JOURNAL OF BACTERIOLOGY, Mar. 1970, p. 731-737
Vol. 101, No. 3

Isolation of Conditionally Putrescine-Deficient Mutants of *Escherichia coli*

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Received for publication 1 December 1969

Mutants defective in the conversion of arginine to putrescine were found by screening clones from mutagenized cultures for inability to produce urea during growth in arginine-supplemented media. Two partially blocked mutants were isolated; one was deficient in arginine decarboxylase and the other was deficient in agmatine ureohydrolase. As predicted from the pattern of putrescine synthesis in *Escherichia coli*, these mutants were conditionally putrescine-deficient. When grown in either minimal or ornithine-supplemented media, conditions which lead to preferential utilization of the ornithine to putrescine pathway, the mutants had normal intracellular polyamine levels. However, when the mutants were placed in arginine-supplemented media, the level of intracellular putrescine was lowered markedly. Under conditions where intracellular putrescine was 1% of normal, the doubling time of the mutants was increased approximately 10%. The putrescine-deficient mutants had wild-type morphology, normal levels of protein and ribonucleic acid (RNA), and stringent amino acid control of RNA synthesis.

The polyamines are found in nearly all organisms at relatively high intracellular concentrations (54). Spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, is commonly found in eukaryotic organisms (19, 45) but has been reported in only two bacterial species, *Pseudomonas aeruginosa* and *Bacillus stearothermophilus* (49, 60). Spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, on the other hand, is found in bacteria, fungi, and higher animals (19, 45). Putrescine, $NH_2(CH_2)_4NH_2$, is a biosynthetic precursor of both spermidine and spermine (41, 53) and thus is found in all organisms capable of synthesizing polyamines. In higher organisms, putrescine is present at relatively low intracellular concentrations (24). In bacteria, on the other hand, putrescine pools are sometimes quite large, approaching 15 μmoles per g (wet weight) in *Escherichia coli* (10).

Despite the widespread occurrence of these compounds and their high intracellular concentration, little is known of their biological function. Polyamines have been observed to interact with various cellular components. They bind electrostatically to nucleic acids (2, 5, 12, 21, 43, 52) and stabilize osmotically fragile forms of bacteria (17, 28–30, 50). Polyamines significantly lower the magnesium requirement for cell-free protein synthesis (4, 20, 31, 56), perhaps through their ability to promote the aggregation of ribosomal subunits (6, 7, 31, 33, 39, 46). Polyamines also stimulate deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase, probably through complexing the inhibitory product of the reaction (1, 13, 25, 42, 48).

To understand the biological function of the polyamines, one must relate these in vitro experiments to the in vivo situation. Approaches to this problem are complicated by two factors. First, polyamines are capable of redistribution in cell-free extracts (5, 51). This means that one must approach studies of intracellular distribution with great caution. Second, at high concentration, polyamines have toxic effects on bacteria (11, 32, 43). Therefore, in experiments in which polyamines are added to cells, one must be aware that they may be exerting pharmacological as well as physiological effects.

One way to approach the question of the in vivo action of polyamines would be to study the physiological effects of starvation of polyamine-requiring mutants. A few cases of polyamine requirements for microbial growth have been documented. However, some of these requirements have been shown to be for stabilization of osmotically fragile organisms (17, 28–30). In other instances, the requirement is too ill defined for the mutants to be used for this type of study (18, 44, 47). Therefore, we set out to isolate putrescine-deficient mutants of *E. coli*.

Because of the complexity of putrescine biosynthesis in *E. coli* (38), it was apparent that

isolation of these mutants would not be a straightforward process. We have shown that *E. coli*, growing on minimal media, possesses two pathways of putrescine biosynthesis. Both routes to putrescine arise from the arginine biosynthetic pathway (Fig. 1).

These pathways are not used equally under all growth conditions (35). Cells growing in unsupplemented minimal media preferentially use the ornithine to putrescine conversion (pathway I). However, during growth in arginine-supplemented media, de novo synthesis of ornithine from glutamate is repressed and feedback-inhibited (15, 27, 57, 59). Since *E. coli* does not contain a mechanism for converting arginine to ornithine, only the arginine to putrescine conversion (pathway II) is utilized under this condition. From these considerations, one would propose that a mutant defective in pathway II would be conditionally putrescine-deficient. Cells growing in minimal media would produce normal levels of putrescine via pathway I. However, cells growing with arginine supplementation would be putrescine-deficient. This prediction is borne out in these studies.

An additional complication which arises is that we know nothing of the physiology of putrescine-deficient strains. For example, is polyamine deficiency lethal and can exogenously supplied polyamines supply this requirement? We have tried repeatedly, without success, to isolate mutants which demonstrate an auxotrophic requirement for putrescine when grown in the presence of arginine or will grow in ornithine- but not arginine-supplemented media. We have, therefore, designed a screening procedure which presupposes nothing about the growth requirements of polyamine-deficient cells but simply tests for functioning of pathway II. We previously showed that urea production by *E. coli* arises solely from putrescine pathway II (34). Therefore, a mutant with a block in either step of this pathway would be unable to produce urea. We test for this by incubating cells with arginine-*guanidino*-^{14}C. Upon subsequent incubation of the culture medium with urease, radioactive CO_2 is produced with wild-type cells but not with mutant strains. Utilizing this screening procedure after nitrosoguanidine mutagenesis, we have isolated two mutants, one blocked in arginine decarboxylase and one in agmatine ureohydrolase.

METHODS AND MATERIALS

Bacterial strains and growth media. The wild-type strain of *E. coli* used in these studies was termed BR9a. This strain was obtained from August Doermann and was selected for use because it produces very low levels of the inducible arginine decarboxylase (14). The putrescine-deficient mutants isolated in this study were termed CJ556 and CJ866. Strain CJ556-2, a derivative of CJ556 isolated by ultraviolet irradiation and penicillin selection (16), requires isoleucine and ornithine for growth. The ornithine requirement is due to a block between glutamate and ornithine in the pathway of arginine biosynthesis.

The minimal medium used in this study was medium E of Vogel and Bonner (58) supplemented with trace elements (3) and 0.2% D-glucose. Amino acid supplementations were at a level of 50 μg ml. Medium tris(hydroxymethyl)aminomethane (Tris)-AF (arginine-free) is a Tris-buffered medium (0.1 M Tris-hydrochloride, *p*H 7.0; 9×10^{-4} M K_2HPO_4; 0.08 M NaCl; 0.02 M KCl; 0.02 M NH_4Cl; 3×10^{-3} M Na_2SO_4; 10^{-3} M $MgCl_2$) enriched as described by Novick and Maas (40) and supplemented with trace elements (3) and 0.2% D-glucose. A concentration of 1.5% Difco agar was added to prepare a solid medium. The liquid cultures were grown with vigorous shaking at 37 C. Growth was monitored spectrophotometrically, and the measurements were converted to cell number by using a factor derived from plate counts.

Mutagenesis and mutant screening. For mutagenesis, a single colony isolate of BR9a was grown overnight in Difco Penassay Broth. A sample of this overnight culture was diluted into fresh medium at a density of 10^8 cells/ml. After the cell number approximately doubled, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (250 μg/ml, freshly prepared) was added to a final concentration of 25 μg/ml. The culture was shaken at 37 C for 30 min. This mutagenesis procedure produced a frequency of mutants incapable of fermenting lactose of approximately 0.3% as judged by plating on Difco EMB-lactose medium. The cells were harvested by centrifugation, washed once in 0.1 M potassium phosphate (pH 7.0), and diluted for plating. Samples corresponding to approximately 200 colonies per plate were spread on Tris-AF media supplemented with 50 μg of ornithine per ml and 150 μg of putres-

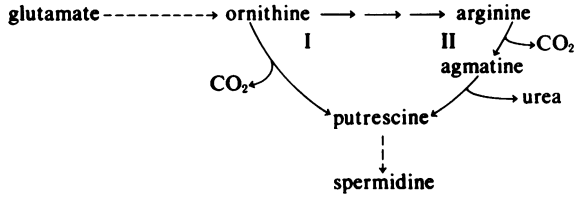

Fig. 1. Two pathways of putrescine biosynthesis in *E. coli*.

cine per ml. The plates were incubated overnight at 37 C.

To screen for mutants blocked in putrescine biosynthetic pathway II, colonies were picked from the above plates and grown overnight in 2.5 ml of Tris-AF supplemented with ornithine and putrescine as above. Samples of these overnight cultures (0.025 ml) were added to 2.5 ml of fresh medium supplemented with 26 μg of arginine-*guanidino*-^{14}C (6.6×10^4 counts per min per μmole) and grown to a density of approximately 5×10^8 cells/ml. The cultures were then centrifuged at 12,000 $\times$ *g* for 5 min, and 1.0-ml samples of supernatant fluid were removed. Urease was then added to these samples at a final concentration of 50 μg/ml. The solution was incubated at 37 C for 1 hr. Trichloroacetic acid was then added to a final concentration of 10%. The radioactive CO_2 was trapped (37), and the content of ^{14}C was measured at an efficiency of 85% in a Beckman CPM 100 scintillation counter.

Enzyme assays. Ornithine decarboxylase, arginine decarboxylase, and agmatine ureohydrolase were assayed as previously described (38). The published agmatine ureohydrolase assay was altered slightly through the use of the urea determination described by Hunninghake and Grisolia (23). Enzyme-specific activity was based on protein concentration determined by the method of Lowry et al. (26), by using bovine serum albumin as a standard. A unit of enzyme activity was defined as that amount which catalyzes the formation of 1 nmole of product per min under the standard assay conditions.

Measurement of polyamine levels. Cultures, with or without arginine supplementation as required, were grown overnight from an initial inoculum of 10^7 cells/ml. For analysis of polyamines, 200-ml cultures were inoculated at a density of approximately 7×10^7 cells/ml from the appropriate overnight culture and were grown to a final concentration of 7×10^8 cells/ml. Cultures were harvested by centrifugation at room temperature, and the cells were washed once in 0.1 M potassium phosphate, *p*H 7.0. Extraction and analysis of polyamines were performed as previously described (36). Polyamine levels were normalized on the basis of cell protein by using the protein determination of Lowry et al. (26) with bovine serum albumin as a standard (1 mg of cellular protein is equivalent to 3.6×10^9 cells).

Celluar protein and RNA content. Samples of growing cultures were precipitated by the addition of trichloroacetic acid to a final concentration of 5%. The precipitates were collected by centrifugation, and the supernatant fluid was discarded. The precipitates were dissolved in 0.5 N NaOH, and protein was determined by the method of Lowry et al. (26). For RNA analysis, 0.5 N perchloric acid was added to the precipitates, and the mixtures were heated at 70 C for 25 min with periodic mixing. After cooling in ice, the precipitates were removed by centrifugation. The supernatant solutions were analyzed for RNA by the orcinol method (8).

Materials. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Aldrich Chemical Co., Milwaukee, Wis., and stored at -20 C in a dark bottle. Urease (2$\times$ crystallized) was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Urease (1 mg/ml) was dissolved in 0.1 M potassium phosphate (*p*H 7.0) containing 10^{-3} M ethylenediaminetetraacetic acid. Arginine-*guanidino*-^{14}C was supplied by New England Nuclear Corp., Boston, Mass.

RESULTS

Isolation of mutants defective in putrescine biosynthesis. As described above, a mutant blocked in either step of putrescine biosynthetic pathway II will not be able to produce urea. With this as a criterion, we screened for putrescine-deficient mutants. Since the existence of an inducible arginine decarboxylase in *E. coli* (14) might complicate the screening procedure, we started with a strain lacking this enzyme (BR9a). After mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, cultures were plated on enriched medium supplemented with ornithine and putrescine. These conditions should allow growth of mutants blocked in pathway II. Clones were then grown in the presence of arginine-*guanidino*-^{14}C. Urea in the culture medium was detected by the appearance of $^{14}CO_2$ after incubation with urease. From 873 single-colony isolates examined with this screening procedure, two mutants deficient in urea production were found. In a typical screening assay, the parental strain produced 705 counts/min of $^{14}CO_2$, and the mutants CJ556 and CJ866 yielded 9 and 0 counts/min, respectively.

From the considerations outlined above, one would predict that a mutant blocked in putrescine biosynthetic pathway II would be conditionally putrescine-deficient. When grown in unsupplemented minimal media, the mutant strain should have the capability of producing putrescine via pathway I. However, in arginine-supplemented media, the mutant must use the defective putrescine pathway II because of feedback inhibition and repression of the arginine biosynthetic pathway. The growth properties of the two mutants were compared with the wild-type strain. In neither case was an absolute putrescine requirement noted. This is illustrated for one of the mutants, CJ556, in Fig. 2. This mutant grew in unsupplemented minimal medium with a doubling time of 48 min, identical to the parental strain under these conditions. When the mutant was supplemented with arginine, growth was exponential and the doubling time was lengthened to 54 min. On the other hand, arginine shortened the doubling time of the parent to 44 min. These experiments have been repeated many times, and the growth of this mutant strain has always slowed by 10% under conditions predicted to create a putrescine deficiency. Use of

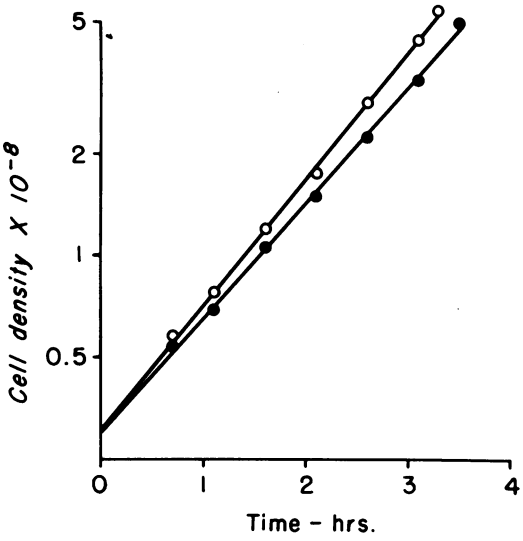

FIG. 2. *Growth of E. coli strain CJ556. Cultures grown overnight on minimal medium, with (●) and without (○) arginine supplementation, were inoculated into fresh medium and their growth was monitored as a function of time. The units of the ordinate are 10^8 cells/ml, an estimate from turbidometric measurement.*

strain CJ556-2, which has a mutation before ornithine in the pathway of arginine biosynthesis, did not accentuate this effect.

Enzyme levels. The levels of the enzymes of putrescine biosynthesis in the mutants were compared with the parental strain. The results of these experiments are summarized in Table 1. As predicted, both mutants showed an enzymatic defect in putrescine biosynthetic pathway II. One mutant, CJ556, showed diminished arginine decarboxylase activity. The other, CJ866, demonstrated a lower specific activity of agmatine ureohydrolase.

It should be pointed out that the levels of the biosynthetic decarboxylases were significantly increased in both mutant strains when grown in arginine-containing medium. Therefore, there seems to be a derepression of these enzymes under putrescine-deficient conditions. This is consistent with recent results of Tabor and Tabor, which showed a derepression of ornithine decarboxylase during putrescine deficiency induced in an arginine-limited chemostat (55). This result is interesting, since no repression of the enzymes of putrescine biosynthesis has been noted in wild-type strains growing in the presence of putrescine or spermidine (D. R. Morris, *unpublished data*).

Polyamine levels. The intracellular levels of putrescine, spermidine, and agmatine were compared in BR9a, CJ866, and CJ556-2, a derivative of CJ556 requiring isoleucine and ornithine for growth. The expected results were obtained (Table 2). Arginine supplementation had no significant effect on the polyamine levels in the parent strain (BR9a). Strain CJ556-2 had wild-type polyamine levels when grown with ornithine supplementation. When this strain was supplemented with arginine, the putrescine level was lowered by approximately 100-fold and there was a slight, but reproducible, increase in spermidine. In CJ866 grown in unsupplemented minimal medium, putrescine levels were normal, and spermidine was approximately 50% of the wild-type level. Under these conditions, there was an appreciable accumulation of agmatine. This is consistent with the defective agmatine ureohydrolase in this strain. When CJ866 was supplemented with arginine, putrescine was lowered fivefold, spermidine increased slightly, and agmatine increased fourfold. These results are consistent with the data on enzyme levels presented above.

Properties of putrescine-deficient cells. Putrescine-deficient strain CJ556-2 was chosen for

TABLE 1. *Enzyme levels in mutants*[a]

Strain	Growth condition	Ornithine decarboxylase	Arginine decarboxylase	Agmatine ureohydrolase
BR9a	−arg[b]	24	16	23
BR9a	+arg	33	17	25
CJ556	−arg	33	1	18
CJ556	+arg	119	4	16
CJ866	−arg	33	21	5
CJ866	+arg	44	20	3

[a] Expressed as enzyme units per milligram of protein.
[b] Without arginine, −arg; with arginine, +arg.

TABLE 2. *Polyamine levels in mutants*

Strain	Growth Condition	Polyamine levels[a]		
		Putrescine	Spermidine	Agmatine
BR9a	−arg[b]	22.1	3.8	<0.3
BR9a	+arg	26.4	4.0	<0.3
CJ556-2	+orn	25.6	3.7	<0.3
CJ556-2	+arg	<0.3	5.5	<0.3
CJ866	−arg	24.8	1.7	4.8
CJ866	+arg	5.0	2.2	20.2

[a] Expressed as nanomoles per 10^9 cells.
[b] Without arginine, −arg; with arginine, +arg; with ornithine, +orn.

further study, since the results would not be complicated by the agmatine accumulation seen with CJ866. As with CJ556, the doubling time of CJ556-2 was increased approximately 10% when the cells were growing under putrescine-deficient conditions (i.e., arginine-supplemented medium). When viewed under the phase-contrast microscope, no alterations in morphology were seen in the putrescine-deficient state. Likewise, the protein and RNA contents of this strain were unaltered when it was putrescine-deficient. The amino acid control of RNA synthesis was assessed by measuring the rate of ^{14}C-uracil incorporation during either isoleucine starvation or phenylalanine starvation induced by the presence of β-2-thienylalanine (200 μg/ml). The strain showed identical stringent control under putrescine-deficient or putrescine-sufficient conditions. From these results, we conclude that lowering the intracellular putrescine concentration to approximately 1% of normal creates no serious physiological aberrations in *E. coli*.

DISCUSSION

Mutants of *E. coli* which do not produce urea are defective in putrescine biosynthetic pathway II. Two of these mutants, each with partial blocks, have been found. One of these (CJ556) has a defective arginine decarboxylase, and the other (CJ866) is altered in its agmatine ureohydrolase. With regard to putrescine levels, these mutants behave as predicted. When grown in unsupplemented or ornithine-supplemented media, they possess normal putrescine levels. In arginine-supplemented media, the cells are putrescine-deficient. These results are consistent with independent experiments, indicating that *E. coli* grown in unsupplemented minimal medium preferentially uses the ornithine to putrescine conversion and that in arginine-supplemented medium the arginine to putrescine conversion is used exclusively (35).

Even though the putrescine concentration drops approximately 100-fold in CJ556-2 grown in arginine-containing medium, the spermidine content of the cells increases approximately 50%. This observation has two ramifications. First, it appears that the very high intracellular putrescine concentrations observed in wild-type cells are greatly in excess of that needed to saturate the propylamine transferase responsible for the conversion of putrescine to spermidine (53). Second, since the rate of spermidine synthesis is in fact stimulated, this reaction sequence may be regulated by the total organic cation concentration in the cell. One might predict, therefore, that one of the steps in spermidine biosynthesis, either that catalyzed by *S*-adenosylmethionine decarboxylase or that catalyzed by propylamine transferase, would be inhibited by excess putrescine. We have not tested this proposal.

The results reported in this paper indicate that 99% of the intracellular putrescine in *E. coli* can be eliminated, with no serious physiological effects on the cells. Putrescine-deficient strain CJ556-2, when placed in arginine-containing medium, shows a 10% longer doubling time, normal morphology, normal protein and RNA content, and wild-type regulation of RNA synthesis. Therefore, it would appear that either a great proportion of the intracellular putrescine is superfluous, or that its function may be replaced by some other cation, perhaps magnesium. We have preliminary results which show that, indeed, putrescine-deficient cells have a higher intracellular magnesium content.

The following question then arises: does *E. coli* specifically require polyamines for growth? The mutants described in this paper do not answer this question, since they are only partially defective in putrescine biosynthesis. In an accompanying paper, Hirshfield and co-workers describe a mutant which has a tight block in agmatine ureohydrolase (22). This mutant has an absolute requirement for putrescine. Unfortunately, as pointed out by these authors, the interpretation of this finding is complicated by the accumulation of agmatine in this type of mutant. However, if growth inhibition in their mutant is due to putrescine deficiency, our studies indicate that the intracellular level absolutely required for growth is far less than that found in wild-type cells. It is possible that the required component may not be putrescine itself but a derivative such as spermidine, the glutathione-spermidine conjugate described by Dubin (9), or some other unknown compound.

ACKNOWLEDGMENTS

We thank Werner K. Maas and his co-workers for discussions of the properties of their mutant before publication.

This investigation was supported by Public Health Service grant GM-13957 from the National Institute of General Medical Sciences and by the University of Washington Graduate School Research Fund (Initiative 171 funds) and the University of Washington American Cancer Society Institutional Grant.

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