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Diaminopimelic Acid Synthesis by Resting Cells of *Escherichia coli* ATCC 13002 : A Double Auxotroph of Lysine and Histidine

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Resting cell suspension of *Escherichia coli* ATCC 13002, a double auxotroph for lysine and histidine grown in the basal salts medium with lysine (250 μg/ml), regained the capacity to synthesize diaminopimelic acid. This concentration of lysine was inhibitory to DAP synthesis in the growing cultures of this organism. The protein biosynthetic inhibitors such as chloramphenicol and streptomycin were found to inhibit the recovery process of DAP synthesis. L-lysine was much more inhibitory than L-methionine and L-threonine+L-isoleucine.

Introduction

Diaminopimelic acid (DAP) has been reported as mucopeptide component of cell wall of certain bacteria (Work 1951) and blue green-algae (Salton 1956). DAP has been shown to be the precursor of lysine (Dewey & Work 1952, Davis 1952). Lysine-requiring mutants of bacteria, which are deficient in enzyme DAP decarboxylase, accumulate DAP in the culture medium.

A mutant of *Escherichia coli*(ATCC 13002; Huang et al. 1960), a double auxotroph of lysine and histidine, was used for the present studies. The effect of exogenous lysine and DAP synthesis in growing cultures of this organism has been already reported (Abdullah et al. 1977). A resting cell system which accumulates significant amounts of DAP in the culture medium has been developed. This paper is concerned with this phenomenon and the regulatory effects of protein biosynthetic inhibitors and exogenous amino acids on DAP synthesis.

Materials and Methods

Organism

Escherichia coli ATCC 13002 was obtained from American Type Culture Collection, Rockville, Maryland, USA. Stocks were maintained on Nutrient Agar (Oxoid CM3) and stored at 4°C. The strain was checked periodically for its auxotrophic requirements for L-lysine and L-histidine.

Medium

The growth medium (Davis & Mingioli 1950) (Basal-Salts medium, with sucrose as carbon source) had the following composition (g/L): K_2HPO_4, 7.0; KH_2PO_4, 3.0; $MgSO_4$ $7H_2O$, 0.10; $(NH_4)_2SO_4$, 1.0; Sucrose, 20.0;

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L-lysine and L-histidine (250 and 25 μg/ml respectively) *p*H of the medium was adjusted to 7.0 with NaOH. Medium was sterilized at 115°C for 20 min. Sucrose was sterilized separately and added at the time of inoculation.

Culture techniques

Cultures were grown at 26°C in Erlenmeyer flasks (250 ml) containing 25 ml of medium and were incubated in an orbital incubator at 250 rev./min (Gallenkamp Ltd, London). Inoculum preparation, inoculation procedure and dry weight measurements of the cells were made as outlined earlier (Abdullah et al. 1977).

Preparation of resting cell suspension

Bacterial cells were grown for 20 hr (logarithmic) at 26°C by shaking, with L-lysine 250 μg/ml and L-histidine 25 μg/ml. Cells were centrifuged for 10 min at 1350×g. Pellets were washed twice with basal salts medium, using sterile conditions. The washed cells were suspended in 20ml of same fresh medium with $CaCO_3$ (0.5% W/V) to buffer the medium.

Estimation of diaminopimelic acid in the medium

Culture samples taken at various growth times were centrifuged at 2300×*g* for 5 min. The clear supernatants were used for DAP estimation following the method of Work (1963).

Results

Demonstration of the accumulation of DAP by the resting cells of E. coli *ATCC 13002*

Resting cell suspensions prepared from the cultures previously grown in lysine and histidine supplement were resuspended in basal salts medium devoid of lysine as outlined in methods. The time course of appearance of DAP in the culture filtrates was followed (table 1). Significant accumulation of DAP occurred at 2 hr reaching maximum by the end of 5 hr. However, there occurred substantial variation of these levels in day to day experiments indicating the need for careful monitoring of the resuspension conditions.

Table 1 *Elaboration of DNA in the medium by the resting cells of* E. coli *ATCC 13002*

Time (hr)	Cell dry weight (mg/ml)	DAP (μg/ml)	μg/DAP/ mg cells
0	1.70	ND	ND
2	1.95	200	103
5	1.25	375	300
20	1.28	470	367

Effect of temperature on extracellular accumulation of DAP by resting cells

The DAP synthesis by resting cells was examined at two different temperatures (26°C and 37°C). The samples taken after 5 and 20 hr of resuspension were analysed for bacterial cell density and DAP produced. Initially DAP accumulation was higher at 37°C although the accumulation was the same at both the temperatures at a later time point (table 2).

Table 2 *Effect of different temperatures on DAP synthesis by resting cells of* E. coli *ATCC 13002*

Temperature	Cell dry weight (mg/ml)		DAP (μg/ml)		μg DAP mg cells	
(°C)	5	20	5	20	5	20 (hr)
26	0.195	0.230	65	850	33	369
37	0.200	0.225	205	870	102	386

Identification of the nature of DAP synthesized by resting cells

Thin-layer chromatography of the culture filtrate was carried out by the method of Rhuland et al. (1955). The dried plates were developed with ninhydrin solution. DAP gave an olive green colour spot which turned yellow with time. The Rf values of culture

filtrate, standard DAP (Both DD and LL-forms) and lysine are given in table 3. The results establish that both DD and LL-isomers of DAP were found in the medium.

Table 3 *Rf values of the standard samples and culture filtrates of* E. coli *ATCC 13002*

Sample	Rf	R-meso-DAP
Standard lysine	0.434	2.0
Standard DAP	0.200	
LL-DAP		1.3
DD-DAP		1.0
Culture filtrate	0.204	

Samples containing (5-10 μg amino acid) were spotted on cellulose TLC plates and chromatograms were developed with the solvent system of Rhuland et al. (1955). The dried plates were sprayed with ninhydrin solution. The Rf and R-meso-DAP values were calculated.

Effect of lysine environment to cells on DAP formation in resting cells

Lysine is known to be a feedback inhibitor of lysine and DAP synthesis by inhibiting some of the earlier enzymes of its biosynthetic pathways (Cohen 1968). Hence we decided to look at the effect of lysine present during the growth phase, on the subsequent formation of DAP by the resting cells. Cells were grown in the basal salts medium containing (*a*) L-lysine - 7.5 μg/ml (optimal concentration for DAP synthesis in growing cultures), (*b*) L-lysine - 250 μg/ml (inhibitory concentration for DAP synthesis in growing cultures). L-histidine concentration was 25μg/ml. The DAP accumulation by resting cells was determined at different time intervals. At 5 hr the DAP accumulation was lower in cells treated with higher concentration of lysine during the growth phase. But by the end of 20 hr, however, these levels tended to catch up the normal levels (table 4).

Table 4 *Effect of lysine environment on DAP synthesis in resting cells of* E. coli *ATCC 13002*

Initial growth medium contained	Cell dry weight (mg/ml)			DAP (μg/ml)		μg DAP/ mg cells	
	0	5	20	5	20	5	20 (hr)
Lysine (7.5 μg/ml)	1.60	1.50	1.40	1040	1160	693	828
Lysine (250 μg/ml)	1.45	1.55	2.00	740	1500	477	750

Effect of protein synthetic inhibitors on DAP formation by resting cells

Since the accumulations of DAP by resting cells could be due to the release of repression of the lysine biosynthetic pathway enzymes, the effect of protein synthesis inhibitors would indicate whether these enzymes are synthesized *de novo* in the absence of lysine. Both chloramphenicol and streptomycin were found inhibitory to DAP when added at the start of resuspension (table 5).

Table 5 *Effect of protein synthetic inhibitors on DAP formation by resting cells of* E. coli *ATCC 13002*

Samples	Cell dry weight (mg/ml)	DAP (μg/ml)	μg DAP/ mg cells	%inhibition of DAP synthesis
Control	1.30	990	762	0
Chloramphenicol (100 μg/ml)	0.97	112	116	84.8
Streptomycin (100 μg/ml)	1.08	330	305	60.0

The samples taken after 20 hr were analysed for cell dry weight and DAP.

These results clearly establish that new protein synthesis is essential for the formation of DAP in resting cells.

Effect of exogenous amino acids on DAP synthesis by resting cells

The effect of end product inhibitors of aspartate family amino acids, when present in the resuspension medium on DAP synthesis, were investigated. Lysine was the most

potent inhibitor, while L-methionine or L-threonine + L-isoleucine were less inhibitory (table 6).

Table 6 *Effect of exogenous amino acids on DAP formation by resting cells of* E. coli *ATCC 13002*

Additions	μg DAP/mg cells		% inhibition	
	5	20	5	20 (hr)
Control (No addition)	212	270	0	0
Lysine	47	32	77.9	88.2
Methionine	130	165	38.7	38.9
Threonine + Isoleucine	107	162	49.6	40.0

The concentration of amino acids added was 3.42×10^{-3} M

Discussion

It has been shown that when *E. coli* ATCC 13002 (lys^-, his^-) was grown in a medium containing high lysine concentration (250 μg/ml), the DAP synthesis, in growing cultures was inhibited (Abdullah et al. 1977). However, when the cells of this organism, recovered from this medium, were allowed to metabolize sucrose, without added lysine and histidine, they accumulated considerable amount of DAP in the medium. Similar observations have been reported by Gilvarg (1958) using lysine auxotroph of *E. coli*.

The results of our investigations have established that DAP was accumulated in considerable quantities and was also the principal amino acid synthesized by the resting cells of *E. coli* ATCC 13002. With the exception of a faint trace of a spot on the chromatogram in the lysine position, no other amino acid was detected. The DAP was found to be a mixture of LL and DD-isomers (table 3). This reflected the presence of diaminopimelate epimerase in the cells as also reported by Rhuland et al. (1955).

A possible explanation for the considerable yield of DAP by the resting cells, might be that when the high lysine environment (which largely inhibit DAP synthesis in growing cultures) is removed there is a considerable derepression of the pathway enzymes. The phenomenon of derepression appears to require new protein synthesis by the resting cells. The addition of streptomycin and chloramphenicol inhibited DAP synthesis in resting cells to the extent of 60% and 84% respectively (table 5). This strong inhibition on DAP accumulation reflects the requirement of *de novo* synthesis of DAP pathway enzymes in resting cells.

The synthesis of amino acids in *E. coli* derived from aspartates is governed by three different aspartokinases (Patte et al. 1967). The activity of these isoenzymes is known to be inhibited by specific amino acid end products (Stadtman et al. 1961). Lysine inhibited DAP synthesis up to 88% in resting cells, while methionine and threonine + isoleucine were less inhibitory (table 6). The effect of added amino acids on the DAP accumulation indicated a severe inhibitory effect of lysine on DAP accumulation and probably reflect a synergistic effect of lysine on both the pathway enzymes, viz., aspartokinase III and dihydropicolinate synthetase. The less effect of methionine and threonine + isoleucine indicates the effect on respective aspartokinases only. These results substantiate that the lysine auxotrophy is strong at the conversion of DAP to lysine.

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