A probable new pathway for the biosynthesis of putrescine in *Escherichia coli**

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Some cultures of *Escherichia coli* BGA8, a mutant unable to synthesize putrescine, showed a change of behaviour and could grow almost equally well in either the absence or the presence of polyamines after repeated periods of polyamine starvation. Experiments *in vivo* with radioactive precursors showed that the bacteria which evaded the polyamine requirement had recovered their ability to synthesize putrescine from glucose or glutamic acid, but not from ornithine or arginine. These results are in agreement with the fact that the polyamine-independent cells were still deficient in the enzymes ornithine decarboxylase and agmatinase. Our findings seem to indicate the existence of a new pathway to synthesize putrescine which does not involve ornithine or arginine as intermediates.

INTRODUCTION

It has been known for many years that putrescine can be formed in *Escherichia coli* by two pathways: the first involves decarboxylation of ornithine, and the second decarboxylation of arginine to produce agmatine, followed by the hydrolytic conversion of this compound into putrescine and urea (Morris & Pardee, 1966; Tabor & Tabor, 1984). Formation of putrescine from agmatine via *N*-carbamoylputrescine has also been reported in *Streptococcus faecalis* (Roon & Barker, 1972) and in *Pseudomonas aeruginosa* (Haas *et al.*, 1984).

Several studies on the decarboxylation of ornithine and arginine have indicated that each of these reactions can be catalysed by two different enzymes: one is a constitutive 'biosynthetic' decarboxylase present in cells grown in neutral minimal medium, and the other is a 'biodegradative' enzyme induced in certain strains cultivated in acid media containing high concentrations of the respective undecarboxylated substrate, ornithine or arginine (Gale, 1946; Morris & Fillingame, 1974; Applebaum *et al.*, 1975, 1977).

The genes corresponding to all the enzymes involved in the biosynthesis of putrescine in *E. coli* have been characterized, and point and deletion mutants for most of these genes have been described (Morris & Jorstad, 1970; Maas, 1972; Hafner *et al.*, 1979). The resulting strains defective in putrescine biosynthesis have been extensively used to investigate the physiological role of polyamines in bacteria.

Working with the polyamine-auxotrophic mutant *E. coli* BGA 8, deficient in ornithine decarboxylase and agmatinase (Goldemberg & Algranati, 1981), we have observed that after a long period of polyamine starvation some cultures were able to 'by-pass' this requirement and showed almost optimal growth rate in media without the addition of polyamines.

The present paper reports our experiments showing that bacteria that escaped exogenous polyamine depriva-

tion contained almost wild-type concentrations of intracellular polyamines, although these cells were still blocked in the two known routes of putrescine biosynthesis. These results strongly suggest the existence of a new pathway leading to putrescine in some strains of *E. coli*.

EXPERIMENTAL

Materials

The bacterial strains used throughout this work are *Escherichia coli* MRE 600 (wild-type strain), MA17 (*thr*⁻, *leu*⁻, *ser*⁻, *his*⁻, *thi*⁻) and BGA8 (*speB*, *speC*, *thr*⁻, *leu*⁻, *thi*⁻). The last is a polyamine-auxotrophic strain deficient in agmatinase (*Spe B*; agmatine amidinohydrolase, EC 3.5.3.11) and in the constitutive ornithine decarboxylase (*Spe C*; L-ornithine carboxylase, EC 4.1.1.17), and therefore it requires polyamines for optimal growth (Goldemberg & Algranati, 1981). *E. coli* MA17 is the polyamine-independent strain from which BGA8 was derived.

The minimal growth medium used (Davis & Mingioli, 1950) was supplemented with 0.5% glucose, thiamin (10 µg/ml), the corresponding required amino acids (100 µg/ml) and, where indicated, putrescine (100 µg/ml).

L-[U-¹⁴C]Ornithine (266 Ci/mol), L-[U-¹⁴C]arginine (406.5 Ci/mol), L-[U-¹⁴C]glutamic acid (262.6 Ci/mol), D-[U-¹⁴C]glucose (258.7 Ci/mol) and L-[1-¹⁴C]ornithine (55.9 Ci/mol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Chloramphenicol was purchased from Sigma and dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride from Aldrich. This reagent was purified as described by Seiler (1983).

Methods

Procedures for polyamine depletion have been previously described (Goldemberg & Algranati, 1977).

* Dedicated to Professor Luis F. Leloir on the occasion of his 80th birthday.

For polyamine analysis of bacterial extracts, cells were harvested and washed at room temperature with buffer A (Tris/HCl, pH 7.8, 0.05 M; NH_4Cl , 0.06 M; magnesium acetate, 5 mM; mercaptoethanol, 6 mM). Bacterial pellets were then extracted with 0.2 M- HClO_4 at 4 °C and, after centrifugation at 10000 g for 10 min, the supernatant fluids were dansylated as described by Dion & Herbst (1970). The resulting dansyl derivatives as well as those of standard polyamine solutions were extracted with toluene and analysed by t.l.c. as described previously (Goldemberg & Algranati, 1977).

Cell extracts used for enzyme assays were prepared from 100 ml cultures grown in supplemented minimal medium without polyamines (except for inducible ornithine decarboxylase determinations). Cells were harvested at mid-exponential phase, collected by centrifugation for 5 min at 10000 g and washed once with buffer A. Bacterial pellets were resuspended in 1 ml of 0.05 M-Tris/HCl buffer, pH 8.2, containing 1 mM-dithiothreitol and 2.4 mM-EDTA, and cells were disrupted by sonication. After centrifugation at 10000 g for 10 min, the supernatant was used to measure enzyme activities.

Ornithine decarboxylase was determined as described by Applebaum *et al.* (1977), with a 0.5 ml reaction mixture containing 0.05 M-Tris/HCl buffer, pH 8.2, 5 mM-L-[1- ^{14}C]ornithine (0.1 $\mu\text{Ci/ml}$), 1 mM-dithiothreitol and 2.4 mM-EDTA.

For assay of inducible ornithine decarboxylase, cell extracts were obtained from 100 ml cultures grown in the medium described by Applebaum *et al.* (1975). Bacteria were collected, washed and then resuspended in 1 ml of 0.05 M-Mes buffer, pH 6.3, containing 1 mM-dithiothreitol and 2.4 mM-EDTA. After sonication and centrifugation at 10000 g for 10 min, the supernatant was used for measurement of the enzyme activity as described above, but with Mes buffer, pH 6.3, in the reaction mixture.

Arginine decarboxylase was determined as described by Hirshfield *et al.* (1970) in a mixture (0.5 ml) containing 4.75 mM-L-[U- ^{14}C]arginine (0.6 $\mu\text{Ci/mol}$).

A unit of decarboxylase activity is defined as the quantity of enzyme that releases 1 nmol of CO_2/h of incubation at 37 °C.

Agmatinase activity was assayed by the method of Morris & Pardee (1966). The urea formed during the reaction was measured as described by Hunninghake & Grisolia (1966). A unit of agmatinase activity is the amount of enzyme that produces 1 nmol of urea/min.

In order to measure biosynthesis of polyamines *in vivo*, 10 ml samples from exponentially growing cultures were incubated with one of the following radioactive precursors under the conditions indicated in each case: L-[U- ^{14}C]ornithine (0.1 $\mu\text{Ci/ml}$), L-[U- ^{14}C]arginine (0.1 $\mu\text{Ci/ml}$), L-[U- ^{14}C]glutamic acid (0.5 $\mu\text{Ci/ml}$) and D-[U- ^{14}C]glucose (1 $\mu\text{Ci/ml}$). When [^{14}C]glucose was used, the concentration of this sugar in the growth medium was lowered to 0.1%. At the end of the incubation period, bacteria were treated with HClO_4 and the resulting extract was dansylated as described above. The labelled dansyl derivatives were separated by t.l.c. (Goldemberg & Algranati, 1977), and the radioactivity of each spot was detected by using a radiochromatogram scanner and then measured by scintillation counting after scraping them off the plates.

To measure the uptake of ornithine and arginine, bacteria were harvested at the mid-exponential phase, washed with minimal growth medium and resuspended

in the same medium containing 200 μg of chloramphenicol/ml. After 30 min at 37 °C, samples of the cell suspension (3×10^6 bacteria) were incubated at 37 °C in the presence of labelled amino acid (0.15 μM , 0.05 μCi) and, at the end of the indicated incubation periods, bacteria were collected on Millipore filters (0.45 μm pore size) and washed at room temperature with 5 ml of minimal medium containing 100 μg of unlabelled ornithine or arginine/ml. The filters were dried and counted for radioactivity in a toluene-based scintillation mixture.

RESULTS

The strain *E. coli* BGA8 is unable to synthesize putrescine and its growth is strongly dependent on addition of putrescine (Fig. 1a) or spermidine to the medium. However, after repeated periods of polyamine starvation, most of the cultures of this strain escape the requirement of polyamines and can grow almost equally well (though with different lag periods), either in the presence or in the absence of these substances (Fig. 1b). For each polyamine starvation period, a fully grown culture of *E. coli* BGA8 was diluted 100–1000-fold in fresh medium without polyamines and allowed to grow overnight. After three to six successive similar treatments, most of the cultures became independent of exogenous polyamines. In view of the unusual physiological behaviour of these cultures, which were designated as *E. coli* BGA8 BP (for 'by-pass'), we have investigated whether these bacteria were able to synthesize polyamines or whether they could replace them in some way.

The intracellular polyamine pools of *E. coli* BGA8 BP as well as those of BGA8 were measured by quantitative t.l.c. Extracts of the polyamine-independent strains MA17 (the parental strain of BGA8) or MRE 600 were also analysed for comparison. Fig. 2 and Table 1 show that, whereas *E. coli* BGA8 contained only traces of putrescine and about one-third of the normal spermidine concentration, the endogenous concentrations of both polyamines in *E. coli* BGA8 BP were similar to those in wild-type strains. The spot running a little ahead of dansyl-spermidine and detected only in the extracts of BGA8 cells (Fig. 2) was previously identified as the dansyl

Fig. 1. Growth of *E. coli* BGA8 (a) and BGA8 BP (b) in polyamine-depleted and supplemented media

Cultures in the absence (●) and presence (○) of putrescine were incubated at 37 °C and their A_{490} was measured at the indicated times.

Fig. 2. Endogenous polyamine contents of *E. coli* BGA8, BGA8 BP and MRE 600

Thin-layer chromatogram of dansylated extracts obtained from bacteria grown in the absence of putrescine. Blanks of reagents (R) and dansyl derivatives of pure polyamines (St) were also analysed. The spot of dansyl-ammonia ('NH₃') is present in all samples. Chloroform/triethylamine (10:1, v/v) was used as developing solvent. Key: P, putrescine; C, cadaverine; S, spermidine. Different volumes of each sample corresponding to a constant number of bacteria were used. The picture was taken under u.v. light.

Table 1. Intracellular polyamine pools in *E. coli* BGA8, BGA8 BP, MRE 600 and MA 17

Bacteria were cultivated in the absence of putrescine, and extracts were dansylated and analysed as described in Fig. 2 and in the Experimental section. All values, which represent averages of at least two determinations, were normalized for 1 ml of culture at $A_{490} = 1$.

Bacterium	Polyamine content (nmol/ml per A_{490} of culture)	
	Putrescine	Spermidine
<i>E. coli</i> BGA8	0.38	0.55
<i>E. coli</i> BGA8 BP	7.47	1.85
<i>E. coli</i> MRE 600	10.08	1.67
<i>E. coli</i> MA 17	6.00	1.22

derivative of γ -aminobutyraldehyde (Goldemberg & Algranati, 1977) formed from ornithine during the dansylation reaction (Seiler, 1971). The spots at the front of thin-layer chromatograms were products of the

reagents, and their different amounts correspond to the size of sample used in each case. The bands near the origin were not yet identified and did not disappear when cell extracts were hydrolysed before dansylation. The identification of putrescine and spermidine in *E. coli* BGA8 BP extracts has been confirmed by using two different developing solvents in the t.l.c. analyses (Goldemberg & Algranati, 1977). Although the presence of other polyamines, such as 2-hydroxyputrescine, norspermidine or homospermidine, which could substitute for normal polyamines, has not yet been ruled out, the results shown in Fig. 2 and Table 1 strongly suggest that *E. coli* BGA8 BP has recovered the ability to synthesize putrescine, either by genetic reversion or by induction of a biosynthetic pathway so far unknown. In order to elucidate these possibilities, we have measured the enzymic activities of ornithine decarboxylase, arginine decarboxylase and agmatinase in the auxotrophic and 'by-pass' bacteria. Data in Table 2 indicate that both *E. coli* BGA8 and 'by-pass' cells contained normal amounts of arginine decarboxylase and very low amounts of constitutive ornithine decarboxylase and agmatinase. Therefore the polyamine-independent growth of *E. coli* BGA8 BP cannot be explained by a reversion in any of the corresponding genes.

Since the behaviour of 'by-pass' cells could also be due to the appearance of an inducible form of ornithine decarboxylase, we have measured this activity, using *Enterobacter aerogenes* as a positive control, because it has been shown that this micro-organism can easily induce the biodegradative enzyme under certain conditions (Wright & Boyle, 1984). The results obtained showed that amounts of inducible ornithine decarboxylase in *E. coli* BGA8 BP were even lower than in strain BGA8 (Table 3).

In order to confirm that 'by-pass' bacteria were unable to synthesize putrescine from either ornithine or arginine, we have performed experiments *in vivo* adding one of these radioactive precursors to growing cultures of *E. coli* BGA8, BGA8 BP and MRE 600. After 1 h incubation, cell extracts were analysed by dansylation and subsequent t.l.c. The radioactive dansyl derivatives detected by scanning of the plates were eluted and counted for radioactivity. Table 4 showed that the conversion of labelled ornithine or arginine into polyamines in *E. coli* BGA8 and BGA8 BP was less than 5% of that occurring in the wild-type strain. These results also rule out a possible conversion via *N*-carbamoylputrescine. The radioactive peak remaining at the origin of the thin-layer chromatograms was identified as the dansyl derivative of agmatine. It could be argued that the very small conversion of radioactive ornithine or arginine into polyamines detected in 'by-pass' cells was an artifact caused by a markedly decreased uptake of these amino acids. This was not the case, since the amounts of ornithine and arginine taken up by *E. coli* BGA8 BP after 5 min incubation were 50–100% of those found in wild-type MRE 600 cells (Figs. 3a and 3b).

On the other hand, the intracellular pools of ornithine and arginine were very similar in *E. coli* BGA8 BP and MRE 600 (results not shown). This fact ruled out the possibility of different dilution of the added radioactive precursors by endogenous pools, which might have explained the results shown in Table 4.

Since we have found that *E. coli* BGA8 BP indeed contained almost normal concentrations of endogenous

Table 2. Ornithine decarboxylase, arginine decarboxylase and agmatinase activities in *E. coli* BGA8, BGA8 BP, MRE 600 and MA 17

Enzyme activities were assayed as described in the Experimental section. All data represent average values from assays carried out in duplicate. Results are also presented as percentages of those in *E. coli* MRE 600.

Bacterium	Enzyme specific activity					
	Constitutive ornithine decarboxylase		Constitutive arginine decarboxylase		Agmatinase	
	(units/mg of protein)	(%)	(units/mg of protein)	(%)	(units/mg of protein)	(%)
<i>E. coli</i> MRE 600	414.0	100	728	100	15.10	100
<i>E. coli</i> BGA8	0.38	0.09	666	91.5	0.33	2.1
<i>E. coli</i> BGA8 BP	1.10	0.26	666	91.5	0.62	4.0
<i>E. coli</i> MA17	288.0	69	473	65	3.20	22.0

Table 3. Inducible ornithine decarboxylase activity in *E. coli* BGA8, BGA8 BP and *Enterobacter aerogenes*

Enzyme activity was assayed at pH 6.3 as detailed in the Experimental section. Average values for duplicate experiments are given. Results are also presented as percentages of that in *E. aerogenes*.

Bacterium	Inducible ornithine decarboxylase	
	(units/mg of protein)	(%)
<i>E. aerogenes</i>	1046	100
<i>E. coli</i> BGA8	11.8	1.1
<i>E. coli</i> BGA8 BP	3.4	0.3

polyamines, even though these bacteria cannot form putrescine from ornithine or arginine, we have investigated the polyamine synthesis *in vivo* from other likely radioactive precursors, such as glucose or glutamic acid. The polyamine-deficient strain BGA8 and the wild type *E. coli* MRE600 were also used for comparison in these experiments.

Figs. 4 and 5 show that 'by-pass' bacteria were able to synthesize polyamines from either glucose or glutamic acid. The conversion of this amino acid into putrescine and spermidine in *E. coli* BGA8 BP was almost as efficient as in the wild-type strain MRE 600 (Fig. 5).

Fig. 3. Uptake of ornithine and arginine into *E. coli* MRE 600 (○) and BGA8 BP (●)

Bacteria were incubated with labelled ornithine (a) or arginine (b), and samples were taken at the indicated times. Details are described in the Experimental section. All values were normalized for 1 ml of culture at $A_{490} = 1$.

Since it could be expected that the addition of an unlabelled metabolite involved in a particular pathway would dilute the intermediate pool, decreasing the specific radioactivity of the final product, we followed the synthesis of putrescine from radioactive glucose in the absence and presence of an excess of unlabelled ornithine. Data in Table 5 demonstrate that radioactive putrescine almost disappeared when unlabelled ornithine was added

Table 4. Conversion of L-[U- 14 C]ornithine or L-[U- 14 C]arginine into polyamines in *E. coli* BGA8, BGA8 BP and MRE 600

Details are described in the Experimental section. Radioactive peaks corresponding to those in thin-layer chromatograms were detected, eluted and counted for radioactivity. Values were normalized for 1 ml of culture at $A_{490} = 1$ and expressed as indicated in Table 2.

Radioactive precursor	Bacterium	Putrescine		Spermidine	
		(c.p.m./ml per A_{490})	(%)	(c.p.m./ml per A_{490})	(%)
Ornithine	<i>E. coli</i> MRE 600	11 155	100	2220	100
	<i>E. coli</i> BGA8	130	1.1	117	5.2
	<i>E. coli</i> BGA8 BP	387	3.5	80	3.6
Arginine	<i>E. coli</i> MRE 600	3860	100	1660	100
	<i>E. coli</i> BGA8	48	1.2	22	1.3
	<i>E. coli</i> BGA8 BP	58	1.5	12	0.7

Fig. 4. T.l.c. of dansylated extracts from cultures of *E. coli* BGA8 (a), BGA8 BP (b) and MRE 600 (c) incubated with labelled glucose

E. coli BGA8 (a), BGA8 BP (b) and MRE 600 (c) were cultivated without polyamines. After addition of [^{14}C]glucose and incubation for 2 h cell extracts were treated and analysed by t.l.c. with chloroform/triethylamine (10:1, v/v) as described in the Experimental section. The corresponding scans are shown. The origin (O) and positions of putrescine (P) and spermidine (S) are indicated by arrows.

to *E. coli* MRE 600 incubated with [^{14}C]glucose. In contrast, the same experiment carried out with *E. coli* BGA8 BP showed only a 50% decrease of putrescine radioactivity. A similar study with added unlabelled arginine has not been carried out, because this amino acid causes the repression of the first enzyme involved in the metabolic conversion of glutamate (Morris & Pardee, 1966) in the wild-type strain and presumably also in *E. coli* BGA8 BP.

DISCUSSION

The biosynthetic pathways so far described leading to putrescine in *Escherichia coli* involve either direct decarboxylation of ornithine or a two-step reaction which includes the conversion of arginine into agmatine and the subsequent hydrolysis of this compound with a concomitant release of urea (Morris & Pardee, 1966). These two metabolic routes have been confirmed in many studies (Tabor & Tabor, 1984), and both of them have to be blocked in order to obtain polyamine-auxotrophic bacteria (Hirshfield *et al.*, 1970; Morris & Jorstad, 1970).

Fig. 5. T.l.c. of dansylated extracts from cultures of *E. coli* BGA8 (a), BGA8 BP (b) and MRE 600 (c) incubated with radioactive glutamic acid

Details are described in the Experimental section and in Fig. 4.

Table 5. Conversion of D-[U- ^{14}C]glucose into radioactive putrescine in the absence and presence of unlabelled ornithine

Procedures are described in the Experimental Section, Table 4 and in the text. Bacterial cultures were incubated for 2 h with radioactive glucose in medium without or with ornithine (100 $\mu\text{g}/\text{ml}$). Radioactive peaks corresponding to putrescine in the thin-layer chromatograms were eluted and counted.

Bacterium	Ornithine addition	Putrescine	
		(c.p.m./ml per A_{490})	(%)
<i>E. coli</i> BGA8	—	22	100
	+	41	186
<i>E. coli</i> BGA8 BP	—	300	100
	+	147	49
<i>E. coli</i> MRE 600	—	610	100
	+	2	0.3

We have been working for several years with some of these double mutants requiring exogenous polyamines for optimal growth, and we have repeatedly observed that some polyamine-depleted cultures after long periods of starvation were able to escape the requirement and could grow normally in the absence of polyamines.

In this paper we present evidence that the 'by-pass' cells had recovered their ability to synthesize putrescine, presumably through a pathway different from those already known. It is noteworthy that in some of our experiments the recovered capacity to grow in the absence of polyamines was due to the fact that bacteria were true revertants in constitutive ornithine decarboxylase or agmatinase genes. On the other hand, the appearance of 'by-pass' organisms in polyamine-deprived cultures of *E. coli* HT411 (which contains deletions in the genes *speA*, *speB* and *speC*) have also been reported (Tabor & Tabor, 1982, 1983). These 'by-pass' cells, which have an additional mutation, do not contain polyamines, but are still able to grow without the addition of these substances.

The 'by-pass' bacteria obtained in our laboratory from cultures of *E. coli* BGA8 contain endogenous concentrations of putrescine and spermidine similar to those found in wild-type strains (Fig. 2 and Table 1). However, we have been able to demonstrate that the recovered capacity to synthesize polyamines was not due to any genetic reversion, since *E. coli* BGA8 BP was still deficient in ornithine decarboxylase and agmatinase (Table 2). The possible induction of a biodegradative ornithine decarboxylase was also ruled out (Table 3).

Experiments performed *in vivo* with radioactive glucose or glutamic acid have indicated that these compounds were precursors of polyamines in *E. coli* BGA8 BP (Figs. 4 and 5) and that ornithine was not an intermediate in this conversion, since the addition of unlabelled ornithine did not cause a marked decrease of the polyamine specific radioactivity, as is the case in wild-type strains (Table 5).

The inability of 'by-pass' cells to synthesize putrescine from ornithine or arginine was also confirmed *in vivo* through the use of these uniformly labelled substrates (Table 4). These results cannot be attributed to a marked decrease in the uptake of ornithine and arginine (Fig. 3), or to a large expansion of their intracellular pools which could have diluted the radioactive precursors.

Although the bacteria described in this paper which are able to evade the polyamine requirement did not arise by genetic reversion, they could have been originated by the induction of a new metabolic pathway or by the selection of cells with a different polyamine-synthetic pathway or carrying an additional mutation which allows the biosynthesis of putrescine. In order to investigate these possibilities, we have done some preliminary experiments to find out whether the 'by-pass' phenotype is stable. After incubation of *E. coli* BGA8 BP in the presence of putrescine, followed by a new period of polyamine starvation, 'by-pass' cells did not lose their capacity to synthesize putrescine, and they continued to behave as autotrophic for polyamines.

We have also explored the effect of different culture conditions such as temperature, aeration and carbon sources on the appearance of the 'by-pass' phenotype.

The results have indicated that it is possible to decrease markedly the lag period in the growth of *E. coli* BGA8 BP by using a temperature of 42 °C, low aeration and mannose instead of glucose as carbon source (A. A. Cataldi & I. D. Algranati, unpublished work).

All the results described in the present paper support the conclusion that *E. coli* BGA8 BP has evolved a mechanism to escape the polyamine requirement, which is not related to any reversion of the mutations in the genes corresponding to ornithine decarboxylase and agmatinase. The recovered capacity of 'by-pass' cells to synthesize putrescine seems to implicate a new pathway not involving ornithine or arginine as metabolic intermediates.

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