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Polyamines and Regulation of Ornithine Biosynthesis in *Escherichia coli*

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The growth rate of several polyamine-deficient mutants of *Escherichia coli* was very low in minimal medium and increased markedly upon the addition of putrescine, spermidine, arginine, citrulline, or argininosuccinic acid. The endogenous content of polyamines was not significantly altered by the supplementation of polyamine-starved cultures with arginine or its precursors. In contrast, these compounds as well as putrescine or spermidine caused a 40-fold reduction in intracellular ornithine levels when added to polyamine-depleted bacteria. In vivo experiments with radioactive glutamic acid as a precursor and in vitro assays of the related enzymes showed that the decrease in ornithine levels was due to the inhibition of its biosynthesis rather than to an increase in its conversion to citrulline or Δ^1-pyrroline-5-carboxylic acid and proline. High endogenous concentrations of ornithine were toxic for the *E. coli* strains tested. The described results indicate that the stimulatory effect of putrescine and spermidine on the growth of certain polyamine-starved bacteria may be partially due to the control of ornithine biosynthesis by polyamines.

Many studies carried out since the isolation of polyamine-auxotrophic mutants of *Escherichia coli* have shown that these polycations play an important role in nucleic acid and protein biosyntheses as well as in the control of cell growth (18, 22). The physiological functions of polyamines have been well established in DNA replication (8, 21), RNA transcription (1), protein synthesis (2, 3), and biogenesis of ribosomal particles (6, 7, 12). Moreover, experiments performed in vivo and with cell extracts have shown that polyamines can stimulate almost every step in polypeptide synthesis and modify the accuracy of translation (2, 16).

Working with the polyamine-auxotrophic mutant *E. coli* MA255 Cunningham-Rundles and Maas (4) made the observation that arginine was able to replace partially the polyamine requirement for growth. This finding, confirmed in our laboratory with other bacterial mutants deficient in ornithine decarboxylase and agmatinase, cannot be attributed to a pathway selection involving a preferential utilization of exogenous arginine in polyamine biosynthesis and therefore has remained without explanation. More recently we have reported that when several double mutants blocked in the biosynthesis of polyamines were grown in the absence of these substances the corresponding bacterial extracts contained appreciable amounts of ornithine, which almost disappeared upon the addition of putrescine or spermidine to the culture medium (9). These preliminary results led us to investigate the possibility that polyamines, in addition to their roles in macromolecular synthesis and cell proliferation, might regulate the internal pool of ornithine.

Since it is well known that arginine causes ornithine depletion by feedback inhibition and repression (14), the stimulatory effect of arginine on the proliferation of various polyamine-deficient strains of *E. coli* could be explained if high concentrations of ornithine were inhibitory for bacterial growth. In this paper we describe experiments showing that polyamines can regulate ornithine biosynthesis in several strains of *E. coli* and that high intracellular levels of ornithine strongly inhibit the growth of these bacteria.

MATERIALS AND METHODS

Chemicals. All the amino acids as well as polyamines, citrulline, argininosuccinic acid, ninhydrin, and 5-dimethylaminonaphthalene-1-sulfonyl chloride were obtained from Sigma Chemical Co., St. Louis, Mo. The last compound was purified as described by Seiler (20). Sulfosalicylic acid was from Mallinckrodt, Inc., St. Louis, Mo. L-[U-^{14}C]ornithine (291 Ci/mol), L-[U-^{14}C]arginine (327 Ci/mol) L-[U-^{14}C]glutamic acid (262.6 Ci/mol), and [^{14}C]carbamylphosphate (12.7 Ci/mol) were purchased from New England Nuclear Corp., Boston, Mass.

Bacterial strains and culture conditions. *E. coli* BGA8 (*speB speC thr leu thi lac sup*) and the wild-type strain *E. coli* MRE600 were used in this work. The former is a polyamine-auxotrophic double mutant deficient in ornithine decarboxylase and agmatinase (10). The minimal medium of Davis and Mingioli (5) was supplemented with 0.5% glucose, thiamine (10 μg/ml), the required amino acids (100 μg/ml), putrescine (100 μg/ml), and other additions as needed in each case. Bacterial growth was monitored by measuring the A_{490} of cultures at different times. For polyamine starvation of cultures *E. coli* BGA8 grown in rich medium was used to inoculate minimal medium without polyamines. After overnight growth at 37°C with shaking these starved cells were checked for a putrescine requirement and used as the inoculum for the cultures described in each experiment.

Measurement of polyamine and amino acid contents. The analysis of polyamines in bacterial extracts was performed by thin-layer chromatography after dansylation as described previously (9). To measure the intracellular levels of amino acids, bacteria were extracted with 0.2 M $HClO_4$ at 4°C and, after neutralization with M KOH and subsequent centrifugation for 10 min at 10,000 × *g*, the supernatant fractions were applied to a 119 CL amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.). The reaction of the effluents with ninhydrin was monitored by measuring the A_{570}. In some cases the endogenous concentration of orni-

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thine was determined after paper electrophoresis of cell extracts with sulfosalicylic acid as described below.

In vivo synthesis of ornithine and polyamines. The in vivo conversion of glutamic acid or arginine into ornithine or polyamines, respectively, was investigated by incubation of aliquots from exponentially growing cultures with the corresponding radioactive precursors under the conditions needed in each case. At the end of the labeling period, bacteria were treated with $HClO_4$ and neutralized with KOH. The resulting extracts were submitted to electrophoresis on Whatman 3MM paper for 3 h at 600 V with 50 mM sulfosalicylic acid (pH 3.2). The radioactive spots were detected with a radiochromatogram scanner (Packard Instrument Co., Inc., Rockville, Md.), eluted, and measured by scintillation counting.

Enzymatic assays. Cells obtained from exponentially growing cultures after centrifugation for 5 min at 10,000 × *g* were washed with a 50 mM Tris hydrochloride buffer with different additions and pHs as needed in each case. Bacteria were resuspended in 1 ml of the same buffer solution and disrupted by sonication. The supernatant fluid collected after 10 min of centrifugation at 10,000 × *g* was used for enzymatic determinations.

Agmatinase activity was measured as described by Morris and Pardee (17); the urea released during the reaction was estimated as described by Hunninghake and Grisolia (11). One unit of agmatinase is the amount of enzyme forming 1 nmol of urea per min at 37°C. Ornithine transcarbamylase (OTC) activity was assayed by the method of Legrain and Stalon (13). Alternatively, we used [^{14}C]carbamylphosphate as a substrate and measured the stable radioactivity remaining after the addition of 0.1 N HCl and heating for 5 min at 100°C; this radioactivity corresponds to the labeled citrulline formed during the reaction. One unit of OTC activity is defined as the amount of enzyme that produces 1 μmol of citrulline per min at 37°C.

The intracellular levels of carbamylphosphate were determined with carbamate kinase from *Streptococcus faecalis* as described by Wanders et al. (23). The ATP produced was measured by coupled enzymatic reactions with hexokinase and glucose-6-phosphate dehydrogenase.

The determination of L-ornithine:2-oxo-acid aminotransferase (OAT) activity was performed by using the radioisotopic assay of Phang et al. (19) with labeled ornithine and by measuring the radioactive Δ^1-pyrroline-5-carboxylic acid formed in the reaction. One unit of OAT activity is the amount of enzyme producing 1 nmol of Δ^1-pyrroline-5-carboxylic acid per h at 37°C.

RESULTS

Growth of *E. coli* BGA8 under different conditions and endogenous concentrations of polyamines and amino acids. Several polyamine-auxotrophic strains of *E. coli* blocked in both pathways of putrescine biosynthesis grow very slowly in minimal medium and show a marked increase in growth rate upon the addition of putrescine or spermidine. We observed that by supplementation of the minimal medium with arginine, citrulline, or argininosuccinic acid it was possible to reduce considerably the doubling time of such bacterial strains, although without reaching the optimal growth rate obtained in the presence of polyamines (Table 1). The minimal arginine concentration able to reduce the mass doubling time to 90 min was 20 μg/ml of culture. These results, which confirmed the earlier observations of Cunningham-Rundles and Maas (4), could indicate that the presence of high concentrations of exogenous arginine or its precursors in the cultures induces some synthesis of polyamines. However, we concluded that this was not the case after determination of polyamine intracellular pools. Table 2 shows that supplementation of polyamine-depleted cultures with a high concentration of arginine almost did not alter the intracellular levels of putrescine or spermidine. In addition, we could rule out the possible contamination of arginine or its precursors with polyamines (data not shown).

TABLE 1. Mass doubling time of *E. coli* BGA8 grown under different conditions

Compound added to medium[a]	Mass doubling time (min)
None	240
Putrescine	75
Arginine	90
Citrulline	90
Argininosuccinic acid	100
Ornithine	270

[a] The culture medium was that described in Materials and Methods. The compounds were added at a concentration of 100 μg/ml.

E. coli BGA8 contains normal amounts of arginine decarboxylase but only a residual amount of agmatinase when grown in minimal medium; the addition of arginine up to 100 μg/ml of culture did not induce any increase in agmatinase activity in *E. coli* BGA8, whereas in the wild-type strain, MRE600, a 60% increase was observed (unpublished results). Therefore, polyamine synthesis was not enhanced in *E. coli* BGA8 when the culture medium was supplemented with arginine. This result was further demonstrated by the fact that this strain was unable to synthesize polyamines from [^{14}C]arginine in the presence of an excess of this unlabeled amino acid, while under similar conditions the formation of radioactive putrescine was clearly evident in wild-type *E. coli* MRE600 (results not shown).

The endogenous content of different amino acids in *E. coli* BGA8 grown in the absence or presence of polyamines was determined in the corresponding bacterial extracts with an amino acid analyzer. Although the intracellular concentrations of several amino acids such as glycine, alanine, and methionine were markedly modified when polyamines were added to the growth medium, ornithine levels showed the most dramatic changes (Fig. 1). The concentration of this amino acid was about 40-fold higher in polyamine-depleted bacteria than in putrescine-supplemented cells (Table 3). Intracellular ornithine levels were also low (about 0.2 nmol/mg of cells) when arginine instead of putrescine was added to polyamine-starved *E. coli* BGA8 or to the wild-type strain grown in minimal medium.

It is interesting to point out that an additional peak, not yet identified, which appeared between serine and glycine in the amino acid analyzer profiles was present only in extracts of bacteria cultivated without polyamines (Fig. 1A).

TABLE 2. Polyamine content in *E. coli* BGA8 grown in different media

Compound added to culture medium[a]	Amt (nmol/mg of cells [wet wt]) of [b]:	
	Putrescine	Spermidine
None	0.26	0.37
Putrescine	7.43	1.34
Arginine	0.27	0.28

[a] Putrescine and arginine were added at 100 μg/ml.
[b] Values are the averages of duplicate determinations.

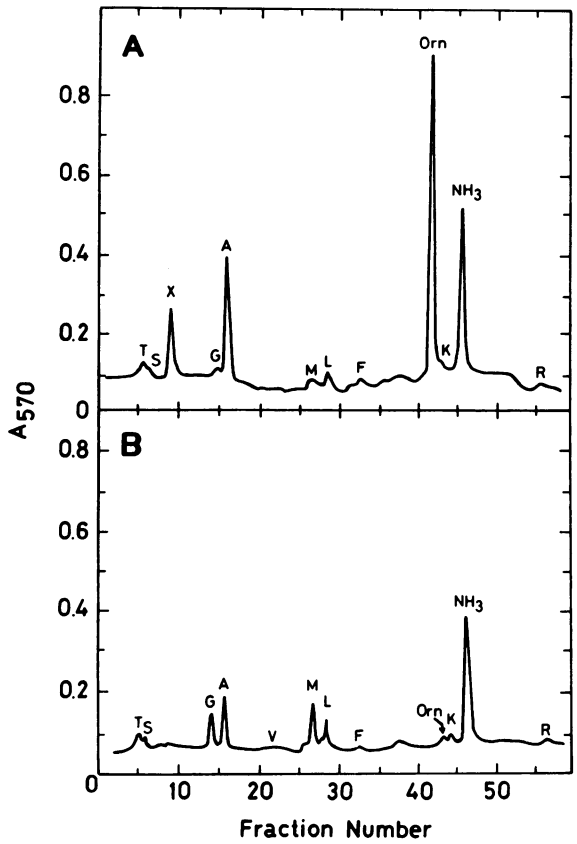

FIG. 1. Analysis of intracellular amino acids in *E. coli* BGA8 cultivated in the absence (A) or presence (B) of putrescine.

To know whether the accumulation of ornithine was the result of polyamine starvation or the low growth rate of polyamine-depleted bacteria, we cultivated *E. coli* BGA8 in a putrescine-containing medium but with acetate instead of glucose as the carbon source. Under these conditions *E. coli* BGA8 grew slowly despite the presence of the polyamine, and the endogenous content of ornithine was very low (about 0.25 nmol/mg of wet cells).

The described results show that high intracellular levels of ornithine occurring in polyamine-starved bacteria can be reduced to normal values upon supplementation of cultures with putrescine, spermidine, or arginine.

Ornithine metabolism in *E. coli* BGA8. The accumulation of ornithine in bacteria cultivated in minimal medium without polyamines might be the result of the increased biosynthesis of this amino acid or its impaired utilization. We investigated these possibilities by measuring the activities of OTC and OAT as well as the intracellular levels of carbamylphosphate and the in vivo conversion of glutamic acid to ornithine.

TABLE 3. Endogenous content of basic amino acids in *E. coli* BGA8 grown in the absence or presence of putrescine

Putrescine	Concn (nmol/mg of cells [wet wt]) of:			
	Lysine	Arginine	Ornithine	Histidine
Absent	0.12	0.17	10.5	ND[a]
Present	0.22	0.20	0.24	ND

[a] ND, <0.05 nmol/mg.

TABLE 4. Enzymatic activities of OTC and OAT and endogenous content of carbamylphosphate in *E. coli* BGA8 grown under different conditions

Compound added to medium	Activity (U/mg of protein) of[a]:		Carbamylphosphate (pmol/mg of cells [wet wt])
	OTC	OAT	
None	3.40	1.18	155
Putrescine	2.07	0.49	88
Arginine	0.32		

[a] Values are the averages of duplicate determinations.

The specific activity of OTC was about 70% higher in extracts of polyamine-starved bacteria than in preparations obtained from cells cultivated in the presence of putrescine (Table 4). In addition, OTC activity was markedly lower in arginine-supplemented bacteria. We also confirmed that citrulline was the product of the reaction. Since all these results were obtained in vitro under optimal conditions for OTC activity, it could be argued that in vivo the opposite situation might prevail if a reduced intracellular level of carbamylphosphate in polyamine-depleted bacteria were limiting for the enzyme activity. However, the endogenous concentration of carbamylphosphate was almost twofold higher in cells cultivated in the absence of polyamines (Table 4). The same ratio of OTC activities was obtained when, instead of bacterial extracts, toluene-permeabilized cells, which might represent a more physiological situation were used (data not shown). OAT specific activity was also clearly higher in extracts obtained from polyamine-starved bacteria (Table 4).

The biosynthesis of ornithine was studied by labeling polyamine-depleted and -supplemented cells with [^{14}C]glutamic acid and analyzing bacterial extracts. Figure 2 shows the radioactive products present in the soluble fractions obtained after treatment of bacteria with perchloric acid. An appreciable amount of labeled ornithine was observed only in samples corresponding to polyamine-starved cells (Fig. 2A), and the formation of ornithine was almost undetectable in bacteria grown in the presence of putrescine (Fig. 2B); in

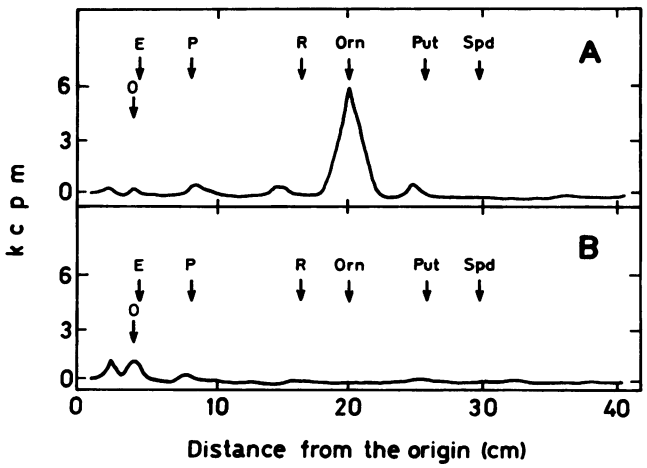

FIG. 2. In vivo conversion of labeled glutamic acid into ornithine. *E. coli* BGA8 cultures grown in the absence (A) or presence (B) of putrescine were incubated for 60 min with L-[U-^{14}C]glutamic acid (1.9 μM; 0.5 μCi/ml). Cell extracts were obtained and analyzed by paper electrophoresis as described in Materials and Methods.

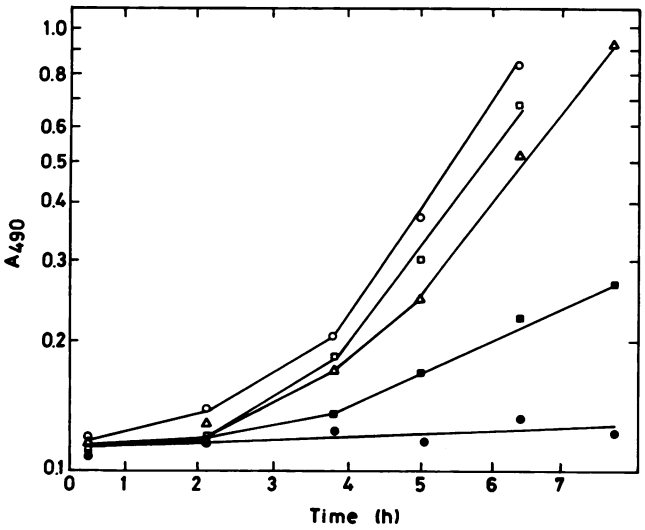

FIG. 3. Effect of ornithine levels on the growth of *E. coli* BGA8 in putrescine-containing medium supplemented with the following concentrations (milligrams per milliliter) of ornithine: 0 (○), 0.2 (□), 1.0 (△), 3.0 (■), and 5.0 (●).

the latter case the bulk of radioactive glutamic acid was incorporated into proteins, and only small amounts of this amino acid and proline were detected.

After eluting and counting the radioactive peaks we calculated that the biosynthesis of ornithine was at least 30 times higher in polyamine-depleted cells than in bacteria supplemented with putrescine or spermidine. Similar results were obtained in experiments performed in the presence of chloramphenicol to block protein synthesis.

Toxic effects of ornithine. The addition of increasing amounts of ornithine to *E. coli* BGA8 cultures in the presence of putrescine allowed us to calculate that when exogenous ornithine levels were as high as 3 mg/ml, the intracellular concentration of this amino acid reached values similar to those observed in bacteria deprived of polyamines and grown in ornithine-free medium (data not shown). The bacterial growth rate was strongly inhibited under these conditions of high endogenous levels of ornithine (Fig. 3), and this effect was observed not only in the polyamine-deficient mutant *E. coli* BGA8 cultivated either in the absence or in the presence of putrescine but also in the wild-type strain. Therefore, this inhibition of growth cannot be related to an uptake competition between ornithine and putrescine.

DISCUSSION

Previous experiments carried out in our laboratory (9) indicated that several polyamine-auxotrophic mutants of *E. coli* cultivated in the absence of these substances contained large amounts of intracellular ornithine which decreased markedly when putrescine or spermidine was added to the culture medium (Fig. 1 and Table 3). These results have now been confirmed and correlated to the slow bacterial growth in the absence of polyamines, since we were able to demonstrate that high intracellular ornithine concentrations caused retardation of growth in such strains of *E. coli* (Fig. 3).

Earlier studies by Cunningham-Rundles and Maas with other polyamine-dependent bacterial strains closely related to the one used in this work showed that arginine could also increase their growth rate in the absence of polyamines (4). This result might be explained by the reduction of intracellular ornithine levels by feedback regulation and repression caused by arginine (14) rather than by induction of putrescine biosynthesis; therefore, we have postulated that polyamines might also play a regulatory role in ornithine metabolism.

The 40-fold increase in the internal ornithine concentration observed in bacteria cultivated without polyamines could indicate that putrescine and/or spermidine enhances the utilization of ornithine or inhibits its biosynthesis. We have investigated all these possibilities by measuring the OTC and OAT activities in extracts of polyamine-depleted and -supplemented bacteria as well as the in vivo conversion of labeled glutamic acid into ornithine in cultures in the absence and presence of putrescine. The specific activities of the enzymes OTC and OAT were about twofold higher in lysates from polyamine-starved cells (Table 4). In addition, the carbamylphosphate pool was also higher in bacteria grown in the absence of polyamines. Although we cannot rule out the possibility that polyamines are needed as activators of ornithine utilization in vivo, all the presented data strongly suggest that polyamines regulate ornithine biosynthesis rather than the conversion of ornithine to citrulline or to Δ^1-pyrroline-5-carboxylic acid and proline. This assumption was confirmed, since the in vivo formation of labeled ornithine from [^{14}C]glutamic acid was clearly evident only in bacteria cultivated in the absence of putrescine (Fig. 2). The increased synthesis of ornithine under conditions of polyamine depletion might be due to derepression of the enzymes involved in the ornithine biosynthetic pathway or to stimulation of the corresponding enzymatic activities. We have so far been unable to distinguish between these possibilities, probably because of the instability of *N*-acetylglutamic acid synthase, the first enzyme in the biosynthesis of ornithine (15); its activity was undetectable in the bacterial extracts assayed.

The results presented in this paper showed that bacterial growth decreased markedly when intracellular ornithine levels were high. This situation prevailed not only in polyamine-starved *E. coli* BGA8 but also in cultures of the same strain grown in the presence of putrescine when high amounts of ornithine (3 to 5 mg/ml) were added to the medium (Fig. 3). Other basic compounds, such as arginine or agmatine, did not produce similar toxic effects at high concentrations.

Ornithine toxicity, not yet studied at the molecular level, can explain the stimulation of growth of polyamine-depleted *E. coli* BGA8 by putrescine, spermidine, arginine, citrulline, and argininosuccinic acid (Table 1), because all these substances seem to restrict ornithine biosynthesis. Polyamines were more efficient in increasing the *E. coli* BGA8 growth rate than was arginine or its precursors. These differential effects might be due to the well-known roles of polyamines in many metabolic processes other than the control of ornithine synthesis (22). This conclusion is supported by the fact that putrescine or spermidine but not arginine can stimulate the growth of another polyamine-auxotrophic mutant, *E. coli EWH319* [*pro thr thi* Δ(*speA-speB*) Δ(*speC-glc*) Δ*speD rpsL25 spc supE44*], which contains low intracellular levels of ornithine in the absence of polyamines (data not shown).

The results of our study seem to indicate that in addition to the multiple functions already described for polyamines, these compounds can regulate the biosynthesis of ornithine and therefore overcome the toxic effects of this amino acid on polyamine-auxotrophic strains of *E. coli*. Arginine, citrulline, and argininosuccinic acid can also control ornithine

formation by feedback inhibition and repression (14), and this fact can explain the ability of arginine and its precursors to partially replace putrescine or spermidine as a growth requirement in certain polyamine-deficient bacterial strains.

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