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Regulatory Factors Affecting α-Amylase Production in *Bacillus licheniformis*

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Possible factors regulating α-amylase synthesis in wild-type *Bacillus licheniformis* and in mutants producing elevated levels of the enzyme were studied in terms of catabolite repression, apparent temperature-sensitive repression, induction, and culture age. The synthesis of α-amylase in the parent strain occurred long after the culture reached the stationary phase of growth as a result of de novo protein synthesis, occurred only at high temperature around 50 C and not below 45 C, appeared to be induced in the presence of oligosaccharides with some linkage of α-1,4-, β-1,4-, β-1,6-glucosyl glucose, or α-1,6-galactosyl glucose, and was repressed by the addition of exogenous glucose or low-molecular-weight metabolites. The addition of cyclic adenosine 3′,5′-monophosphate stimulated α-amylase accumulation in growing cultures of the parent strain, but neither shortened the long lag period prior to the start of α-amylase synthesis nor mitigated the repressive effect of glucose. Mutant strains derived from the parent strain showed variation in the pattern of α-amylase synthesis, and some of them such as F-12s and F-14 produced α-amylase constitutively and without sensitivity to catabolite repression or transient repression from the moment of cell growth. These results are discussed in relation to possible regulatory mechanisms that might account for the observed characteristics of α-amylase synthesis in this facultative thermophilic microorganism.

There are a large number of reports dealing with the synthesis of bacterial α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) (3, 6, 9, 15), one of the typical extracellular enzymes, and considerable attention has also been given to the mechanism that controls its synthesis (2, 8, 13, 14, 17). There is, however, disagreement as to whether α-amylase is produced by *Bacillus subtilis* in logarithmic (3, 6) or stationary phase (9). The latter might suggest catabolite repression (8, 13), but Coleman (2) argued that α-amylase synthesis is not subject to this repression but rather is controlled by the nucleic acid precursor pool size. Since he gave no explanation about the dissimilarity in expression of growth genes and exoenzyme structural genes, the weak point in his theory, as indicated by Schaeffer (13), is that many genes unconcerned with ribosome synthesis become expressed during growth. However, since the amount of enzyme detected in the autolyzate or lysozyme-lysed solution of the bacterial cells was very small (15), the time lag observed by Nomura et al. (9) between cell growth and α-amylase appearance in the culture medium was suggested to mean that the expression of the amylase gene may be regulated in a manner different from the expression of the genes specifying the cellular proteins.

Another question about α-amylase synthesis concerns "induction" by starch or lower-molecular-weight oligosaccharides. It is known empirically that higher yields of amylase can be obtained on media comprised of complex raw materials containing starch, such as maize, barley, wheat, or malt, than on artificial defined media (1, 10), and that the rate of the enzyme synthesis is greater when starch rather than glucose is used as the sole source of carbon (4, 6, 15). Hence, the commercial production of *B. subtilis* α-amylase is now accomplished by use of high concentrations of starch (8 to 12%) (9). These observations have been regarded as evidence for inducibility of α-amylase synthesis.

On the other hand, however, some strains of *Bacillus* have been reported to produce α-amylase when grown on media containing glucose or other monosaccharides as the sole source of carbon or energy (3, 6, 8, 15). Accordingly, no convincing evidence has been obtained to indicate that α-amylase production is really inducible. Such a confused situation has arisen partly

because of the diversity of strains, growth media, and experimental conditions employed, and partly because α-amylase synthesis is controlled by complicated regulation mechanisms which also slightly differ with the strains used.

Saito previously reported that a strain of *B. licheniformis* isolated from soil produces a thermostable α-amylase which has an extremely broad pH activity, showing high activity even in the alkaline range (11). In studying this strain, it was found that the enzyme was synthesized at high temperature (approximately 50 C) in the presence of starch, but not at temperatures below 45 C. Also, the addition of glucose to growing cultures caused a complete inhibition of α-amylase synthesis. An attempt to obtain mutants with higher amylase yield was successful, and some were found to produce the enzyme during growth on a medium containing either starch or glucose as the sole carbon source, indicating resistance to catabolite repression. The purpose of this work was to investigate factors affecting α-amylase formation by using the parent and mutant strains of *B. licheniformis*. The results are discussed in relation to possible regulatory mechanisms that might account for the observed characteristics of synthesis of this enzyme.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in all experiments were the parent strain of *Bacillus licheniformis* 584 (11), its derivatives including mutants A1, C1, N69, N108, N116, F-4s, F-5s, F-12s, and F-14, and a strain of *B. subtilis* 29 newly isolated from soil in Japan. All the mutants of *B. licheniformis* were derived from parent strain 584 in the following way, and were related to different patterns of amylase production. Cells of the parent strain in the logarithmic phase of growth were treated with 200 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 40 min at 37 C. The treated cells were washed with 0.05 M phosphate buffer (pH 7.5) by centrifugation, grown overnight on brain-heart infusion broth at 37 C, and spread on AH-agar plate (11) containing 0.5 or 2% glucose in petri dishes, which were then incubated for 16 h at 50 C. An iodine solution (ca. 0.4 ml) was poured under the agar of the plates, and colonies with amylase-positive halos were selected as mutants, since colonies of the parent strain could not form a halo on AH-medium containing more than 0.5% glucose under the same conditions.

Growth media. The culture medium contained 4% soluble starch, 0.5% $(NH_4)_2HPO_4$, 0.5% yeast extract (Difco), 0.2% sodium citrate, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.008% $CaCl_2 \cdot 2H_2O$. This medium will be referred to hereafter as the standard growth medium. In appropriate experiments, soluble starch of the standard growth medium was replaced with 2% sorbitol. These media were adjusted to pH 7.2 before autoclaving.

Growth of bacteria. Two drops of bacterial cell-suspension were inoculated into a large test tube (2.2-cm diameter by 19-cm length) containing 10 ml of the standard growth medium. The inoculated test tube was aerobically incubated at an appropriate temperature on a shaker adjusted to 300 rpm. The growth was followed by variation in optical density at 690 nm with a Klett-Summerson photoelectric colorimeter. The production of α-amylase was determined by periodically assaying the culture fluid. To determine optimum temperatures for growth and α-amylase formation, an L-shaped tube containing the standard growth medium (5 ml) was adapted to a temperature gradient incubator. In the induction experiments, solutions of monosaccharides and oligosaccharides were sterilized by membrane filtration (0.45-μm pore size, 25-mm diameter; Millipore Corp.), whereas solutions of polysaccharides were autoclaved and then added aseptically to the cultures to give a final concentration of 0.5 or 1%. Induction is considered to result from the carbohydrate added when the α-amylase activity per milliliter of the culture fluid increases at least 15-fold. For determination of the number of spores, the culture was heated for 15 min at 80 C, diluted with saline, plated on brain-heart infusion agar, and incubated at 37 C.

Amylase activity. After an appropriate time of incubation, each culture was centrifuged at 5,000 × *g* for 15 min, and the supernatant fraction was assayed for α-amylase activity by measurement of dextrinizing power, using amylose as a substrate, according to the method of Fuwa (7) with slight modifications. Dilution of enzyme solution was made in 0.02 M tris(hydroxymethyl)aminomethane-acetate buffer (pH 8.0). In the standard assay the reaction mixture containing 0.25 ml of enzyme solution, 0.25 ml of 0.5 M glycine-sodium hydroxide buffer (pH 9.0), and 0.5 ml of 0.2% amylose was incubated at 50 C for 10 min. The reaction was stopped with 1.0 ml of 1.5 N acetic acid and 1.0 ml of iodine reagent (0.2% iodine and 2.0% potassium iodide). Distilled water (17 ml) was added to make a volume of 20 ml, and absorbance at 690 nm was measured on a Klett-Summerson photoelectric colorimeter fitted with a no. 69 filter. Consequently, one unit of amylase activity (D.P.) was defined as the amount of enzyme that produces 10% reduction in the intensity of the blue color of the amylose-iodine complex under the conditions used. Blanks lacking enzyme were run with each batch of assays. The activity was proportional to the enzyme concentration unless the change in absorbance exceeded 0.6.

Other procedures and chemicals. Anionic polyacrylamide disc-gel electrophoresis and immunodiffusion analysis were performed as described previously (11, 12). When intracellular α-amylase activity was to be determined, the cells were harvested by centrifugation, extensively washed with 0.05 M tris(hydroxymethyl)aminomethane-acetate buffer (pH 8.0) and resuspended in the same buffer at approximately a 10-fold higher concentration of cells. The cells were

disrupted by sonic oscillation with a Marusan 20-kc Sonifier at −3 C for 5 min at about 70 W.

The extract was centrifuged at 100,000 × *g* for 30 min, and the supernatant fluid was assayed for amylase activity.

Rifampin, nalidixic acid, and tetracycline-hydrochloride (Achromycin) were purchased from Boehringer Mannheim Japan (Tokyo), Nakarai Chemicals, Ltd. (Kyoto), and Lederle Japan, Ltd. (Tokyo), respectively. Cyclic adenosine 3′,5′-monophosphate (cyclic AMP) was a generous gift from J. Ishiyama. The other materials were of reagent grade and from commercial sources.

RESULTS

Time course of α-amylase formation in growing cultures of parent and mutant strains. The progressive relationship of bacterial growth and α-amylase secretion was examined when parent strain 584 and mutant strains of *B. licheniformis* were cultivated aerobically in a 4% starch medium at 50 C (the parent strain 584 and mutant C1) or 46 C (mutants F-12s and F-14) for a long time. It was found in every case that logarithmic growth continued for about 4 h, and then after a lag for 4 to 8 h, during which the bacterial concentration doubled, a final stationary phase was quickly reached. During the stationary phase, the cell concentration remained almost constant for about 24 h and then began to gradually decrease during the remainder of the experimental period. When parent strain 584 was cultivated in the standard medium at 50 C, α-amylase began to appear in the culture medium about 48 h after the culture started, and its maximum accumulation occurred at the end of the experimental period (96 to 120 h) (Fig. 1a). With mutant C1, in which α-amylase synthesis is not inhibited by 0.5% glucose, the lag observed for parent strain 584 was shortened by 1 day, and the secretion of α-amylase started 1 day earlier than in parent strain 584 with more of the enzyme at the maximum accumulation (Fig. 1a). There was, however, still a pronounced lag period before the appearance of the enzyme in the culture fluid. Moreover, no significant intracellular amylase activity was found in the cells of the wild type and mutant C1 before and after the onset of amylase secretion. Therefore, it is not likely that α-amylase is present in the cells before and is simply released on lysis. α-Amylase synthesis of some catabolite repression-resistant mutants, F-12s or F-14, started at the onset of the growth, proceeded in a linear manner during the logarithmic phase, and continued at a maximum rate during the stationary phase until the rate of cell lysis decreased (Fig. 1b).

Effect of addition of various inhibitors on α-amylase formation by the parent strain.

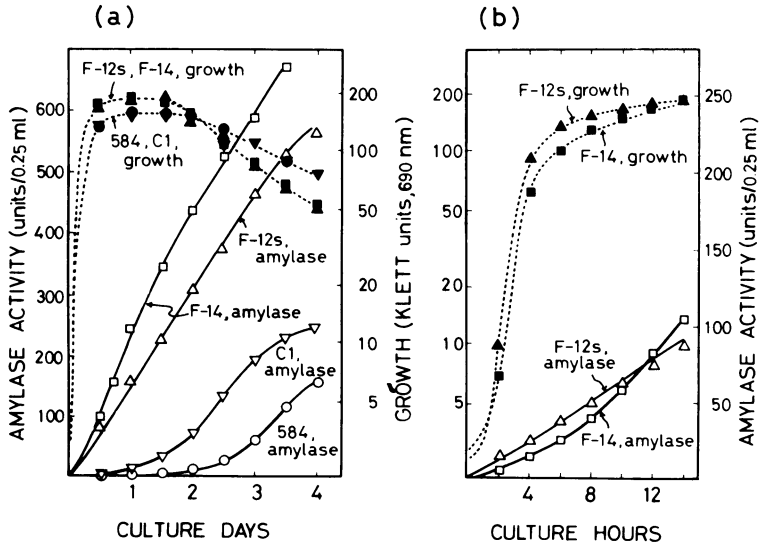

FIG. 1. *α-Amylase formation in growing culture of B. licheniformis. Cultures were aerobically grown on the standard growth medium in tubes (22 by 190 mm) with shaking at 50 C (the parent strain 584 and mutant C1) or at 47 C (mutants F-12s and F-14). Culture tubes were removed prior to inoculation and at various times during the experimental period, and α-amylase was assayed. The activities per 0.25 ml of culture fluid are represented by the solid lines: parent strain 584, ○; mutant C1, ▽; mutant F-12s, △; and mutant F-14, □. The growth was expressed as Klett units after a 1:10 dilution with saline and is represented by dashed lines: parent strain, ●; mutant C1, ▼; mutant F-12s, ▲; and mutant F-14, ■.*

The addition of some antibiotics inhibitory to ribonucleic acid and protein synthesis, such as rifampin and tetracycline at a concentration of 100 μg per ml, to the culture of the parent strain which is actively secreting α-amylase caused a cessation of further enzyme secretion within 2 h. The enzyme synthesis was also stopped immediately by the discontinuance of aeration or by the addition of respiratory inhibitors such as 5 mM 2,4-dinitrophenol or KCN to the cultures (Fig. 2a). This might be due to lack of sufficient supply of adenosine 3'-triphosphate during α-amylase synthesis. Of course, the addition of these antibiotics or inhibitors to the culture in the logarithmic phase of growth not only stopped further growth but also caused gradual cell lysis. These results suggest that the appearance of α-amylase in the culture fluid after 2 days of growth involves de novo protein synthesis, though cell lysis is occurring slowly. Interestingly, the addition of nalidixic acid, an inhibitor of deoxyribonucleic acid synthesis, showed a curious effect on α-amylase formation different from those of inhibitors for ribonucleic acid and protein synthesis, and suppressed completely the subsequent enzyme synthesis when added at a concentration of 100 μg per ml to the culture after 16 to 20 h of growth. However, when the reagent was added to the culture after 24 h of growth, only a slight inhibitory effect was observed on amylase synthesis, which occurred normally after 2 days of growth. The addition of this reagent after 28 or 32 h of growth did not affect α-amylase synthesis at all.

Effect of temperature on α-amylase formation. The effects of cultural temperature on growth and α-amylase synthesis in the parent and some mutant strains of *B. licheniformis* were examined in a temperature gradient incubator and compared with those for *B. subtilis* 29. In parent strain 584 (Fig. 3a), the optimum temperatures for growth and α-amylase formation were 45 to 46 C and 50 C, respectively, whereas in *B. subtilis* 29 the optimum temperature for growth (42 C) was higher by 5 C than that for amylase formation (37 C) (Fig. 3b). In the mutant strains (C1 and N116) of *B. licheniformis*, both optimum temperatures coincided with each other at 45 to 47 C (Fig. 3a). Although the temperature curves for α-amylase synthesis were significantly different between parent and mutant strains of *B. licheniformis*, the portions of both curves above 51 C completely overlapped each other. This experiment seems to have clearly demonstrated the temperature dependence of α-amylase synthesis in *B. licheniformis*.

Furthermore, the effect of temperature shift on growth and α-amylase formation in the parent strain of *B. licheniformis* was examined by transferring the cultures from 35 to 50 C and from 50 to 35 C at different growth phases (Fig. 4a to h). When the parent strain was transferred from 35 to 50 C, an abrupt increase in

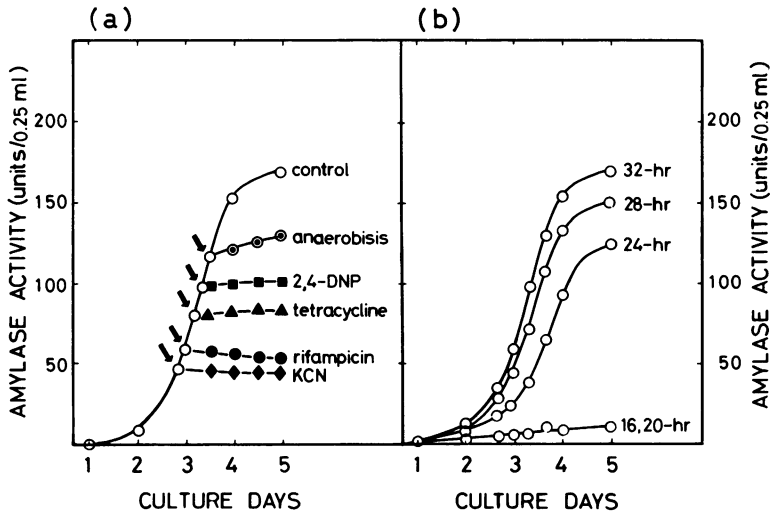

FIG. 2. *Inhibition of α-amylase synthesis in growing cultures of the parent strain 584 by various inhibitors or by discontinuance of aeration. Cultures were aerobically grown on the standard growth medium in tubes (22 by 190 mm) with aeration at 50 C. (a) Times of inhibitor-additions and aeration-discontinuance are shown by arrows: control, ○; rifampin (100 μg/ml), ●; tetracycline (100 μg/ml), ▲; KCN (5 mM), ◆; 2,4-dinitrophenol (5 mM), ■; and discontinuance of aeration, ◉. (b) Times of addition of nalidixic acid (100 μg/ml) are shown on the figure.*

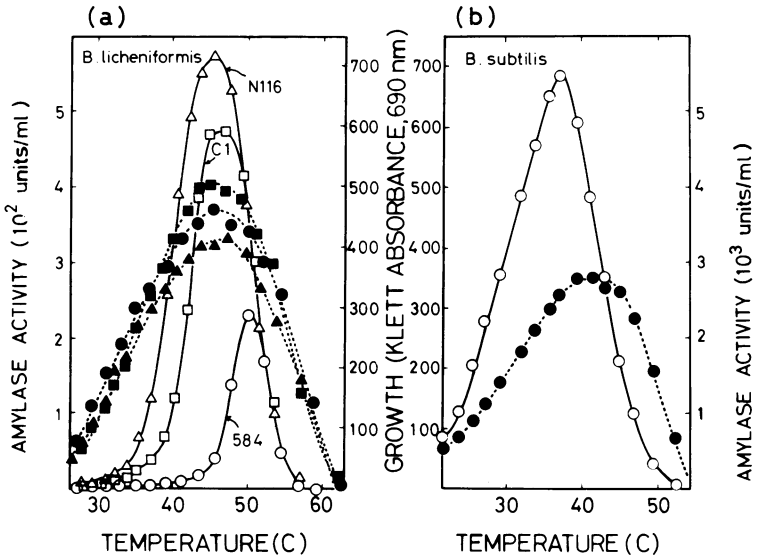

FIG. 3. *Temperature dependence of α-amylase synthesis in growing cultures of B. licheniformis and B. subtilis. L-shaped tubes containing 5 ml of the standard growth medium were incubated by rocking in a temperature gradient incubator for 5 days. (a) B. licheniformia: α-amylase activity is expressed by the solid lines: parent strain 584, ○; mutant C1, □; and mutant N116, △. The growth after incubation for 6 h is expressed as Klett units by dashed lines: parent strain 584, ●; mutant C1, ■; and mutant N116, ▲. (b) B. subtilis 29: α-amylase activity* (-O-) *and growth* (--●--) *were determined as in* (a).

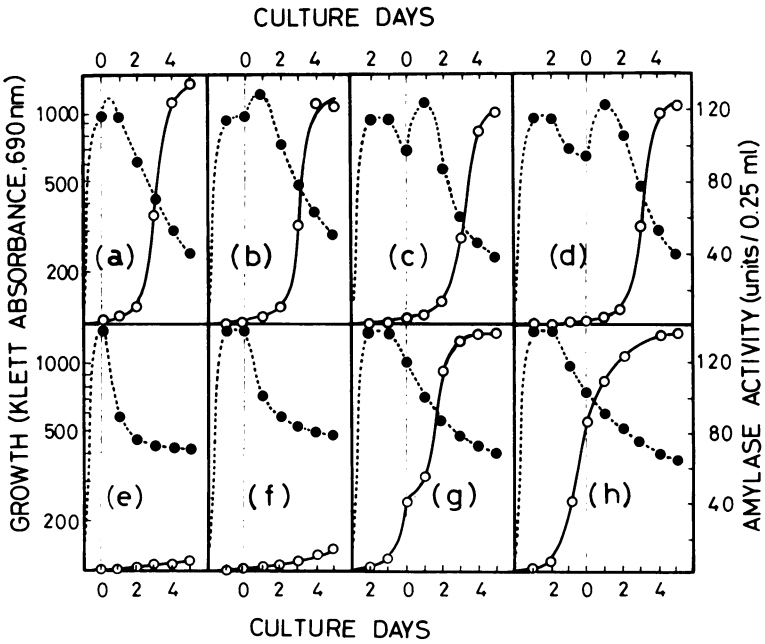

FIG. 4. *Effect of temperature shift on α-amylase synthesis in parent strain 584. Cultures were transferred at zero time on culture days from 35 to 50 C (a to d) or from 50 to 35 C (e to h) at various stages of the growth phases. The growth was expressed as Klett units at 690 nm. Symbols:* -O-, *α-amylase activity (U/0.25 ml);* --●--, *growth.*

mass always occurred after the transfer, but no significant increase in the extracellular enzyme began until day 3 after transfer to 50 C regardless of the period of prior culture at 35 C (Fig. 4a to d). Conversely, when the culture was shifted from 50 to 35 C, there was no increase in growth but there was some promotion of cell lysis, and the cultures in which α-amylase synthesis had not started at the time of transfer to 35 C did not accumulate amylase at 35 C, whereas only cultures in which significant α-amylase secretion had already occurred at the time of the transfer continued further amylase secretion (Fig. 4e to h).

Induction or stimulation effect of various carbohydrates on α-amylase synthesis. Since the parent strain of *B. licheniformis* can grow well on a medium containing sorbitol as the sole carbon source, but forms little α-amylase, stimulation or induction of the enzyme was determined by adding 0.5% individual carbohydrates to a 48-h culture pregrown on sorbitol medium at 50 C and measuring the resulting α-amylase activity after 3 days.

Polysaccharides such as glycogen, starch, and dextrin induced α-amylase formation when added to a culture pregrown on sorbitol medium (Table 1). Oligosaccharides including cellobiose, maltose, melibiose, and raffinose, but neither oligosaccharides nor monosaccharides, caused α-amylase induction. Induction of α-amylase also resulted from addition of maltooligosaccharides; maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose, all isolated from a *B. licheniformis* 584 amylase digest of amylose, have been found to be superior to maltose as inducers of α-amylase synthesis in *B. licheniformis*. Therefore, α-amylase synthesis in the parent strain of *B. licheniformis* seems to be induced or stimulated by the addition of compounds having linkages of α-1,4-, β-1,4-, and β-1,6-glucosyl glucose, or α-1,6-galactosyl glucose. Another experiment using the parent and various mutant strains with different patterns of α-amylase secretion also confirmed the induction or the stimulation by cellobiose. To each culture pregrown on sorbitol medium was added 1% cellobiose, and amylase activity was assayed after incubation for 24 h. The parent strain synthesized α-amylase only when cellobiose was added (Table 2). Mutant strains such as F-5s, F-12s, and F-14, in which α-amylase formation is insensitive to 2% glucose, were found to be able to synthesize the enzyme even when grown on a medium containing sorbitol as the sole carbon source. All the preparations of α-amylase elaborated by growing cultures in the presence of these different inducers were ascertained to be identical by polyacrylamide disc-gel electrophoresis and immunochemical methods.

TABLE 1. *Effect of various carbohydrates on α-amylase formation*

Carbohydrate[a]	α-Amylase activity formed (U/0.25 ml)
Polysaccharides	
Dextrin	136
Glycogen	305
Inulin	0
Soluble starch	192
Trisaccharides	
Melezitose	8
Raffinose	40
Disaccharides	
Cellobiose	69
Gentiobiose	9
Lactose	15
Maltose (technical)	105
Melibiose	48
Sucrose	1
α,α-Trehalose	4
Turanose	1
Monosaccharides and polyols	
D-Arabinose	0
L-Arabinose	2
D-Arabitol	2
D-Xylose	4
L-Rhamnose	0
D-Fructose	3
D-Galactose	2
D-Glucose	0
D-Mannose	3
L-Sorbose	0
D-Dulcitol	0
Inositol	3
D-Sorbitol	2
Maltooligosaccharides	
Maltose (purified)	77
Maltotriose	192
Maltotetraose	356
Maltopentaose	272
Maltohexaose	232
Maltoheptaose	200

[a] Concentration of each carbohydrate added was 0.5%. Additions were made to 48-h cultures growing on 2% sorbitol medium at 50 C, in which 4% starch in the standard growth medium was replaced by 2% sorbitol, and amylase activities in the culture fluids were assayed after 72 h.

Catabolite repression of α-amylase synthesis. Amylase formation in the parent strain of *B. licheniformis* was immediately suppressed by the addition of 0.5% glucose, glycerol, acetate, or succinate, and no measurable enzyme synthesis occurred when glucose and starch

TABLE 2. *Induction or stimulation of α-amylase synthesis by cellobiose*[a]

Addition	α-Amylase activity (U/0.25 ml) of strain:				
	584	C1	F-5s	F-12s	F-14
Before addition	0 (2)[b]	4 (2)	34 (2)	31 (1)	21 (1)
After 24 h					
− Cellobiose	0 (3)	11 (3)	76 (3)	89 (2)	59 (2)
+ Cellobiose	28 (3)	94 (3)	133 (3)	168 (2)	155 (2)

[a] Addition of 1% cellobiose was made to 24- or 48-h cultures growing on 2% sorbitol medium at 50 C, and α-amylase activity in the culture fluid was determined after further incubation for 24 h.

[b] Numbers in parentheses represent the day of the culture.

were added simultaneously to non-induced cultures of the parent strain (Fig. 5a). These observations suggest that the secretion of α-amylase in the parent strain is sensitive to catabolite repression. The addition of cyclic AMP to a growing culture of the parent strain at various times stimulated the enzyme formation by about 40 to 70% (Fig. 5b) but could neither shorten the lag period after which the enzyme synthesis starts nor result in an alleviation of the repressive effect by glucose (Fig. 5a). This stimulatory effect of cyclic AMP on α-amylase formation, however, was not observed in mutants A1 and C1, in which α-amylase formation is not repressed by 0.5% glucose but is sensitive to 2% glucose, nor in mutants F-4s, F-5s, F-12s and F-14, in which α-amylase formation is entirely insensitive to 2% glucose (Table 3). It should also be noted that mutants such as F-12s and F-14 could synthesis α-amylase when grown on a medium containing glucose as the sole carbon source.

Effect of cell age and culture fluid age on α-amylase formation. As indicated already, the enzyme secreted by the parent strain of *B. licheniformis* appeared in the culture fluid of the standard medium after 2 or 3 days of growth. This observation led us to experiments in which cells of various age were transferred into culture fluids that had previously supported growth for various times. In the parent strain the combination of 3-day-old cells and 2-day-old culture fluid was the best for amylase formation when followed for a further 4 h (Table 4). The enzyme formation in this combination was increased significantly by the addition of 0.5% starch but repressed by addition of 0.5% glucose, again showing a severe catabolite repression. On the other hand, in the same experiment with mutant C1, the best combina-

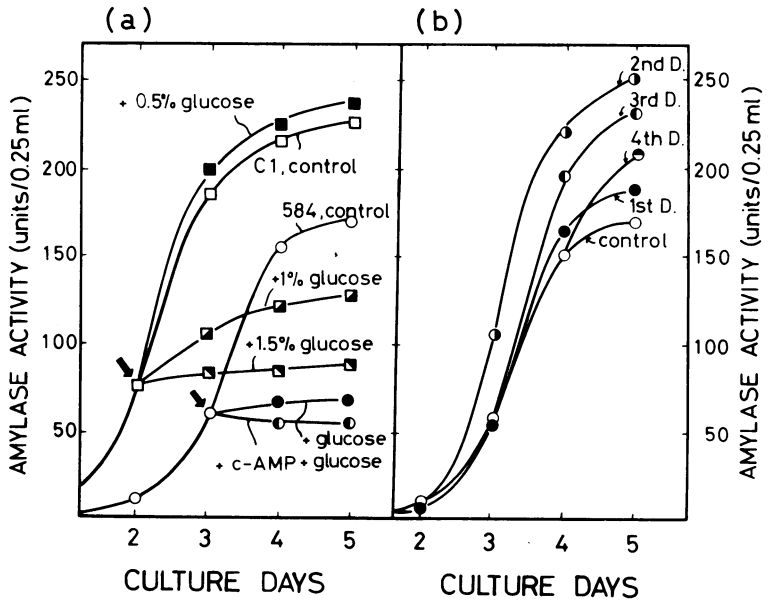

FIG. 5. *Effects of glucose and cyclic AMP on α-amylase synthesis in growing cultures. Cultures were grown on the standard growth medium at 50 C. (a) Symbols:* ○, *parent strain 584, control;* ●, *strain 584, 0.5% glucose added (arrow) on day 3;* ◐, *strain 584, 0.5% glucose and 5 mM cyclic AMP added on day 3;* □, *mutant C1, control;* ■, *mutant C1, 0.5% glucose added (arrow) on day 2;* ◪, *mutant C1, 1% glucose added on day 2;* ◹, *mutant C1, 1.5% glucose added on day 2. (b) Cyclic AMP (5 mM) was added to the culture of parent strain 584 on day 1,* ●, *on day 2,* ◑; *on day 3,* ◐; *on day 4,* ◓; *control,* ○.

TABLE 3. *Effect of glucose and cyclic AMP on α-amylase formation*[a]

Strain	α-Amylase activity (U/0.25 ml)				
	Control, day 2[b]	Control, day 3	Glucose (0.5%), day 3	Glucose (2%), day 3	Cyclic AMP[c] (5 mM), day 3
584	9	58	13	9	105
A1	85	190	180	73	193
C1	76	187	194	84	188
N69	68	189	75	65	168
N108	81	164	79	75	223
N116	80	167	76	78	178
F-4s	98	174	175	270	181
F-5s	87	166	180	229	151
F-12s	305	462	478	560	470
F-14	433	582	693	764	577

[a] Additions (made on day 2) were made to 48-h cultures growing on the standard growth medium at 50 C, and α-amylase activities in the culture fluids were determined after 24 h.

[b] Culture day.

[c] Cyclic-AMP was added without glucose.

TABLE 4. *Effect of cell age and culture fluid age on α-amylase formation*[a]

Culture	Age of cells (days)	α-Amylase formed (U/0.25 ml)				
		0[b]	1	2	3	4
584	1	0.7	2.9	4.0	2.3	1.5
	2	0.8	2.8	4.3	2.3	2.0
	3	0.7	2.9	4.9	1.9	1.6
	3, Starch (0.5%)[c]			7.0		
	3, Glucose (0.5%)[c]			1.2		
Mutant C1	1	1.0	6.3	4.8	3.8	
	2	1.5	11.3	7.9	4.4	
	3	1.9	2.7	1.7	1.3	
	4	1.5	2.4	1.3	1.1	
	2, Starch (0.5%)[c]				10.6	
	2, Glucose (0.5%)[c]				9.6	

[a] Culture fluids were membrane filtered (0.45-μm pore size, 47-mm diameter; Millipore Corp.) after removal of cells by centrifugation. Cells obtained from the culture broths of various ages were washed twice with 0.05 M tris(hydroxymethyl)aminomethaneacetate buffer (pH 7.2) by centrifugation, and were suspended in the fresh or culture fluid to make a reading of 150 Klett units at 690 nm. Cell suspensions thus prepared were incubated at 50 C for 4 h by rocking, and were then assayed for α-amylase activity.

[b] Age (days) of culture fluid.

[c] Addition.

tion for α-amylase formation was 2-day-old cells and 1-day-old culture fluid (Table 4). In this case, however, α-amylase formation was increased by the addition of either 0.5% starch or glucose, and no catabolite repression was observed at this concentration. Thus, the data seem to clearly indicate that age of the culture fluid is the important factor in promoting α-amylase synthesis in *B. licheniformis*.

DISCUSSION

Although it had been thought that α-amylase release in the wild type and mutant C1 begins with cell lysis, we found that is not the case, because no intracellular amylase activity was found in the cells throughout the growth phase, even during the lag period and lysis. This fact, together with the results obtained from the addition of inhibitors, strongly suggests that the appearance of α-amylase in the culture medium of *B. licheniformis* 584 has the characteristics of de novo protein synthesis during the period of secretion, as already demonstrated in earlier work with other bacterial strains (3, 5, 6, 17), and seems to be controlled by induction, catabolite repression, apparent temperature-sensitive repression, and culture age.

Nomura et al. (9) reported a distinct separation of several hours between the end of the logarithmic growth of *B. subtilis*, during which no α-amylase secretion was observed, and the phase during which the enzyme was secreted in significant amounts. In the case of *B. licheniformis* 584, however, a much longer lag of about 2 days was observed between the two phases. This finding, together with the fact that the addition of 0.5% glucose, glycerol, or acetate completely repressed α-amylase synthesis in *B. licheniformis* 584 suggests that α-amylase synthesis in this bacterium can be subject to severe catabolite repression, supporting the proposals of Schaeffer (13) and Meers (8).

Although there was a weak but significant stimulation of amylase synthesis by exogenous cyclic AMP in the wild type, this nucleotide could neither overcome glucose repression nor shorten the lag. Moreover, no stimulative effect of cyclic AMP was found with such mutants as A1, C1, F-4s, F-5s, F-12s, or F-14, which were all derived from parent strain 584 and are probably partly or completely released from catabolite repression by mutation in a regulatory catabolite repression gene. These facts may simply mean that uptake of cyclic AMP by this bacterium is very poor, or they could mean that there is some indirect effect of cyclic AMP not related to catabolite repression.

In any case, amylase synthesis of the parent strain seems to be subject to catabolite repression in the early stages of the culture growth at high temperature (50 C), but may be released from the repression and become active in the presence of various inducers as growth proceeds and the intracellular level of low-molecular-

weight metabolites decreases. Although soluble starch was preferably used as the carbon source in the standard medium, starch itself cannot be an inducer in nature because it is apparently too large to enter the cell. Possibly the natural inducers may be some low-molecular-weight maltooligosaccharides or maltodextrins that were present in soluble starch or were derived from starch by amylase that had been formed only in trace amounts up to that time. Actually, maltooligosaccharides such as maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were found to be superior to starch as inducers of the enzyme synthesis in the parent strain 584 (Table 1). This seems to have some relation to the fact that α-amylase of *B. licheniformis* hardly acts on maltooligosaccharides with molecular weights lower than that of maltopentaose, as reported previously (11). This would be also in accord with a report of Welker and Campbell (16), in which maltooligosaccharides (maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose) isolated from technical-grade maltose were found to stimulate α-amylase formation in *B. stearothermophilus*. Thus, the results obtained from Tables 1 and 2 should be regarded as evidence for inducibility of α-amylase synthesis in *B. licheniformis* 584 for the first time. Although many people tried to demonstrate that α-amylase synthesis in bacteria is inducible, they merely observed the stimulative effect of starch, in which the rate of α-amylase synthesis was greater when starch rather than glucose was used as the sole source of carbon (1, 6, 10, 15).

The reason why they could not prove clearly the inducibility of α-amylase synthesis may be that they had preferably used only such "constitutive" or "semiconstitutive" strains that can utilize various saccharides to form α-amylase. Since the ability to synthesize α-amylase from glucose as the sole source of carbon has been regarded as "constitutive," the strains used by Fukumoto et al. (6) and Coleman and Elliott (3), which were reported not to be subject to catabolite repression but could form α-amylase when grown on a medium containing glucose as the sole source of carbon, could be considered constitutive. Likewise, the strain used by Meers (8) also could be considered to be constitutive but still subject to catabolite repression.

The results in Table 4 suggest that something is being either produced or removed in the culture fluid and that change in fluid permits α-amylase synthesis. Since the culture fluid from mutant C1 responds more quickly and there is some effect of cell age, as well as age of the fluid with mutant C1, an inducible or derepressible element appears to be involved in the change in the medium. Formation of that element may be partially constitutive in the C1 strain.

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