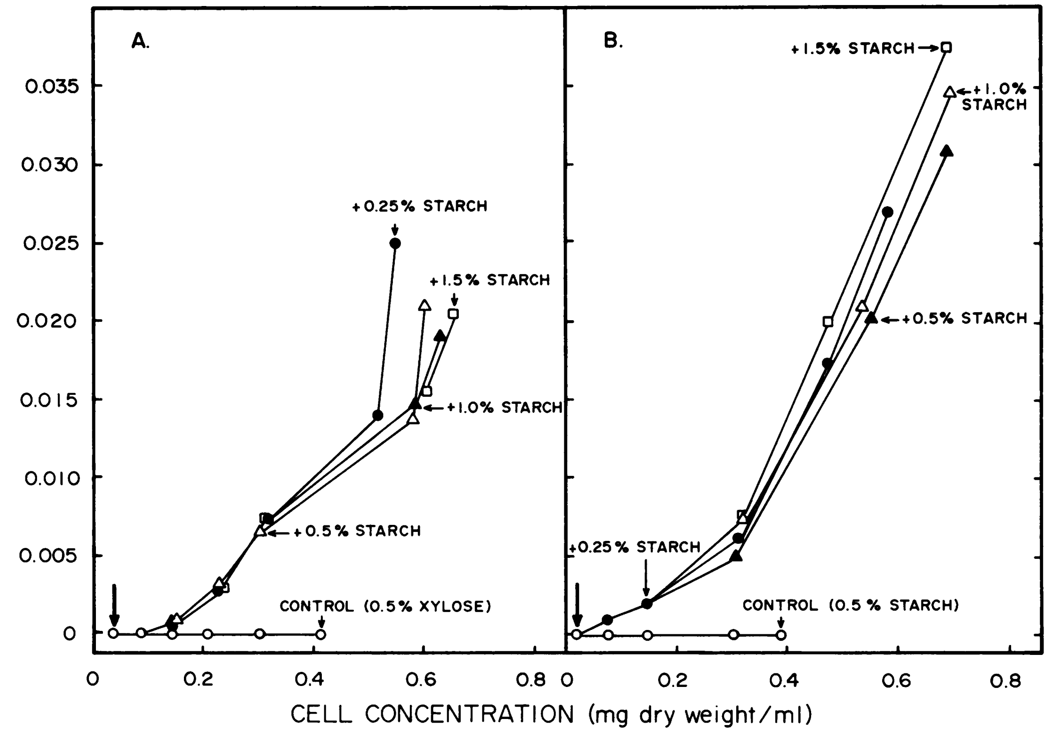

FIG. 1. Effect of starch on the differential rate of pullulanase synthesis in *C. thermohydrosulfuricum* wild type (A) and mutant Z21-109 (B). Experiments were conducted in serum bottles containing 50 ml of TYE medium. The large arrows indicate when starch was added to cells growing on 0.5% xylose.

TABLE 4. Comparison of amylolytic enzyme activities in *C. thermohydrosulfuricum* wild-type and mutant strains[a]

Strain	Growth substrate(s)	Growth (OD_{660})[b]	Pullulanase activity (U/ml)	Glucoamylase activity (U/ml)
Wild type	1% Starch	1.23	0.23	0.030
	0.5% Starch + 1.5% glucose	1.40	0.00	0.000
	1% Xylose (or 1% glucose)	0.95 (1.05)[c]	0.00	0.000
Catabolite repression-resistant mutant Z67-143	1% Starch	1.16	0.23	0.030
	0.5% Starch + 1.5% glucose	1.35	0.23	0.020
	1% Xylose (or 1% glucose)	1.05 (1.07)	0.00	0.000
Hyperproductive mutant Z21-109	1% Starch	1.30	0.43	0.048
	0.5% Starch + 1.5% glucose	1.30	0.24	0.026
	1% Xylose (or 1% glucose)	0.95 (1.08)	0.00	0.000

[a] Cells were cultivated in pressure tubes containing 10 ml of TYE medium supplemented with the carbon sources indicated at 65°C without shaking for 24 h. Enzyme activities were measured in cell suspensions washed with water.

[b] OD_{660}, Optical density at 660 nm.

[c] The values in parentheses are the values obtained when 1% glucose was used.

drates containing maltobiose units. Table 2 shows the effects of maltose on the expression of glucoamylase and pullulanase by *C. thermohydrosulfuricum* in medium containing various carbon sources as growth substrates. Our results indicate that both glucoamylase production and pullulanase production are more severely repressed by glucose than by xylose or lactose.

Isolation of catabolite repression-resistant mutants. These studies were initiated in order to test the assumption that glucoamylase synthesis and pullulanase synthesis are regulated by induction and catabolite repression mechanisms. Table 3 shows that 2-deoxyglucose (17, 24) served as a nonmetabolizable catabolite repressor in *C. thermohydrosulfuricum* because the species grew on glucose medium supplemented with 2-deoxyglucose but not on starch medium containing more than 0.005% 2-deoxyglucose or on 2-deoxyglucose alone. Therefore, mutants were isolated by mutagenesis with nitro-*N*-nitrosoguanidine, enrichment with 2-deoxyglucose, and selection of colonies with large halos on starch-glucose agar plates that were stained with iodine. About 800 colonies were tested for alteration in the regulational nature of enzyme synthesis, and two major kinds of mutants were chosen and characterized further.

The glucoamylase and pullulanase activities of wild-type

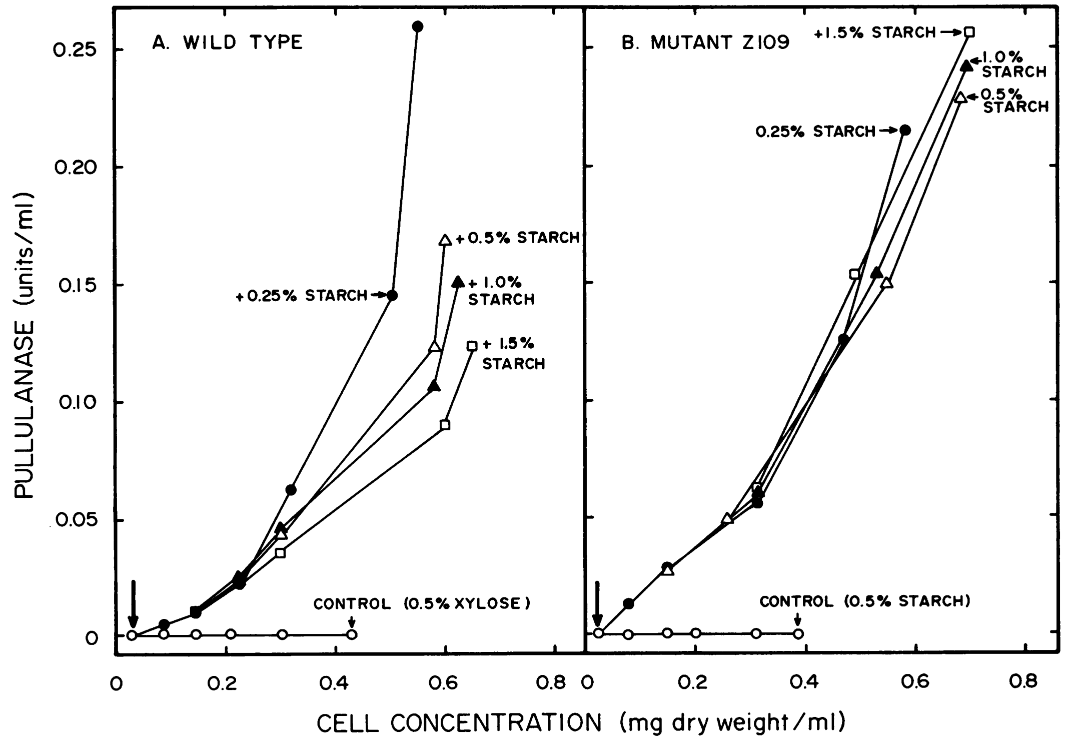

FIG. 2. Effect of starch on the differential rate of glucoamylase synthesis in *C. thermohydrosulfuricum* wild type (A) and mutant Z21-109 (B). The experimental procedures were the same as those described in the legend to Fig. 1.

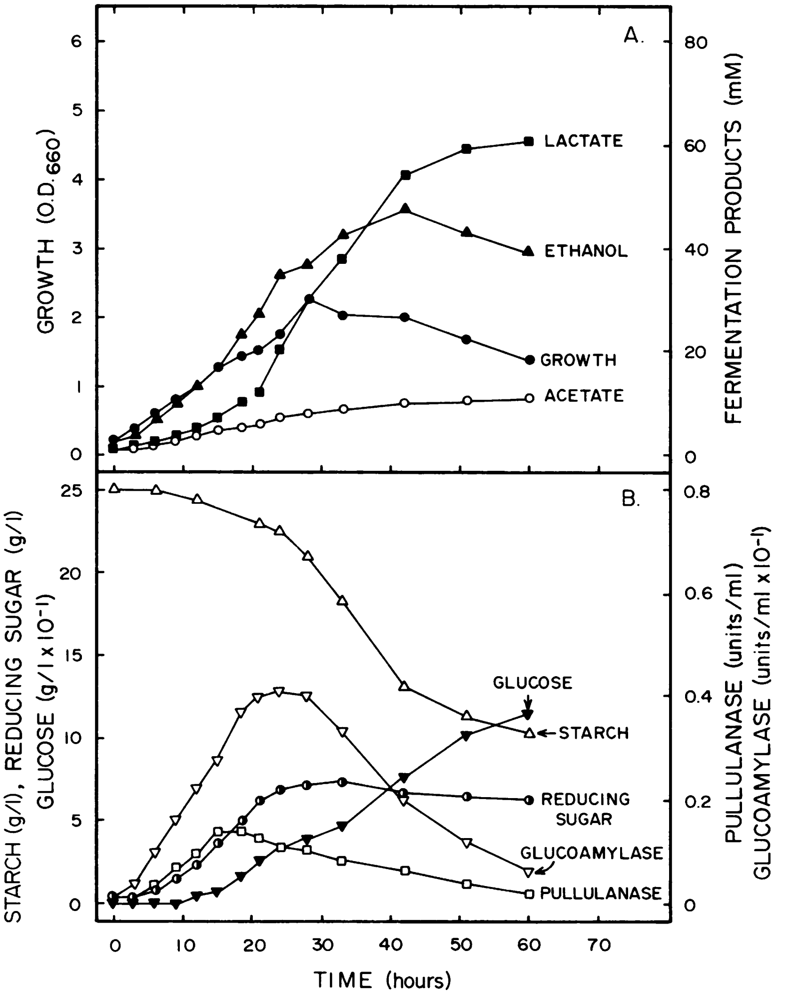

FIG. 3. Starch metabolism time course of *C. thermohydrosulfuricum* wild-type strain 39E in a gassed, pH-controlled fermentor. Experiments were conducted in a fermentor containing 650 ml of TYE medium and 2.5% soluble starch, which was continuously gassed with N_2-CO_2 (95:5) and controlled at pH 6.0. O.D.$_{660}$, Optical density at 660 nm.

and mutant strains grown on various carbon sources are compared in Table 4. These mutants were confirmed to be stable by testing amylase productivities after at least 10 culture transfers on various carbon sources. Our data show that both mutant Z67-143 and mutant Z21-109 were catabolite repression resistant because they produced the same amount of glycoamylase and pullulanase on medium containing 0.5% starch and 1.5% glucose as produced by the wild type on medium containing 1% starch. Notably, mutant Z21-109 produced about twofold more amylase on starch medium than the wild type, and it was classified as a hyperproductive mutant.

TABLE 5. Comparison of growth and fermentation products in *C. thermohydrosulfuricum* wild-type and mutant strains[a]

Strain	Growth substrate	Growth (OD_{660})[b]	μ_{max} (h^{-1})[c]	Substrate consumption (μmol, as glucose)	Amt of end products (μmol/tube)			
					Ethanol	Acetate	Lactate	H_2
Wild type	Glucose	0.97	0.36	278	498	50	83	48
	Starch	0.80	0.17	270	461	26	54	39
Catabolite repression-resistant mutant Z67-143	Glucose	0.39	0.36	139	306	43	48	28
	Starch	1.00	0.16	248	390	35	25	40
Hyperproductive mutant Z21-109	Glucose	1.40	0.37	278	551	27	47	34
	Starch	1.18	0.28	271	472	39	36	44

[a] Cells were cultivated in pressure tubes containing 10 ml of TYE medium supplemented with substrate at a concentration of 0.5% at 65°C without shaking for 24 h.

[b] OD_{660}, Optical density at 660 nm.

[c] μ_{max}, Maxiumum growth rate.

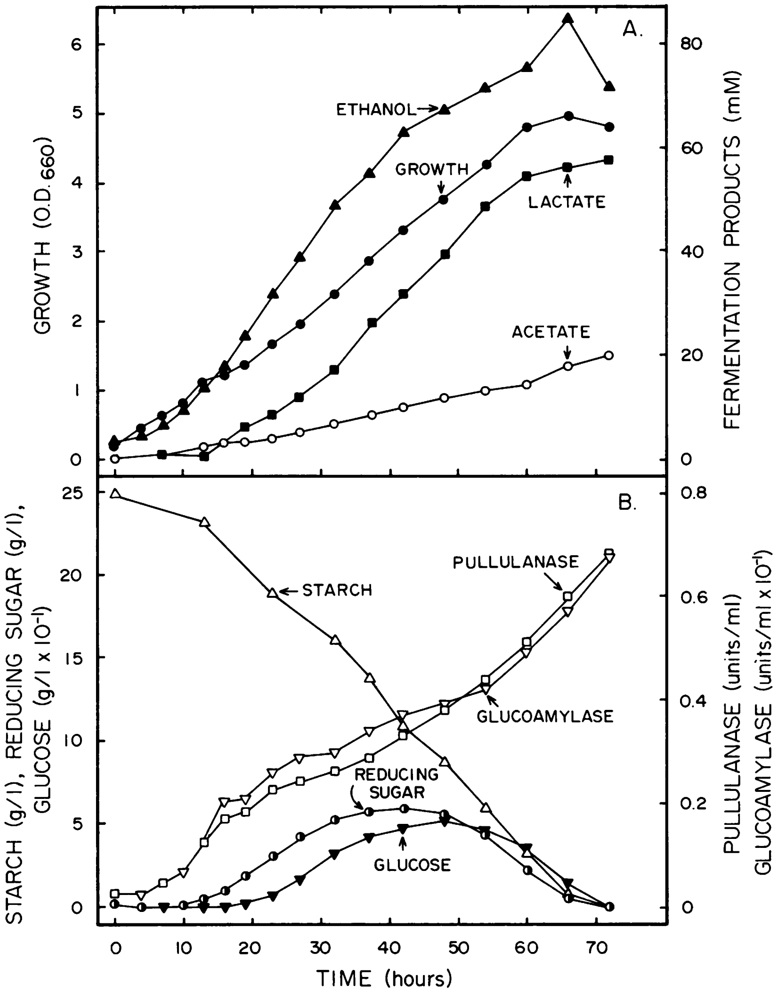

FIG. 4. Starch metabolism time course of *C. thermohydrosulfuricum* mutant strain Z21-109 in a gassed, pH-controlled fermentor. The experimental procedures were the same as those described in the legend to Fig. 3. O.D.$_{660}$, Optical density at 660 nm.

Induced synthesis and catabolite repression of amylase synthesis. The effect of starch concentration on the differential rate of amylase synthesis was compared in the wild type and mutant strain Z21-109 (Fig. 1 and 2). Our data indicate that an inducer (e.g., starch) was required for expression of both pullulanase (Fig. 1) and glucoamylase (Fig. 2) because in both wild-type and mutant strains the enzymes were synthesized only after starch was added to cells growing on xylose. The differential rate of enzyme synthesis decreased during the logarithmic growth phase for both activities according to the increase in starch concentration in the wild type but not in the mutant, indicating that there was catabolite repression caused by glucose accumulation from the action of glucoamylase and pullulanase during starch fermentation (Fig. 3 and 4).

To eliminate the possibility of regulation of amylase synthesis by repression (i.e., constitutive but catabolite repressible), carbon-limited chemostat studies were performed. Both glucoamylase and pullulanase were expressed by both wild type and mutant Z21-109 at higher levels in starch-limited chemostat cultures than in batch cultures, but not under glucose- or xylose-limited chemostat conditions (data not shown), indicating that there was catabolite repression by glucose which accumulated from the action of glucoamylase and pullulanase during the batch starch fermentation time course.

General characterization of mutants. The mutants were similar to the wild type in morphology, kinds of catabolic end products, and sporulation. Table 5 shows that the mutants had higher growth rates and yields on starch medium than the wild type, due to the enhanced amylase activities and (to a lesser extent) to improved resistance to lysis in the stationary phase (microscopic observation). It is noteworthy that mutant Z21-109 produced more ethanol than the wild type as a consequence of decreased lactate production. Other experiments were performed in order to investigate whether the catabolite repression-resistant mutants were altered specifically in only glucoamylase and pullulanase production or also in the production of other saccharide-transforming enzymes. Table 6 shows that mutant Z21-109 was catabolite repression resistant for various saccharide-transforming enzymes in addition to glycoamylase and pullulanase because it produced glucose isomerase, isomaltase, and lactase under conditions that repressed enzyme synthesis in the wild type.

Comparison of starch metabolism time courses in wild-type and hyperproductive mutant strains. We wanted to assess both catabolite repression resistance and the potential im-

TABLE 6. Comparison of saccharide-transforming enzyme activities in *C. thermohydrosulfuricum* wild type and mutant strain Z21-109[a]

Enzyme	Growth substrate(s)	Sp act (U/mg of cells)	
		Wild type	Mutant Z21-109
Glucose isomerase	0.5% Glucose	0.05	0.05
	0.5% Xylose	0.45	0.42
	0.5% Xylose + 1.5% glucose	0.05	0.21
Isomaltase	0.5% Glucose	0.00	0.00
	0.5% Isomaltose	0.44	0.76
	0.5% Isomaltose + 1.5% glucose	0.03	0.14
Lactose	0.5% Glucose	0.03	0.12
	0.5% Lactose	1.46	1.67
	0.5% Lactose + 1.5% glucose	0.18	1.33

[a] Cells were grown in serum bottles containing 50 ml of TYE medium and the growth substrates indicated at 60°C for 24 h without shaking. Only glucose isomerase activity was assayed anaerobically.

provement of mutants for starch transformation process applications. Figures 3 and 4 show the fermentation time courses of *C. thermohydrosulfuricum* wild type and mutant Z21-109, respectively, grown on 2.5% starch under conditions of continuous gassing and pH control at 6.0. In the wild-type strain (Fig. 3), starch was not completely utilized under the conditions used. Reducing sugar and glucose accumulated continuously during the entire culture period. Growth and end product formation nearly ceased, even in the presence of large quantities of reducing sugar and glucose in the medium, and this was followed by decreases in glucoamylase and pullulanase activities due to extensive cell lysis. In the mutant strain (Fig. 4), starch metabolism was significantly improved compared with the wild type in terms of starch utilization, amylase production, and ethanol production. Growth of the mutant continued until starch was completely utilized. Reducing sugar and glucose accumulation increased in the early growth phase and disappeared at the end of fermentation. The decrease in the ethanol concentration observed during the stationary growth phase was the result of evaporation caused by gassing.

DISCUSSION

In general, our results prove that amylase synthesis is regulated and is a rate-limiting step during growth of thermoanaerobes on starch. Furthermore, we developed techniques to obtain hyperproductive, catabolite repression-resistant amylase mutants which enhanced the overall starch metabolism physiology of *C. thermohydrosulfuricum* and its ethanol productivity.

Our data demonstrate that glucoamylase synthesis and pullulanase synthesis are induced by maltose and other carbohydrates containing maltose units and are subject to catabolite repression in *C. thermohydrosulfuricum*. Essentially nothing is known about the mechanisms of regulation of saccharidase synthesis in thermophiles or anaerobes. Therefore, our findings advanced our fundamental understanding of regulational mechanisms involved in saccharide fermentations by these poorly studied microbes. Our data also help explain the basis for enhancement of glucoamylase and pullulanase activities by cocultures of *C. thermohydrosulfuricum* and *C. thermosulfurogenes* (12). The catabolite repression caused by glucose accumulation during starch fermentation by *C. thermohydrosulfuricum* was eliminated by the consumption of glucose by both species in coculture.

Recently, ethanol-resistant mutants of *Clostridium thermocellum* (8) and *C. thermohydrosulfuricum* were isolated (15). However, isolation of mutants with regulational alterations in saccharidase production has not been reported previously in thermoanerobes. We have also isolated constitutive or derepressed mutants of *C. thermosulfurogenes* in β-amylase production (14).

We previously demonstrated that the glucoamylase and pullulanase of *C. thermohydrosulfuricum* (11) and the β-amylase of *C. thermosulfurogenes* (10) are extremely thermostable and thermoactive. Therefore, achievement of strain improvements with amylase mutants suggests that a genetic approach may be useful for advancing the practical potential of using thermoanaerobic fermentations for industrial production of amylases as well as ethanol from starch.

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