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Biochemistry

Biosynthesis of polyamines in ornithine decarboxylase, arginine decarboxylase, and agmatine ureohydrolase deletion mutants of *Escherichia coli* strain K-12

(ribosomes/ribosomal proteins/streptomycin resistance/putrescine/spermidine)

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ABSTRACT ***Escherichia coli* K-12 mutants that carry deletions in their genes for ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) (*speC*), arginine decarboxylase (L-arginine carboxy-lyase, EC 4.1.1.19) (*speA*), and agmatine ureohydrolase (agmatinase or agmatine amidinohydrolase, EC 3.5.3.11) (*speB*) can still synthesize very small amounts of putrescine and spermidine. The putrescine concentration in these mutants was found to be 1/2500th that in *spe*$^+$ cells. The pathway of putrescine synthesis appears to be through the biodegradative arginine decarboxylase, which converts arginine to agmatine, in combination with a low agmatine ureohydrolase activity—1/2000th that in *spe*$^+$ strains. These results suggest that even such low levels of polyamines permit a low level of protein synthesis. Evidence is presented that the polyamine requirement for the growth of the polyamine-dependent *speAB,speC* deletion mutants, which are also streptomycin resistant, is not due to a decreased ability to synthesize polyamines.**

Polyamines are among the few metabolites whose function remains to be elucidated. *Escherichia coli* lacks spermine but contains high concentrations of putrescine and spermidine. Spermidine is derived from putrescine, whereas putrescine is derived directly from the decarboxylation of ornithine by ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) and is derived indirectly from arginine. In the latter case, arginine is decarboxylated by arginine decarboxylase (L-arginine carboxy-lyase, EC 4.1.1.19) to agmatine, which is then hydrolyzed to putrescine by agmatine ureohydrolase (agmatinase or agmatine amidinohydrolase, EC 3.5.3.11) (1) (see Fig. 1). *E. coli* generally has two ornithine and arginine decarboxylases: the biosynthetic decarboxylases, which have high pH optima, and the biodegradative decarboxylases, which have low pH optima (2); however, in some *E. coli* strains, the biodegradative ornithine decarboxylase is absent. It has been suggested that the function of the biodegradative decarboxylases is to raise the pH when cells are grown under acidic conditions (3). There is no evidence for the existence of a biodegradative agmatine ureohydrolase.

Our studies on the regulation of polyamine biosynthesis in *E. coli* K-12 have shown that antizyme, an acidic protein of *E. coli*, is a noncompetitive inhibitor of the biosynthetic, but not the biodegradative, ornithine and arginine decarboxylases (4). Extension of these studies showed that the two basic ribosomal proteins S20/L26 and L34 share this same property (5). These latter studies suggested a possible connection between protein synthesis and the regulation of polyamine biosynthesis.

A significant contribution to our understanding of polyamine metabolism has been made recently through the construction of *E. coli* strains carrying deletion mutations in the genes encoding the biosynthetic arginine decarboxylase (*speA*), the biosynthetic ornithine decarboxylase (*speC*), agmatine ureohydrolase (*speB*), and *S*-adenosylmethionine decarboxylase (*speD*) (6). These deletion mutants have relatively low growth rates in minimal medium but do not require polyamines for growth (6). They were found to become polyamine dependent when they were made streptomycin resistant by the introduction of a particular *rpsL* (*strA*) mutation (7). However, these streptomycin-resistant polyamine-dependent strains give rise to phenotypic revertants that are polyamine independent (8). Polyamines generally have been considered to be essential for growth and have been associated with protein synthesis. The existence of such polyamine-independent deletion mutants raised the possibility that polyamines may not be essential for the growth of *E. coli* and may not be critical in protein synthesis. Therefore, we have examined whether these various *spe* deletion mutants are in fact unable to synthesize polyamines.

To evaluate the potential of putrescine biosynthesis in such mutants, we have used triple mutants—i.e., *speAB,speC* deletion mutants. The advantage of using these strains is that the presence of a functional *S*-adenosylmethionine decarboxylase, the product of the *speD* gene, permits the conversion of putrescine to spermidine and thereby provides additional confirmation for any observed synthesis of putrescine.

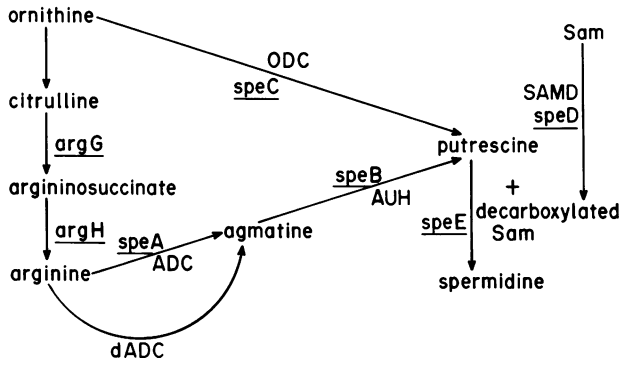

FIG. 1. The pathway for polyamine biosynthesis in *E. coli* K-12. ODC (*speC*), ornithine decarboxylase; ADC (*speA*), arginine decarboxylase; dADC, biodegradative arginine decarboxylase; AUH (*speB*), agmatine ureohydrolase; *argG*, argininosuccinate synthetase; *argH*, argininosuccinate lyase; SAMD (*speD*), *S*-adenosylmethionine decarboxylase; Sam, *S*-adenosylmethionine; *speE*, spermidine synthetase.

MATERIALS AND METHODS

Materials. All the chemicals used were of the highest purity available; deionized, redistilled water was used for the preparation of growth media and buffers. L-[1-^{14}C]Ornithine

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(58 mCi/mmol; 1 Ci = 37 GBq) and L-[1-^{14}C]arginine (56 mCi/mmol) were purchased from Moravek Biochemicals (City of Industry, CA); L-[U-^{14}C]arginine (288 mCi/mmol), from ICN; L-[2,3-^{3}H]ornithine (20 Ci/mmol), from New England Nuclear; and L-[2-^{3}H]methionine (10 Ci/mmol), [1,4-^{14}C]putrescine dihydrochloride (107 mCi/mmol), and [^{14}C]spermidine trihydrochloride (105 mCi/mmol), from Amersham. We confirmed the purity of the radioactive amino acids by HPLC, and we purified the [^{14}C]putrescine used in the isotope dilution experiment by preparative HPLC.

Bacterial Strains. The *E. coli* K-12 strains used in these studies are described in Table 1. Strain construction was carried out as indicated in Table 1 and as described (12, 13).

Cell Growth. Cells were grown at 37°C with vigorous shaking in minimal medium 56 (14) supplemented with glucose at 0.4%, thiamine at 1 μg/ml, and threonine at 100 μg/ml. Where required, 3 μg of spermidine or 25 μg of arginine were added per ml.

When cells were grown in the presence of radioactive precursors, they were usually labeled by transferring 3 ml of a stationary-phase culture into 30 ml of medium 56 containing either 30 μCi of L-[2,3-^{3}H]ornithine or 9 μCi of L-[U-^{14}C]arginine or 30 μCi of L-[2-^{3}H]methionine and incubated usually for 6 hr at 37°C with shaking, at which time they reached close to the end of their logarithmic phase of growth.

At the end of the incubation period, a small sample from each labeling experiment was put aside to check for possible contamination. The cells were harvested at ambient temperature either by filtration through Millipore 0.25-μm filters or by centrifugation at 8000 × *g* for 10 min and were washed twice with saline.

HPLC. Separation of polyamines was achieved by using the ion-pair partition HPLC system of Seiler and Knodgen (15). Polyamines were detected fluorometrically after separation by reaction with *o*-phthalic dicarboxaldehyde (16).

Extraction, Identification, and Quantitation of Polyamines and Amino Acids by HPLC. The polyamines were extracted from the cell pellets with 10% ice-cold CCl_3COOH overnight. After centrifugation at 15,000 × *g*, the CCl_3COOH was extracted from the supernatant fluid with ether, and the aqueous phase was then mixed with an equal volume of the primary HPLC buffer.

Polyamines and protein-bound arginine were also determined in another portion of each cell suspension after hydrolysis for 12 hr at 110°C with 6 M HCl. The HCl was evaporated at 55°C *in vacuo*, and the dry sample was dissolved in primary HPLC buffer. Putrescine and spermidine were identified by comparing their HPLC elution profiles with the elution profiles of reference radioactive and/or nonradioactive putrescine and spermidine. Arginine and agmatine also were identified on the HPLC elution profiles after comparison with reference radioactive arginine and with enzymatically decarboxylated radioactive arginine, respectively (see below). The concentration of the polyamines was determined from standard curves prepared from the values obtained by HPLC of polyamine solutions of defined concentrations.

Quantitation of Putrescine by Isotope Dilution. Because of the low amount of putrescine in the *speAB,speC* deletion mutants, the putrescine concentration was determined by isotope dilution. KL515 was grown in 500 ml of minimal medium 56 supplemented with 0.4% glucose and 1 μg of thiamine per ml at 37°C with vigorous shaking until the absorption reached A_{600} = 0.7. The cells were harvested by centrifugation at 10,000 × *g* for 10 min at ambient temperature and were washed twice with saline. To the cell pellet was added 56,000 cpm (0.236 nmol) of [1,4-^{14}C]putrescine, and the pellet was extracted with CCl_3COOH (see above). The final aqueous phase from which the CCl_3COOH and ether had been removed was mixed with primary HPLC buffer and subjected to preparative ion-pair partition HPLC.

The central portion of the HPLC fraction containing the radioactive putrescine was isolated and desalted by adsorption to a Dowex 50W-X8 column (8 × 1 cm), which was washed sequentially with water and 2 M HCl and from which the putrescine was finally eluted with 6 M HCl. This radioactive putrescine was collected, the HCl was removed *in vacuo* at 55°C, and the dry sample was dissolved in primary HPLC buffer. The putrescine was quantitated as described above after refractionation by HPLC.

Quantitation of RNA. RNA was isolated from cells grown to A_{600} = 0.7 by the method of Zengel *et al.* (17), except that no carrier RNA was added to the lysis buffer and the DNA was eliminated by incubation with DNase (RNase free). The RNA was determined spectrophotometrically.

Purification of Arginine Decarboxylase and Preparation of Agmatine. The biosynthetic arginine decarboxylase that was used for the preparation of radioactive agmatine was purified 2200-fold from *E. coli* MA255 (ornithine decarboxylase- and agmatine ureohydrolase-negative) by the method of Wu and Morris (18). L-[U-^{14}C]Agmatine was prepared by incubating L-[U-^{14}C]arginine with the purified biosynthetic arginine decarboxylase in 25 mM Tris·HCl, pH 8.4/5 mM $MgCl_2$/50 μM pyridoxal phosphate/1 mM dithiothreitol. The incubation mixture was deproteinized at 90°C for 15 min. After centrifugation at 20,000 × *g* for 20 min and analysis by HPLC, it was found that >90% of the radioactive arginine had been converted to radioactive agmatine.

Table 1. *E. coli* K-12 strains used in the present investigation

Strain	Genotype*	Comments and source
HT414	F$^-$ *thr-1 thi-1 Δ(speA-speB)97 Δ(speC-glc)63 hsd$^-$ rpsL9 supE44 lacY1*	Polyamine dependent (Spe$^-$); Tabor *et al.* (7)
KL515	As HT414 but *spa-1*	Spontaneous polyamine-independent (Spe$^+$) derivative of HT414 (S.B. and K.B.L., unpublished data)
KL522	As KL515 but *argG6 zgi-203::Tn10*	PlCMclr100·SK2262 (*argG6 zgi-203::Tn10*) (from S. Kushner via B. Bachmann†) × KL515 → Tet^R; this work
KL523	As KL515 but *argH::Tn5*	PlCMclr100·CBK230 (*argH::Tn5*) (from C. Berg) × KL515 → Kan^R; this work
KL527	F$^-$ *gyrA$^-$ Δ(speA-speB)97 Δ(speC-glc)63* λ$^-$	MG1655 $\xrightarrow{\text{spont}}$ Thy$^-$ $\xrightarrow{\text{spont}}$ Nal^R (*gyrA$^-$*) × EWH331 (6) → Thy$^+$ Nal^R Spe$^+$; this work
MA255	F$^-$ *thr-1 thi-1 leuB6 speB2 speC3 lacY1 gal-6 rpsL133 xyl-7 mtl-2 tonA2? supE44?* λ$^-$	Cunningham-Rundles and Maas (9) via B. Bachmann†
MG1655	F$^-$ λ$^-$	Guyer *et al.* (10)

Tet^R, tetracycline resistant; Kan^R, kanamycin resistant; Nal^R, nalidixic acid resistant; spont, spontaneous mutation; Thy$^+$, thymidine independent.

*Genotypic symbols are as in ref. 11 except for *spa*, which is the locus corresponding to the mutation to Spe$^+$ in KL515.

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Enzyme Assays. Cells were grown until they reached A_{600} = 1.0. The cultures were rapidly chilled by pouring over crushed ice, and cells were collected by centrifugation at 10,000 × *g* for 15 min. The cell pellets were suspended in the appropriate buffers and disrupted by sonication. Enzyme activities were assayed in the supernatant fluid obtained at 10,000 × *g*. We developed a very sensitive and specific assay for the determination of agmatine ureohydrolase activity. In this method, rather than rely on the indirect determination of the $^{14}CO_2$ evolved from uniformly labeled agmatine (through the action of urease), we isolated and identified the radioactive products by HPLC. In addition to the higher radioactivity available in the uniformly labeled putrescine and in the spermidine, this method provided a direct confirmation of the synthesis of putrescine and spermidine. Various volumes of extracts of strains MG1655 or KL515 were incubated in a final volume of 55 μl of 10 or 100 μM L-[U-^{14}C]agmatine (56 mCi/mmol)/50 mM Hepes (pH 7.4)/5 mM $MgCl_2$/50 μM pyridoxal phosphate/1 mM dithiothreitol. Incubation was performed for 1 hr at 37°C, and the reaction was stopped by the addition of 100 μl of ice-cold 10% CCl_3COOH. The mixture was chilled in ice for 30 min and centrifuged at 15,000 × *g* for 10 min; the supernatant fluid was fractionated by HPLC as described above. Biodegradative arginine and ornithine decarboxylases as well as biosynthetic arginine decarboxylase were assayed by the method of Morris and Boeker (2). The biosynthetic ornithine decarboxylase was assayed as described (19).

RESULTS

Conversion of Radioactive Ornithine, Arginine, and Methionine to Putrescine and/or Spermidine in the *speAB,speC* Deletion Mutant KL515 (Spe$^+$). To determine whether or not KL515 can synthesize any polyamines, cells were grown in the presence of L-[2,3-^{3}H]ornithine, L-[U-^{14}C]arginine, or L-[2(N)-^{3}H]methionine, and the extracts were analyzed by HPLC. Cells grown in the presence of radioactive ornithine or arginine gave rise to three radioactive peaks corresponding to putrescine, agmatine, and spermidine, whereas cells grown in the presence of radioactive methionine gave rise to one radioactive peak, corresponding to spermidine (Fig. 2). The elution patterns remained unchanged after hydrolysis of the cell extracts with 6 M HCl.

The Phenotypic Reversion to Polyamine Independence in Strain KL515 Is Not Due to Increased Polyamine Biosynthesis. We next asked whether or not the spontaneous reversion of the polyamine-requiring strain (HT414) to polyamine independence (KL515) is associated with a change in the polyamine-synthesizing capacity.

Strain KL515, its immediate parent HT414, and the independently derived *speAB,speC* deletion mutant KL527 (Spe$^+$), were grown in the presence of [^{3}H]ornithine for 6 hr. The percentage of [^{3}H]ornithine converted to polyamines and the putrescine/spermidine ratios in the three mutant strains were similar (Table 2). These results are directly comparable and express rates of conversion of ornithine to polyamines. When strain MG1655 was labeled under the same conditions, it converted a much higher proportion of the [^{3}H]ornithine to polyamines; therefore, a significantly smaller proportion of the [^{3}H]ornithine was converted to arginine (Table 2).

The Pathway of Polyamine Biosynthesis in the *speAB,speC* Deletion Mutants. We could not detect any biosynthetic ornithine or arginine decarboxylase activity in extracts of various *speAB,speC* deletion mutants nor any biodegradative ornithine decarboxylase activity, even when such mutants were grown under conditions that induce this enzyme (20). On the other hand, extracts of KL515 were found to convert agmatine to putrescine (Table 3), indicating that they have agmatine ureohydrolase, although the activity of this enzyme

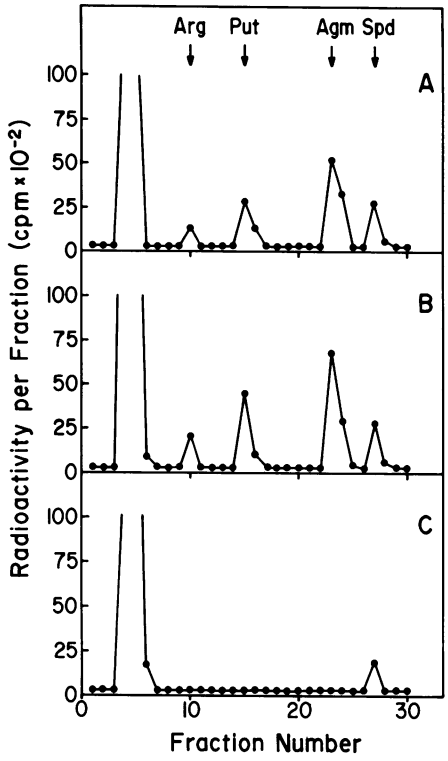

FIG. 2. Conversion of [^{3}H]ornithine, [^{14}C]arginine, and [^{3}H]-methionine to polyamines by strain KL515, which was grown in the presence of L-[2,3-^{3}H]ornithine (*A*), L-[U-^{14}C]arginine (*B*), or L-[2-^{3}H]methionine (*C*) as described, and the CCl_3COOH extracts of the cell pellets were analyzed by HPLC. One ml (1 min) fractions were collected, and the radioactivity was measured by liquid scintillation spectroscopy. The initial large peak is composed of neutral and acidic metabolites. Put, putrescine; Agm, agmatine; Spd, spermidine.

is 1/2000th that present in MG1655. Table 3 also indicates that both strain MG1655 and the *speAB,speC* deletion mutant KL515 express equivalently low levels of biodegradative arginine decarboxylase activity when they are grown in minimal medium 56 at neutral pH.

Therefore, it would appear that in the *speAB,speC* deletion mutants, putrescine can be synthesized from arginine by a two-step mechanism, through the decarboxylation of arginine to agmatine by the biodegradative arginine decarboxylase followed by the conversion of agmatine to putrescine by agmatine ureohydrolase (Fig. 1).

To acquire additional evidence that polyamines are synthesized in these mutants through the biodegradative arginine decarboxylase, the biosynthesis of arginine was prevented by introducing an *argG* point mutation or an *argH* insertion mutation into the *speAB,speC* deletion mutant KL515. The

Table 2. Conversion of tracer [^{3}H]ornithine to putrescine (Put), spermidine (Spd), and arginine by *E. coli* mutants compared with MG1655

Strain	% of total intracellular radioactivity			
	Put	Spd	Arg	Put/Spd
MG1655	8.5	3.7	78	2.3
KL527	0.52	0.38	95	1.4
HT414	0.54	0.45	91	1.2
KL515	0.48	0.4	95	1.2

The Spe$^-$ HT414 strain was grown in the presence of [^{3}H]ornithine and 3 μg of spermidine per ml. The effect of the added spermidine on the conversion of [^{3}H]ornithine to polyamines was determined by growing KL515 in the presence of [^{3}H]ornithine with and without 3 μg of spermidine per ml. The ratio of these values was used to normalize the value obtained for HT414.

Table 3. Biodegradative ornithine and arginine decarboxylase and agmatine ureohydrolase activities in strains MG1655 and KL515

	Activity, nmol of product per mg of protein per hr		
		Biodegradative decarboxylase	
Strain	Agmatine ureohydrolase	Arginine	Ornithine
MG1655	20	48	0
KL515	0.009	77	0

Cell extracts were prepared and assayed for the relevant enzyme activities as described. No activity could be detected when these extracts were assayed for the biosynthetic ornithine and arginine decarboxylases.

resulting derivatives, designated KL522 and KL523, respectively, were arginine dependent.

When these three strains were grown in the presence of [^{3}H]ornithine, the conversion of radioactive ornithine to arginine was found to be inhibited 85% in KL522, whereas it was inhibited 97% in KL523 (Table 4). The differential inhibition of arginine biosynthesis from ornithine is reflected in a correspondingly differential inhibition of polyamine biosynthesis from radioactive ornithine in these mutants. Furthermore, Table 4 shows that, when these mutants were grown in the presence of radioactive arginine, they all yielded the same amounts of polyamines, indicating that the extent of conversion of arginine to polyamines in the three strains is equivalent (see Fig. 1). Consequently, the biosynthesis of polyamines from ornithine in these mutants is dependent upon the existence of a functional pathway for arginine biosynthesis from ornithine.

The Putrescine Content of Strain KL515. After the quantitation of putrescine by isotope dilution (see *Materials and Methods*), its specific activity was found to average 6.1 mCi/mmol, compared to 107 mCi/mmol for the carrier-free [^{14}C]putrescine that had been added to the *E. coli* extract. The decrease of the specific activity by a factor of 17.54 indicates the presence of 4.14 nmol of putrescine in the total sample. Correction for the 0.236 nmol of carrier-free radioactive putrescine added yields 3.9 nmol of putrescine in 2.8×10^{11} KL515 cells compared to 10,000 nmol in an equivalent number of cells of strain MG1655, which we determined independently by HPLC. These results indicate that the putrescine content of the *speAB,speC* deletion strain KL515 is approximately 1/2500th that of MG1655. This value corresponds to ≈8000–8400 putrescine molecules per KL515 cell or 16,000 molecules of putrescine plus spermidine per cell (see Table 2).

The RNA Content of Strain KL515. To relate our findings to other cell constants, we have collated some interrelated information. When KL515 was grown in the absence of polyamines, its total RNA content was found to be 0.84×10^{-13} g of RNA per cell as compared to 1.0×10^{-13} g of RNA per cell for MG1655; under these conditions, *speAB,speC* deletion mutants grow at about one-third the normal growth rate (4) (or that of MG1655 as we have determined). The total RNA content of KL515 increases to 1.0×10^{-13} g of RNA per cell when it is grown in the presence of polyamines, where its growth rate is similar to that of MG1655. It is generally accepted (21, 22) that an *E. coli* cell contains 15,000–20,000 ribosomes and ≈160,000–180,000 tRNA molecules. For the *speAB,speC* deletion mutant KL515, these values should be adjusted downwards by 16%. Some possible correlations among these values are presented in the *Discussion*.

Table 4. The effect of mutations in the arginine biosynthetic pathway on the conversion of ornithine and arginine to putrescine (Put) and spermidine (Spd)

	% of total intracellular radioactivity					
	Precursor [^{3}H]ornithine			Precursor [^{14}C]arginine		
Strain	Put	Spd	Arg	Put	Spd	Arg
KL515	0.4	0.3	78	0.2	0.2	90
KL522	0.15	0.1	12	0.2	0.2	91
KL523	<0.01	<0.01	2	0.3	0.25	93

Cells were grown in the presence of either 30 μCi of L-[2,3-^{3}H]ornithine or 9 μCi of L-[U-^{14}C]arginine as described except that the growth media were supplemented with 25 μg of nonradioactive arginine per ml.

DISCUSSION

We have provided evidence that although the *speAB,speC* deletion mutants of *E. coli* strain K-12 were designated as being polyamine-free (6), they actually contain low levels of polyamines. The evidence includes (*i*) the conversion of uniformly labeled ornithine and arginine to radioactive putrescine and spermidine, (*ii*) the conversion of [^{3}H]methionine to radioactive spermidine but not to putrescine, (*iii*) the identification of the enzymes required for polyamine synthesis and quantitation of their activities, (*iv*) the decrease in polyamine synthesis from ornithine in proportion to the inhibition of arginine biosynthesis, and (*v*) the quantitation of the total putrescine content of such cells. It is also apparent that the Spe$^-$ HT414 strain has the same ability to synthesize polyamines as that of the spontaneous Spe$^+$ KL515 derivative and utilizes this potential *in vivo* (Table 2).

The ability of the *speAB,speC* deletion mutants to convert agmatine to putrescine indicates either that the deletion introduced into the *speB* locus does not cause total loss of agmatine ureohydrolase activity or that there exists a second, much less active, agmatine ureohydrolase. The low level of agmatine ureohydrolase activity in the *speAB,speC* deletion mutants (1/2000th that of MG1655) compares favorably with the low concentration of intracellular polyamines (1/2500th that of MG1655). Supportive evidence was acquired through the use of *argG* and *argH* mutants. The decrease in the levels of polyamine synthesis from ornithine in these mutants was found to approximate the extent to which the biosynthesis of arginine had been inhibited. One potential alternative approach to further clarifying this issue, that of deleting the biodegradative arginine decarboxylase and studying its effects on polyamine production, was not used because the gene for this enzyme has not been mapped. These results indicate that mutants that lack biosynthetic arginine decarboxylase can synthesize agmatine through their biodegradative arginine decarboxylase; the agmatine can then be converted to putrescine.

Our results indicate that the number of polyamine molecules per KL515 cell, grown in the absence of polyamines, approximates the number of ribosomes but is much lower than the number of tRNA molecules and much higher than the number of mRNA molecules (21, 22). If, for example, a physiological function of polyamines is the consequence of a minimum 1:1 relationship between polyamines and RNA, two types of associations for the polyamines become plausible: the ribosomes and the mRNA.

The *speAB,speC* deletion mutants that we examined have the same low potential for polyamine synthesis, regardless of whether they are streptomycin sensitive or resistant and/or polyamine dependent. Resistance to streptomycin is associated with well-defined mutations in the *rpsL* gene, specifically in two short regions of the corresponding ribosomal protein S12—namely, in position 42 and in amino acids

located between positions 85 and 91 (23). It is possible that the site of a particular mutation in the ribosomal protein S12 may help to define the level of polyamine dependence for protein synthesis in that particular mutant. Such an explanation would be consistent with the fact that *speAB;speC* deletion mutants carrying different *rpsL* mutations have varying degrees of polyamine requirements (7).

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