Thymineless Death in *Escherichia coli*

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For filament forming strains of *Escherichia coli*, it is shown that susceptibility to thymine starvation was dependent upon the nature of the plating media. In such bacteria, part of the induced damages was found to be repairable if the cells after starvation were held in a non-nutrient medium. For non-filament forming bacteria, no such phenomena were observed.

Introduction

There is little doubt that thymine starvation affects the DNA of thymine requiring bacteria [1-5]. However, the process of killing is fairly complicated and remains to be clarified. The genes responsible for the control of sensitivity to thymine starvation have not been identified, even though claims have been but forward for at least one gene [6]. Besides, whether the cells die when still in incubation medium or on the surface of agar plates is yet to be established [7]. In an attempt to provide some answer to these questions, the phenomenon of thymineless death was studied with bacteria having different mutations, by varying the conditions of platings and treatment of post-starved cells, etc. We have come to the conclusion that the process of thymineless death has two pathways in filament forming bacteria; the damage caused through one of which was repairable, whereas for the nonfilament forming bacteria there was one pathway and the induced damages were not repairable under conditions not supporting growth. A part of killing was taking place on agar surface.

Materials and Methods

Bacterial strains

Bacterial strains used are listed in Table I. The requirements were: 2 μg/ml for thymine, 20 μg/ml for arginine, methionine, tryptophan and proline, 10 μg/ml for uracil. For *E. coli* B_{s-1} (strain 6), the methionine requirement was 40 μg/ml and for *E. coli* B_{s-12} (strain 23) the thymine requirement was 20 μg/ml. All these strains had been routinely maintained in this laboratory.

Bacterial growth and thymineless incubation

Bacteria were grown in M9 minimal medium (NH_4Cl — 1 mg/ml, $MgSO_4$ — 0.13 mg/ml, KH_2PO_4 — 3 mg/ml, Na_2HPO_4 — 6 mg/ml, pH 7.2) supplemented with the requirements as necessary at 37 °C in a shaker bath. Glucose at 5 gm/l was used as sole carbon source.

0.1 ml of overnight culture was diluted in 10 ml fresh medium and allowed to grow. Cells at desired titre were collected in the log phase of growth. For thymineless incubation, cells were resuspended in M9 medium containing all requirements except thymine. For controls, the bacteria were resuspended in M9 buffer *i. e.*, M9 medium less glucose and requirements. Thymineless incubations were done with air bubbling at 37 °C. Viability was assayed by spreading 0.1 ml of bacterial suspension at suitable dilution on nutrient agar or on supplemented minimal agar surface. Visible colonies were counted after about 18 hours of incubation at 37 °C.

In case of change of media, cells were centrifuged and resuspended in new medium without any further washing.

Results and Discussions

Survival of different bacteria under the condition of thymine starvation is shown in Fig. 1. The origin and the genetic characters of all strains are given in Table I, along with their designations. The following pairs of *E. coli* mutants were used in experiments: hcr^+ and hcr^- (strains 7 and 8), pol A^+ and pol A^- (strains 3 and 4), fil^+ and fil^- (strains 18 and 26). Besides these, three other strains (number 1, 6 and 23) also were utilized. Except for the strains differing in *fil* characteristics, the pairs of mutants amongst themselves were of alomst identical sensitivity to thymine starvation. In case of strains 18 and 26 (26 being the fil^- isolate of strain 18), strain 26 was significantly more resistant than strain 18. Further, it may be seen that only the
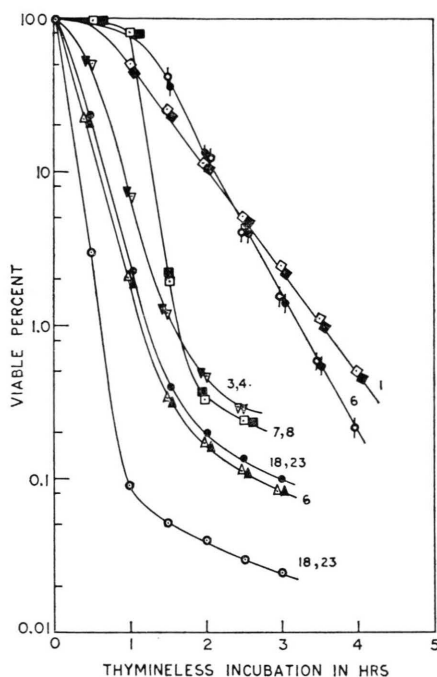
Fig. 1. Survival of different strains of *E. coli* on thymine starvation. Open symbols refer to platings on nutrient agar and filled up symbols on minimal agar plates. Numbers indicate strain designation in this laboratory.

Table I. Bacterial strains with relevant characteristics.

Strain	Our desig-nation	Characteristics	Source
E. coli 15	1	*fil⁻ thy⁺ arg⁻ ura⁻ met⁻ tryp⁻ pro⁻*	D. Freifelder
E. coli K12 (W 3110)	3	*thy⁻ pol* A⁺ *fil⁻*	Bryn Bridges
E. coli K12 (P 3478)	4	*thy⁻ pol* A⁻ *fil⁻*	Bryn Bridges
E. coli B$_{s-1}$	6	*thy⁻ met⁻ fil⁻ hcr⁻*	D. Billen
E. coli B/r	7	*thy⁻ tyrp⁻ hcr⁺ fil⁻*	D. Billen
E. coli B/r	8	*thy⁺ tryp⁻ hcr⁻ fil⁻*	D. Billen
E. coli B	18	*thy⁻ fil⁺ uvr⁻*	D. Cummings
E. coli B$_{s-12}$	23	*thy⁻ fil⁺ uvr⁻*	D. Cummings
E. coli B	26	*thy⁻ fil⁻ uvr⁻*	Isolated here from 18

N. B. — *fil⁺* indicates filament formers and *fil⁻* nonfilament formers. Filament forming capacity was assayed by growing the cells in complete medium after thymine starvation. This was determined by direct microscopic observation.

filament forming cells showed variation in sensitivity depending on the type of assay media used. Filament formers were more sensitive on nutrient

than on minimal agar plates. The results indicate that part of killing was taking place on agar plates. The existence of a possible restoration phenomenon for the filament forming cells on minimal agar plates was also indicated. Possibly the restoration phenomena could work only if the medium of growth after challenge was not enriched. Similar recovery phenomenon for these types of cells has been reported for X-irradiated bacteria [8].

In order to test the validity of the existence of restoration processes on minimal agar surface, bacteria belonging to strain 18, after 0 and 60 min of thymine starvation were layered on minimal agar plates, supplemented with varying amounts of nutrient broth in such a way that the final concentrations of the nutrient broth in plates were 0 (minimal), 1, $1/2$, $1/4$, $1/8$, $1/16$th of the concentration used in normal nutrient agar plates. Each set had been supplemented with glucose (0.5% w/v) and thymine (2 μg/ml). There was a sharp increase in viability for the thymine starved cells as the concentration of nutrient was decreased from normal to $1/4$th of normal, reaching a plateau at $1/8$th of the normal. There was only a slight rise in viable number for control cells (Fig, 2). The finding justified the contention about the restoration process for these bacteria.
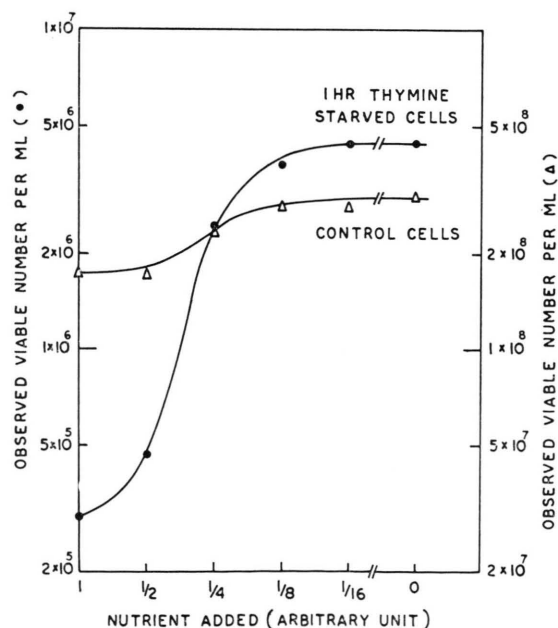

Fig. 2. Influence of nutrients added to the minimal agar plates on the viability of thymine starved (●) and control (△) *E. coli* B (strain 18).
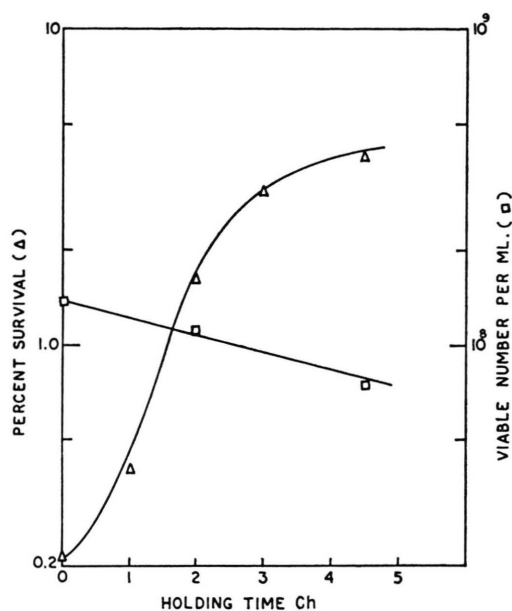
Fig. 3. Variation in the survival of control (□) and thymine starved (△) *E. coli* B (strain 18) on holding on solid non-nutrient agar layer.

Fig. 4. Increase in viability of control (curve E) and 1 hr thymine starved (curves A, B, C, D) *E. coli* B (strain 18) and *E. coli* B_{s-12} (strain 23) on transfer to M9 buffer. Open symbols refer to platings on nutrient agar and filled up symbols on minimal agar surfaces.

For further corroboration of the results obtained above, filament forming bacteria after thymine starvation were held in nonnutrient solid agar. After different periods of holding, nutrients were poured on top of that layer. Experimental details were as below:

Cells after the requisite period of thymine starvation were suspended in 3 ml (Difco) agar solution (0.7% w/v) at 42 °C and then poured on sterile petri dishes within 15 sec. The agar solution took less than 5 min to solidify at room temperature, giving a very thin layer. The bacteria thus held, were kept at 37 °C. At different periods of incubation, the petri dishes were taken out and 20 ml nutrient agar was slowly poured over the solid surface without disturbing the bottom layer. After solidification, the plates were incubated as usual and the visible colonies were counted after 18 hours. Function of the thin soft agar layer was to hold the bacteria in fixed positions even if they divided. The results are shown in Fig. 3. Control cells when plated using the same technique of double layering did not show any increase in viability; in fact, there was a slight decrease.

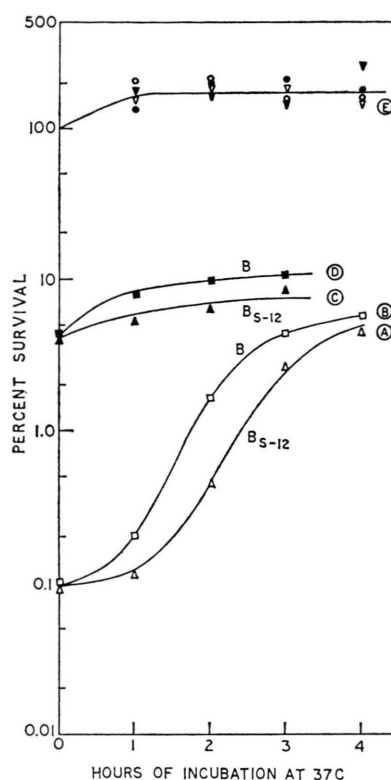Next, the viability of the bacteria on incubation in a buffer after thymine starvation was followed.

Fig. 4 shows the results. When strains 18 and 26 were starved for 1 hr and then incubated at 37 °C in buffer, there was a rapid increase in viability for both strains. Control cells starved in buffer did not show any significant increase in viability during starvation. To check whether this recovery was the same as our observed minimal agar recovery, bacteria during different periods of buffer incubation were plated on both minimal and nutrient plates. The results shown in Fig. 4 indicate that with the progress of buffer recovery, there was gradually decreasing difference between the observed viable counts in the minimal and nutrient agar plates, ultimately giving nearly the same number (*cf.* Curves 4 A, C and 4 B, D). This clearly indicates that minimal agar recovery was of the same nature as that of buffer recovery.

The observed buffer recovery could not be stopped by chloramphenicol (concentration up to 100 $\mu g/ml$); there was only a little depression. Dif-

ferent concentrations of caffeine (2 to 8 mg/ml) could not stop the buffer recovery. Caffeine at 2 mg/ml and chloramphenicol at 20 μg/ml when present simultaneously also could not stop the recovery. Recovery took place even in the presence of acriflavine (1 μg/ml) in buffer. However, in all these cases, there were some depressions in the level of recovery.

The only gene that we could establish to be involved with the susceptibility of bacteria to thymine starvation was *fil*. It has been clearly shown that filament forming bacteria were more sensitive to thymineless incubation than their nonfilament forming counter parts. The failure of the other workers to implicate *fil* gene with the susceptibility to thymine starvation [9] could be due to the nature of the plating media used. It has been specifically shown here that the distinction in sensitivity between filament formers and nonfilament formers vanishes in rich medium. We thus can claim in conformity with some other published results [10, 11] that there are two pathways for the killing induced by thymine starvation. The damage induced through one of the pathways was repairable under favourable conditions. This pathway producing repairable damages was absent in nonfilament forming bacteria.

Some recovery from the damages induced by thymine starvation have already been reported for bacteria in complete growth medium [7, 12]. The recovery observed in the present investigations is independent of growth medium, takes place in buffer and is apparently similar to the liquid holding recovery in case of UV irradiated cells, even though the known inhibitors of dark repair such as caffeine, acriflavin were without any affect on the phenomena. During thymine starvation, the protein synthesis remains unaffected and results in the accumulation of initiator proteins. Thus when thymine starved cells are put in growth medium, DNA synthesis would start immediately with multiple forks [13, 14]. This rapid DNA synthesis and subsequent fast cell division would continue until the cells reach their normal DNA/cell mass ratio. Thus the observed recovery in growth medium might not be wholly due to actual repair.

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