Thymineless Death in *Bacillus subtilis*: Correlation Between Cell Lysis and Deoxyribonucleic Acid Breakdown

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Received for publication 3 January 1974

*Bacillus subtilis* carrying an inducible defective phage is several times more sensitive to thymineless death than a mutagenized derivative that behaves as a nonlysogen. When the integrity of the deoxyribonucleic acid (DNA) of both strains was examined during thymine starvation by transformation experiments, sedimentation studies, and measurements of acid-soluble DNA degradation products, it was shown that extensive DNA breakdown occurred only in the lysogenic strain. During thymine starvation of this strain, there is a progressive proclivity to lysis, followed by leakage of DNA and DNA degradation products. Such leakage was not observed in the nonlysogen. A correlation between proclivity to lysis and extensive DNA degradation is indicated.

Growing cultures of thymine auxotrophs deprived of thymine lose viability in a characteristic manner. This phenomenon is known as thymineless death (TLD) (5). Sensitivity to TLD varies in different microorganisms. Factors such as prophages or bacteriocins which are induced during thymine starvation enhance the rate of TLD (7, 17, 21, 22, 37). On the other hand, the efficiency of repair mechanisms may be an important factor in the relative resistance of some microorganisms (3, 6, 20). However, the primary cause of cellular death is still unknown. There is considerable evidence that single-strand breaks and deoxyribonucleic acid (DNA) degradation occur in chromosomal and episomal DNA of thymine-starved cultures (10, 11, 14, 23, 27, 28, 40). There is no direct evidence that this damage is the lethal factor in TLD. It was shown that repair of nucleolytic damage that occurs under certain conditions during thymine starvation (31) or as a result of X-ray irradiation (39) is very efficient. On the other hand, studies on a recA mutant of *Escherichia coli* showed it to be 100% resistant to TLD during prolonged thymine starvation (15) despite the fact that DNA degradation occurs spontaneously in recA mutants under normal conditions (25). Studies on DNA degradation in rec DNA" mutants indicated that extensive degradation is not the primary cause of cellular death (4).

In an attempt to gain information on the factors involved in TLD, we isolated and studied mutants showing varying degrees of resistance to thymine deprivation. This paper is concerned with the effect of thymine starvation on the integrity of the bacterial DNA, and the correlation between cell lysis and DNA degradation in a lysogenic strain of *B. subtilis* and in an apparently nonlysogenic derivative.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Growth conditions. Cultures were grown in MinCATT medium: mineral medium (38) supplemented with 0.5% glucose, 0.5% Casamino Acids (Difco), 20 μg each of thymine, tryptophan, and adenine per ml, 0.002% glutamic acid, and 0.0075% asparagine. MinCATT medium is MinCATT without thymine; MinTT is MinCATT without Casamino Acids and supplemented with 0.002% yeast extract. Viable counts were carried out on plates containing MinTT medium with 1.5% agar (Difco). Culture samples were diluted in mineral medium, and 0.05, 0.02, and 0.01-ml amounts were placed on dry plates (2 drops of each volume per plate). Bacterial growth was determined by optical density (OD) measurements in a Klett-Summerson colorimeter with filter 42.

Chemicals. Mitomycin C was from Sigma Chemical Co. Rifampin was a kind gift of Ciba-Geigy.

Transformation procedure. Preparation of competent cultures and transformation media were carried out as described previously (2).

Preparation of transforming-cell extracts. Culture samples (2 ml) were collected and suspended in 1 ml of saline-ethylenediaminetetraacetic acid (EDTA; 0.1 M, pH 8.0) with 500 μg of egg lysozyme (Nutritional Biochemicals) and incubated for 2 to 5 min at 37 C. A 200-μg amount of Pronase (Calbiochem) was added, and the samples were kept overnight at 4 C. Before use in transformation experiments, the extracts were heated at 70 C for 15 min to inactivate the...
enzymes. The extracts were used as the DNA source at a dilution of 1:10 to 1:20 within 1 to 3 days.

Thymine starvation procedure. Cultures, grown overnight on MinCATT agar plates at 30 °C, were suspended in MinCATT medium at an OD of 50 Klett units and incubated with shaking at 37 °C. The culture was diluted with fresh medium after each doubling. After two to three doublings, the culture was filtered, washed with MinCATT medium, and suspended in the same medium at an OD of 80 Klett units.

Mutagenesis and selection of TLD-resistant mutants. A method was designed to select for conditional TLD-resistant mutants. An exponential culture was spun and resuspended in the same volume of tris(hydroxymethyl)aminomethane-maleic buffer (0.05 M, pH 6.0) with 100 μg of nitrosoguanidine per ml. The suspension was incubated for 30 min at 30 °C, washed, suspended in MinCATT medium, and incubated overnight with shaking at 30 °C. The culture was diluted into fresh medium at an OD of 50 Klett units and allowed to double twice. It was then diluted to an OD of 50 Klett units, incubated at 48 °C for 90 min, washed, transferred to MinCATT medium, and incubated at 37 °C for additional 90 to 120 min. Survival from TLD was approximately 0.1 to 1%. Appropriate dilutions were spread on blood agar base medium (Difco), left at room temperature overnight, and then transferred to 48 °C for 4 to 5 h. Colonies smaller than the average size were picked and tested for inability to grow at 48 °C and for sensitivity to TLD at the permissive and restrictive temperature.

Labeling procedure. For alkaline sedimentation studies, cellular DNA was labeled by growth for three generations in MinCATT medium containing 5 μg of cold thymine and 10 μCi of [3H]thymidine per ml (5 Ci/mmol) or 5 μCi of [14C]thymidine per ml (50 mCi/mmole, New England Nuclear Corp.).

Sedimentation studies. Cells, centrifuged in the cold, were suspended in 1 ml of ice-cold saline-EDTA (0.1 M, pH 8.0) containing 500 μg of egg lysozyme, and kept in ice for 5 min.

Linear alkaline sucrose gradients (12 ml; 5 to 20% [wt/vol] sucrose; 0.1 M NaOH; 10-4 M EDTA; and 0.5% sodium dodecyl sulfate) were overlaid with 0.1 ml of 0.5 M NaOH and 0.5% sodium dodecyl sulfate. A 0.05-ml volume of cell suspension containing about 5 × 106 cells was layered onto each gradient, and the gradients were kept for 20 min at room temperature to allow for cell lysis. The gradients were then centrifuged for 2 h at 37,000 rpm at 20 °C in a Spinco SW41 rotor. About 30 fractions of 25 drops each were collected and assayed for acid-insoluble radioactivity.

Determination of radioactivity. The procedure for determination of radioactivity was essentially as described previously (30). For total radioactivity, a washed culture sample was heated in 5% trichloroacetic acid for 20 min at 90 °C, cooled, and centrifuged 10,000 × g for 10 min. The supernatant was extracted four times with ether to remove the trichloroacetic acid, and 0.1 ml was added to 5 ml of scintillation fluid. Two volumes of toluene, 1 volume of Triton, 0.4% 2,5-diphenyloxazole, and 0.3% 2,4-bis-(5-phenylazoyl)-benzene] were added to dry filters. Radioactivity was measured in a Packard liquid scintillation spectrometer, model 3310.

DNA synthesis. Incorporation of [3H]adenine into alkali-stable, acid-precipitable material was used as a measure of DNA synthesis. A 0.5-ml volume of culture sample was added to 1 ml of 1 M KOH and kept overnight at room temperature; 0.5 ml of 50% trichloroacetic acid was added to each sample. The samples were chilled and diluted further with 2 ml of cold 5% trichloroacetic acid. Precipitates were collected on membrane filters and counted as above.

RESULTS

Effect of thymine starvation and MC treatment on B. subtilis strains E10 and 109. Figure 1a shows the survival curve of a thymine-requiring mutant, strain E10, and of its temperature sensitive (ts) derivative, strain 109, when they are incubated in complete medium lacking thymine. The loss of viability in strain E10 is exponential for three 10-min periods during 150 min of incubation. Strain 109 is several times more resistant. Figure 1b shows OD measurements of both strains taken during thymine starvation. It can be seen that strain E10 increases in mass about threefold within 120 min of starvation and starts lysing, whereas strain 109 increases its mass fourfold during 3 h of starvation with no signs of lysis. When the same cultures are treated with mitomycin C (MC), a similar pattern is observed (Fig. 1c). Treating B. subtilis 168 with MC (13, 26; M. Haas, Ph.D. thesis, Univ. of California, Berkeley, 1968), starving it for thymine (23, 33), or irradiating it with ultraviolet light (16) induces defective prophages such as PBSX (26, 34) and PBSH (13; M. Haas, Ph.D. thesis), resulting in cell lysis. Strain E10 is supposed to be cured of phage PBSX (12), and therefore the lysis of this

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strain during thymine starvation could be due to the induction of phage PBSH. Strain 109, on the other hand, seems to have lost the ability to be induced and behaves as a nonlysogen.

**Biological activity of DNA from thymine-starved cultures.** In the following experiment, the integrity of the DNA from thymine-starved strains E10 and 109 was examined by using cell lysates, carefully prepared to minimize nucleolytic damage, as a DNA source in transformation experiments. Transformation to adenine and histidine independence is seen in Fig. 2a and b. It can be seen that the transforming activity in lysates from strain E10 decreased with time of incubation in the absence of thymine, whereas those of strain 109 did not lose activity during 5 h of starvation. Similar results were obtained when transformants to both tryptophan and histidine independence, which are about 70% linked, were scored (results not shown).

**Sedimentation studies.** The integrity of the DNA from thymine-starved cultures was studied further by examining the pattern of DNA sedimentation in alkaline sucrose gradients. It can be seen in Fig. 3a that DNA from strain E10, incubated for 90 min without thymine, shows a peak in the same position as the control peak and a considerable amount of DNA with smaller sedimentation values. In strain 109 (Fig. 3b), the DNA from the thymine-starved culture is very similar in size to that of the control culture, with a small fraction of lower molecular weight. Strain E10 starts lysing after 90 min of starvation; therefore, only the 90-min sample was taken for sedimentation studies.

**Degradation and release of DNA.** Figure 4 shows the extent of DNA degradation to acid-soluble material and the amount of DNA (acid-insoluble fraction) released to the medium during thymine starvation. Exponential cultures were labeled for 3 min with [3H]thymine, washed, and chased for 20 min with nonradioactive thymine. [14C]thymine was added to the culture for 3 min, and then the culture was washed and suspended in thymine-free medium. It can be seen (Fig. 4a) that the newly replicated [14C]-labeled DNA is degraded to acid-soluble material in both strains E10 and 109 prior to the [3H]-labeled DNA of behind the replicating fork, and to a greater extent. In strain E10, the degradation is almost three times more extensive than that observed in strain 109. Figure 4b shows the amount of radioactive material found extracellularly during thymine starvation. Within 150 min, about 70% of the labeled newly replicated DNA of strain E10 is found extracellularly, whereas only about 10% of the label is found extracellularly in strain 109. Most of the total extracellular label is comprised of acid-insoluble material.

**Cell lysis and DNA degradation.** In the previous experiments, it was shown that in an E10 culture, within 2 h of thymine starvation, a considerable amount of DNA is found extracellularly. This suggested that lytic enzymes associated with phage maturation cause weakening of the permeability barriers, allowing leakage before cell lysis is apparent.

The integrity of the cell wall can be examined by OD measurements of a culture suspended and incubated in phosphate buffer (24). Figure 5a shows the increase in OD in cultures E10 and 109 during thymine starvation; Fig. 5b shows OD measurements of culture samples incubated in phosphate buffer. Curve A shows the rate of turbidity loss at the beginning of starvation, being the normal rate for young actively grow-
FIG. 2. Transforming activity of cell extracts. Culture samples were taken during thymine starvation for the preparation of cell extracts. Extract (0.05 to 0.1 ml) was added to 1 ml of competent culture of strain BD25, and the mixture was incubated for 30 min at 37°C. Drops (0.01 to 0.05 ml) of appropriate dilutions were placed on minimal plates. Transformants were scored for adenine independence (separate experiments) (●) (average data for adenine, ○); and histidine independence (○) (average of several experiments). (a) Strain E10. (b) Strain 109.

FIG. 3. Alkaline sucrose gradient profile of DNA from (a) strain E10 and (b) strain 109. Samples from the exponential culture (\(^{3}H\)) and from cultures after 90 min of thymine starvation (\(^{14}C\)) were mixed, treated with lysozyme, and layered on sucrose gradients. Symbols: ●, \(^{3}H\); ○, \(^{14}C\).

Effect of readdition of thymine to thymine-starved strain 109. It has been suggested (20) that the lethal event in TLD could occur upon readdition of thymine when DNA replication is resumed and before repair of damaged regions is complete. Although excessive DNA breakdown was not observed in thymine-starved cultures of strain 109, some degradation, which may be
tion began multiplying without delay. When 0.2 µg of thymine per ml is added to a starved culture, the rate of DNA synthesis is slower than that observed with 5 µg, indicating that only the resumption of DNA replication at a normal rate can rescue the surviving fraction from TLD (Fig. 6b).

**Correlation between lysogeny and sensitivity to TLD.** Strain 109 was isolated, after mutagenesis, as a temperature-sensitive mutant whose defect was not identified. This strain, at the same time, lost its lysogenic properties (Fig. 1). Experiments were done to examine (i) whether non-inducibility is associated with the ts mutation and (ii) its effect on the pattern of TLD. Ten spontaneous revertants to ts* behaved exactly as strain 109 ts in the absence of thymine (results not shown), indicating that the two phenomena are not associated. When strain 109 was transformed to ts* with DNA from strain E10, two out of ten transformants examined regained the sensitivity to thy-

**Fig. 4.** Degradation of DNA at and behind the replicating fork. Exponential-phase cultures of strain E10 and 109 in MinCATT medium were washed and suspended in MinCAT medium (lacking thymine); 10 µCi of [3H]thymidine per ml (5 Ci/mmol) was added for 3 min. The cells were washed and suspended in MinCATT medium and incubated for 20 min at 37°C. The cells were then washed, suspended in MinCAT medium with 5 µCi of [14C]thymine per ml (50 mCi/mmol), and incubated for 3 min at 37°C. The cultures were washed of radioactivity, suspended in MinCAT medium, and incubated at 37°C. Small volumes were removed for measurements of radioactivity in (a) the acid-soluble fraction and (b) total extracellular radioactive material. Strain E10: ●, 14C; ○, 3H. Strain 109: ▲—▲, 14C; △—△, 3H. Extracellular acid-insoluble material, strain E10: ×—×, 14C; •—•, 3H; strain 109: ■, 14C; □, 3H.

**Fig. 5.** Growth and proneness to lysis in thymine-starved cultures. (a) Optical density: left curve, strain 109; right curve, strain E10. (b) Culture samples A through D were filtered, suspended in warm phosphate buffer, and incubated with aeration at 37°C; incubation time is as in (a). Optical density measurements (Klett-Summerson, filter 42) are expressed as percentages of the turbidity at time zero.
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DISCUSSION

This communication shows that the pattern of TLD in a nonlysogenic derivative of B. subtilis is significantly different from that observed in the lysogenic one. The latter strain dies faster and its DNA is considerably degraded, whereas in the nonlysogen the integrity of its DNA is affected only slightly. These results suggest a correlation between the induction of phage during thymine starvation and DNA degradation. Progressive DNA breakdown could be a direct result of nucleolytic activity associated with phage functions during maturation, or an indirect consequence of the physiological changes that occur in the cell as a result of the phage lytic enzymes.

The role of nucleolytic activity associated with phage functions in DNA degradation in B. subtilis, after MC induction, was suggested by Okamoto et al. (26). In their study, about 40% of the degradation seems to be very specific in forming only 22S fragments, which are packed in the phage head. During the induction of bacteriophage lambda by MC or thymine starvation, new nucleolytic activity was observed (19). On the other hand, there is evidence that inhibition of DNA synthesis in DNA mut mutants (4), by nalidixic acid (30) or by thymine starvation (32), causes preferential degradation of DNA at the replicating point. In our study, progressive degradation to acid-soluble material was observed mainly in the thymine-starved

FIG. 6. Thymine-starved strain 109: effect of readdition of thymine on (a) resumption of cell division, (b) DNA synthesis. (a) After 90 min of thymine starvation, the culture was divided into three portions: (i) untreated, (ii) 5 μg of thymine added per ml, (iii) 0.2 μg of thymine added per ml. The cultures were incubated further, and samples were withdrawn for viable counts. (b) At the beginning of thymine starvation 0.5 μCi of [3H]adenine per ml (1.1 mCi/mmol) and 5 μg of cold adenine were added. Samples were taken for the measurement of radioactivity incorporated into alkali-stable DNA fraction.
lysogenic strain, and only a negligible amount of degradation was found in the "cured" derivative. In the latter strain, 5 h of thymine starvation did not have any effect on the transforming activity of its DNA. There was not even a reduction in the degree of linked transformation, indicating that the extent of DNA breakdown is minimal. It is possible that in both strains inhibition of DNA synthesis causes nucleolytic damage. However, the progressive weakness in the permeability barriers observed during thymine starvation in the lysogenic strain (Fig. 5) allows the depletion of the nucleotide pool to the medium, thus preventing any possible repair. In the nonlysogenic strain which does not lyse, hardly any loss of radioactive label was found extracellularly. There is evidence that repair of DNA breaks by DNA polymerase I takes place in the absence of thymine (3, 31, 39). A correlation between poor repair of lesions in ultraviolet light-irradiated DNA and cell lysis was suggested by Kantor and Barnhart (18).

There is conflicting evidence concerning the extent of DNA breakdown during thymine starvation (1, 35, 40). It seems reasonable to assume that, in addition to the nucleolytic activity which may operate whenever DNA synthesis stops (4), secondary factors which favor or interfere with immediate repair will affect the final result.

DNA degradation was suggested by several authors to be the cause of TLD (20). If DNA breakdown occurs in strain 103 during thymine starvation, it is very limited. Readdition of thymine to starved cultures, at low concentration, shown to reduce drastically the velocity of replication (2), should have increased the chances of repair and allowed at least partial recovery from TLD. Recovery was not observed and the rate of TLD continued, as in the absence of thymine, suggesting that DNA damage may not be the primary factor causing death in the nonlysogenic strain of *B. subtilis*.

ACKNOWLEDGMENTS

This investigation was supported by grants from The Central Research Fund of the Hebrew University and from the Advancement of Mankind Foundation.

LITERATURE CITED


