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Regulation of Nitrogen Catabolic Enzymes in *Bacillus* spp.

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The levels of the inducible nitrogen catabolic enzymes arginase (L-arginine amidinohydrolase, EC 3.5.3.1) and alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase [deaminating], EC 1.4.1.1) from *Bacillus licheniformis* and histidase (L-histidine ammonia-lyase, EC 4.3.1.3) from *Bacillus subtilis* and the ammonia assimilatory enzymes from *B. licheniformis* were determined in cultures grown in the presence of different nitrogen sources. Although the levels of these enzymes were dependent upon the nitrogen source present, induction of the catabolic enzymes in response to the addition of inducer occurred even in the presence of preferred nitrogen sources. Intracellular pool sizes of ammonia, glutamate, glutamine, and α-ketoglutarate were measured in continuous cultures of *B. licheniformis* growing in the presence of different nitrogen sources. A comparison of the pool sizes of these metabolites with the ammonia assimilatory enzyme levels showed that the pools of the metabolites did not change in a manner consistent with their use as regulators of the synthesis of any of these enzymes.

Control of the synthesis of the enzyme systems responsible for utilization of various nitrogen-containing compounds as a function of the nitrogen source used for growth has been termed nitrogen catabolite repression (35). This control system allows microorganisms growing rapidly and efficiently on "preferred" nitrogen sources to bypass the synthesis of unneeded enzymes that would catabolize nitrogen-containing nutrients and allows the induction of these enzymes when the preferred nitrogen sources are not available. Preferred nitrogen sources are rapidly or readily metabolizable and yield high growth rates. A nitrogen source which is not in the preferred category requires the synthesis of a sequence of enzymes for catabolism of that source, and growth is slow. In the enteric organisms, growth in the presence of ammonia (a preferred compound) and histidine (a nonpreferred compound) as nitrogen sources leads to rapid growth (ammonia utilization) and the repression of the *hut* enzymes (reviewed in reference 35).

In all microorganisms that can grow in minimal media, a relationship exists between growth rate and the spectrum of nitrogen sources that support growth. Thus, these microorganisms have preferred nitrogen sources, and it has therefore been logical to assume that a control mechanism exists to allow for efficient growth. The phenomenon of nitrogen catabolite repression has been shown to be a control mechanism in enteric organisms such as *Escherichia coli*, *Klebsiella aerogenes*, and *Salmonella typhimurium* (reviewed references in 3 and 35) and in yeasts (11) and fungi (24). The phenomenon has not been extensively investigated in *Bacillus* spp. and other gram-positive bacteria. Recently, it was shown that an inducible glutaminase (L-glutamine aminohydrolase, EC 3.5.1.2) from *Bacillus licheniformis* is expressed at the same level in the presence of its inducer (glutamine) regardless of the presence or absence of ammonia (7). We present data to show that the inducible catabolic enzymes arginase (L-arginine amidinohydrolase, EC 3.5.3.1) and alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase [deaminating], EC 1.4.1.1) from *B. licheniformis* and histidase (L-histidine ammonia-lyase, EC 4.3.1.3) from *Bacillus subtilis* are not regulated by nitrogen catabolite repression.

MATERIALS AND METHODS

Cultures and growth conditions. *B. subtilis* NP-19 *trpC* (*B. subtilis* 168 derivative obtained from J. F. Kane University of Tennesse, Memphis) and *B. licheniformis* A5 were used in this study. *B. subtilis* NP-19 was stored on nutrient agar plates, and *B. licheniformis* A5 was stored as a frozen spore suspension. All studies with both organisms were done in the minimal salts A medium previously described (33), supplemented with 15 mM glucose and a 20 mM concentration of the indicated nitrogen source. Sodium citrate (4 mM) and tryptophan (0.25 mM) were included in the medium for *B. subtilis* NP-19. Batch cultures were grown in 1 liter of medium at 37°C on a rotatory shaker set at 250 rpm. Cell density was measured turbidimetrically with a Klett-Summerson colorimeter equipped with a no. 54 (green) filter.

Measurement of enzyme activities in crude extracts. Cells used for the measurement of enzyme activities were harvested by centrifugation at 18,000 × *g* for 10

TABLE 1. Effect of the nitrogen source on arginase and alanine dehydrogenase levels in *B. licheniformis* A5[a]

Nitrogen source	μ[b]	Level[c]			
		Arginase		Alanine dehydrogenase	
		Basal	+ Arginine[d]	Basal	+ Alanine[d]
Ammonia	60	31	1,500	91	1,030
Glutamine	60	94	1,055	103	775
Urea	60	45	1,950	57	990
Glutamine + urea	60	36	1,200	ND[e]	ND
Glutamate (Na^+)	60	92	ND	250	1,340
Arginine	65		1,100	25	740
Ornithine	70	1,590	1,880	ND	ND
Nitrate	90	94	ND	100	1,150
Alanine	120	ND	ND		1,460

[a] *B. licheniformis* A5 was germinated and grown in the minimal salts A medium (33) as described in the text. All data were obtained from midexponential cultures.

[b] Doubling times, in minutes.

[c] Expressed in nanomoles $minute^{-1}$ milligram of $protein^{-1}$. Harvesting and assay conditions are described in the text. Each result is the average of at least two different experiments, the final average being less than ±25%.

[d] Inducers, arginine or alanine, were added to a final concentration of 20 mM.

[e] ND, Not determined.

min at 4°C. Glutamate dehydrogenase (L-glutamate: $NADP^+$ oxidoreductase [deaminating], EC 1.4.1.4) (27) and glutamate synthase (L-glutamate:$NADP^+$ oxidoreductase [transaminating], EC 1.4.1.13) (31) were assayed spectrophotometrically. Crude extracts were prepared by washing harvested cells four times in 50 mM Tris-hydrochloride (pH 8.0) buffer containing 5 mM 2-mercaptoethanol, 1 mM EDTA, and 200 mM KCl. The cells were suspended (at 1 to 2% of the original culture volume) in the same buffer without KCl and were broken by two passages through an Aminco French pressure cell at 20,000 lb/in^2. Cell debris was removed by centrifugation at 18,000 × *g* for 20 min at 4°C.

Histidase was assayed by monitoring the formation of urocanate at 277 nm as described by Chasin and Magasanik (5). Alanine dehydrogenase was assayed spectrophotometrically as described by McCowen and Phibbs (25). Crude extracts for both histidase and alanine dehydrogenase were prepared as described above except that EDTA was eliminated from the buffers and cells were broken by sonic disruption (10 times for 30 s with 30-s intervals for cooling) with a Bronwill Biosonik IV sonic oscillator set at 50% power.

Arginase was assayed by the procedure of Ratner (29) except that assays were conducted at pH 9.0 in 10 mM Tris-hydrochloride buffer for 10 min. Crude extracts were prepared as described above except that 50 mM Tris-hydrochloride buffer (pH 9.0) containing 200 mM KCl was used for washing the cells and the cells were broken by sonication (as above) in 50 mM Tris-hydrochloride buffer (pH 9.0). Glutamine synthetase (L-glutamate:ammonia ligase, EC 6.3.1.2) was assayed by the radiochemical method of Prusiner and Milner (28) as modified by Donohue and Bernlohr (10). Cell extracts were prepared as described above except that cells were washed in a 10 mM morpholinepropanesulfonic acid buffer (pH 6.8) containing 10 mM $MnCl_2$ and 200 mM KCl and were broken by sonication in the same buffer without KCl.

In all cases, the determination of enzyme activities was done shortly after the preparation of cell extracts. Protein was determined by the 230/260 method of Kalb and Bernlohr (17).

Measurement of metabolite pools. The preparation of cell extracts for pool studies was done as described previously (33). Ammonia and glutamine were determined by the radiochemical method of Kalb et al. (18). Glutamate and α-ketoglutarate were determined using the fluorometric assay described by Lowry and Passonneau with a Turner model III fluorometer (21). Assays were done at 37°C. Standard solutions of glutamate and α-ketoglutarate were standardized with a Cary 118 recording spectrophotometer.

Materials. All biochemicals were purchased from Sigma Chemical Co. [*U*-^{14}C]glutamate (296 mCi/mmol) was obtained from New England Nuclear Corp. and purified before use (28). All other chemicals used were reagent grade. Double-distilled water was used to prepare all solutions.

RESULTS

Arginase and alanine dehydrogenase levels in *B. licheniformis* A5. Table 1 shows specific activities obtained for arginase and alanine dehydrogenase from cells grown in the presence of various nitrogen sources. The basal levels of the two enzymes were dependent on the nitrogen source present, varying 3-fold for arginase (ornithine is an inducer of the arginine utilization pathway [20]) and 10-fold for alanine dehydrogenase. When 20 mM inducer was added to the medium, arginase and alanine dehydrogenase

TABLE 2. Effect of the nitrogen source on histidase levels in the absence and presence of histidine in *B. subtilis* NP-19[a]

Nitrogen source	μ[b]	Histidase levels[c]	
		Without histidine	With histidine[d]
Glutamine	75	3.7	32.2
Arginine	75	4.0	31.0
Glutamate	120	4.0	28.3
Ammonia	120	3.8	59.1
Histidine	700		24.4

[a] *B. subtilis* NP-19 was grown in the supplemented minimal salts A medium (33) as described in the text. All data were obtained from midexponential cultures.

[b] Doubling time, in minutes.

[c] As described in Table 1, footnote *c*.

[d] Histidine was added to a final concentration of 20 mM.

TABLE 3. Effect of the nitrogen source on growth, pools, and enzyme levels in *B. licheniformis* A5 grown in continuous culture[a]

Nitrogen source	Dilution rate (ml/min)	Sp act[b]			Intracellular pool[c]			
		Glutamine synthetase	Glutamate dehydrogenase	Glutamate synthase	Ammonia	Glutamate	Glutamine	α-Ketoglutarate
Urea	7.0 ± 0.2	240	174	260	5.7 ± 1.8	44 ± 11	10.5 ± 3.5	0.8 ± 0.2
$(NH_4)_2SO_4$	6.3 ± 0.5	85	350	400	10.8 ± 2.3	70 ± 22	1.6 ± 0.5	1.2 ± 0.4
Glutamine	6.0 ± 0.2	11	300	160	5.5 ± 2.0	42 ± 8	17.0 ± 4.3	1.6 ± 0.3
Arginine	5.0 ± 0.1	250	30	160	6.1 ± 1.6	63 ± 7	21.1 ± 4.9	1.2 ± 0.2
Glutamate (Na^+)	4.6 ± 0.6	240	6	11	7.3 ± 2.8	150 ± 30	11.2 ± 4.2	2.9 ± 0.6
Nitrate	3.7 ± 0.4	220	11	180	1.7 ± 0.8	68 ± 10	11.1 ± 2.7	1.2 ± 0.2
Ornithine	3.5 ± 0.1	240	10	14	2.7 ± 1.1	60 ± 8	11.9 ± 3.1	0.5 ± 0.1
γ-Aminobutyrate	1.6 ± 0.1	247	13	26	3.2 ± 1.0	111 ± 11	11.4 ± 2.4	0.2 ± 0.1
Glutamate (K^+)	1.9 ± 0.1	200	10	10	4.2 ± 1.2	130 ± 10	6.5 ± 4.6	0.5 ± 0.1

[a] Continuous culture experiments were done as described by Schreier et al. (31). All values were obtained from cells grown to a density of 10^8 cells per ml, which corresponded to the midexponential phase seen in batch culture. The cultures were maintained in this steady state for the equivalent of 10 doubling times.

[b] Expressed in nanomoles $minute^{-1}$ milligram of $protein^{-1}$. Harvesting and assay conditions are described in the text.

[c] Expressed as the millimolar intracellular concentration as determined in reference 33. Results are the averages of at least triplicate analyses of extracts prepared from two separate sets of cultures.

levels both increased to the levels obtained when the inducer was used as the sole source of nitrogen. Since both enzymes are regulated by carbon catabolite repression (20, 25), the values reported in Table 1 were intermediate levels which would become elevated further if glucose were not used as the carbon source (data not shown). Addition of alanine to cultures of *B. licheniformis* utilizing ammonia or another preferred nitrogen source led to the induction of alanine dehydrogenase without a reduction in growth rate (Table 1). The arginase results were consistent with those obtained by Broman et al., using *B. licheniformis* ATCC 14580 (4), and Baumberg and Harwood, using *B. subtilis* 168 (1). The results clearly indicated that these enzymes do not respond in a manner consistent with regulation by the nitrogen catabolite repression mechanism (35).

Histidase levels in *B. subtilis* NP-19. The *hut* enzymes have been used as the model system for the study of nitrogen catabolite repression in *Klebsiella* spp. (35). Since *B. licheniformis* A5 will not utilize histidine as either a nitrogen source or a carbon source, we repeated the above study with a derivative of *B. subtilis* 168. Histidase levels obtained from cells grown in the presence of various nitrogen sources are shown in Table 2. Basal levels were the same regardless of the nitrogen source used. When 20 mM histidine was added to the medium, an increase in the histidase level was seen which was similar to that obtained when histidine was used as the sole nitrogen source. This was in agreement with earlier studies done by Chasin and Magasanik (5) which established that histidase is under carbon catabolite repression control. The values reported in Table 2 were intermediate levels which would become elevated further if glucose were not used as the carbon source (data not shown). Addition of histidine to cultures of *B. subtilis* led to the induction of histidase without a reduction in growth rate (Table 2). The histidase from *B. subtilis* does not, therefore, respond to the nitrogen catabolite repression control mechanism as described for the enteric bacteria (35).

Regulation of glutamine synthetase, glutamate synthase, and glutamate dehydrogenase in *B. licheniformis* A5. Since the ammonia assimilatory enzymes (particularly glutamine synthetase) have been shown to be involved in the nitrogen catabolite repression control mechanism in enteric organisms (35), we studied the levels of these enzymes in *B. licheniformis* grown under various cultural conditions.

The levels of glutamine synthetase, glutamate synthase, and glutamate dehydrogenase from cells grown in continuous culture in the presence of different nitrogen sources are shown in Table 3. Continuous cultures were used to insure that all values obtained were steady-state levels. This was necessary since it has been shown that enzyme levels and metabolite pool sizes, which are described below, change with culture age (6, 9). The observed dilution rates were dependent on the individual nitrogen source used for growth. The activity of glutamine synthetase

varied 20-fold, being low when cells were grown in the presence of glutamine or ammonia and high under the other conditions. Glutamate synthase activity varied approximately 40-fold and was at its highest level when ammonia and urea were used as nitrogen sources, at an intermediate level when cells were grown in the presence of glutamine, arginine, or nitrate, and at the lowest level under the other conditions. Glutamate dehydrogenase activity varied 60-fold. The level was elevated in cells grown in the presence of glutamine, ammonia, or urea as the sole nitrogen source and was very low under the other conditions. Our data indicated that the levels of the enzymes were regulated by the nature of the nitrogen source present in the medium, but no obvious metabolic relationship was apparent.

Intracellular pool sizes of ammonia, glutamate, glutamine, and α-ketoglutarate. To determine whether the activities of glutamine synthetase, glutamate synthase, and glutamate dehydrogenase from *B. licheniformis* were related to the levels of ammonia, glutamine, glutamate, or α-ketoglutarate, we measured the intracellular pools of these metabolites in cells grown in continuous culture in the presence of various nitrogen sources (Table 3). The ammonia pool varied approximately sixfold, with the low concentrations being obtained from cells grown in the presence of a nitrogen source which produced a slow dilution rate. The level of glutamine varied approximately 13-fold, the lower value being obtained from cells grown in the presence of ammonia. The glutamate pool remained relatively high under all conditions. α-Ketoglutarate pools varied over a range of approximately 14-fold.

A comparison of the pool size of any one metabolite, or combinations of these pools, with any one of the enzyme levels (Table 3) showed that the pools of these metabolites did not change in a manner consistent with their use as repressors (corepressors) of any of the ammonia assimilatory enzymes.

DISCUSSION

Arginase and histidase are catabolic enzymes which respond to both carbon control and nitrogen control in enteric bacteria (14, 15, 22, 35). These enzymes are synthesized at very low levels in the presence of excess ammonia even if their respective inducers are present (14, 15, 22, 35). In *Bacillus* spp., arginase, histidase, and alanine dehydrogenase have all been shown to be under carbon catabolite repression control, and this phenomenon in the *Bacillus* spp. has been fully documented (13), even though this organism does not make cyclic AMP (2). However, our data, along with those of others (1, 4, 5), indicated that nitrogen catabolite repression as a control mechanism does not appear to be involved in the regulation of these enzymes in *Bacillus* spp. Our results indicated that basal levels were regulated by the nature of the nitrogen source present, and the mechanism of this control is unknown.

Our results for the levels of glutamine synthetase, glutamate synthase, and glutamate dehydrogenase and the intracellular concentrations of ammonia, glutamine, glutamate, and α-ketoglutarate showed that even though the metabolites and enzyme levels changed in response to the nature of the nitrogen source present in the medium, the metabolite levels were not related in a direct manner to the levels of the enzymes. These results, however, do not rule out complex relationships between the metabolite pools and the enzyme levels. Metabolite pool studies of this kind have not been described for investigation of glutamate synthase and glutamate dehydrogenase in the enteric bacteria. However, a direct relationship between α-ketoglutarate/glutamine ratios and glutamine synthetase levels has been shown by Senior (32) in *E. coli* and extended by Magasanik and Rothstein in *Klebsiella* sp. (23). This is not apparent in *B. licheniformis*. It has been proposed that the glutamine pool is responsible for the level of glutamate synthase in *B. subtilis* NP-19; however, that study with batch cultures had fewer nitrogen sources (8). *B. subtilis* yields a different glutamate synthase activity profile when grown in the presence of the same variation of nitrogen sources shown in Table 3 (26). The control of glutamate dehydrogenase in *B. subtilis* (19) is different from that in *B. licheniformis* (27).

It has been shown that the control system(s) responsible for sporulation and production of protease and antibiotics in *Bacillus* spp. responds to the nitrogen source present in the medium and is related to growth rate (16, 30, 31). Since we have shown, in this report, that the "classical" nitrogen catabolite repression control system does not exist in *Bacillus* spp., some other mechanism for relating nitrogen source and growth rate with sporulation and concomitant metabolism must be proposed. This work is continuing in this laboratory and in the laboratory of others (12, 34).

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