Participation of The Lysine Pathway in Dipicolinic Acid Synthesis in Bacillus cereus T

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A possible involvement of the diaminopimelic acid-lysine pathway in dipicolinic acid (DPA) synthesis has been suggested on the basis of the similarities of certain proposed lysine and DPA precursors (Martin and Foster, 1958). In addition, the in vitro synthesis of DPA has been demonstrated by the addition of known lysine precursors to extracts prepared from sporulating Bacillus megaterium (Bach and Gilvarg, 1966). Results of isotope experiments employing Penicillium are also consistent with possible precursors of lysine functioning in DPA synthesis (Hodson and Foster, 1966) although other experiments with the mold suggest an alternative scheme may be involved (Tanenbaum and Kaneko, 1964).

Further evidence for the participation of the lysine pathway in DPA synthesis in sporulating bacteria is presented in the present paper. It was found that certain lysine auxotrophs of Bacillus cereus T which are altered at an early step in the pathway synthesize spores which contain low levels of DPA. In addition, a change in the property of aspartokinase, presumably the first enzyme in the lysine pathway in this organism, can be detected when comparing extracts of vegetative versus sporulating cells. This change, a densensitization to lysine, is consistent with the functioning of this enzyme in DPA synthesis.

Vegetative cells of B. cereus T growing in G medium (Gollakota and Halvorson, 1960) were treated with N-methyl-N'-nitro-N-nitroso-guanidine (Aldrich Chemical Co.) as previously described (Levisohn and Aronson, J. Bacteriol., in press).

The cells were plated on supplemented media (CDGS of Nakata, 1964) and lysine auxotrophs were picked by replica plating. A total of six lysine auxotrophs were found and they all had reversion frequencies to prototrophy (1 in 10^6-10^7) which were consistent with an initial single step mutation. All of the mutants were able to grow and sporulate without added diaminopimelic acid (DAP). The amount of DAP in the young cells and spores was normal suggesting that all of the mutants were sufficiently leaky to provide the necessary DAP but no lysine (see Fig. 1).

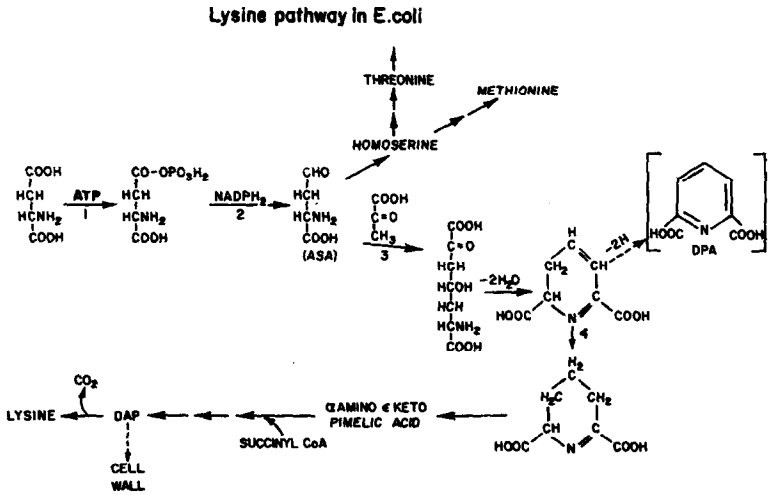

Fig. 1. A portion of the lysine pathway and related branch points. Scheme drawn from data of Umbarger and Davis (1962); Yugari and Gilvarg (1965); Farkas and Gilvarg (1965).
Dashed arrow refers to hypothetical conversion of dihydrodipicolinic acid to DPA. 1. aspartokinase 2 aspartic β semialdehyde (ASA) dehydrogenase 3. condensing enzyme 4. dihydrodipicolinic acid reductase.

Two of these auxotrophs formed spores which were less refractile in the phase microscope than the wild type and these spores contained low levels of DPA (#21 and #47 in Table 1).

Table 1

Comparison of the levels of dipicolinic acid and certain lysine biosynthetic enzymes in Bacillus cereus T lysine auxotrophs

Mutant	Requires lysine to grow*	gDPA ($\times 10^{-15}$) /spore°	ASA dehydrogenase□	condensing enzyme+	aspartokinase△
21	+	16	128	.040	2-3
21 revertant	-	116	-	-	32
47	+	21	1	.037	28
16	+	110	40	.040	25
51	+	140	35	.035	29
7	+	108	37	.042	31
wild	-	112	51	.038	27

*Tested by streaking on CDGS agar medium (Nakata, 1964)
All lysine auxotrophs grow without added diaminopimelic acid (DAP)

°DPA determined by method of Janssen et. al. (1958); spore counts in a Petroff-Hauser chamber

+o-aminobenzaldehyde assay of Yugari and Gilvarg (1965). Values represent ΔA_{540}/min/mg protein. Protein determination by method of Lowry et. al. (1951). Cells were grown on limiting lysine in synthetic medium (Nakata, 1964) supplemented with 0.2% Difco lysine assay medium. The cells were washed twice with 0.2 M tris pH 7.8 at 4C, broken in a French pressure cell and the crude extract (10,000rpm/10 min supernatant) dialyzed overnight at 4°C.

△Units as per Stadtman et. al. (1961). Values represent units/mg protein. Background value obtained in absence of aspartate is subtracted. Freshly prepared extracts treated with streptomycin (10mg/ml) for 60 min at 0°C. Supernatant used for assay. Values represent averages of at least two experiments.

□Assayed in reverse direction with NADP according to procedure of Black and Wright (1955). Streptomycin supernatants of freshly prepared extracts were heated at 60°C/5 min. After cooling, the insoluble proteins were removed by centrifugation and the supernatant fluid used for the assay. Results are mμmoles NADPH formed/min/mg protein.

This low concentration of DPA was found in spores formed in G medium, G medium when further supplemented with $CaCl_2$ to 200 μg/ml or in a synthetic medium (CDGS) supplemented with 10 μg/ml L-lysine. In addition, these spores were heat sensitive with less than 1% survival after 20 minutes at 80°C as compared to greater than 95% survival for the wild type. In the case of mutant 21,

addition of DPA (500 μg/ml) to G medium at the commencement of spore formation or at the time of DPA synthesis increased the DPA content per spore by only a factor of two. These treated spores were still very heat sensitive. Three of the other lysine auxotrophs tested produced spores containing normal amounts of DPA (Table 1).

An explanation for the two classes of lysine auxotrophs would be based on the site of the mutation (Fig. 1). Those blocked prior to the proposed shunt to DPA would be low producers while those blocked after the shunt would produce normal amounts. The three enzymes functioning prior to the shunt have been assayed in crude extracts of the various mutants (Table 1). The cultures were grown on limiting lysine (4 μg/ml) in a phosphate buffered salts medium (CDGS) supplemented with 0.2% Difco lysine assay medium (LAM). The amino acid mixture recommended for CDGS medium (leucine, valine, threonine, methionine, glutamate and histidine) was usually omitted since crude extracts from cells grown with the amino acids present contained very low levels of aspartokinase activity. This low activity was due to the presence of an inhibitor in the crude extracts which could be removed by precipitation with 10 mg/ml of streptomycin. After the A_{660} mμ had stopped increasing, the cultures were incubated an additional 45-60 minutes to ensure derepression of the relevant enzymes. All of the extracts contained normal levels of condensing activity suggesting that the lysine biosynthetic enzymes had indeed been derepressed (assuming that the three enzymes are part of the same operon).

One of the two lysine auxotrophs which contained a low level of DPA, (#21) had a low aspartokinase activity while the other, #47, had a very low level of the ASA dehydrogenase (Table 1). Several revertants of #21 and #47 have been picked and they all produce spores with normal DPA levels. The one revertant of #21 which has been assayed contains a normal level of aspartokinase (Table 1). All lysine auxotrophs with a normal DPA content in their spores had normal levels of the three enzyme activities.

In Escherichia coli, the condensing enzyme (Yugari and Gilvarg, 1962) and

one of the aspartokinases (Stadtman et. al., 1961) are inhibited by lysine. If the lysine pathway functions in DPA synthesis, it is obvious that either the lysine concentration must be very low at the time of DPA synthesis or there must be a change in the regulatory properties of one or more of the key enzymes. Bacillus cereus T is usually grown in a medium containing yeast extract. Further supplementation of this G medium with 100 μg/ml of L-lysine at various times during spore formation did not affect the rate or amount of DPA produced. It is clear, therefore, that some alteration in regulation must occur.

In B. cereus, the condensing enzyme is not lysine sensitive whereas the kinase prepared from vegetative cells is completely inhibited (Table 2).

Table 2

Aspartokinase in young and sporulating cells of Bacillus cereus T

Growth medium	Extraction buffer	units*/mg protein - lysine	units*/mg protein + lysine (2 μmoles)
S-LAM+-young cells	Tris°	26	0
S-LAM-young cells	Tris-SH□	23	21
S-LAM-old cells	Tris	0	-
S-LAM-old cells	Tris-SH	19	19
G medium△-young	Tris	3.5	0
G medium-young	Tris-SH	2.5	2.8
G medium-old	Tris	0	0
G medium-old	Tris-SH	7.5	7.5

*Units as in Stadtman et. al. (1961). Extracts prepared as described in footnote to Table 1. Background value obtained without aspartic acid is always subtracted

°.02 M tris-HCL, pH 7.8 at 4°C

□.02 M tris-HCL, pH 7.8 at 4°C plus 0.03 M mercaptoethanol

+S-LAM: CDGS medium minus amino acids (Nakata, 1964) supplemented with 0.2% Difco lysine assay medium. Young cells incubated for 20 hr at 30°C (cells grow at linear rate). Old cells incubated for 33 hr at 30°C (most cells contain dull phase white structure).

Δ Yeast extract-glucose-salts medium (Gollakota & Halvorson, 1960). Same values are obtained in extracts from young cells when G medium is further supplemented with up to 100 μg/ml L-lysine. Young cells grown for 7.5 hr at 30°C (exponential phase). Old cells incubated for 17 hr at 30°C (commencement of DPA synthesis).

No threonine or methionine sensitive activity could be detected even if the cells were grown with homoserine in place of threonine and methionine.

It was initially observed that no kinase activity was found in extracts prepared in tris buffer from sporulating cells (at the time of DPA production). If the extraction buffer also contained mercaptoethanol, however, kinase activity insensitive to lysine inhibition was found (Table 2). If extracts from young cells were prepared in the presence of mercaptoethanol or if extracts from young cells in tris buffer were treated with mercaptoethanol the kinase activity also became insensitive to lysine inhibition. The results indicate that at the time of DPA synthesis the only functional kinase is insensitive to lysine inhibition. Preliminary experiments suggest that the lysine insensitive activity appears 1-2 hours prior to the commencement of DPA synthesis.

A similar pattern of activity was found in G medium but with much lower levels of activity (Table 2). Cells grown in the synthetic medium with or without lysine (100 μg/ml) synthesize DPA at the same rate suggesting that the repressed enzyme levels are not rate limiting.

While the desensitization ensures a continued flow of carbon through the lysine pathway, this alteration alone is not sufficient to assure that the DPA bypass will function (Fig. 1). When C^{14} aspartic acid was added to sporulating cells at the time of DPA synthesis, very little of the radioactivity was converted to lysine as compared to the conversion to other amino acids synthesized from aspartate or the conversion to lysine occuring in young cells. These experiments suggest that an efficient shunt does indeed exist. To ensure the flow of carbon to DPA, either an enzyme functioning after the proposed shunt must be sensitive to lysine inhibition or possibly one of the late enzymes becomes inactive at the time of DPA synthesis. The dihydrodipicolinic acid may then more readily accumulate and be converted to DPA.

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