Difluoromethylornithine irreversibly inactivates ornithine decarboxylase of *Pseudomonas aeruginosa*, but does not inhibit the enzymes of *Escherichia coli*

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(Received 7 April 1981/Accepted 19 June 1981)

DL- α -Difluoromethylornithine, an enzyme-activated irreversible inhibitor of eukaryotic ornithine decarboxylase and consequently of putrescine biosynthesis, inhibited ornithine decarboxylase in enzyme extracts from *Pseudomonas aeruginosa* in a time-dependent manner with $t_{1/2}$ 1 min, and also effectively blocked the enzyme activity *in situ* in the cell. Difluoromethylornithine, however, had no effect on the activity of ornithine decarboxylase assayed in enzyme extracts from either *Escherichia coli* or *Klebsiella pneumoniae*. However, the presence of the inhibitor in cell cultures did partially lower ornithine decarboxylase activity intracellularly in *E. coli*. Any decrease in the intracellular ornithine decarboxylase activity observed in *E. coli* and *Pseudomonas* was accompanied by a concomitant increase in arginine decarboxylase activity, arguing for a co-ordinated control of putrescine biosynthesis in these cells.

Putrescine is synthesized in *Pseudomonas aeruginosa* and in *Escherichia coli* via two biosynthetic pathways. Ornithine is decarboxylated by ornithine decarboxylase to yield putrescine directly, and arginine can be decarboxylated by arginine decarboxylase to form agmatine, which is then converted into putrescine (Morris & Fillingame, 1974; Tabor & Tabor, 1976; Rahman *et al.*, 1980; Mercenier *et al.*, 1980; Kallio *et al.*, 1981; Pegg & Williams-Ashman, 1981). *E. coli* possesses two distinct forms of both arginine decarboxylase and ornithine decarboxylase. One form acts in a biodegradative fashion, the other in a biosynthetic manner (Morris & Fillingame, 1974; Kallio *et al.*, 1981). All these decarboxylases have been characterized and are pyridoxal phosphate-dependent enzymes (Morris & Fillingame, 1974; Pegg & Williams-Ashman, 1981).

The two forms of arginine decarboxylase of *Escherichia coli* have been shown to be susceptible to inhibition by DL- α -difluoromethylarginine (compound R.M.I. 71897), a specific enzyme-activated irreversible inhibitor, both on the isolated enzymes and in culture (Kallio *et al.*, 1981). The putrescine-biosynthetic pathway of eukaryotic cells has been successfully inhibited *in vitro* (Metcalf *et al.*, 1978), *in situ* in cell cultures (Mamont *et al.*, 1978a,b; Hölttä *et al.*, 1979; Luk *et al.*, 1981), in infective protozoa *in vivo* (Bacchi *et al.*, 1980; McCann *et al.*, 1981) and in animal tissues *in vivo* (Seiler *et al.*, 1978; Danzin *et al.*, 1979; Prakash *et al.*,

1980; Fozard *et al.*, 1980; Luk *et al.*, 1980) by inhibition of ornithine decarboxylase activity with DL- α -difluoromethylornithine (compound R.M.I. 71782). This compound is a specific enzyme-activated irreversible inhibitor, and appears to be one of the most potent inhibitors of putrescine formation in eukaryotic cells.

In the studies reported in the present paper DL- α -difluoromethylornithine was used to inhibit ornithine decarboxylase activity in bacteria. We find that DL- α -difluoromethylornithine is a potent irreversible inhibitor of ornithine decarboxylase of *Pseudomonas aeruginosa* both *in vitro* and *in vivo*, but has no effect *in vitro* on the enzyme from *E. coli* or *Klebsiella pneumoniae*. However, DL- α -difluoromethylornithine added to the growth medium of *E. coli* cells prevents the normal induction of *E. coli* ornithine decarboxylase activity seen when the cells enter the exponential phase of growth.

Experimental

Chemicals

DL-[1- 14 C]Ornithine (58 mCi/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). L-[U- 14 C]Ornithine (274 mCi/mmol) and [1,4- 14 C]putrescine were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). DL-[1- 14 C]Arginine (20 mCi/mmol) was purchased from C.E.A. Research Products International (Mt.

Prospect, IL, U.S.A.). GTP (trisodium salt) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest grade commercially available and were used without further purification. DL- α -difluoromethylornithine (compound R.M.I. 71782) and DL- α -methylornithine (compound R.M.I. 71633) were synthesized at the Centre de Recherche, Merrell International, Strasbourg, France (Metcalf *et al.*, 1978).

Preparation of enzyme extracts

Wild-type strains of *E. coli* (M.R.C. 59), *E. coli* (M.R.C. 62), *Ps. aeruginosa* (A.T.C.C. 9027) and *K. pneumoniae* (A.T.C.C. 8047) were grown in minimal media (Davis & Mingioli, 1950) at 37°C with vigorous aeration. Cells were harvested by centrifugation and washed once with phosphate-buffered saline, pH 7.2 (0.3 M-NaCl, 10 mM-Na₂HPO₄ and 3 mM-KH₂PO₄). Cells were resuspended in 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithiothreitol and 0.1 mM-EDTA and disrupted in a Branson Cell Disrupter 350 set at power setting 2–3.5 for 2 min. All sonications were done in a salt/ice bath. The cell debris was removed by centrifugation at 20000g for 30 min. The supernatant solution was used for measuring the activities of the biosynthetic ornithine decarboxylase and arginine decarboxylase. Partially purified biodegradative ornithine decarboxylase from *E. coli* was purchased from Sigma Chemical Co. and was dissolved in 5 ml of 30 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-pyridoxal phosphate and 0.4 mM-dithiothreitol. The preparation was stored in small samples at –20°C, and before use it was diluted 1:10 with 10 mM-Tris/HCl buffer, pH 7.0, containing 1 mM-dithiothreitol and 0.1 mM-EDTA.

Assay for biosynthetic ornithine decarboxylase activity

Ornithine decarboxylase activity was measured by the release of ¹⁴CO₂ from L-[1-¹⁴C]ornithine at 37°C (Jänne & Williams-Ashman, 1973) in a reaction mixture (1 ml) containing 100 mM-Tris/HCl buffer, pH 8.25, for the *E. coli* enzyme and pH 7.5 for the *Ps. aeruginosa* enzyme, 0.04 mM-pyridoxal phosphate, 1 mM-dithiothreitol, 1 mM- or 5 mM-L-ornithine, 1.5–2.5 μ Ci of DL-[1-¹⁴C]ornithine and enzyme. The incubation was conducted for 20 min.

Assay for biodegradative ornithine decarboxylase activity

The biodegradative ornithine decarboxylase from *E. coli* was assayed as described above, except that it was done at pH 7.0, its pH optimum as described by Applebaum *et al.* (1975), and the incubation was conducted for 15 min.

Assay for arginine decarboxylase activity

Biosynthetic arginine decarboxylase was measured by a modification of the previously described method of Wu & Morris (1973). The incubation mixture (1 ml) contained 100 mM-Tris/HCl buffer, pH 8.25, 0.04 mM-pyridoxal phosphate, 4 mM-MgSO₄, 1 mM-dithiothreitol, 0.5 mM-L-arginine, 0.83 μ Ci of L-[U-¹⁴C]arginine and the enzyme. The incubations were conducted for 20 min at 37°C.

Definition of enzyme activity and determination of protein

In both the ornithine decarboxylase and the arginine decarboxylase assays, the ¹⁴CO₂ produced was trapped and its radioactivity counted as described previously (Jänne & Williams-Ashman, 1973; McCann *et al.*, 1975). Reactions were linear with time and proportional to the amount of enzyme protein added. One unit of enzyme activity was defined as the amount catalysing the release of 1 μ mol of CO₂/h under the standard assay conditions. The specific enzyme activity was defined as activity units/mg of protein. The protein concentrations were determined by the method of Böhlen *et al.* (1972) or Bradford (1976), with crystalline bovine serum albumin used as a standard.

Determination of the time-dependent irreversible inhibition of enzyme activity

Equal volumes of enzyme extract, 300 mM-Tris/HCl buffer (pH 7.5 or 8.25, containing 3 mM-dithiothreitol and 0.12 mM-pyridoxal phosphate) and various concentrations of the inhibitor solution in water, were mixed. The mixtures were then incubated in closed test tubes at 37°C, and at various times 20 μ l samples were transferred to the final assay mixture, resulting in 50-fold dilution of the inhibitor. This transfer effectively stopped any further enzyme inhibition.

Assay of the formation of putrescine in vitro

The enzymic synthesis of putrescine was measured under the following conditions. Preincubation was done at 37°C in the presence and in the absence of 2 mM-DL- α -difluoromethylornithine. The mixtures (0.25 ml) contained 150 μ l of enzyme preparation, 0.08 μ mol of pyridoxal phosphate, 2 μ mol of dithiothreitol, 200 μ mol of Tris/HCl buffer, and the inhibitor or water. After 20 min 0.25 ml of 0.081 mM-L-[U-¹⁴C]ornithine was added. After another 20 min the reaction was stopped with 0.5 ml of 1 M-HCl. Proteins were removed by centrifugation (20000g for 30 min) and the clear supernatants were subjected to electrophoresis. Samples and standards (10 μ l) were applied to silica-gel plates (EM Laboratories) and electrophoresis was done for 30 min with a pyridine/acetic acid/citrate buffer,

pH 4.8 (Fischer & Bohn, 1957). The plates were cooled with circulating water at 0°C. At 600 V there was a current of 70 mA. After electrophoresis, the plates were dried for 10 min at 100°C, and radioactive spots were located by using a scanner (TLC Scanner; Berthold, Wildbad, Germany). Standards were revealed by spraying the plates with ninhydrin [0.2% (w/v) in acetone]. Spots were scraped off the plates and their radioactivities separately counted in 20 ml of Aquasol scintillation liquid.

Results

Effect of DL- α -difluoromethylornithine on bacterial ornithine decarboxylase activity *in vitro*

Incubation of the ornithine decarboxylase obtained from *Ps. aeruginosa* with DL- α -difluoromethylornithine (40–500 μ M) results in an irreversible and time-dependent loss of the enzymic activity. As described in the Experimental section, the inactivation of ornithine decarboxylase was followed for at least two half-lives, and analysis of the inhibition data was performed by the method of Kitz & Wilson (1962) as further developed by Jung & Metcalf (1975). The inactivation process was progressive with time and followed pseudo-first-

order kinetics. Fig. 1 shows the half-life of the enzyme activity plotted against the reciprocal of the inhibitor concentration, from which it was possible to calculate the dissociation constant K_i (1400 μ M) and the half-life of the enzyme activity at an infinite concentration of DL- α -difluoromethylornithine (1 min); $t_{1/2}$ at 10 μ M concentration of the compound was 130 min.

The substrate, L-ornithine (1 mM), protected the enzyme against the time-dependent inactivation by DL- α -difluoromethylornithine, indicating the probable involvement of the active site in inactivation of the enzyme. The presence of dithiothreitol in the preincubation medium and the absence of any lag time before the onset of inhibition argue against the possibility of inactivation via an affinity-labelling mode by a diffusible alkylating species (Rando, 1974; Metcalf *et al.*, 1978).

However, 1 mM-DL- α -difluoromethylornithine had no inhibitory effect *in vitro* on the biosynthetic ornithine decarboxylase activity of either *E. coli* 59 or *E. coli* 62, nor that of *K. pneumoniae* or the biodegradative enzyme of *E. coli*. At 5 mM, DL- α -difluoromethylornithine does not inhibit the biosynthetic *E. coli* 59 ornithine decarboxylase *in vitro*, even when preincubated for 6 h. The non-inhibition of the biosynthetic enzyme of *E. coli* by DL- α -difluoromethylornithine was not influenced by any increase in the concentration of the pyridoxal phosphate in the assay mixture. Final concentrations of pyridoxal phosphate of 0.4 or 4.0 mM still did not allow any inactivation of ornithine decarboxylase by 1.3 mM-DL- α -difluoromethylornithine, the standard assay final concentration of pyridoxal phosphate normally being 0.04 mM. When GTP (1.2 mM), a known activator of *E. coli* ornithine decarboxylase (Hölttä *et al.*, 1972), was added to the assay mixture with DL- α -difluoromethylornithine (1.2 mM) and preincubated for 20 min at 37°C before addition of any ornithine substrate, there again was no inhibition of the enzyme activity. At 50 mM, DL- α -difluoromethylornithine did cause 64% inhibition in the enzyme activity when present during the assay, but 50-fold dilution of the enzyme/inhibitor solution before the assay abolished the inactivation, i.e. the inhibition was reversible. As might be expected, DL- α -difluoromethylornithine at concentrations up to 10 mM also had no effect *in vitro* on arginine decarboxylase activity obtained from *E. coli* 59 or *Ps. aeruginosa*.

The striking difference between the effects of DL- α -difluoromethylornithine on the *Ps. aeruginosa* and *E. coli* ornithine decarboxylase was confirmed by measuring the formation of putrescine *in vitro*. Extracts from early-stationary-phase cells were preincubated for 20 min in the presence and in the absence of 2 mM-DL- α -difluoromethylornithine, after which 20.4 μ mol of L-[U- 14 C]ornithine was added.

Fig. 1. Dependence of the half-life of *Ps. aeruginosa* ornithine decarboxylase on the concentration of DL- α -difluoromethylornithine

The half-lives of the enzyme activity at different concentrations of DL- α -difluoromethylornithine (DFMO) (0.04–0.4 mM) were determined as described in the Experimental section and analysed by the procedure of Kitz & Wilson (1962).

After a 20 min incubation, the formation of putrescine and release of CO₂ were measured as described in the Experimental section. The formation of putrescine was clearly stoichiometric to the amount of CO₂ released in all cases (Table 1). In *Ps. aeruginosa* the low yield of putrescine was obviously related to the inhibition of ornithine decarboxylase activity, whereas in *E. coli* the formation of putrescine was not affected because ornithine decarboxylase was not inhibited. It is noteworthy that we have also found that DL- α -methylornithine, a potent competitive inhibitor of mammalian ornithine decarboxylase activity (Mamont *et al.*, 1976), inhibited ornithine decarboxylase from *Ps. aeruginosa* *in vitro* with K_i 6.5 mM (K_m 0.6 mM) at pH 7.5, but again had no effect on the *E. coli* 59 biosynthetic enzyme at concentrations up to 10 mM.

Effect of DL- α -difluoromethylornithine on bacterial ornithine decarboxylase and arginine decarboxylase in vivo

To determine if DL- α -difluoromethylornithine would inhibit ornithine decarboxylase activity in whole dividing cells, *Ps. aeruginosa* and *E. coli* were grown in media (Davis & Mingioli, 1950) for 5 h in the presence of several DL- α -difluoromethylornithine concentrations. No effect on bacterial growth rate was observed at any concentration of the compound. As illustrated in Fig. 2(a), a DL- α -difluoromethylornithine concentration as low as 0.05 mM caused 93% inhibition of ornithine decarboxylase activity in *Ps. aeruginosa*, but had no effect on this enzyme activity in *E. coli* cells (Fig. 2b). However, with higher concentrations of the compound there was 30–46% inhibition of ornithine decarboxylase activity in *E. coli* cells (Fig. 2b). This suggests some interference in the synthesis of the enzyme, since DL- α -difluoromethylornithine has no direct effect *in vitro* on *E. coli* ornithine decarboxylase activity. In this experiment it became evident that these bacteria seem to be able to compensate for the inhibition of putrescine via

ornithine decarboxylase by an increase in the arginine decarboxylase activity. When arginine decarboxylase activity from the same extracts was measured, a clear enhancement was observed when the inhibition of ornithine decarboxylase exceeded 40% (Figs. 2a and 2b). Extensive dialysis of the extracts (20 h at 4°C in 100 mM-Tris/HCl buffer, pH 8.25, containing 1 mM-dithiothreitol and 0.1 mM-EDTA) did not abolish the inhibition of ornithine decarboxylase or the stimulation of arginine decarboxylase activity.

Ps. aeruginosa cells were grown to early stationary phase in Davis & Mingioli (1950) media. Samples were then removed and grown in the presence or in the absence of DL- α -difluoromethylornithine, and during the exponential phase of growth samples of the cells were withdrawn at time points indicated in Fig. 3. At a concentration of 0.5 mM a rapid inhibition of ornithine decarboxylase activity was observed in *Ps. aeruginosa* cells (Fig. 3), suggesting that there was good penetration of the compound into the cells. A 50% stimulation of arginine decarboxylase activity was seen immediately after 1 h of growth.

In a similar experiment, when 5 mM-DL- α -difluoromethylornithine was added to *E. coli* growth medium it abolished the normal increase of ornithine decarboxylase activity seen when the cells enter the exponential phase of growth, but it did not decrease the enzyme activity normally present in early-stationary-phase cells (Fig. 4). There was a constant, but moderate, stimulation of arginine decarboxylase activity in the cells throughout the exponential phase of the growth (Fig. 4). Although moderate inhibition of *E. coli* ornithine decarboxylase activity was observed at 0.5 mM-DL- α -difluoromethylornithine concentration, there was no stimulation of arginine decarboxylase activity (results not shown).

To investigate the manner of how DL- α -difluoromethylornithine inhibits the increase in *E. coli* ornithine decarboxylase activity *in vivo* but still has

Table 1. *Effect of DL- α -difluoromethylornithine on ornithine decarboxylase activity and formation of putrescine in Ps. aeruginosa and E. coli in vitro*

Extracts from early-stationary-phase cells were preincubated without substrate in the absence or in the presence of 2 mM-DL- α -difluoromethylornithine (DFMO). After 20 min L-[U-¹⁴C]ornithine was added, and the reaction was stopped with 1 M-HCl after 20 min. The CO₂ released and putrescine formed were determined as described in the Experimental section.

Bacterial source	Concn. of DFMO (mM)	Ornithine decarboxylase activity (μ mol/h per mg of protein)	Putrescine formation (μ mol/h per mg of protein)
<i>E. coli</i> 59	0	0.011	0.010
<i>E. coli</i> 59	2	0.010	0.011
<i>Ps. aeruginosa</i>	0	0.047	0.039
<i>Ps. aeruginosa</i>	2	<0.003	<0.003

no effect on the enzyme *in vitro*, cells from early stationary phase were grown for 4 h in the presence of 5 mM-DL- α -difluoromethylornithine, -DL- α -methylornithine or -L-ornithine, and samples of cells were taken every hour. As illustrated in Table 2, the effect of L-ornithine on ornithine decarboxylase activity was clearly similar to the effect of DL- α -difluoromethylornithine. The addition of L-ornithine in the growth medium, however, did not stimulate arginine decarboxylase activity, as did DL- α -difluoromethylornithine, but instead decreased the activity by 32–52%. DL- α -Methylornithine, which is structurally a closer analogue of DL- α -difluoromethylornithine than is ornithine, had practically no effect on ornithine decarboxylase (Table 2) or arginine decarboxylase activity (results not shown). Extensive dialysis of the extracts (20 h at 50 mM-Tris/HCl buffer, pH 8.25, containing 1 mM-dithiothreitol, 0.1 mM-EDTA and 4 μ M-pyridoxal phosphate) did not abolish the inhibition or stimulation of enzyme activities.

To determine if the partial inhibition of ornithine decarboxylase activity in *E. coli* cells *in vivo* is due to poor penetration of DL- α -difluoromethylornithine or perhaps due to the fact that the cells might contain another DL- α -difluoromethylornithine-sensitive ornithine decarboxylase, dialysed and non-dialysed extracts obtained from cells grown in the absence or in the presence of 5 mM-DL- α -difluoromethylornithine for 3 h were then incubated *in vitro* with 5 mM-DL- α -difluoromethylornithine for 2 h. Again, there was no inhibition of ornithine decarboxylase activity in any of these extracts.

Discussion

The results have clearly shown that the *E. coli* and *Klebsiella* ornithine decarboxylases are markedly different from all eukaryotic ornithine decarboxylases yet studied with respect to their interaction with DL- α -difluoromethylornithine (Mamont *et al.*, 1978a,b; Seiler *et al.*, 1978; Bacchi *et al.*, 1980; McCann *et al.*, 1981), in that the two enterobacterial enzymes are completely unaffected by this

Fig. 2. Inhibition of ornithine decarboxylase and stimulation of arginine decarboxylase by several concentrations of DL- α -difluoromethylornithine *in vivo* in (a)

Pseudomonas aeruginosa cells and (b) *E. coli* cells

The cells were grown for 5 h in Davis & Mingioli (1950) minimal medium in the presence and in the absence of various concentrations of DL- α -difluoromethylornithine (DFMO), and thereafter assayed for enzymic activities as described in the Experimental section. ●, Ornithine decarboxylase activity; ■, arginine decarboxylase activity.

Fig. 3. Effect of 0.5 mM-DL- α -difluoromethylornithine on exponentially growing *Ps. aeruginosa* cells

Ps. aeruginosa cells were grown in Davis & Mingioli (1950) minimal medium in the presence (○ and □) and in the absence (● and ■) of 0.5 mM-DL- α -difluoromethylornithine for 6 h. Every 1 h a sample of the cells was withdrawn from the culture and enzyme activities were measured as described in the Experimental section. ● and ○, Ornithine decarboxylase activity; ■ and □, arginine decarboxylase activity.

Fig. 4. Effect of 5 mM-DL- α -difluoromethylornithine on exponentially growing *E. coli* cells

Details are as indicated in Fig. 3 legend. ● and ■, Control cells; ○ and □, cells growing in the presence of 5 mM-DL- α -difluoromethylornithine. ● and ○, Ornithine decarboxylase activity; ■ and □, arginine decarboxylase activity.

Table 2. Effects of DL- α -difluoromethylornithine, DL- α -methylornithine and L-ornithine on *E. coli* ornithine decarboxylase activity *in vivo*

E. coli was grown from early stationary phase to exponential phase of growth in Davis & Mingioli (1950) medium in the absence or in the presence of 5 mM-DL- α -difluoromethylornithine (DFMO), -DL- α -methylornithine (α -MO) or -L-ornithine. At the times indicated, samples were withdrawn from the cultures and ornithine decarboxylase activity was measured as described in the Experimental section.

Compound in the medium	Ornithine decarboxylase activity (μ mol/h per mg of protein)				
	0h	1h	2h	3h	4h
None	0.28	0.93	1.34	1.36	1.22
DFMO	0.28	0.44	0.58	0.69	0.68
α -MO	0.28	0.81	1.27	1.23	1.25
Ornithine	0.28	0.66	0.77	0.84	0.67

normally potent substrate analogue. It is obviously not a general phenomenon for bacterial enzymes, as we have demonstrated that the *Pseudomonas* enzyme is rapidly and irreversibly inactivated by DL- α -difluoromethylornithine (Fig. 1). The enterobacterial ornithine decarboxylases may have evolved quite separately from their corresponding arginine decarboxylases, as we have demonstrated that the arginine analogue, DL- α -difluoromethylarginine, is an effective irreversible inhibitor of all the arginine decarboxylases from *E. coli*, *Klebsiella* and *Pseudomonas* (Kallio *et al.*, 1981).

The fact that DL- α -difluoromethylornithine still does diminish the ornithine decarboxylase activity in *E. coli in vivo* is an interesting finding, as this phenomenon must result from some regulatory mechanism, as L-ornithine itself produces a similar effect (Table 2). This could be due to an effect at the level of enzyme synthesis, or to any one of a number of other regulatory mechanisms that have previously been shown to control ornithine decarboxylase activity, i.e. enzyme turnover, induction of an 'antizyme'-like molecule or a post-translational shift from an active to an inactive form of the enzyme (for references see McCann, 1980). What is curious, and so far unexplained, is why the other α -methyl derivative, DL- α -methylornithine, did not show a similar effect. Peter *et al.* (1979) have also shown that DL- α -difluoromethylornithine will partially lower putrescine and spermidine concentrations in *E. coli*, demonstrating that polyamine biosynthesis is indeed affected by the presence of millimolar concentrations of the analogue in the medium. This indicates that in *E. coli*, as well as in *Pseudomonas*, DL- α -difluoromethylornithine is transported into the cell from the medium. The com-

pound by itself has no effect on the growth rate of *E. coli* or *Ps. aeruginosa*, even when a concentration of 10mM is incubated with exponentially growing cells for more than 12h. It should be possible, however, to use an appropriate combination of inhibitors to shut off the synthesis of putrescine in bacterial cells, and thus test whether a diminished growth rate results as a consequence of polyamine depletion, as is the case with polyamine-deficient mutants of *E. coli* (Hafner *et al.*, 1979; Tabor *et al.*, 1980).

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