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Regulation of Arginine and Proline Catabolism in *Bacillus licheniformis*

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The enzymes in the arginine breakdown pathway (arginase, ornithine-δ-transaminase, and Δ'-pyrroline-5-carboxylate dehydrogenase) were found to be present in *Bacillus licheniformis* cells during exponential growth on glutamate. These enzymes could be coincidentally induced by arginine or ornithine to a very high level and their synthesis could be repressed by the addition of glucose, clearly demonstrating catabolite repression control of the arginine degradative pathway. The strongest catabolite repression control of arginase occurred when cells were grown on glucose and this control decreased when cells were grown on glycerol, acetate, pyruvate, or glutamate. The proline catabolite pathway was present in *B. licheniformis* during exponential growth on glutamate. The proline oxidation and the Δ'-pyrroline-5-carboxylate dehydrogenase in this breakdown pathway were induced by L-proline to a high level. The Δ'-pyrroline-5-carboxylate dehydrogenase was found to be under catabolite repression control. Arginase could be induced by proline and arginine addition induced proline oxidation, suggesting a common in vivo inducer for these convergent pathways.

Bacillus licheniformis is able to grow on a minimal medium and sporulate endotrophically. Since storage compounds are not formed, it has been suggested that the oxidation of endogenous amino acids during sporulation serves as the primary energy source for the formation of the spore (2).

Ramaley and Bernlohr have shown the presence of the arginine breakdown enzymes in sporulating *B. licheniformis* cells (18). These enzymes were barely detectable in vegetative cells growing on glucose. It was postulated that the degradative pathway of arginine proceeds directly to ornithine via an arginase, as no arginine deiminase was detected in cell-free preparations of post-logarithmic-phase cells. Ornithine is deaminated by ornithine-δ-transaminase to glutamic-γ-semialdehyde which cyclizes spontaneously to Δ'-pyrroline-5-carboxylate, and this compound is oxidized by Δ'-pyrroline-5-carboxylate dehydrogenase to glutamate. De Hauwer et al. (7) have shown the induction of this pathway in *B. subtilis* cells growing on glycerol and L-arginine or L-ornithine. Also, Middelhoven (13) has shown the induction of the first two enzymes in this pathway by arginine or ornithine in stationary-phase *Saccharomyces cerevisiae* cells. Recently, we demonstrated that this pathway can be induced by L-arginine in cells of *B. licheniformis* growing on glutamate and we showed catabolite repression by glucose (8).

The phenomenon of catabolite repression observed in nonsporeforming organisms appears to be geared for efficient growth, allowing the cell to metabolize the preferred substrate (12). Since we observed the arginine degradative pathway to be under catabolite repression control during vegetative growth and present during sporulation, we wanted to study the role played by catabolite repression in relation to the mechanism of sporulation and also to determine under what growth conditions the catabolite repression could be relieved in *B. licheniformis*. Such a role has been postulated by Schaeffer et al. (20).

In addition, it has been shown (7) that proline induces the proline catabolic pathway in *B. subtilis*. Proline is oxidized by proline oxidase to Δ'-pyrroline-5-carboxylate and this intermediate is converted to glutamate by a Δ'-pyrroline-5-carboxylate dehydrogenase.

These two catabolic pathways have a common

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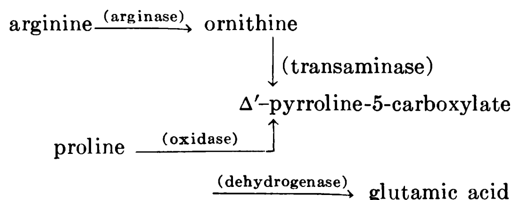
intermediate, Δ'-pyrroline-5-carboxylate, as follows:



We have investigated the reciprocal induction of the two pathways. In *B. licheniformis* cells growing on glutamic acid as the sole carbon and nitrogen source, proline induces arginase and arginine induces proline oxidase activity. It is suggested that the common intermediate, Δ'-pyrroline-5-carboxylate, may be the in vivo inducer and that this system is another example of product induction.

MATERIALS AND METHODS

Growth conditions. B. licheniformis strain A-5 was used throughout this investigation. Spores were prepared by growing *B. licheniformis* for 72 hr at 37 C in the glucose (20 mM) minimal salts medium described by Bernlohr and Novelli (3) supplemented with 50 mM ammonium lactate. These spores were harvested by centrifugation in a Sorvall refrigerated centrifuge and were washed several times at 3,000 × *g* for 20 min with distilled water to separate the lysed cell wall material from the spores. The washed spore suspension was pipetted into sterile tubes (containing approximately 10^{10} spores per ml), pasteurized at 60 C for 90 min, and stored at 5 C. The minimal salts medium used in all experiments was described by Bernlohr and Novelli (3). In the induction and repression studies, this simple salts medium was supplemented with one or more of the following carbon and nitrogen sources as noted: 120 mM glycerol, 100 mM sodium pyruvate, 50 mM ammonium lactate, and 50 mM sodium glutamate (used as carbon and nitrogen source). Cultures on sodium acetate and sodium pyruvate were grown in 50-ml lots and the remaining cultures were grown in 200-ml lots.

A typical experimental procedure consisted of the following. Four 125-ml Erlenmeyer flasks containing 25 ml of 40 mM sodium pyruvate plus 50 mM ammonium lactate salts medium were inoculated with approximately 10^9 spores and were germinated at 37 C by shaking in an Eberbach water-bath shaker (120 one-inch strokes per min). After 16 hr, these cultures were combined into one standard inoculum. A 4-ml amount of this inoculum (8%) was used to inoculate 250-ml Erlenmeyer flasks containing 46 ml of growth medium at a final concentration of 50 mM sodium pyruvate and 50 mM ammonium lactate plus or minus 15 mM L-arginine. Growth occurred at 37 C under the conditions described above. Glucose was added to give a final concentration of 30 mM, and samples were taken at times indicated in the figures. These samples were centrifuged at 29,000 × *g* at 0 C for 10 min in a Sorvall refrigerated centrifuge. The supernatant solutions were discarded and cells were suspended to 5 ml with fresh salts medium.

The cultures grown in 200-ml lots (1,000-ml flask) were incubated in a warm room at 37 C on Eberbach reciprocal shakers at 110 one-inch strokes per min. A Klett-Summerson colorimeter with a no. 54 filter was used to determine growth, and the *p*H was determined with a Beckman Zeromatic *p*H meter. All the cell-free preparations were prepared by breaking the cells in a sonic oscillator (Measuring & Scientific Equipment, Ltd., London, England) at 2 C for 1 min and were centrifuged in a Sorvall refrigerated centrifuge at 22,000 × *g* for 10 min. Supernatant solutions were dialyzed against two changes of 2 liters of 3 mM KH_2PO_4 buffer (*p*H 6.5) for 5 hr. These dialyzed extracts were used in the enzyme assays. The same procedure was used with the different carbon sources. The spores were germinated and the experiments were performed in the same medium in each case. In the experiments in which proline oxidation was measured, whole cells were used and cell samples were centrifuged as above. The supernatant solution was discarded and the cells were washed with cold distilled water by centrifugation at 29,000 × *g* at 0 C for 10 min. The supernatant solution was discarded and the cells were suspended in 5 ml of 5×10^{-2} M tris (hydroxymethyl) aminomethane (Tris)-chloride buffer, *p*H 7.5.

Enzyme assays. Arginase was assayed by the appearance of ornithine from arginine by the method of Chinard as modified by Ratner (19). Ornithine-δ-transaminase and the Δ'-pyrroline-5-carboxylate dehydrogenase were assayed according to the method described by Ramaley and Bernlohr (18). The proline oxidation was measured in whole cells by the procedure described by De Hauwer et al. (7).

A modification of the modified copper-Folin system of McDonald and Chen (11) for determining proteolytic enzymes was employed because of its sensitivity in assaying small amounts of enzyme. A 2.5-g amount of acid-washed casein (Difco) plus 0.5 g of $NaHCO_3$ were added to 90 ml of distilled water, the *p*H was adjusted to 8.2 with saturated KOH, and the solution was diluted to 100 ml with distilled water. The mixture was heated to 80 C so that the casein would go into solution and was filtered through Whatman no. 1 filter paper. Thus, 1 ml of filtrate contained approximately 25 mg of casein plus 5 mg of $NaHCO_3$.

The assay mixture contained 1.0 ml of acid-washed casein (25 mg), enzyme preparation, and distilled water to a final volume of 2.0 ml. The reaction was stopped after 10 min at 37 C by the addition of 2 ml of 10% trichloroacetic acid, giving a final concentration of 5%. The mixture was centrifuged in a Sorvall refrigerated centrifuge at 12,000 × *g* for 5 min, the supernatant solution was collected, and a maximum of 1 ml or less was used for analysis by the following Folin procedure. A 0.5-ml amount of 0.3 N NaOH and 5 ml of copper sulfate reagent (1 ml of 2% copper

sulfate, 1 ml of 4% sodium potassium tartrate made to 100 ml with 4% sodium carbonate in 0.1 N NaOH) were added and mixed thoroughly with a vortex stirrer. This mixture was incubated for 20 min at 37 C; then 0.5 ml of 1.0 N Fisher's phenol reagent (distilled water-phenol reagent, 1:1) was added, mixed, and incubated at room temperature for 15 min for color development. The samples were read in a Klett-Summerson colorimeter with a no. 66 filter. The standard curve for tyrosine was prepared as above and was linear over the range of 0.04 to 0.3 μmoles.

Definition of enzyme unit. In the enzyme assays described above, one unit of activity is defined as the appearance or disappearance of 1 μmole of product or substrate, respectively, in 1 min at 37 C. Proline oxidation is given in μmoles per min per mg of protein.

Chemical analysis. All protein concentrations were measured by the method of Lowry et al. (10).

Chemicals. L-Arginine was purchased from Calbiochem, L-proline from Mann Research Laboratory, and Δ'-pyrroline-5-carboxylate was a gift from Robert Ramaley. All other reagents and chemicals were of reagent grade purity.

RESULTS

Kinetics of catabolite repression of the arginine breakdown pathway. The three enzymes on the metabolic pathway from arginine to glutamic acid in *B. licheniformis* are arginase, ornithine-δ-transaminase, and Δ'-pyrroline-5-carboxylate dehydrogenase (18). These enzymes are not found in cells grown on glucose as carbon source but do appear in the cell during sporulation (glucose is absent). It was found (8) that these enzymes are present in cells growing on glutamate and that L-arginine can induce them to much higher levels. Glucose addition causes a repression of this induction.

Figure 1 presents a time course of this phenomenon in relation to arginase synthesis and repression. Growth rates of *B. licheniformis* on glutamate and glucose are 120 min and 65 min, respectively. Figures 2 and 3 present the results of similar experiments in which the activities of the transaminase and the dehydrogenase were monitored. A comparison of these figures allows us to conclude that the three activities are coincidently induced and that the profiles of repression and dilution of the enzymes in the cell are also coincident. Thus, all three enzymes can be induced by arginine, but the control of each may be separate as the rates of induction and repression are different. It is also clear that the base levels (minus inducer) of these enzymes are easily demonstrable.

The effect of various carbon sources on arginase induction. The stringency of catabolite repression has been related to growth rate (12, 20). It is possible to grow *B. licheniformis* on different

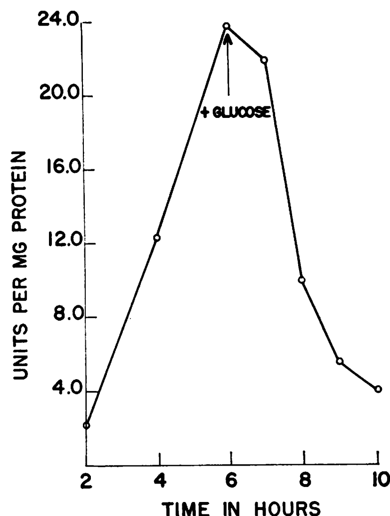


FIG. 1. *Induction and repression of arginase in cells growing on 50 mM glutamate (generation time, 120 min). Arginine was added at zero-time (final concentration, 15 mM). Glucose was added at 6 hr to a concentration of 30 mM (generation time, 65 min).*

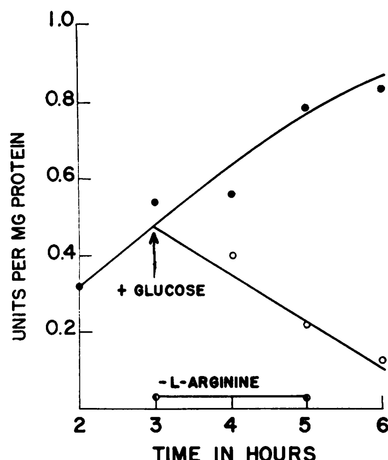


FIG. 2. *Induction and repression of ornithine-δ-transaminase in cells growing on 50 mM glutamate (generation time, 120 min). Arginine was added (to 15 mM) at zero-time (● and ○) and glucose was added at 3 hr to 30 mM (○); generation time, 65 min.*

carbon sources and measure the kinetics of induction of arginase. Figure 4 presents the results of experiments with glycerol as carbon source. On this medium, basal levels of the enzyme were barely detectable but arginine induced arginase to a low but significant level. In this and all other studies of this type, the experiments were terminated

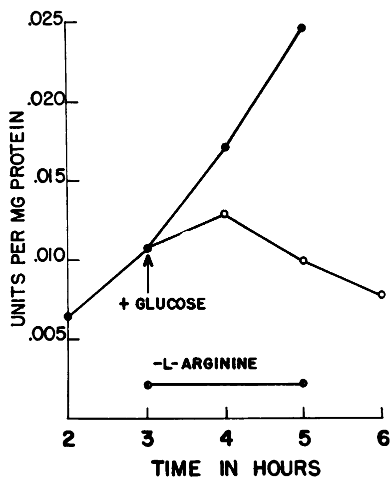


FIG. 3. *Induction and repression of the arginine-induced Δ′-pyrroline-5-carboxylate dehydrogenase. Conditions are the same as in Fig. 2.*

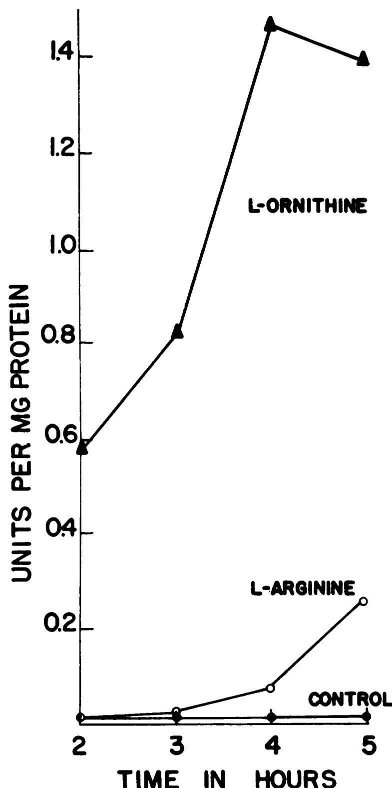


FIG. 4. *Induction of arginase in cells growing on 120 mM glycerol (generation time, 65 min). L-Ornithine (15 mM; ▲) or L-arginine (15 mM; ○) was added at zero-time. At 5 hr, the cultures were at mid-log phase.*

when the cultures had reached the middle of the logarithmic phase of growth. Thus, it is assumed that the enzymes were products of growing cells. It should be noted that L-ornithine was a better inducer than L-arginine.

Figures 5 and 6 present the data from similar experiments in which acetate (Fig. 5) or pyruvate (Fig. 6) was used as the carbon source. In these experiments, L-arginine and L-ornithine appeared to be equivalent inducers. Table 1 summarizes the data from many experiments and includes the results of experiments with glucose and glutamate as the carbon source. It is clear from these data that the extent of catabolite repression by a carbon source is closely related to its ability to promote rapid growth.

The proline catabolic pathway. The enzymes reported to be responsible for the degradation of proline to glutamate are proline oxidase and a Δ′-pyrroline-5-carboxylate dehydrogenase (7). By use of a manometric assay, it was possible to measure the ability of whole cells to oxidize proline (Table 2). In cells growing on glutamate, proline addition (20 mM) caused an approximately eightfold increase in this activity. However, proline-enhanced oxygen uptake activity was also

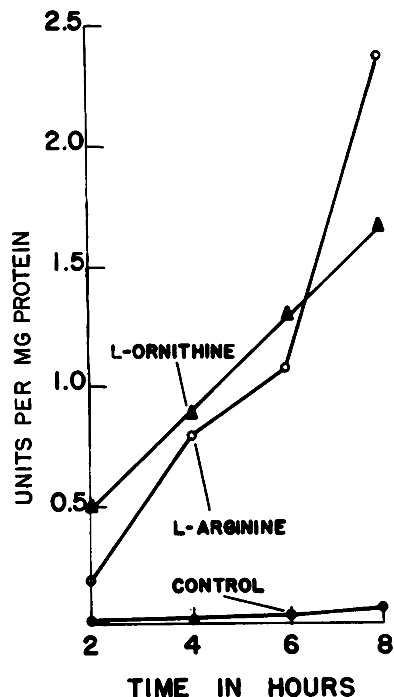


FIG. 5. *Induction of the arginase in cells growing on 50 mM acetate (generation time, 90 min). Conditions are the same as in Fig. 4.*

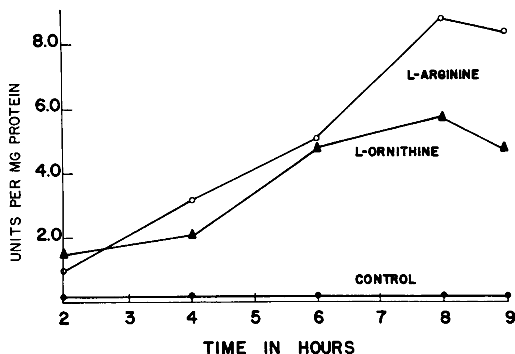


FIG. 6. *Induction of arginase in cells growing on 50 mM pyruvate (generation time, 95 min). Conditions are the same as in Fig. 4.*

TABLE 1. *Catabolite repression of arginase during growth on various carbon compounds*

Carbon source	Doubling time	Arginase activity (μmoles per min per mg of protein)[a]		
		No inducer	Plus L-ornithine	Plus L-arginine
	min			
Glucose (20 mM)	65	<0.01	0.34	0.025
Glycerol (50 mM)	65	0.02	1.47	0.075
Acetate (50 mM)	90	0.02	1.48	1.08
Pyruvate (50 mM)	95	0.15	3.50	4.10
Glutamate (50 mM)	120	0.25	24.2	28.8

[a] Values are the maximum obtainable in growing cells.

increased in cells grown on glucose plus proline, but to a level that was one-half of that seen in glutamate-grown cells. These data are consistent with the catabolite repression of proline-induced proline oxidation but are not completely satisfactory.

Δ′-Pyrroline-5-carboxylate dehydrogenase activity was induced to very high levels by proline in cells growing on glutamate. Figure 7 shows the time course of this induction and the dramatic repression caused by glucose. The kinetics of both induction and repression of this activity are different from the kinetics of arginine induction of the same activity (Fig. 3). It has been demonstrated that proline and arginine induce different dehydrogenases in *B. subtilis* (7), and our data are not inconsistent with this phenomenon in *B. licheniformis*. We have not, however, investigated this point. Since we do not know which of the two enzymes (oxidase or dehydrogenase) is rate-limiting in the cell and because the oxidase could not be measured directly, we cannot assume that

TABLE 2. *Apparent glucose repression of induced proline oxidation*

Carbon source	Rate of oxygen uptake[a] (μmoles per min per mg of protein)
Glutamate (50 mM)	0.00
Glutamate (50 mM) plus proline (15 mM)	0.059
Glucose (20 mM)	0.00
Glucose (20 mM) plus proline (15 mM)	0.027

[a] Endogenous rates of O_2 uptake of 0.0075 for glutamate-grown cells and 0.0030 for glucose-grown cells have been subtracted from the above values.

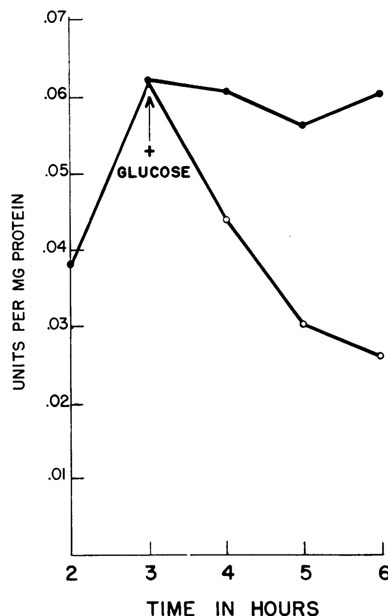


FIG. 7. *Induction and repression of the proline-induced Δ′-pyrroline-5-carboxylate dehydrogenase in cells growing on 50 mM glutamate. Proline was added at zero-time to a concentration of 15 mM (●). Proline was added (15 mM) to a separate culture (○) at zero-time and glucose was added (40 mM) at 3 hr. Generation times were 120 min on glutamate ± proline and 65 min in the presence of glucose.*

the oxidase is induced by proline. The data on dehydrogenase are clear, however, and allow the suggestion that the pathway is induced by proline and is under catabolite repression control in *B. licheniformis*.

Relationship between the arginine and proline pathways. The arginine and proline pathways are convergent and share a common intermediate,

TABLE 3. *Induction of arginine and proline catabolic enzymes*[a]

Inducer	Specific activity (μmoles per min per mg of protein)			
	Arginase	Ornithine-δ-transaminase	Δ′-Pyrroline-5-carboxylate dehydrogenase	Proline oxidase
None plus glucose (20 mM)	<0.001	<0.001	<0.001	<0.001
None	0.20	0.025	0.003	0.007
Arginine (15 mM)	28.8	0.795	0.025	0.032
Proline (15 mM)	4.56		0.056	0.059

[a] Log-phase cells growing on the salts plus 50 mM glutamate medium. Values are the maximum obtainable in growing cells.

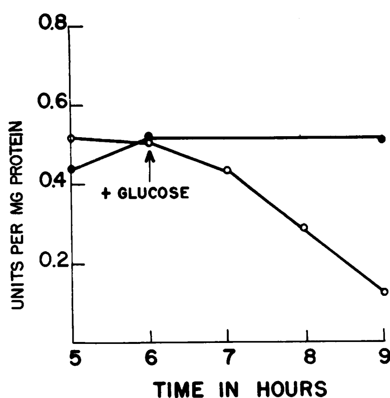


FIG. 8. *Occurrence and repression of the extracellular protease in cells growing on 50 mM glutamate. Glucose (30 mM) was added at 6 hr to one (○) of a pair of cultures. The specific activity is given in terms of milligrams of cell protein. If this experiment is plotted in terms of milligrams of extracellular protein, the minus glucose curve increases and the plus glucose curve remains horizontal after addition of glucose.*

Δ′-pyrroline-5-carboxylate. There are several reports in the literature (5, 6, 14, 15) that show that, in other convergent pathways, the in vivo inducer is one of the common metabolites along the path. We have examined the ability of proline to induce arginase and arginine to induce proline oxidation. Table 3 shows the results of these experiments and includes the activities of other enzymes for ease of comparison. The addition of 15 mM arginine to the glutamate-salts medium not only increased the arginase, ornithine-δ-transaminase, and Δ′-pyrroline-5-carboxylate dehydrogenase activities many-fold, but caused a four- to fivefold increase in proline oxidation. Under the same conditions, proline (15 mM) produced an eightfold increase in proline oxidation and a large increase in arginase activity. It is possible that the apparent induction of proline oxidation by arginine may be an indirect effect caused by increased levels of Δ′-pyrroline-5-carboxylate dehydrogenase. It is clear, however, that proline causes the induction of the enzymes for arginine degradation.

Control of the extracellular protease. During this investigation, it became clear that sporulating cells could use catabolite repression as an efficient mechanism for the control of amino acid turnover and oxidation in relation to sporulation only if a proteolytic activity was induced and repressed coincidently. *B. licheniformis* produces a protease during sporulation but not during growth on glucose (1). The protease is produced during growth on glutamate and the synthesis of this activity can be repressed by glucose (Fig. 8). Thus, the control of protease appears to be similar to that of the amino acid catabolic pathways.

DISCUSSION

We have shown that the entire degradative pathway of arginine to glutamic acid is under catabolite repression control. When *B. licheniformis* was grown on glucose supplemented with arginine, arginase was barely detectable until glucose was exhausted from the medium and the sporulation cycle initiated (8). The enzymes arginase, ornithine-δ-transaminase, and Δ′-pyrroline-5-carboxylate dehydrogenase were present in cells growing on glutamate and could be induced to a higher level by the addition of arginine. The most efficient catabolite repression of arginase occurred when cells were grown on glucose. The degree of repression decreased progressively when the cells were grown on glycerol, acetate, pyruvate, and glutamate, supplemented with arginine. The proline catabolic pathway was shown to be under apparent catabolite repression control. Proline oxidation and a Δ′-pyrroline-5-carboxylate dehydrogenase activity were induced by proline to a high level when cells were grown on glutamate. Ramaley and Bernlohr (16, 17) reported that arginine or ornithine induced arginase in *B. licheniformis* cells grown on glucose. These results may be explained, as shown in this study, by the fact

that the sample used for enzyme preparation was taken at a time when the glucose level was very low or depleted, thus releasing catabolite repression of this enzyme. Early sampling time is necessary for the observation of catabolite repression by glycerol, and possibly explains why De Hauwer et al. (7) did not note catabolite repression of this pathway when *B. subtilis* was grown on glycerol supplemented with arginine.

During growth of *B. licheniformis* on glucose, arginine is synthesized from glutamic acid (18) and catabolic activities are absent. After vegetative growth, sporulation metabolism is initiated and protease activity appears. Also, this organism synthesizes a polypeptide antibiotic, bacitracin, only during sporulation when the cells have been grown on glucose (4). The antibiotic is synthesized by cells growing on glutamate or pyruvate and this synthesis is repressed by glucose (*unpublished data*).

Soon after the synthesis of protease, amino acid catabolic pathways are endogenously induced and the addition of exogenous amino acids causes significant elevations in the levels. We have shown that these enzymes can be induced in cells that are not sporulating and that this synthesis is under catabolite repression control. Therefore, we assume that, although the presence of these enzymes may be necessary for endotrophic sporulation, the control systems utilized by sporulating cells do not preclude the synthesis and possible use of these enzymes during growth on some media. It is clear that the sporulating cell does not have exclusive control over these systems, and that models of development must include this possibility.

We will now discuss the in vivo inducer of the arginine and proline catabolic pathways. The phenomenon of product induction was first described by Palleroni and Stanier (15), who showed that the first three enzymes in the catabolic pathway of tryptophan in *Pseudomonas fluorescens* were induced by an intermediate in the pathway, L-kynurenine. Ornston (14) and Cánovas and Stanier (5) studied the regulation of the synthesis of enzymes catalyzing the conversion of catechol or protocatechuic acid to β-ketoadipic acid in *P. putida*, and again described the occurrence of product induction. A similar mechanism of induction has been suggested by Dagley and Wood (6) to account for the unexpected spectrum of *Pseudomonas* catabolic enzymes that degrade phenylacetic acid and *p*-hydroxyphenylacetic acid. The induction of the histidine pathway in a number of microorganisms appears to be under similar control (9).

We do not consider it likely, in the work reported here, that proline is the in vivo inducer of arginase. In fact, ornithine induces arginase as well as (Fig. 5 and 6) or better (Fig. 4) than arginine. As seen in Table 3, the enzyme having the lowest activity of the four is Δ'-pyrroline-5-carboxylate dehydrogenase. Thus, it might be expected that Δ'-pyrroline-5-carboxylate would accumulate intracellularly and function as the in vivo inducer. This hypothesis cannot be tested directly because *B. licheniformis* cells, grown on glutamate or glutamate and proline, do not grow when inoculated into media containing Δ'-pyrroline-5-carboxylate (15 mM) as the sole carbon source. As the enzymes for the total metabolism of this compound are present in the cell, we assume that it cannot promote growth because of a permeability barrier.

An alternative hypothesis to explain the data in Table 3 is that cells grown in the presence of either proline or arginine synthesize large amounts of the other amino acid endogenously, thus producing both "inducers" from either. This hypothesis is considered unlikely. We have measured the arginase activity in two arginine-minus strains of *B. subtilis* 168 (blocked between ornithine and arginine) grown on 50 mM glutamate plus 1 mM arginine. In both cases, the arginase activity was very high (50 to 150 μmoles per min per mg of protein), whereas the activity in wild-type *B. subtilis* 168 grown on the same medium was 10-fold lower. This is consistent with the idea that a molecule other than arginine is the inducer. Thus, it is our suggestion that the pathways of both arginine and proline breakdown are induced by the common intermediate in these convergent systems, Δ'-pyrroline-5-carboxylate.

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