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Oxygen and Nitrate in Utilization by *Bacillus licheniformis* of the Arginase and Arginine Deiminase Routes of Arginine Catabolism and Other Factors Affecting Their Syntheses

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Bacillus licheniformis has two pathways of arginine catabolism. In well-aerated cultures, the arginase route is present, and levels of catabolic ornithine carbamoyltransferase were low. An arginase pathway-deficient mutant, BL196, failed to grow on arginine as a nitrogen source under these conditions. In anaerobiosis, the wild type contained very low levels of arginase and ornithine transaminase. BL196 grew normally on glucose plus arginine in anaerobiosis and, like the wild type, had appreciable levels of catabolic transferase. Nitrate, like oxygen, repressed ornithine carbamoyltransferase and stimulated arginase synthesis. In aerobic cultures, arginase was repressed by glutamine in the presence of glucose, but not when the carbon-energy source was poor. In anaerobic cultures, ammonia repressed catabolic ornithine carbamoyltransferase, but glutamate and glutamine stimulated its synthesis. A second mutant, derived from BL196, retained the low arginase and ornithine transaminase levels of BL196 but produced high levels of deiminase pathway enzymes in the presence of oxygen.

Two pathways of arginine catabolism have been found in *Bacillus licheniformis* (3, 5, 12, 18, 19). These are the arginase pathway and the arginine deiminase pathway, and they are represented in Fig. 1. Various features of the regulation of these routes have been studied. Both are induced by arginine (5, 13, 18) and repressible by glucose (12). Furthermore, oxygen has a striking effect on the synthesis of the enzymes involved in these pathways: good aeration of cultures favors the arginase route, whereas high levels of the deiminase pathway enzymes are produced in poorly oxygenated cultures (3). These two routes are not mutually exclusive, however, because poorly oxygenated cultures containing arginine or glutamate and arginine, as sole carbon and nitrogen sources, produce relatively high levels of both routes (3). *B. licheniformis* is not the only bacterium to possess more than one pathway for arginine degradation. Pseudomonads of the *fluorescens* group have an arginine deiminase (24, 25) and an arginine decarboxylase (A. Mercenier, J. P. Simon, and V. Stalon, Arch. Int. Physiol. Biochim., in press). In such strictly aerobic organisms, higher levels of arginine deiminase and catabolic ornithine carbamoyltransferase are produced in oxygen-starved cultures (10; Mercenier et al., Arch. Int. Physiol. Biochem., in press). The ATP-producing function of the pathway is therefore considered important as in the well-studied case of *Streptococcus faecalis* (2).

These observations raise a number of questions. What are the physiological roles of the two arginine-catabolic pathways of *B. licheniformis*? Which signals trigger the syntheses of the corresponding enzymes? How do the pathways compare as providers of nitrogen? Is the arginine deiminase route important for ATP production when oxygen is scarce?

We have attempted to throw some light on these problems by the isolation of mutants. We have also looked further into the regulation of the pathways by studying the effects of various nutrients on the levels of enzymes involved in the catabolism of arginine in the presence and the absence of oxygen.

MATERIALS AND METHODS

Strains. The wild-type strain was *B. licheniformis* ATCC 14580. Strain BL196 was isolated by the following procedures. About 10^{10} spores were irradiated with UV light to a survival ratio of 1/200. The spores were then centrifuged, washed with salts medium 154 (25), and allowed to germinate in 50 ml of medium containing 154, 20 mM glucose, 10 mM alanine, 20 mM ornithine, and 10 mM ammonium sulfate. Germination was allowed to carry on for 3 h at 37°C. Aeration was assured by shaking of culture flasks. The cells were

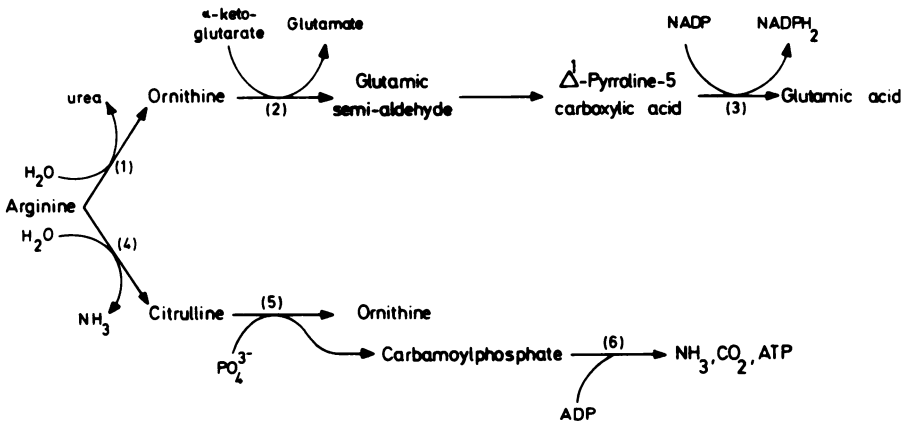

FIG. 1. *Pathways of arginine catabolism in B. licheniformis. (1) Arginase (EC 3.5.3.1); (2) ornithine transaminase (EC 2.6.1.13); (3) pyrroline dehydrogenase (EC 1.5.1.12); (4) arginine deiminase (EC 3.5.3.6); (5) ornithine carbamoyltransferase (EC 2.1.3.3); (6) carbamate kinase (EC 2.7.2.2). NADP, Nicotinamide adenine dinucleotide phosphate.*

then centrifuged, washed twice, resuspended in 25 ml of medium containing glucose, ornithine, and medium 154, and shaken at 37°C for 1 h. After this time, penicillin was added to the concentration of 10,000 U/ml. Two hours later, the remaining cells were washed twice and spread over petri plates containing 154 medium, 20 mM glucose, 20 mM ammonia, and 2% agar. After aerobic growth at 37°C, 540 colonies were obtained. Four of these strains were unable to grow on a medium containing 154, glucose, and ornithine or 154, glucose, and arginine in the presence of oxygen.

Strain BL196 is one of these strains. Previous investigators obtained a similar mutant by a method in many points similar to ours (5). Strain BL196-2 was isolated as a spontaneous "revertant" of BL196, which has recovered the ability to grow on glucose and arginine plus 154 in the presence of oxygen. One difficulty in working with BL196 was the ease with which mutants such as BL196-2 were obtained. This prevented the precise measurement of certain generation times.

Growth conditions and media. All cultures contained the salts medium 154 and 0.0001% thiamine. Although aerobic cultures of *B. licheniformis* require no vitamins for growth, we found that thiamine is necessary for growth in anaerobiosis.

The concentrations of other nutrients, used in the various cultures, were as follows: glucose, 20 mM in aerobic cultures and 40 mM in anaerobic cultures; amino acids, 20 mM; ammonium sulfate, 10 mM; potassium nitrate, 40 mM.

Aerobic cultures were grown in 1-liter flasks containing 100 ml of culture medium and shaken vigorously.

Anaerobic cultures were grown in special flasks designed to allow a current of pure nitrogen to be blown through them and to be hermetically closed thereafter. Such flasks were equipped with a cell for optical density measurement as in somewhat similar flasks described previously (27). Methylene blue was used to detect remaining oxygen in test cultures. Our conditions were adjusted empirically so that any remaining oxygen was used up by the cells before visible growth. In flasks that were opened after this time, the medium became blue once again. This, and the more restrictive conditions of growth obtained under these conditions, led us to believe that our conditions of anaerobiosis were rather good.

Aerobically grown cells were used to inoculate the anaerobic cultures. This enabled the cells to use up remaining oxygen very quickly, and allowed the use of a single "preculture" for a series of cultures on various media. The number of cells in the inoculum was very low (about optical density 0.002, or 2×10^6 cells per ml) to allow time for the cells to adapt to the new conditions and for dilution of repressible enzymes. This procedure of inoculation produced more coherent results among independent cultures on identical media than when separate, aerobic or anaerobic precultures were used. Cells were harvested during exponential growth.

Enzyme assays. Specific activity is defined as the number of micromoles of product formed per hour per milligram of protein. Arginase and ornithine carbamoyltransferase were assayed as mentioned previously (3). Ornithine transaminase measurement has been described elsewhere (7). Our assay differed in only two respects: the buffer used was tris(hydroxymethyl)-aminomethane-hydrochloride at pH 8 and at the final concentration of 33 mM, and the α-ketoglutarate concentration used was 13.3 mM. Protein content was measured by the method of Lowry et al. (15).

Assays of arginine metabolites in the culture medium. Arginine was assayed by the method of Sakaguchi (20). Urea and citrulline were assayed as described by Archibald (1). Ornithine was transformed to citrulline by incubation with 400 U of *Escherichia coli* ornithine carbamoyltransferase and 20 mM carbamoylphosphate, and ornithine concentration was obtained by subtracting total citrulline obtained after incubation minus citrulline concentration before incubation. Ammonia was measured by oxidation of reduced nicotinamide adenine dinucleotide, in the

presence of α-ketoglutarate and glutamic dehydrogenase, as described by Reitz and Rodwell (21).

RESULTS

Growth under anaerobiosis. A typical anaerobic growth pattern is shown in Fig. 2. The exponential phase was rather short as compared with total growth yield, and enzyme levels varied with time of harvest, as shown in Table 1. The effects on enzyme levels presented in this paper were reproducible over at least three experiments. Thiamine was necessary for growth in anaerobic cultures in the absence of nitrate and improved growth when nitrate was supplied. Thiamine does not seem to affect aerobic growth.

Regulation of the pathways. Table 2 shows the generation times of *B. licheniformis* on various media and the levels of arginase and ornithine carbamoyltransferase produced by these cultures. Several aspects of the regulation of these enzymes have already been mentioned. Their inducibility by arginine is shown by the results of experiments 4 and 21, as compared with experiments 1 and 18. The glucose repression of arginase appears in experiment 4, as compared with experiment 11. In the anaerobic cultures containing arginine, arginase levels were very low. The same was true for ornithine transaminase (data not shown). Under these conditions, however, the catabolic ornithine carbamoyltransferase levels were high. In well-aerated cultures on identical media, arginase was produced, and ornithine carbamoyltransferase levels were low. The repressor effect of glucose was not apparent under these conditions. We take this to be due to a better aeration than that in previous work (3, 13). On the basis of these data alone, it would seem that the arginase route is the physiologically important one in the presence of oxygen. It is well known that glutamate, the product of this route, can be used, under these conditions, as sole source of carbon, energy, and nitrogen (19). The results of experiments 5 through 8 show that, in addition to regulation by glucose, arginase is also subject to nitrogen repression. When glucose was supplied in the medium, arginase was repressed by glutamine. The same effect was obtained with ammonia and glutamate, when added together, although separately they had little influence. In the absence of a good carbon-energy source, arginase levels were high regardless of the other nitrogen sources present in the medium (experiments 11 through 15). The slight variations which appeared could be due to the effects of these nutrients as carbon sources. Generation times show that ammonia, glutamine, and glutamate are all better nitrogen sources than arginine. Glutamine and arginine were poor sources of carbon-energy, but growth was improved when they were combined. Glutamate was a relatively good source of carbon. In anaerobic cultures, the pattern was very different. For one thing, no growth occurred on amino acid-containing media unless a suitable carbon source was added. We have used glucose for this purpose. Under these conditions, the arginine deiminase pathway would seem to be the physiologically significant route of catabolism. In one respect, its regulation seems classical: ammonia, a product of the pathway, acts as a repressor of

TABLE 1. *Specific activities of ornithine carbamoyltransferase on glucose-arginine medium, as a function of optical density in anaerobiosis*[a]

Optical density at 660 nm	Ornithine carbamoyltransferase sp act
0.100	415
0.200	535
0.305	765
0.420	920
0.510	800
0.615	860
0.5 (lysis)	1,140

[a] The results were obtained with independent cultures inoculated with cells from an aerobically grown culture on the same medium. One optical density unit equals 10^9 cells per ml.

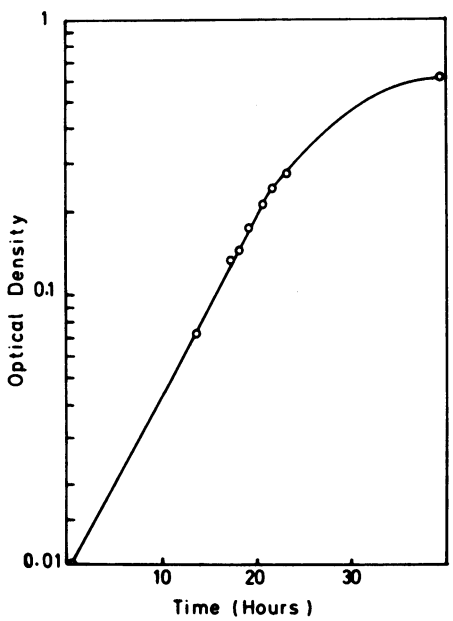

FIG. 2. *Typical anaerobic growth pattern of B. licheniformis, on a medium containing 40 mM glucose, 20 mM arginine, thiamine, and salts medium 154.*

TABLE 2. *Specific activities of the arginase and ornithine carbamoyltransferase on various media and culture conditions*

Expt no./growth conditions	Generation time (min)	Arginase sp act	Ornithine carbamoyltransferase[a] sp act
Aerobic cultures			
1. Glucose + NH_4^+	60	1	22
2. Glucose + glutamate	60	1	16
3. Glucose + glutamine	54	5	39
4. Glucose + arginine	105	160	3
5. Glucose + arginine + NH_4^+	70	135	18
6. Glucose + arginine + glutamate	75	130	45
7. Glucose + arginine + glutamate + NH_4^+	75	50	60
8. Glucose + arginine + glutamine	60	50	10
9. Glutamate	90	3	17.6
10. Glutamine	210	3	17.5
11. Arginine	225	850	<1
12. Arginine + glutamate	100	790	3.2
13. Arginine + NH_4^+	225	980	<1
14. Arginine + glutamate + NH_4^+	105	790	81
15. Arginine + glutamine	140	760	<1
16. Pyruvate + arginine	138	490	2
17. Alanine + arginine	144	510	2
Anaerobic cultures			
18. Glucose + NH_4^+	240	1–4	7
19. Glucose + glutamine	430	1.3–5.0	9
20. Glucose + glutamate	>600	ND[b]	ND
21. Glucose + arginine	240	2–16	240–460
22. Glucose + arginine + NH_4^+	240	5–16	72–150
23. Glucose + arginine + glutamate	255	7–17	1,420–3,820
24. Glucose + arginine + glutamine	300	4–11	1,800–4,100
25. Glucose + arginine + NO_3	90	85	7
26. Arginine + NO_3	∞		

[a] When no arginine is present in the culture medium, the anabolic ornithine carbamoyltransferase is synthesized. When arginine is added, the measured activity is due mainly to the catabolic enzyme, although some anabolic activity may be present.

[b] ND, Not determined.

the catabolic ornithine carbamoyltransferase, as shown by experiments 21 and 22. We have found this to be true for carbamate kinase as well (data not shown).

More striking, however, are the unexpected effects of glutamate and glutamine on the catabolic transferase: they caused a spectacular derepression of this enzyme.

Equally surprising is the poor growth obtained with these amino acids when used alone with glucose.

Generation times also show that arginine and ammonia are equally good nitrogen sources in anaerobic cultures. Under these conditions, the ATP-forming function of the deiminase route does not seem to be essential for growth.

Another interesting point is the effect of nitrate on the two enzymes. Nitrate can be used by *B. licheniformis* both as a source of nitrogen and as a substrate for anaerobic respiration (26). The respiratory nitrate reductase of this organism has been extensively studied (23). Experiment 25 shows that nitrate causes derepression of arginase and repression of ornithine carbamoyltransferase in the absence of oxygen. This effect of nitrate is similar to that of oxygen. *B. licheniformis* would not, however, grow on arginine alone with nitrate as the ultimate electron acceptor. We noticed no effect of nitrate on enzyme levels in aerobic cultures.

Arginine metabolites produced in aerobic and anaerobic cultures. Although we have left aside the study of intermediate conditions of oxygenation that may be of interest, we have been able to establish conditions in which *B. licheniformis* produces the enzymes of one pathway to the almost total exclusion of those involved in the other route. To see if these variations in enzyme levels are a good reflection of what happens in intact cells, the following experiment was performed. The organism was grown on salts–glucose–arginine–thiamine medium in the presence and in the absence of oxygen. The concentration of arginine was growth yield limiting (2 mM). At various stages throughout growth, samples of the cultures were

taken and centrifuged at low temperature. The concentrations of arginine, ornithine, citrulline, ammonia, and urea in the supernatant medium were measured (Fig. 3).

In the oxygenated cultures, ornithine accumulated at the beginning of growth, then disappeared as arginine became scarce. Very little ammonia was found, but urea was produced in large amounts and remained present when the cells began to lyse for lack of nitrogen (*B. licheniformis* has no urease, according to taxonomic studies [14]). A small amount of citrulline accumulated in the medium and was not used.

In anaerobic cultures, the pattern was different: as arginine vanished, ornithine accumulated, but did not disappear when the cells had used all of the arginine and began to lyse. Ammonia accumulated during the early stages of growth, but disappeared as the arginine concentration became low. The small amount of urea produced was the result of low arginase activity. Citrulline concentration was low, but increased slightly throughout growth. The experiments cannot be used for strictly quantitative information because of the limited sensitivity of the methods used and because a certain amount of arginine metabolites may remain inside the cells. Qualitatively, however, they do show that the utilization of arginine is very different in the presence and in the absence of oxygen. The patterns obtained are compatible with a preferred use of the arginase route in the presence of oxygen and of the deiminase pathway in anaerobic cultures.

Mutants of the pathways. We wondered whether the arginine deiminase route might be induced in the presence of oxygen in mutants unable to degrade arginine via the arginase pathway. In other words, are the needs for nitrogen and the presence of arginine sufficient signals for the induction of the arginine deiminase route? Earlier investigators (5) isolated mutants of *B. licheniformis* which were unable to grow on arginine, as a nitrogen source, in the presence of oxygen. These turned out to be regulatory mutants, in which none of the arginase pathway enzymes could be induced. The parent strain, however, was unable to assimilate ammonia, so the lack of growth on arginine did not prove the inability of these mutants to utilize the arginine deiminase route under these conditions. *B. licheniformis* strain ATCC 14850 is able to use ammonia as nitrogen source, and we have selected a mutant for its inability to grow on glucose plus ornithine in the presence of oxygen. Several characteristics of this mutant are presented in Tables 3, 4, and 5.

Strain BL196 grew normally on minimal me-

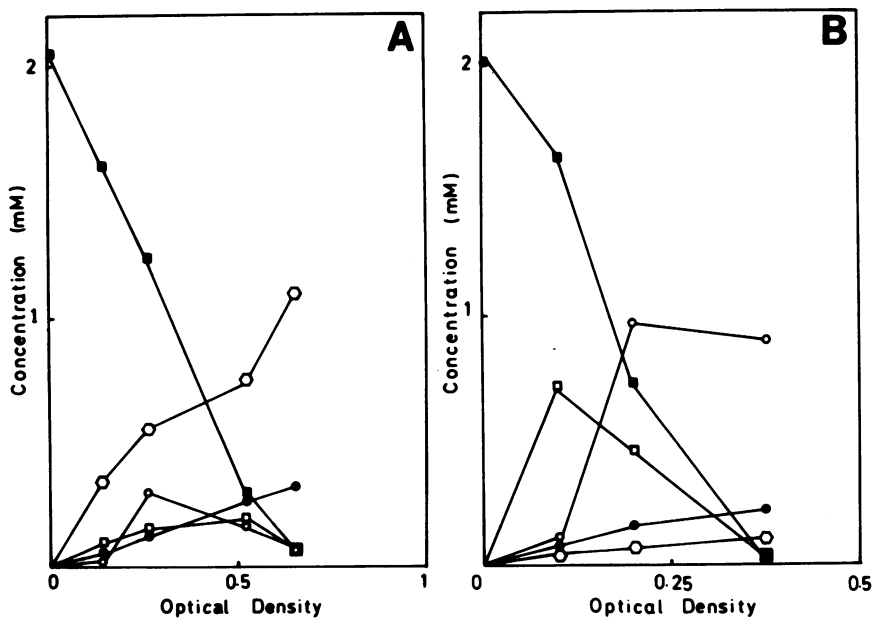

FIG. 3. *Appearance of arginine metabolites in the centrifuged culture medium of cultures grown on a medium containing 154, glucose, arginine and thiamine, in the presence (A) and in the absence (B) of oxygen. Results are plotted as micromoles of metabolite per milliliter of medium versus optical density of culture. In the presence of oxygen, samples were taken from one culture at different stages throughout growth. The results for anaerobiosis were obtained from separate cultures. Symbols: ⬡, urea; ●, citrulline; ○, ornithine; □, ammonia; ■, arginine.*

TABLE 3. *Generation times of mutants BL196 and BL196-2 as compared with the wild type*

Growth conditions	Generation time (min) of:		
	Wild type	BL196	BL196-2
Glucose + NH_4^+, aerobic	60	60	60
Glucose + ornithine, aerobic	105	>700	>700
Glucose + arginine, aerobic	105	>700	80
Arginine, aerobic	210	∞	∞
Glucose + NH_4^+, anaerobic	240	240	240
Glucose + arginine, anaerobic	240	240	240

TABLE 4. *Arginase and ornithine carbamoyltransferase levels in strain BL196*

Expt no./growth conditions	Arginase sp act	Ornithine carbamoyltransferase sp act
Aerobic cultures		
1. Glucose + NH_4^+	<1	
2. Glutamate + arginine	20	0.4
Anaerobic cultures		
3. Glucose + NH_4^+	<1	5
4. Glucose + arginine	<1	350
5. Glucose + arginine + NH_4^+	<1	65
6. Glucose + arginine + glutamate	<1	1,800
7. Glucose + arginine + glutamine	<1	3,100

TABLE 5. *Ornithine transaminase levels in aerobic cultures of strains BL196 and BL196-2 as compared with the wild type*

Growth conditions	Ornithine transaminase sp act		
	Wild type	BL196	BL196-2
Glucose + NH_4^+	0.4	0.6	0.6
Glutamate + arginine	37.0	0.9	0.2

dium, but failed to grow on arginine or on glucose plus arginine in the presence of oxygen. On glutamate plus arginine, it produced extremely low levels of arginase and ornithine transaminase. It is not simply a permease mutant because *B. licheniformis* possesses separate permeases for arginine and ornithine (5). It does not seem to produce high levels of anabolic ornithine carbamoyltransferase, like those isolated for arginine hydroxamate resistance in *B. subtilis* (11). Mutant BL196 grew normally on glucose plus arginine under anaerobiosis, and produced normal levels of catabolic ornithine carbamoyltransferase under these conditions.

The existence of this mutant confirms the importance of the arginase route in the presence of oxygen. It also shows that the deiminase route is sufficient for normal growth in the absence of oxygen.

Tables 3, 5, and 6 show the characteristics of another mutant, isolated by spontaneous reversion of BL196 on glucose plus arginine in the presence of oxygen. Strain BL196-2 grows faster than the wild type on this medium. It still does not grow on arginine alone or on glucose plus ornithine. Enzyme analysis shows that the arginase route is still repressed in this mutant, but the catabolic ornithine carbamoyltransferase is present at high levels. The other deiminase pathway enzymes were also found in these extracts. The route seemed to have lost its control by oxygen, but it remained inducible by arginine, and the carbon source seems to have some importance in its regulation, as shown by experiments 7 through 16 of Table 6. Glutamine and glutamate retained a stimulating effect in anaerobic cultures but had no effect in the presence of oxygen. Clearly, the deiminase route can function as an excellent nitrogen provider in the presence of oxygen, but the need for nitrogen and the presence of arginine are not sufficient signals for its induction.

DISCUSSION

We have studied the regulation of enzymes

TABLE 6. *Arginase and ornithine carbamoyltransferase levels in strain BL196-2*

Expt no./growth conditions	Arginase activity	Ornithine carbamoyltransferase activity
Aerobic cultures		
1. Glucose + NH_4^+	1	26
2. Glucose + arginine	1.3	3,700
3. Glucose + arginine + NH_4^+	1.4	4,000
4. Glucose + arginine + glutamate	1	3,500
5. Glucose + arginine + glutamine	1	3,800
6. Glucose + arginine + glutamate + NH_4^+	1	4,500
7. Arginine + glutamate	16	6,400
8. Arginine + glutamine	3	12,600
9. Arginine + pyruvate	19	10,400
10. Arginine + alanine	15	15,300
Anaerobic cultures		
11. Glucose + NH_4^+	1	10
12. Glucose + arginine	1	2,400
13. Glucose + arginine + NH_4^+	1	2,400
14. Glucose + arginine + glutamate	1	5,300
15. Glucose + arginine + glutamine	1	10,200

belonging to both arginine catabolic pathways of *B. licheniformis* under conditions of vigorous aeration and in anaerobic cultures. The use of extreme conditions such as these has enabled us to study separately the importance of each route because the well-oxygenated cultures produce very little catabolic ornithine carbamoyltransferase and the anaerobic cultures produce practically no arginase.

Studies with mutant BL196 show that the arginase pathway is absolutely necessary for growth on arginine in well-aerated cultures and that it cannot be replaced by the deiminase route under these conditions, except in certain mutants. In anaerobiosis, however, an arginine pathway-deficient mutant grows normally on glucose plus arginine in the absence of oxygen.

As a source of carbon-energy and nitrogen, the arginase pathway presents a rather classical pattern of regulation. Its repression by glucose is well known. Now we see that glutamine, a better source of nitrogen than arginine, is also a repressor of arginase. Nitrogen catabolite repression is a widespread phenomenon among microorganisms. Among bacteria, the case of *Klebsiella aerogenes* has been most extensively studied and is now resolved at the molecular level (16, 17). In this organism, the arginine-utilizing enzymes are subject to both carbon and nitrogen catabolite repression, with both cyclic AMP receptor protein-cyclic AMP and non-adenylated glutamine synthetase acting to stimulate their synthesis. The regulation of arginase in *B. licheniformis* has certain similarities. Here also, both carbon-energy and nitrogen compounds can act as repressors. However, glucose represses arginase, regardless of whether other nitrogen nutrients are added in the medium. Furthermore, arginase repression by glutamine, or by ammonia plus glutamate, occurs only in media containing a good carbon-energy source. It should be stressed that glutamine, and not ammonia, seems to cause the repression. Glutamine synthetase may well play a role in this regulation. This enzyme is necessary for sporulation in *B. megaterium* (22) and *B. subtilis* (8), and in the latter organism it has been shown to regulate its own synthesis (4) and that of histidase (8). The *B. licheniformis* enzyme does not undergo adenylylation, however (6, 9).

The regulation of the deiminase route is more difficult to understand. The only well-established function of the route in *B. licheniformis* is its ammonia-providing function. The regulation of the pathway indicates that its role could be more complex, however. Although ammonia causes repression of the catabolic transferase, which might be regarded as some form of feedback repression, the effects of glutamine and glutamate are more surprising. The slow growth rates obtained with these excellent nitrogen sources in the presence of glucose were equally unexpected. We are, as yet, unable to explain these phenomena.

Is the ATP-providing function of the arginine deiminase route physiologically important for the cell? Growth on glucose plus ammonia is as good as growth on glucose plus arginine in anaerobic cultures. Under these conditions, at least, the importance of arginine as an ATP provider seems negligible. Work in this laboratory indicates that this function of the route may be more important when other carbon sources are used. It does not seem that the deiminase route of arginine catabolism in *B. licheniformis* can be used as a source of carbon. In anaerobic cultures, no growth occurs unless an additional carbon source is added. In well-oxygenated cultures, the mutant BL196-2, which contains derepressed levels of the three known enzymes of the deiminase route, still does not grow on arginine alone. It could be expected that in intermediate cases of oxygen limitation, when both pathways are present, the ornithine formed by both routes would be utilized via ornithine transaminase and pyrroline dehydrogenase. The effect of nitrate on both routes is similar to that of oxygen: it represses the deiminase route and derepresses the arginase pathway in anaerobic cultures. The effects of oxygen and nitrate are probably connected to respiration in general and not due to a direct effect of these compounds.

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