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INDUCTION AND REPRESSION OF AN EXTRACELLULAR PROTEINASE IN *VIBRIO PARAHAEMOLYTICUS*

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SUMMARY An extracellular proteinase, tentatively called gelatinase, of *Vibrio parahaemolyticus* was found to be an inducible enzyme. The induction required de novo protein synthesis and occurred when gelatin or its partial hydrolysates were added to a chemically defined medium. The maximum ratio between the induced and uninduced levels was approximately 40.

Enzyme production was severely repressed in the early phase of growth, followed by an abrupt increase in its differential rate of synthesis. A mechanism of repression similar to so-called catabolite repression was involved in this phenomenon. However, the fact that cyclic 3′, 5′-adenosine monophosphate did not overcome this repression, in contrast to classical catabolite repression, suggests that these two repressions may be distinct.

INTRODUCTION

Many microorganisms are known to produce enzymes extracellularly, and some of these exoenzymes have been purified and extensively studied with respect to their chemical and enzymological properties (Boyer et al., 1960). However, the regulation of synthesis or secretion of these enzymes have rarely been described. One of the striking features of the regulation of exoenzymes is the severe repression of their synthesis during the early stage of growth. The repression is often so strong that enzyme production during early growth phase appears to be completely absent and increases abruptly at a later stage of growth. Although, this phenomenon has been reported for many exoenzymes (Pollock, 1962), no definite conclusion has been established concerning the mechanism of the repression.

In an attempt to study the regulatory aspects of the synthesis and secretion of exoenzymes, we found that the synthesis of a proteinase, tentatively called gelatinase, in *Vibrio parahaemolyticus* is controlled by at least two distinct mechanisms, namely induction by substrate and/or its products and repression caused by metabolizable carbon sources in the growth medium. This report describes these two aspects of regulation, and presents the evidence that the repression is responsible for the severe repression observed in the early phase of growth. The paper also shows an evidence that the repression may be distinct from classical catabolite repression (Magasanik, 1961; Paigen and Williams, 1970) mainly established

in lactose system in *Escherichia coli*.

MATERIALS AND METHODS

1. *Bacterial strains*

The bacterial strains of *Vibrio parahemoyticus* used in this study, were designated as strains 101 and 101G. Strain 101 is strain EB-101 obtained from The Research Institute for Microbial Diseases, Osaka University, Osaka. Strain 101G is a spontaneous mutant isolated by repeated serial transfers in media containing gelatin as the sole carbon source. The mutant has a much shorter growth lag in this medium than strain 101. Two more strains were used in some experiments. These were strains V-1 and V-2, provided by Dr. K. Hisatsune, Department of Food Microbiology, Tokushima University Medical School. All these strains were maintained at room temperature in Brain Heart Infusion Agar (Difco) supplemented with 2.5% sodium chloride.

2. *Media*

The basal mineral medium, designated as medium V, contained 10^{-1}M K_2HPO_4, $5\cdot10^{-1}$M NaCl, $2\cdot10^{-2}$M $(NH_4)_2SO_4$, $3\cdot10^{-4}$M $MgSO_4$, 10^{-5}M $CaCl_2$, 10^{-6}M $ZnCl_2$ and 10^{-6}M $FeSO_4$. The medium was adjusted to pH 8.0 with HCl. Carbon sources were sterilized by filtration and added at a concentration of 0.2%, unless otherwise noted. In some experiments, starch was used as a carbon source and added to the basal medium at a concentration of 1% after boiling it in a water bath for 10 minutes. Gelatin and casamino acids were sterilized by autoclaving and usually added at a concentration of 1%. In the medium used in experiments on the effect of cyclic AMP, the phosphate concentration in medium V was doubled, in order to stabilize the pH in the presence of cyclic AMP. This medium was designated as medium 2PV.

3. *Amino acid mixture*

A mixture of amino acids with a similar composition to gelatin (Tani, 1955; Block et al., 1956) was prepared and used to test inducibility. It contained (in mM): alanine, 9.65; arginine, 4.76; aspartic acid, 4.81; cysteine, 0.08; glutamic acid, 8.15; glycine, 34.20; histidine, 0.58; hydroxyproline, 10.40; isoleucine, 1.14; leucine, 2.36; lysine, 3.56; methionine, 0.60; pheynylalanine, 1.39; proline, 13.90; serine, 3.04; threonine, 1.68; tyrosine, 0.50 and valine, 2.39. All amino acids were of the L-configuration.

4. *Growth measurements*

Cells were grown in flasks aerobically with vigorous shaking. Unless otherwise mentioned, all cultures were grown at 25 C to minimize thermal inactivation of the enzyme. Growth was followed by measuring optical density at 550 mμ in a Shimazu, Bausch & Lomb Spectronic 20 Calorimeter by means of a test tube fitted to the side of the flask. Bacterial density is expressed as mg dry weight of cells per ml of culture. One unit of optical density corresponds to 0.667 mg dry weight of cells per ml. Dry weight was determined by the method of Coleman and Elliott (1962). A correction was made at optical densities above 0.500, where the optical density was not linearly proportional to the weight of cells.

5. *Enzyme assays*

1) Gelatinase

Gelatinolytic activity in the cultural supernatant was measured by viscometry based on the method of Gallop et al. (1957). The Ostwald-Fenske viscometers were used and the flow time of medium V was approximately 60 seconds at 37 C. Two grams of gelatin powder were dissolved in 100 ml of medium V by warming to 60 C and used as substrate. Five ml of the substrate solution were placed in a viscometer which had been equilibrated to 37 C in a water bath. The reaction was started by adding 5 ml of enzyme solution, i.e. cultural supernatant suitably diluted with medium V. Flow times were measured every 20 minutes, and specific viscosities (η_{sp}) were calculate from the following equation;

$$\eta_{sp} = \eta_{rel} - 1 = \frac{\eta}{\eta_m} - 1 = \frac{T}{T_m} - 1$$

in which, η_{sp}, η_{rel}, η and T are for specific viscosity, relative viscosity, viscosity and flow time of the reaction mixture, and η_m and T_m are for viscosity and flow time of medium V alone.

An independent test showed that the specific viscosity calculated in this way was practically proportional to the concentration of gelatin in medium V. Furthermore, a plot of the logarithm of the specific viscosity against time gave a straight line (Fig. 1), suggesting that the reaction proceeds with first-order kinetics under these conditions. The velocity constant of the reaction can be calculated as follows;

$$k = \frac{2.3 \log (\eta_{sp_1}/\eta_{sp_2})}{t_2 - t_1}$$

where, η_{sp_1} and η_{sp_2} are the specific viscosities of the reaction mixture at times t_1 and t_2 minutes. In this situation, the velocity constant must be proportional to the enzyme concentration, and this relationship was confirmed when k was less than 0.01 min^{-1}. So, the velocity constant can be used as a measure of enzyme activity and one unit was defined as the activity showing a velocity constant of $1 \times 10^{-4} min^{-1}$.

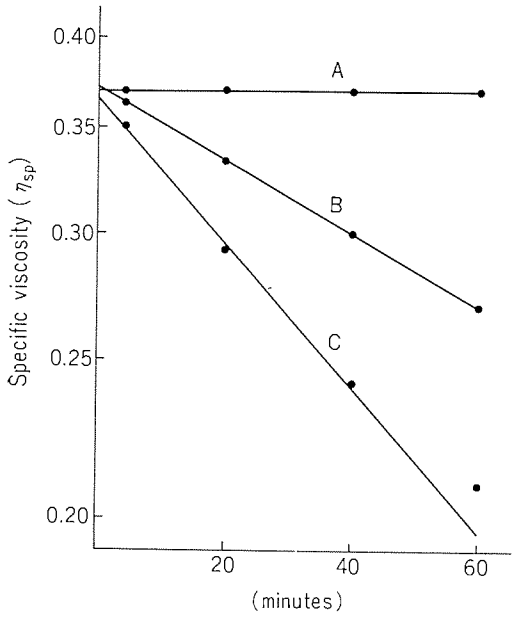

FIGURE 1. *A semilogarithmic plot of the specific viscosity versus time for determination of gelatinase activity. No enzyme (A), 10.5 units/ml (B) and 20.8 units/ml (C).*

2) Amylase

Amylase was assayed as described previously (Tanaka et al., 1969). Activity is expressed as mg of starch decomposed per hour at 37 C.

3) Histidase

Histidase was assayed by a modification of the method of Mehler and Tabor (1953). The assay mixture contained in a total volume of 3.0 ml: 0.1 M L-histidine, 0.05 M Tris buffer (pH 9.0), 0.001 M $MgSO_4$ and an aliquot of the test sample. The mixture was incubated for 10 min at 30 C then the reaction was stopped by adding 0.5 ml of 6 N HCl. The amount of urocanic acid produced was measured photometrically in a Hitachi spectrophotometer (model 101) at 267.5 mμ, the absorption peak of urocanic acid at this acidic pH (less than 1.0). The basal value of absorption was subtracted and enzyme activity is expressed as mμ moles of urocanate produced per minute, adopting the reported value for the molar extinction coefficient of urocanic acid, 18,800 (Mehler and Tabor, 1953). One unit of optical density corresponds to 18.6 mμ moles of urocanic acid produced. The assay curve was linear within 0.800 unit of optical density.

Cells were hervested, washed in medium V and resuspended in the original volume of 0.05 M Tris buffer (pH 9.0) containing 0.001 M $MgSO_4$. Toluene, at a concentration of 2%, was added to accelerate destruction of cells. The mixture was incubated for 30 minutes with gentle shaking at 37 C, and centrifuged for 20 min at 10,000×g at 0 C to remove cell debris. The clear supernatant was used for assay. This organism is very fragile even when suspended in buffer, so that this procedure can release all the enzyme activity into the supernatant.

6. *Chemicals*

Gelatin and casamino acids (technical), an acid hydrolyzed casein, were products of Difco Laboratories, Detroit, Michigan, U.S.A. Gelysate, a pancreatic digest of gelatin, was obtained from Baltimore Biological Laboratory, Baltimore, Maryland, U.S.A. Casein (acc. to Hammarsten) was from E. Merck Co., Darmstadt, Germany, rabbit serum albumin was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Polypepton, a pancreatic digest of casein, was from Daigo Eiyo-kagaku Co., Osaka and soluble starch (reagent grade) was from Junsei Pure Chemicals, Co., Tokyo. Highly purified gelatins were kindly made available by Dr. T. Isemura (The Institute for Protein Research, Osaka University) from Nitta Gelatin Co., Osaka. These preparations are designated in the text as gelatins 1, 2 and 3. Their molecular weights were approximately 94,000, 128, 100 and 201,000, respectively (Nitta Gelatin Co., personal communication). Pronase P, 70,000 PUK/g, was obtained from Kaken Kagaku Co., Tokyo, chloramphenicol powder (Paraxin) was from Yamanouchi Pharmaceutical Co., Tokyo, and adenosine 3′,5′-monophosphate (cyclic AMP) from Boehringer Mannheim Co., Mannheim, Germany.

RESULTS

1. *Kinetics of gelatinase production in strain 101*

The organism grew well in basal mineral medium (medium V) supplemented with simpe carbon compounds such as glucose or glycerol, as the sole carbon and energy source. However, extracellular gelatinase only appeared in the cultural supernatant when gelatin was added to the growth medium. Fig. 2 shows the kinetics of gelatinase production, where enzyme activity is plotted against the dry weight of cells per ml of culture. Cells of strain 101 grown overnight in glycerol medium, were washed and inoculated into medium V supplemented with 0.2% glycerol and 1.0% gelatin. Growth was followed at 25 C and 5 ml samples were withdrawn every 2 hr. The samples were chilled and centrifuged, and the gelatinase activity in the resulting supernatant was determined (curve A). A very low differential rate (66 units/mg dry weight) was observed in the early phase of growth, followed by a high rate of synthesis in a later stage of growth (1046 units/mg dry weight.) Similar results (curve B) were obtained when glycerol was replaced by 1.0% Gelysate. With the latter, the transition from the low to the high rate of synthesis was less marked. Apparently, in these growth conditions, enzyme synthesis was severely repressed at an early stage of growth when the added carbon source, glycerol or Gelysate in these cases, would be actively metabolized. Thus these carbon sources seem to be responsible for the observed repression, though it is known that carbon sources such as glycerol are usually not very effective in repression of enzyme synthesis

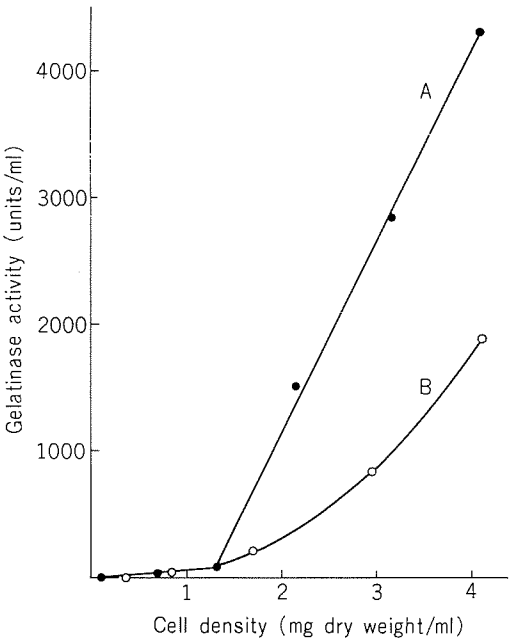

FIGURE 2. *Kinetics of gelatinase production in strain 101. Cells were grown overnight on glycerol, washed and transferred to medium V containing either glycerol (0.2%) and gelatin (1%) (Curve A), or Gelysate (1%) and gelatin (1%) (Curve B).*

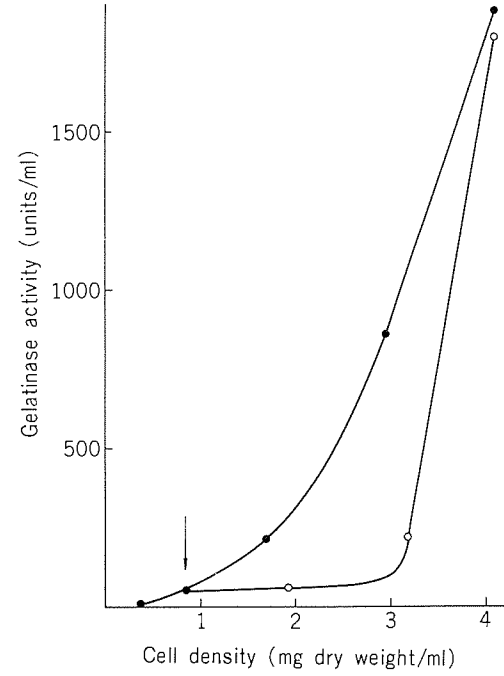

FIGURE 3. *Repression by adding glucose to a culture of strain 101 producing enzyme. Growth conditions were as for Fig. 2B. When the cell density reached 0.84 mg dry weight/ml, glucose (0.2%) was added (as indicated by the arrow) to half the culture (○); the other half served as a control (●).*

(Paigen and Williams, 1970).

With strain 101, glycerol or other readily utilizable carbon source could not be omitted from the medium, because the organism showed a long growth lag, often more than 18 hr, in medium V containing solely 1.0% gelatin as carbon source.

2. *Repression by adding carbon sources to cultures producing enzyme.*

To test whether the readily utilizable carbon sources in the growth medium are responsible for the observed severe repression in the early phase of growth, 0.2% glucose was added to a growing culture just after gelatinase production had started (Fig. 3). Growth conditions were same as for Fig. 2B. When the cell density reached 0.84 mg dry weight per ml, the culture was divided into two parts; glucose was added to one while the other served as a control. The gelatinase activity in cultural supernatant was followed every two hours by sampling of 5 ml. Addition of glucose strongly repressed enzyme synthesis as compared with that in the control culture. The repression continued for at least 4 hr, which corresponded to about two doublings of cell mass, then rapidly disappeared.

The same was true for many other utilizable carbon sources, including glycerol. Various carbon sources were added at concentrations of 0.2% to growing cultures of strain 101, using the same procedures as for Fig. 3. The specific activity of the enzyme was 57 units per mg dry weight at the time of addition of these carbon soucrces. After 4 hr, enzyme activities were determined and the specific activities, expressed as units per mg dry weight, are shown in Table 1. The column on the far right of the table shows the generation times cited from a previous publication (Tanaka et al., 1969), observed when these carbon sources were utilized as the sole carbon and energy source in medium V at

TABLE 1. *Repression of gelatinase by adding various carbon sources to cultures of strain 101 producing the enzyme*

Carbon source added[a]	Specific activity of gelatinase[b]		Generation time[c]
	(units/mg dry weight)	(%)	(min)
None	371	100.0	—
Casamino acids	119	32.0	—
Glucose	142	38.4	51
Galactose	157	42.3	53
Fructose	161	43.4	58
Mannose	164	44.2	51
Mannitol	184	49.6	58
Maltose	189	51.1	57
L-Arabinose	196	52.8	59
Glycerol	252	68.0	72
Succinate	278	75.0	74
Pyruvate	327	88.2	67
Lactose	357	96.2	no growth
Sucrose	367	98.7	no growth

The growth conditions were as for Fig. 2B.

a 1% casamino acids and 0.2% of other carbon sources were added when the cell density reached 0.84 mg dry weight per ml of culture.

b Gelatinase activity was determined 4 hr after adding each carbon source.

c Measured at 37C. Cited from previous paper (Tanaka et al., 1969).

37 C. In general, more readily utilizable carbon sources repressed enzyme synthesis more severely while non-utilizable carbon sources, i.e. lactose and sucrose, caused little repression. These general characteristics are similar to those of the repression commonly known as catabolite repression, in which one or more of the common metabolites of carbohydrates are believed to act as the effector(s) of repression (Magasanik, 1961 ; Paigen and Williams, 1970). Casamino acids, at a concentration of 1.0%, caused the same extent of repression as glucose.

3. *Kinetics of gelatinase production in strain 101G*

The above data suggest that severe repression of gelatinase production in the early phase of growth is due to the carbon sources present in the growth medium. Therefore, it was expected that de-repression of enzyme synthesis would occur if the readily utilizable carbon source, e.g. glycerol, could be omitted from the growth medium. This " de-repressed " state was obtained by using strain 101G as test organism. Strain 101 was repeatedly transferred to medium V supplemented with 1% gelatin as sole carbon source. Finally the organism grew in this medium after only a short lag period. A colony, named strain 101G, was isolated from this culture, which retained all the characteristics of the original strain except that its growth lag in 1% gelatin was much shorter than that of strain 101.

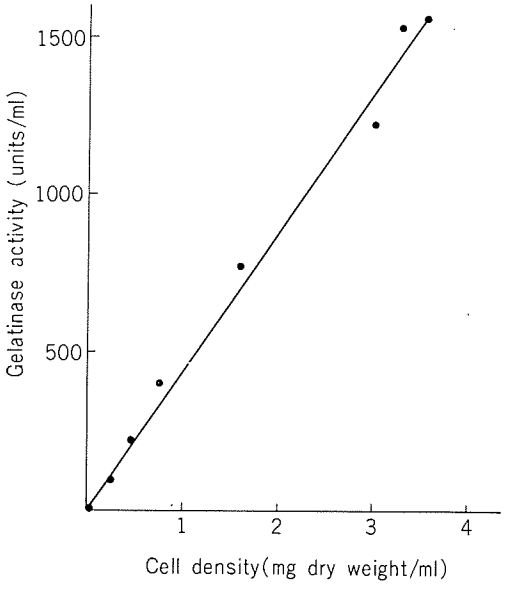

FIGURE 4. *Kinetics of gelatinase production by strain 101G. Cells were grown for 16 hours in medium containing glycerol and gelatin, washed and transferred to medium V containing only gelatin (1%) as substrate.*

Fig. 4 shows the kinetics of gelatinase production of strain 101G. Cells grown for 16 hr at 25 C in medium V containing glycerol and gelatin, were washed and transferred to medium V containing only 1% gelatin as carbon source. Five ml samples were withdrawn and centrifuged every two hours, and their enzyme activity was determined. As expected, the differential rate of gelatinase synthesis in this medium was almost constant throughout the growth period. The monophasic nature of the kinetics suggests that there is a balance throughout the growth cycle between induction and possible repression caused by digested products of gelatin. The fact that the differential rate of enzyme synthesis (approximately 437 units/mg dry weight) was lower than the maximum rate in Fig. 2A (1046 units/mg dry weight)., also suggested the persistence of repression by digested gelation.

4. *Repressibility of strain 101G*

Strain 101G was as sensitive to repression by carbohydrates, as the parent, strain 101. Cells of both strains were grown in glycerol medium, washed and transferred to medium V containing 1% gelatin with or without other carbon sources, i.e. glycerol (0.2%), glucose (0.2%) and casamino acids (1%), respectively. Growth was followed at 25 C and when the cell density reached 0.36 mg dry weight per ml, the enzyme activity in the medium was measured (Table 2). Glucose exerted about 90–95% repression and even in glycerol, about 75% repression was observed. These values were almost the same with both strain 101 and 101G, suggesting that the difference between these two strains was

TABLE 2. *Repression by carbon sources added to medium with gelatin*

Strain[a]	Carbon source other than gelatin[b]	Specific activity of gelatinase[c]	
		(units/mg dry weight)	(%)
101G	None	258.0	100.0
101G	Glycerol	58.1	22.5
101G	Glucose	12.8	5.0
101G	Casamino acids	6.4	2.5
101	Glycerol	60.9	23.6
101	Glucose	24.4	9.5
101	Casamino acids	5.7	2.2

a Cells were grown with glycerol and washed before transfer to the test medium.
b Glycerol (0.2%), glucose (0.2%) or casamino acids (1%) was added to medium V containing gelatin (1%).
c Activities were determined when the cell density reached 0.36 mg dry weight per ml of culture.

not due to the difference in their sensitivities to repression by carbon sources. No value for strain 101 could be obtained in gelatin medium, because of the lack of growth. Table 2 also shows that 1% casamino acids exerted the greatest repression of gelatinase synthesis. In another experiment (not shown), the degree of repression was found to be almost constant, even when the concentration of casamino acids was reduced to 0.2%.

5. *Repression in a cell suspension*

When cells were suspended in gelatin medium at a concentration of 0.667 mg dry weight (20 times the ordinary inoculum size in growth medium), significant gelatinase production occurred on incubation at 25 C. The synthesis was sensitive to chloramphenicol and no enzyme production was observed when gelatin was omitted from the medium. This system had the advantage that enzyme synthesis could be detected at a very early phase of growth, i.e. in the first 60 min, though the increase in cell mass during the incubation was small, being approximately 0.086 mg dry weight with strain 101G.

The amount of enzyme synthesized in the above conditions depended on the carbon source with which the cells had been grown before being suspended in gelatin medium. Table 3 shows the enzyme syntheses of cells previously grown in glycerol (0.2%), glucose (0.2%) or casamino acids (1%). Cells of strain 101G were harvested from exponentially growing cultures in medium V supplemented with each of the carbon sources, washed and suspended in medium V containing 1% gelatin. After incubation for 60 min, enzyme activity was determined. Cells previously grown with glucose had about 20% of the specific activity of those grown with glycerol, suggesting that the previ-

TABLE 3. *Effects of previously utilizing carbon sources on subsequent induction of gelatinase in strain 101G*

Previous carbon source[a]	Specific activity of gelatinase[b]	
	(units/mg dry weight)	(%)
Glycerol	10.8	100.0
Glucose	2.1	19.4
Casamino acids	4.7	43.5

a Cells grown with the carbon source listed, i.e., glycerol (0.2%), glucose (0.2%) or casamino acids (1%), were harvested at the exponential growth phase (0.360 mg dry weight per ml), washed and resuspended at a concentration of 0.667 mg dry weight per ml in medium V containing solely gelatin as substrate.
b Activities were determined after incubation for 60 min at 25C.

ous carbon sources or their metabolites bound on or in the cells may be involved in repression of enzyme synthesis. Again, casamino acids caused repression though less than glucose.

6. *Effect of cyclic AMP on the repression of gelatinase*

Recently, Perlman and Pastan reported that cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) reverses the catabolite repression and the transient repression of β-galactosidase and tryptophanase synthesis in *Escherichia coli* (Perlman and Pastan, 1968 a; 1968 b; Pastan and Perlman, 1968; 1969; Perlman et al., 1969; Varmus et al., 1970). If the mechanism of repression of gelatinase by various carbon sources is the same as that of so-called catabolite or transient repression, cyclic AMP should reverse the former repression, too. Table 4 shows the effect of cyclic AMP on the repression of gelatinase synthesis under two different experimental conditions. Similar procedures to those for Fig. 3 were used with strain 101 except that medium V was replaced by medium 2PV. When the cell density reached 0.734 mg dry weight per ml, the culture was divided into three parts; one served as a control and 0.2% glucose with or without 5 mM of cyclic AMP was added to the others. After two hours incubation, the enzyme activities were determined, and the ratio of the increase in enzyme activity to the increase in cell mass was calculated. No recovery from repression was detected in the presence of cyclic AMP.

Similar results were obtained with strain 101G, using the same procedures as for Table 2, except that medium 2PV was used instead of medium V. Cells of strain 101G were grown in gelatin-glycerol medium for 16 hours at 25 C, washed and transferred to medium 2PV containing 1% gelatin with or without 0.2% glucose or pyruvate. When the cell density reached 0.200 mg dry weight per ml, the cultures grown in gelatin-glucose or in gelatin-pyruvate medium were divided into two portions, and 5 mM of cyclic AMP was added to one. After incubation for 1 hour, enzyme activity was measured. Again, there was no increase in activity on adding cyclic AMP.

TABLE 4. *Effect of cyclic AMP on repression of gelatinase*

Strain	Carbon source (0.2%)	Cyclic AMP (5 mM)	Δ-Enzyme activity/Δ-Cell mass[c]	
			(units/mg dry weight)	(%)
101	None[a]	−	248.0	100.0
101	Glucose	−	53.2	21.4
101	Glucose	+	52.7	21.2
101G	None[b]	−	412.0	100.0
101G	Glucose	−	32.2	7.8
101G	Glucose	+	33.4	8.1
101G	Pyruvate	−	131.5	31.9
101G	Pyruvate	+	115.6	28.1

a Procedures were similar to those for Table 1. Basal medium was medium 2PV containing glycerol (0.2%) and Gelysate (1%). Glucose was added when the cell density reached 0.734 mg dry weight per ml. Cyclic AMP, when used, was added with glucose.

b Procedures were similar to those for Table 2. Basal medium was medium 2PV containing gelatin (1%). Glucose or pyruvate was added to this medium. Cells were grown for 16 hr in gelatin-glycerol medium before transfer to this medium. Cyclic AMP was added when the cell density reached 0.200 mg dry weight per ml.

c The rate of enzyme synthesis is expressed as the increase in enzyme activity divided by the increase in cell mass, in two hours (strain 101) or 1 hr (strain 101G) on incubation after adding cyclic AMP.

Another series of experiments (not shown), indicated that the repression by casamino acids was also completely resistant to cyclic AMP.

Cyclic AMP is a mediator of the repression of other enzyme systems in this organism. These include an extracellular enzyme, amylase (Tanaka et al., 1969) and an inducible intracellular enzyme, histidase, catalyzing the conversion of histidine to urocanic acid (unpublished observations). Cells of strain 101 harvested after overnight culture with 0.2% maltose, were washed and inoculated into medium 2PV containing 1% starch as substrate. The culture was divided into three portions when the cell density reached 0.200 mg dry weight per ml. One portion was grown further as a control, and 0.2% glucose with or without 5 mM of cyclic AMP was added to the other portions. The ratios of increase in amylase activity to increase in cell density during incubation for 2 hr are shown in Table 5. Cyclic AMP overcame the repression both by glucose and starch itself, resulting in about 15-fold and 4-fold increases in the rate of synthesis, respectively.

TABLE 5. *Effect of cyclic AMP on repression of amylase in strain 101*

Carbon source added[a] (0.2%)	Cyclic AMP[b] (5 mM)	Δ-Enzyme activity/Δ-Cell mass[c] (units/mg dry weight)	(%)
None	−	5.8	100.0
Glucose	−	1.3	22.4
Glucose	+	16.3	281.5

Cells were grown overnight on maltose (0.2%), washed and transferred to medium 2PV containing starch (1%).

a Glucose was added when the cell density reached 0.200 mg dry weight per ml.

b Cyclic AMP was added with glucose.

c The rate of enzyme synthesis: expressed as the increase of enzyme activity divided by the increase of cell mass, on two hour incubation after adding cyclic AMP. One unit of enzyme activity is defined as the amount decomposing 1.0 mg of starch per hour.

Similar findings were obtained on histidase. Strain 101 was gorwn in medium 2PV supplemented with 0.05% L-histidine and 0.2% carbon sources, i.e. glycerol or glucose. When growth reached 0.200 mg dry weight per ml, the culture with glucose was divided, and 5 mM of cyclic AMP was added to one portion. After further incubation for 60 min, all three cultures were treated with buffer containing toluene. Then histidase activity was determined as described in MATERIALS AND METHODS. The differential rates during 60 minutes after adding cyclic AMP are shown in Table 6. The derepressed level (10.5 mμ moles/min/mg dry weight) was about 2.4 and 1.5 times the repressed levels with glucose and glycerol, respectively. This effect on histidase could be detected even at a concentration of 0.5 mM of cyclic AMP (data not shown).

Cyclic AMP at the concentration used in the above experiments, did not increase the enzyme activity of either amylase or histidase, in vitro. A commercial preparation of cyclic AMP (Kojin) at a concentration of 5 mM caused approximately 35% inhibition on gelatinase activity, in vitro. This inhibition was probably not due to cyclic AMP per se, since another commercial preparation of cyclic AMP obtained from Boehringer Mannheim Co., did not have

TABLE 6. *Effect of cyclic AMP on repression of histidase in strain 101*

Carbon source[a] (0.2%)	Cyclic AMP[b] (5 mM)	Differential rate[c] (units/mg dry weight)	(%)
Glycerol	−	7.1	100.0
Glucose	−	4.4	61.9
Glucose	+	10.5	147.8

a Medium 2PV containing L-histidine (0.05%) was supplemented with glycerol or glucose.

b Cyclic AMP was added when the cell density reached 0.200 mg dry weight per ml.

c The differential rate of enzyme synthesis was determined from 0 to 60 min after adding cyclic AMP. One unit of enzyme activity is defined as the amount producing 1.0 mμ mole of urocanic acid per minute.

any inhibitory effect. All the experiments described in the text were performed with the latter preparation.

All these observations suggest that the repression of gelatinase synthesis in this organism is not mediated via cyclic AMP, at least under physiological state.

7. *Inducibility of gelatinase synthesis*

Gelatinase was only produced when gelatin was present in the growth medium. Table 7 shows the effects of various compounds on synthesis of the enzyme. Strain 101 was grown in medium V supplemented with 1% concentrations of these compounds and 0.2% glycerol. After 20 hr of growth at 25 C, the enzyme activity in the supernatant was determined. Besides Bacto-gelatin (Difco), three fractions of purified gelatin (gelatin 1, 2 and 3 in the table) were also tested. All these gelatins were equally effective. In contrast, casein had no effect on gelatinase synthesis. A pancreatic hydrolysate of gelatin, commercially available as Gelysate (BBL), was also effective but the enzyme level induced was about 25% of that induced with gelatin. Similar results were observed with a cell suspension system described below. A pancreatic hydrolysate of casein, available as Polypepton (Daigo), was completely inactive. Neither an acid hydrolysate of casein, called casamino acids (Difco), nor an amino acid mixture with a similar composition to gelatin described in the MATERIALS AND METHODS, had any effect.

TABLE 7. *Inducibility of gelatinase in strain 101*

Addition (1%)[a]	Specific activity of gelatinase[b]	
	(units/mg dry weight)	(%)
Bacto-Gelatin (Difco)	1230	100.0
Gelatin 1[c]	1280	104.2
Gelatin 2[c]	1435	116.7
Gelatin 3[c]	1080	87.7
Gelysate (BBL)	298	24.2
Amino acid mixture[c]	10	0.8
Casein (Merck)	19	1.5
Polypepton (Daigo)	22	1.8
Bacto-Casamino acids (Difco)	10	0.8
None	35	2.8

a Test materials were added to medium V containing glycerol (0.2%).
b Activities were determined after 20 hr of growth.
c See MATERIALS AND METHODS.

Preliminary experiments on cell suspensions showed that after digestion with Pronase P (50 μg/ml, for 14 hr at 37 C) gelatin was as effective as untreated gelatin, while casein after digestion with Pronase P (100 μg/ml, for 4 hr at 37 C) had no effect. Furthermore, digests of gelatin prepared by incubating 1% gelatin for 2 hr at 37 C with a crude preparation of gelatinase (approx. 400 units/ml, final concentration) produced by this organism, was still as effective as Gelysate in inducing the enzyme. The digest had lost most of the viscosity specific to intact gelatin and supported good growth of this organism when added to medium V after autoclaving. It also showed no absorption peak corresponding to intact gelatin on gel filtration with Sephadex G–100.

Enzyme production on growth in the presence of gelatin was completely blocked by 20 μg/ml of chloramphenicol, suggesting that the appearance of the enzyme activity in the medium involves de novo synthesis of protein.

These observations suggest that the enzyme is inducible and that the actual effective inducer, if any, will have a peptide structure, such as that present in gelatin but probably not in casein. The inducibility was the same in strains 101, 101G, V–1 and V–2.

8. *Growth on gelatin*

The growth of this organism in medium V containing 1% gelatin as the carbon and energy source varied with the growth conditions and with the strain used. As described previously, strain 101 pregrown on glycerol showed a long growth lag (often more than 18 hr) in gelatin medium both at 25 C and 37 C with shaking. A little shorter growth lag (6–7 hr) was observed

when strain 101 was pre-grown for 20 hr at 25 C in medium containing gelatin in addition to glycerol. In the other hand, both pre-adapted and non-adapted cells of strain 101G started to grow within 30 min in gelatin medium. Growth curves were somewhat irregular, so that the exact generation times could not be determined, but the doubling times were roughly 130–140 min and 120 min at 25 C and 37 C, respectively. Strain 101G also grew on purified gelatin 1, 2 and 3 with a similar generation time and a little longer lag time than on Bacto-gelatin (Difco). Strain 101 could utilize gelatin for growth, in medium containing other readily utilizable carbon sources, i.e. glycerol, glucose, casamino acids etc., in addition to gelatin. The growth curves were diauxic and carbon sources other than gelatin were utilized first. However, unless the concentration of carbon sources other than gelatin in the medium was above 0.05%, utilization of gelatin after other carbon sources were finished, was markedly delayed.

9. *Some characteristics of the gelatinase*

The term " gelatinase " has been tentatively used throughout this paper, although the exact substrate specificity of the enzyme has yet to be determined. A preliminary experiment showed that the caseinolytic activity of the cultural supernatant derived from a highly induced culture with gelatin, was very low, suggesting that the " gelatinase " is relatively specific for gelatin or related substances. The organism did not grow with 1% casein as the sole carbon source or utilize casein in medium containing glycerol. Furthermore, the lack of induction of " gelatinase " by casein or its digest showed specificity to inducers. The great difference between the induced and uninduced levels suggests that the majority of the gelatinolytic activity in a highly induced culture can be ascribed to an enzyme controlled by a single regulatory unit.

The enzyme was relatively unstable. Table 8 shows the stability of the enzyme at 25 C and 37 C, with and without shaking in air. Inac-

TABLE 8. *Stability of gelatinase in a crude preparation*[a]

Temperature	Stabilizer added	Per cent residual activity[b]	
		with shaking	without shaking
25C	None	26	100
	Rabbit serum albumin (1.0%)	76	—
	Gelatin (1.0%)	100	—
	Gelysate (1.0%)	100	—
	Casein (1.0%)	100	—
	(0.8%)	100	—
	(0.6%)	100	—
	(0.4%)	100	—
	(0.2%)	100	—
	(0.1%)	100	—
37C	None	0	53
	Casein (1.0%)	93	—
	(0.5%)	88	—
	(0.2%)	86	—
	(0.1%)	60	—

a Diluted cultural supernatant containing approximately 15 units/ml of gelatinase was used.
b Per cent residual activity after incubation for 2 hours with or without shaking.

tivation on shaking at 25 C could be prevented by adding 1% gelatin to the medium. This effect was not restricted to gelatin (a substrate), for casein and rabbit serum albumin were also effective. Casein was effective at a concentration of as low as 0.1%. The fact that ethylenediaminetetraacetate (EDTA) at a concentration of 0.1 mM strongly inhibited enzyme activity, suggests that the enzyme requires a divalent cation for activity, although no activation was observed on adding various cations to a crude enzyme preparation after dialysis in the cold for about 48 hr until the reaction for chloride ion present in the original medium V became negative. The dialyzed preparation had about 1.6 times higher gelatinase activity than before dialysis. This was due to the inhibitory effects of sodium chloride present in medium V at a concentration of 3%. Some of the cations tested (0.1 mM) exhibited inhibitory effects on enzyme activity, notably Cd^{++}, Hg^{++} and Cu^{++}. Relatively high concentrations (10 mM) of cysteine, mercaptoethanol and sodium thioglycollate were also inhibitory.

DISCUSSION

The preferential synthesis of extracellular enzymes in a late stage of growth has frequently been reported, and is believed to be a feature common to many, though not all, exoenzymes (Pollock, 1962). This feature was often shown by peculiar, biphasic kinetics, similar to those shown in Fig. 2A. Other typical examples have been reported (Bernlohr, 1964; Hofsten and Tjeder, 1965; Coleman and Grant, 1966; Coleman, 1967). The mechanism of this phenomenon is uncertain, though several hypotheses have been proposed. Lampen (1965) supposed that repression of amylase in *Bacillus subtilis* in the early phase of growth was due to a sort of metabolite repression which was overcome in a later stage of growth. On the other hand, Coleman (1967) considered that the catabolite repression or related mechanisms was not involved in the phenomenon and proposed a mechanism based on regulation at the level of supply of nucleic acid precursors in synthesis of m-RNA specific for each of the exoenzymes, i.e. amylase, ribonuclease and proteinase in *B. subtilis*, though he did not mention what mechanism was involved in the control of the precursor supply per se. The present results show that a metabolite repression similar to classical catabolite repression (Magasanik, 1961; Paigen and Williams, 1970), is involved in repression in the early growth phase, since the kinetics of enzyme synthesis in medium containing only gelatin as carbon source is monophasic and almost proportional to increase in the number of cells with strain 101G (Fig. 4). The monophasic character of the kinetics does not mean that strain 101G is resistant to repression, since enzyme production in the presence of readily utilizable carbon sources was as severely repressed in strain 101G as in strain 101 (Table 2). The important factor seems to be rather the ability of strain 101G to utilize gelatin slowly as carbon source in the absence of other readily utilizable carbon sources. Gelatin will supply carbon sources at a limited rate and also will supply sufficient inducer(s) to induce the enzyme, so that a steady state will be established between induction and repression throughout the growth cycle. The exact nature of the difference between strain 101G and 101 is uncertain, but probably induction in strain 101G will be a little more efficient than in strain 101, because of minor increases in either the constitutive level of gelatinase or the transport system of inducer(s). The former possibility is less likely, since this mechanism would have no selective advantage favoring strain 101G over strain 101 on serial transfer in gelatin media.

The effects of cyclic AMP on catabolite and transient repression of various catabolic enzymes have been extensively studied in the last three years by many investigators (Perlman and Pastan, 1968 a; b; Perlman et al., 1969; Crombrugghe et al., 1969; Jacquet and Kepes, 1969; Tao and Schweiger, 1970; Goldenbaum et al., 1970; Moses and Sharp, 1970). The following enzymes have been shown to be

sensitive both to catabolite repression and to derepression by cyclic AMP: β-galactosidase, galactokinase, glycerokinase, tryptophanase, D-serine deaminase, thymidine phosphorylase, lactose permease, L-arabinose permease, α-glycerophosphate permease and enzyme II of the phosphoenolypruvate-dependent phosphotransferase system specific to fructose in *E. coli*. Several cases in other organisms have also been reported, i.e. β-galactosidase in *Salmonella typhimurium* F lac, *Serratia marcescens* F lac, *Proteus inconstans* P lac, *Aerobacter aerogenes*, and galactokinase in *S. typhimurium* (Crombrugghe et al., 1969). The present paper adds two more examples, i.e. histidase and extracellular amylase in *V. parahaemolyticus*. From these observations, it is believed that the effect of cyclic AMP is common to catabolic enzymes which are sensitive to catabolite repression, and also that cyclic AMP is a mediator or a regulatory signal in catabolite and transient repression exerted by metabolizable carbon sources (Crombrugghe et al., 1969).

In contrast, the present communication shows an example of an enzyme which is sensitive to repression by utilizable carbon sources but resistant to the effect of cyclic AMP. The repression was completely resistant to cyclic AMP added to the growth medium at a concentration of 5 mM, under both types of experimental conditions (Table 4). This conclusion is based on the following observations: (a) The concentration of cyclic AMP used in most experiments (5 mM) is sufficient to overcome the repression as shown for various enzymes in *E. coli*, in which with the most sensitive enzyme, even 1 mM of cyclic AMP was effective (Crombrugghe et al., 1969). With the present system, both 2 and 10 mM of the nucleotide were also ineffective. (b) Cyclic AMP (5 mM) completely overcame the repression of two other enzymes, an extracellular and an intracellular enzymes, in this organism (Table 5 and 6), suggesting that the nucleotide is as effective in this organism as in *E. coli*, and also that sufficient nucleotide is transported to exert an effect into the cells. Moreover, 0.5 mM cyclic AMP still was effective to overcome the repression of histidase in this organism. (c) The possibility that the enzyme is extraordinarily susceptible to repression and a relatively minor decrease in the intracellular concentration of cyclic AMP causes severe repression, is unlikely, since several different levels of the enzyme were observed by the presence of various carbon sources and the repression exerted by one of the less effective carbon sources, i.e. pyruvate, still was not reversed by cyclic AMP (Table 4). All these observations suggest that the repression of gelatinase synthesis by carbon sources is insensitive to cyclic AMP in physiological state. This could be decided conclusively by isolation of a pleiotropic carbohydrate-negative mutant deficient in adenyl cyclase (Perlman and Pastan, 1969). Attempts to isolate such a mutant are now in progress.

Assuming that the repression of gelatinase is distinct from classical catabolite and transient repressions, one possible mechanism is inhibition of entrance of inducer across the cell membrane. Perlman et al., (1969) reported that 5 mM of cyclic AMP did not overcome the inhibition of entrance of thiomethyl-β-D-galactoside (TMG) by glucose in *E. coli*, and Tao and Schweiger (1970) described that the induction lag of galactokinase induced by D-fucose in the presence of glucose was not shortened by adding cyclic AMP. Crombrugghe et al., (1969) also reported that, in *E. coli*, cyclic AMP overcame the repression of glycerokinase and α-glycerophosphate permease elicited by glycerol itself, but did not overcome the complete repression caused by the presence of glucose in addition to glycerol. They considered that glucose inhibited the entrance of glycerol into the cell, so that induction was completely abolished. However, in the case of gelatinase, some of the present data do not support the above idea; namely (a) a carbon source in the external medium is not required to express the repression in the cell suspension system (Table 3), and (b) it is rather unlikely that the many carbohydrates listed in Table 1 inhibit entrance of inducer while only lactose and sucrose do not.

These facts are only compatible with the above idea when inhibition of entrance of the inducer is assumed to occur during the process of transport of carbohydrates across the membrane. An alternative possibility that repression is exerted at the level of enzyme synthesis, seems equally probable. Mutants which are defective in components of the phosphoenolpyruvate-dependent phosphotransferase system which has been shown to be involved in transport of several carbohydrates (Tanaka and Lin, 1967; Tanaka et al., 1967; Kaback, 1970), will be helpful to clarify the mechanism, and studies on this are now in progress in this laboratory.

There are also reports that the synthesis of extracellular proteinases by various microorganisms is repressed by carbohydrates in the medium (Litchfield and Prescott, 1970 a; b; Hofsten and Tjeder, 1965) and also by amino acids added singly or as mixture (Chaloupka and Krecková, 1962; Chaloupka et al., 1963 a; b; Neumark and Citri, 1962; Hofsten and Tjeder, 1965; May and Elliott, 1968; Levisohn and Aronson, 1967; Sashital and Zimmerman, 1968). However, the mechanism of the repression is unknown, though some authors consider that with amino acids it involves end-product or feed-back repression (Sashital and Zimmerman, 1968; McDonald and Chambers, 1966). The facts that gelatinase is strongly repressed by carbohydrates and casamino acids, and that both these repressions are resistant to cyclic AMP, suggests that a common mechanism may be involved in these repressions.

The gelatinase appears to be inducible, since no enzyme production occurs unless gelatin or its hydrolysate are added to the medium, even after metabolizable carbon sources have been exhausted and possible metabolite repression probably abolished. The maximum ratio of the induced to uninduced levels is 40, and this induction requires de novo protein synthesis. No induction occurs with partially digested casein contrary to the case with partially digested gelatin, suggesting that the induction is specific to some peptide structures of gelatin. The very low caseinolytic activity of the fully induced preparation also suggests that the enzyme shows substrate specificity. These results are not unlikely since gelatin is know to have a rather peculiar amino acid composition for an animal protein (Block et al., 1956). The lower inductive effect of gelatin digests (Table 7) does not necessarily mean that these digests have low efficiency as inducers, for the following complicating factors may be involved in the induction by gelatin digests: (a) repression caused by amino acids (b) early consumption of inducers and (c) early consumption of stabilizing substances. On the other hand, the occurrence of induction by gelatin digests does not always mean that " intact " gelatin is inert to induce the enzyme. Further work is required on these questions.

Inducible proteinases have rarely been described. Recently, Lichfield and Prescott (1970) reported that an endopeptidase and an aminopeptidase produced by *Aeromonas proteolytica* were induced by the presence of enzymatic digests of casein in the medium, but the induced levels were 3.0 to 3.5 times the level produced in the presence of acid-hydrolyzed casein, and the induction seemed to be less specific, since both endopeptidase and aminopeptidase were induced to the same extent. McDonald and Chambers (1966) also reported that a proteinase of *Micrococcus* sp. was " induced " by several amino acids added singly to the growth medium. However, a more probable interpretation is that rather amino acids which did not cause " induction " repressed a constitutive proteinase in the organism.

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