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Methionineless Death in *Escherichia coli*

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Methionine auxotrophs of strains derived from *Escherichia coli* 15 lose their colony-forming ability when deprived of this amino acid. Late addition of methionine to liquid cultures did not restore plating efficiency but permitted growth of surviving cells. This phenomenon, termed methionineless death (*mld*), was not observed with methionine auxotrophs of *E. coli* strains B, W, or K_{12}, nor was a similar amino acidless death observed with corresponding auxotrophs of *E. coli* 15 for arginine, tryptophan, proline, isoleucine, and leucine. *Mld* was not dependent upon the genetic site determining methionine auxotrophy, nor did it affect the decarboxylation of methionine or the stability of methionyl-transfer ribonucleic acid synthetase activity of starved cells. Death was not altered by the presence of spermine or spermidine but was abolished by the methionine analogue, α-methylmethionine. Simultaneous starvation of another amino acid in a multiple auxotroph also significantly reduced *mld*, suggesting a possible role of protein synthesis. The onset of *mld* is correlated with a lower net increase of deoxyribonucleic acid.

The cessation of growth resulting from deprivation of an amino acid auxotroph of its requirement is rarely accompanied by a loss in colony-forming ability. Thus, a strain of *Bacillus megaterium* dies in the absence of histidine (12), and a form of tryptophanless death in *Bacillus subtilis* has been described (1, 8). In an extension of studies on amino acid antagonist death in *Escherichia coli* WWU (10), we observed that omission of methionine, but not of the other amino acids required by this organism (arginine, tryptophan, or proline), led to a pronounced loss of viability. Investigation into the possible causes of this phenomenon is the subject of the present paper.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains employed in this study are listed in Table 1.

Growth medium. The fortified medium (FM) employed was made up in medium A of Davis and Mingioli (4) containing 11 mM glucose with the following supplements given in final mM concentration: L-alanine, 3.25; L-aspartic acid, 0.45; L-asparagine, 1.5; L-cysteine, 0.58; L-glutamic acid, 2.7; glycine, 2.1; L-histidine; 0.32; L-leucine, 2.1; L-isoleucine, 1.2; L-lysine, 3.1; L-serine, 1.6; L-threonine, 1.7; L-valine, 2.8; L-tyrosine, 0.33; L-phenylalanine, 1.7; L-methionine, 0.80; L-arginine, 0.57; L-tryptophan, 0.27; L-proline, 1.2; uracil, 0.36; and thymine, 0.055.

Viability determinations. Bacteria were grown overnight in FM at 37 C in a New Brunswick gyratory incubator at 400 rev/min. Cells from the overnight culture were centrifuged, washed with medium A minus glucose, diluted approximately 20-fold in FM, grown at 37 C, and harvested when in exponential phase growth at a concentration of 10^8 to 5×10^8 cells per ml by filtration on a membrane filter (0.45-μm pore size). They were then washed, suspended in medium A minus glucose, and transferred to tubes containing the experimental medium to give a concentration of approximately 10^7 to 5×10^7 cells per ml. At times indicated in individual experiments, culture samples were diluted in cold medium A minus glucose, and appropriate dilutions were spread on nutrient agar (Difco) plates. Colonies were counted after overnight incubation at 37 C.

Decarboxylation of methionine. To the main compartment of a Warburg flask was added 2 ml of an iced cell suspension made up in FM and containing 1×10^9 cells, 200 nmoles of L-methionine-*1*-^{14}C (876 counts per min per nmole), and 100 μg of chloramphenicol. The center well contained a filter-paper wick and 0.2 ml of 2 M KOH. After incubation at 37 C the reaction was stopped by adding, from a side arm, 0.2 ml of 2 N H_2SO_4. After a few minutes, 0.2 ml of 47 mM Na_2CO_3 was added from the other side arm. The vessel was allowed to stand for approximately 5 min, and then the filter-paper wick and the KOH contents of the center well were quantitatively transferred with 0.8 ml of water to a scintillation vial. A 10-ml amount of scintillation fluid (containing 2 volumes of toluene-phosphor and 1 volume of Triton X-100) was added, and the samples were counted in a liquid scintillation spectrometer.

Incorporation of methionine into protein. Exponential phase cells were incubated for 3 hr at 37 C in fortified medium lacking various growth requirements or without methionine but with 5 mM DL-α-methylmethionine. At this time L-methionine-*1*-^{14}C (4,240 counts per min per mmole) to give a final concentration of 100 μM and the missing growth requirements were added. The incubation was continued, and at various

TABLE 1. *Bacterial strains employed in this study*

E. coli strain	Growth requirements[a]	Source
15-WWU	Thy, ura, arg, met, trp, pro	R. C. Bockrath
15-TAU-bar	Thy, ura, arg, met, trp, pro	P. C. Hanawalt
15-70V3 met$^-$	Thy, met	T. R. Breitman
15-TUM	Thy, ura, met	S. S. Cohen
B333	Thy, leu, met, pro	M. Cashell
B45	Met	S. S. Cohen
W122-33	Met	C. W. Tabor
K_{12} 58-161 RC_{str}	Met	A. Peterkofsky
K_{12} 58-161 RC_{rel}	Met	A. Peterkofsky
K_{12} leu$^-$ met$^-$	Leu, met	A. Peterkofsky

[a] Abbreviations: (thy) thymine, (ura) uracil, (arg) arginine, (met) methionine, (trp) tryptophan, (pro) proline, and (leu) leucine.

time intervals samples were removed and treated with 6 volumes of ice-cold 5% trichloroacetic acid. The samples were heated at 90 C for 10 min and cooled, and the hot acid-insoluble material was collected on membrane filters. The filters were washed with 5% trichloroacetic acid, placed in scintillation vials, and dried in a vacuum oven. To each vial, 5 ml of toluene scintillation fluid was added, and radioactivity was measured in a scintillation counter.

Assay of methionyl-tRNA synthetase activity. Exponential phase cells were inoculated into FM minus methionine at 37 C to give a concentration of 5×10^7 cells per ml. The culture was incubated at 37 C, and at timed intervals samples (400 ml) were removed and the cells harvested by centrifugation. The cells were washed with medium A minus glucose and suspended in 10 ml of 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer (*p*H 7.4) containing 10 mM dithiothreitol. Cells were disrupted by treatment for 1 min with a Bronson S-75 sonifier as described previously (3). Cell debris was removed by centrifugation at $30,000 \times g$ for 30 min.

The assay was carried out as described by Muench and Berg (9) with the exceptions that Tris was substituted for sodium cacodylate and dithiothreitol was substituted for reduced glutathione.

An equal volume of bacterial extract in 0.1 M Tris (*p*H 7.4) and 10 mM dithiothreitol containing the equivalent of 6×10^8 cells/ml was added to a mixture containing (per ml): Tris (*p*H 7.0), 200 μmoles; KCl, 20 μmoles; $MgCl_2$, 20 μmoles; adenosine triphosphate, 2 μmoles; bovine serum albumin, 0.4 mg; L-methionine-*l*-^{14}C, 200 nmoles (6.32 Ci/mole); dithiothreitol, 8 μmoles; and *E. coli* transfer ribonucleic acid (tRNA), 20 mg.

Determination of total DNA. A culture of strain WWU, grown overnight in FM, was diluted in FM containing thymine-*2*-^{14}C (27.8 Ci/mole) to give final concentrations of approximately 2×10^7 cells per ml and 18 μM thymine. This culture was grown at 37 C and 400 rev/min in a New Brunswick gyratory incubator for four to five generations to a concentration of 5×10^8 cells per ml to label the deoxyribonucleic acid (DNA)-thymine to a specific activity which was at least 93% of that of the ^{14}C-thymine in the medium. These exponential phase cells were harvested on membrane filters, washed at room temperature with medium A, suspended in ice-cold medium A, and transferred to tubes in an ice bath containing experimental medium and thymine-*2*-^{14}C (27.8 Ci/mole) to give final concentrations of 5×10^7 cells per ml and 18 μM thymine. Portions (1 ml) from each tube were incubated at 37 C and 400 rev/min in a New Brunswick gyratory incubator. These were removed at timed intervals and cooled to 0 C, and an equal volume of 10% trichloroacetic acid was added. After standing at least 10 min, they were filtered on membrane filters. The filters were washed with 5% trichloroacetic acid, placed in scintillation vials, and dried in a vacuum oven. To each vial, 5 ml of toluene scintillation fluid was added, and radioactivity was measured in a scintillation counter at a counting efficiency of approximately 80%.

RESULTS AND DISCUSSION

Effect on cell viability of methionine deprivation. Removal of methionine from cultures of methionine auxotrophs of *E. coli* 15 results in loss of colony-forming ability (Fig. 1). Starvation of auxotrophs of *E. coli* 15 for each of six requirements—arginine, tryptophan, proline, isoleucine, leucine, and uracil—did not result in loss of viability. The simultaneous deprivation of methionine and another amino acid (arginine, tryptophan, proline) markedly reduced the loss in viability (Fig. 1A). The simultaneous omission of methionine and uracil was lethal but to a much lesser extent than with omission of methionine alone.

As shown in Fig. 1B, methionineless death (*mld*) occurs in cultures depleted of methionine by growth. Addition of methionine to a culture undergoing *mld* did not result in an immediate restoration of plating efficiency but prevented further death and permitted those cells that were viable to commence growth (Fig. 1B). Individual clones from a culture deprived of methionine for 5 hr were as sensitive to *mld* as was the parent culture, indicating that the survivors are not *mld* resistant mutants. Replacement of methionine with α-methylmethionine, an analogue which neither supports protein synthesis (11) nor reacts with *S*-adenosylmethionine synthetase (5, 7), resulted in no loss of viability (Fig. 1A). Qualitatively, similar results as shown in Fig. 1 were obtained when medium A was used.

Survey of sensitivity of other methionineless auxotrophs to methionineless death. All methionine auxotrophs listed in Table 1 that are derivatives of *E. coli* 15 (WWU, TAU-bar, 70V3 met$^-$, and TUM) undergo *mld*. Strains B333 and B45 derived from *E. coli* B; strain W 122-33, a mutant of *E. coli* W; 58-161 RC_{str} and 58-161 RC_{rel} derived from K_{12}; and K_{12} leu$^-$met$^-$ were

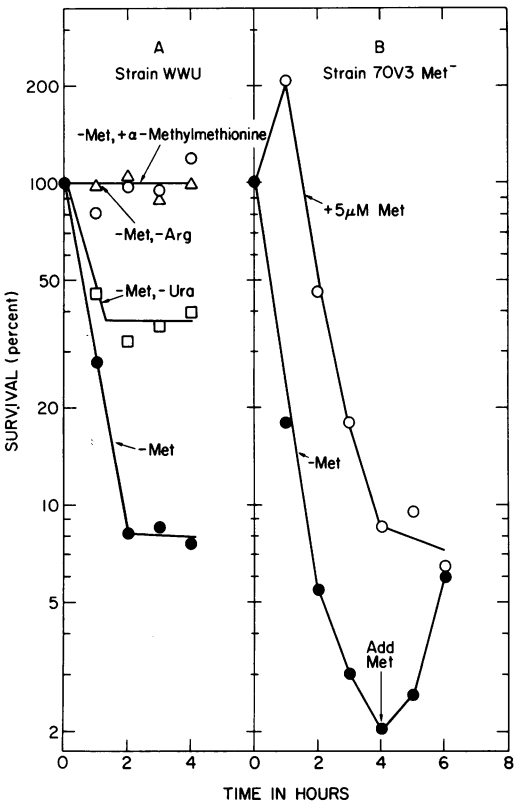

FIG. 1. *Viability of Escherichia coli cultures under conditions of methionine deprivation. A, strain WWU at an initial concentration of 1.2 × 10⁷ cells/ml was incubated in fortified medium minus methionine (●), minus methionine and uracil (□), minus methionine and arginine (○), minus methionine plus 2 mM DL-α-methylmethionine (△). B, strain 70V3 met⁻ at an initial concentration of 2.5 × 10⁷ cells/ml was incubated in fortified medium minus methionine (●), or with 5 μM methionine (○). At 4 hr, 8 mM methionine was added to the minus methionine culture to give a final methionine concentration of 800 μM.*

not susceptible to *mld*. In addition, these results eliminated the possibility that the thymine requirement of the *E. coli* 15 strains or the RC locus played a role in the sensitivity of *E. coli* 15 strains to *mld*.

The possibility that the enzymatic defect resulting in methionine auxotrophy was involved in *mld* was ruled out in growth experiments (Table 2) showing that derivatives of *E. coli* 15 (TUM and 70V3 met⁻), sensitive to *mld*, were blocked at a site different from that of WWU but the same as that of three methionine auxotrophs derived from three different wild-type strains that were not susceptible to *mld*.

Relationship of metabolic products of methionine to methionineless death. Methionine has a central role in the metabolism of the cell. In addition to its incorporation into protein, it is converted to *S*-adenosylmethionine, which is involved in methylation reactions and the synthesis of polyamines. The possibility was considered that methionine or one of its metabolic products could stabilize an enzyme which was involved in the production of *S*-adenosylmethionine, the synthesis of polyamines, or the incorporation of methionine into protein. In the absence of methionine this enzyme could be irreversibly inactivated and would not be available for an essential function when methionine was reintroduced into the medium. As shown in Table 3, spermine or spermidine, compounds not synthesized in the absence of methionine, did not affect the pattern of *mld*. This result left open the possibility that during methionine deprivation a defect developed in the utilization of methionine for the formation of spermidine. To investigate this possibility, the ability of cells to decarboxylate methionine was measured (Fig. 2). It had been suggested by H. Tabor and C. W. Tabor (*personal communication*) that essentially all of the decarboxylation of methionine can be accounted for by the formation of "decarboxylated adenosylmethionine" from *S*-adenosylmethionine. The finding that methionine-starved cells have as great, if not

TABLE 2. *Growth of methionine auxotrophs on two methionine precursors and survival under methionineless conditions*

Strain	Growth in medium containing		Survival under methionineless conditions
	Cystathionine	Homocysteine	
15-WWU	–	–	–
15-TUM	–	+	–
15-70V3 met⁻	–	+	–
B333	–	+	+
W122-33	–	+	+
K_{12} 58-161 RC_{rel}	–	+	+
K_{12} leu⁻ met⁻	+	+	+

TABLE 3. *Effect of spermine and spermidine on methionineless death*

Time (hr)	% Survival[a]		
	Methionineless conditions	Methionineless conditions with 10^{-4} M spermine	Methionineless conditions with 10^{-4} M spermidine
0	100	100	100
1	240	245	239
2	24	38	29
3.5	12	14	12
5	14	6	6.7

[a] Initial cell concentration was 1.5 × 10⁷ cells/ml.

slightly greater, capacity to decarboxylate methionine (Fig. 2), together with the lack of effect of polyamines on *mld* (Table 3), indicates that a defect in methionine conversion to polyamines is not a prime cause of *mld*.

Effect of methionine starvation on incorporation of methionine into protein. The possibility was next examined that a block in methionine incorporation into protein was responsible for *mld*. It had been observed that the turbidity of cultures remained essentially constant during methionine deprivation. On addition of methionine, a lag phase was noted before turbidity increased. The duration of this lag phase was directly related to the time that the cultures had been under methionine-depleted conditions. In a gross sense, these results indicated that cultures which had been starved of methionine had decreased capacities to synthesize protein. This phenomenon was investigated more specifically by analyzing the capacity of cells to incorporate methionine into protein after different growth requirements were withheld for 3 hr (Fig. 3). Only cells deprived of methionine alone lost the capacity to incorporate this amino acid into protein. Simultaneous starvation for methionine and another amino acid, starvation for an amino acid other than methionine, or replacement of methionine with α-methylmethionine, conditions under which there is little or no loss in viability (Fig. 1A), result in essentially no loss of incorporation of methionine into protein.

The specificity of this loss in protein synthetic capacity and the observation that α-methylmethionine can inhibit methionyl-tRNA synthetase (11) raised the possibility that this enzyme might be inactivated during methionine starvation and stabilized by the methionine analogue. However, there was no change in methionyl-tRNA synthetase activity during 4 hr of methionine deprivation (Fig. 4).

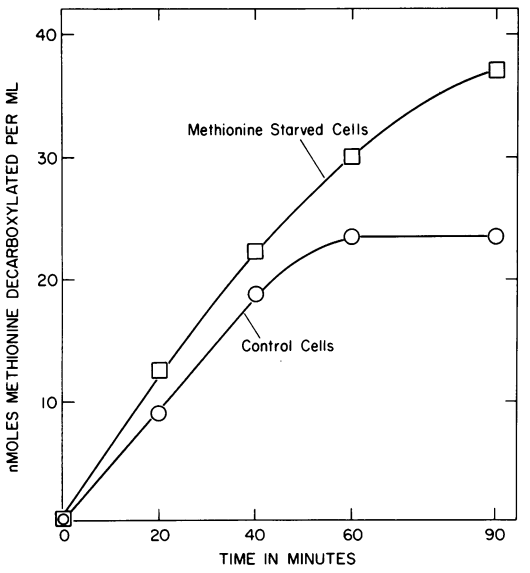

FIG. 2. *Comparative abilities of cells of strain WWU to decarboxylate methionine when starved of methionine for 3 hr* (□) *or not starved* (○).

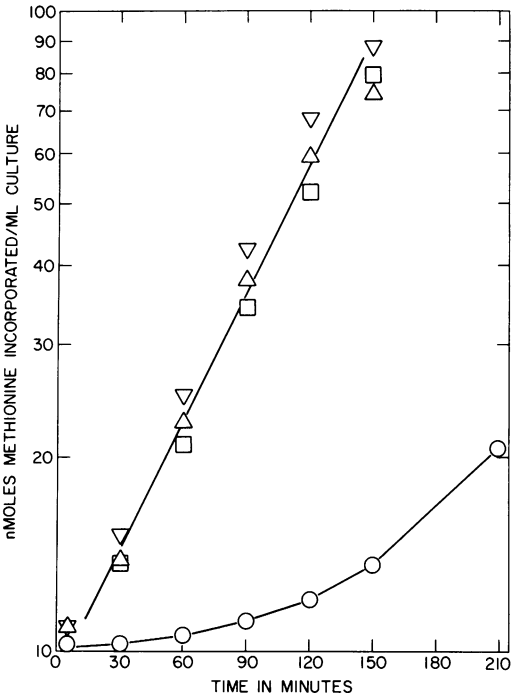

FIG. 3. *Capacity of cultures of strain WWU to incorporate methionine into protein after a 3-hr starvation for different growth requirements. Starvation conditions were: minus methionine* (○), *minus arginine* (□), *minus arginine and methionine* (△), *minus methionine plus 5 mM* DL-*α-methylmethionine* (▽). *Essentially the same results as with minus arginine were obtained with the following starvation conditions: minus proline, minus methionine and proline, minus tryptophan, and minus methionine and tryptophan.*

Effect of methionine deprivation on total DNA. Specific effects of methionine deprivation on DNA synthesis and stability have been reported. Thus, Billen and Hewitt (2) found that, in *E. coli* 15 strains, the DNA synthesized in the absence of methionine was more slowly replicated than was that synthesized in the absence of other amino acids. These authors reported that when an auxotroph requiring methionine and other amino acids was deprived of more than one nutrient, the extent of DNA synthesized during unbalanced growth was similar to that during deprivation of either nutrient alone. We have examined the effect of methionine deprivation on total DNA and have observed a direct correla-

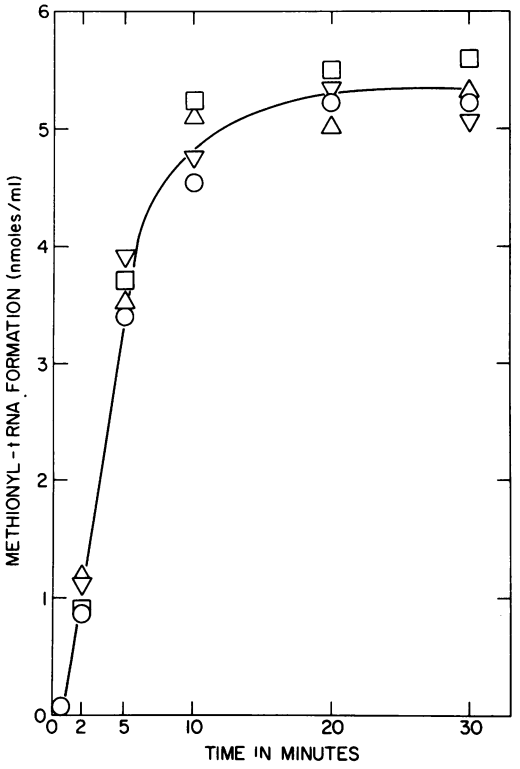

FIG. 4. *Effect of methionine starvation on methionyl-transfer ribonucleic acid synthetase activity. Cultures of strain WWU were starved of methionine in fortified medium for 0 hr* (○), *1 hr* (△), *2 hr* (□), *and 4 hr* (▽).

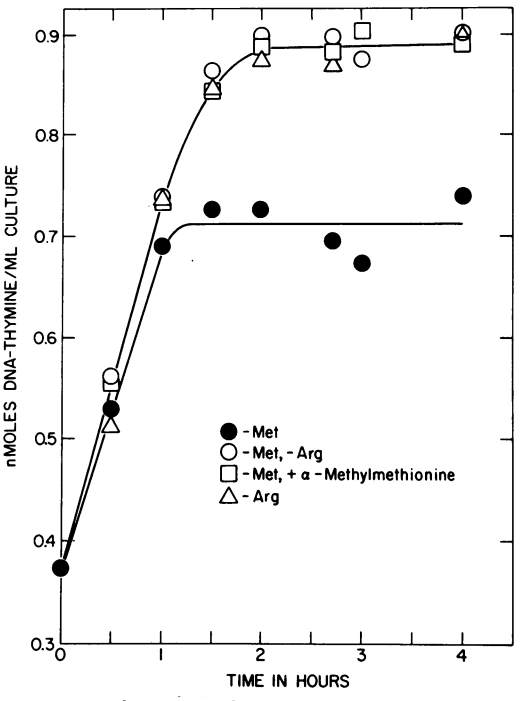

FIG. 5. *Changes in the deoxyribonucleic acid content of strain WWU during incubation in fortified medium lacking amino acid requirements. Where indicated,* DL-*α-methylmethionine was present at a concentration of 2 mM.*

tion between those conditions causing *mld* and those causing a decrease in DNA levels (Fig. 5). It would therefore appear that the observations of Billen and Hewitt are not related directly to *mld*. In their case the concurrent deficiency of other required amino acids did not decrease the effects of methionine deficiency (2), whereas in our case it counteracted the result of a methionine deficiency on both viability (Fig. 1) and DNA levels (Fig. 5). This lower net increase in DNA could result from DNA degradation such as that found by Lark (6) in *E. coli* 15 strains grown in ethionine or norleucine. However, it is not clear to what extent the breakdown of DNA under these conditions may be associated with utilization of these analogues for synthesis of unnatural protein. The methionine analogue, α-methylmethionine, which is not a substrate for protein synthesis (11) or for *S*-adenosylmethionine synthetase (5, 7), can prevent the lower net increase of DNA synthesis during methionine deficiency (Fig. 5). Further experiments on the status of DNA under methionineless conditions are necessary.

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