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Radiosensitivity of Spores of DNA Polymerase Deficient Mutants of *Bacillus subtilis*

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The radiosensitivity to γ-ray of the spores and the vegetative cells of *Bacillus subtilis* mutants with the reduced DNA polymerase activity, was compared with that of their parent strains. Both the spores and the vegetative cells of the mutants were approximately 2 times as sensitive to γ-ray as those of the parent strains. It is suggested that the differences in radiosensitivity between the mutant spores and the parent spores are at least partly attributable to the genetic differences between the strains in themselves. The spores of the mutants employed were biologically "normal" in many respects with the exception of the sensitivity to chloramphenicol and the cell size.

INTRODUCTION

The spores of bacteria are much more resistant to ionizing radiations than their progenitor vegetative cells and also ordinary cells of most of the non-spore-forming bacteria.[1,2] It has been known that DNA molecule is the principal target responsible for radiation damages in the spores and in the vegetative cells, and that difference between the spores and the vegetative cells with respect to the resistance to ionizing radiations reflects the difference of the state of DNA between the spores and the vegetative cells.[3~5] The radioresistance of the spores, however, has not yet been studied from the genetical point of view.

In order to get further information about the mechanism involved in the radioresistance of the bacterial spores, we examined in the present study the radioresistance of genetically different strains of spore-forming bacteria: spores and vegetative cells of the mutants of *Bacillus subtilis* having the reduced DNA polymerase activity, were compared each other with respect to the radioresistance of the colony-forming ability.

MATERIALS AND METHODS

Bacterial strains: *Bacillus subtilis* Marburg 168Tt (thy$^-$, try$^-$) and HA101 (his$^-$, leu$^-$, met$^-$) were used as parent or wild type strains. Strains 160C5 (thy$^-$, try$^-$) and HA160 (his$^-$, leu$^-$, met$^-$) are the mutant strains of *B. subtilis* strains 168Tt and HA101, respectively. These mutant strains have impaired DNA polymerase activity (see Table 1), and are sensitive to ultraviolet light (UV) and methyl methanesulfonate (MMS).[6,8] Of these strains HA160 and 160C5 were isolated by Gass *et al.*[6] and Munakata,[7] respectively.

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Table 1. DNA polymerase activities in crude extracts of various strains of *Bacillus subtilis*.

Strains	Template		Origin or reference
	Calf thymus DNA	Endogenous DNA	
168Tt	5,107 cpm	1,754 cpm	Farmer & Rothman (15)
HA101	4,989	2,247	Gass *et al.* (6)
160C5	1,152	222	Munakata (7)
HA160	1,344	357	Hill *et al.* (8)

Preparation of spores and vegetative cells: Spores and vegetative cells were prepared by growing the strains at 37°C in the Schaeffer medium.[9] Vegetative cells were harvested by centrifugation after reaching the late log-growing phase. Spores were formed in the same medium by 36 hrs' incubation. After being harvested, spores were purified by treatment with 500 μg/ml lysozyme for 40 min and then 1 % sodium lauryl sulfate for 20 min at 37°C in order to remove remaining vegetative cells and debris of sporangia. The spores prepared like this were finally washed ten times with distilled water.

Measurement of sensitivity to γ-ray: Spores or vegetative cells, suspended in 1/15 M phosphate buffer (pH 7.2) at the concentration of 10^8 cells/ml, were exposed to ^{60}Co γ-ray irradiation at cold temperature in air (dose rate: about 100 Krad/hr). The survivors were counted by using nutrient agar plate method.

Measurement of heat resistance: Spore suspension in phosphate buffer which was prepared to have an optical density (660 mμ) of 0.54, was incubated at 80°C. At intervals, aliquot samples were taken and the viable counts were made using nutrient agar plate method.

Germination of spores: Spores were germinated by shaking at 37°C in the Demain medium,[10] supplemented with 100 μg/ml L-tryptophan and 10 μg/ml thymine. Turbidity (optical density at 560 mμ) decrease of the culture was chased as a measure of germination.

Assay of DNA polymerase activity: Crude extracts for assay were prepared by treatment with lysozyme-Brij as described by Gass *et al.*[6] The assay mixture (0.5 ml) contained 65 mM Tris-HCl (pH 8.0), 6.5 mM $MgCl_2$, 25 μM dATP, dGTP and dCTP, 5 μM TTP, 0.5 μCi ^{3}H-TTP, calf thymus DNA as template and the extracts as enzyme.

Measurement of content of dipicolinic acid (DPA): The content of DPA in the cells was measured according to the method described by Janssen *et at.*[11]

RESULTS AND DISCUSSION

The dose-survival curves of spores and vegetative cells of various strains of *B. subtilis* are shown in Fig. 1. In both the spores and the vegetative cells, the colony-forming ability of the MMS-sensitive mutant strains (160C5 and HA160) with the reduced DNA polymerase activivity was approximately 2 times as sensitive to γ-ray as that of the parent and wild type strains (168Tt and HA101). It may be due to the limited repairing ability of cells of the mutant strains, as reported in the DNA

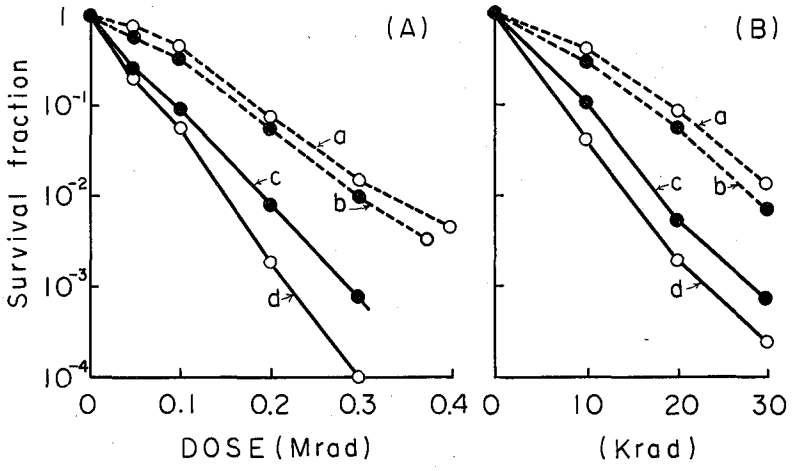

Fig. 1. Dose-survival curves of spores (A) and vegetative cells (B) of *B. subtilis*.
(a) strain 168Tt; (b) strain HA101; (c) strain HA160; (d) strain 160C5.

polymerase deficient mutant *pol Al* of *Escherichia coli*.[12] These facts suggest that the differences between the spores of the mutants and those of wild type strains in regard to the response to ionizing radiation are at least partly attributable to the genetic differences between the strains in themselves. Furthermore, it seems that the differences between the wild type strains and the mutants in radiosensitivity found with the spores parallel those found with the vegetative cells. Further studies along

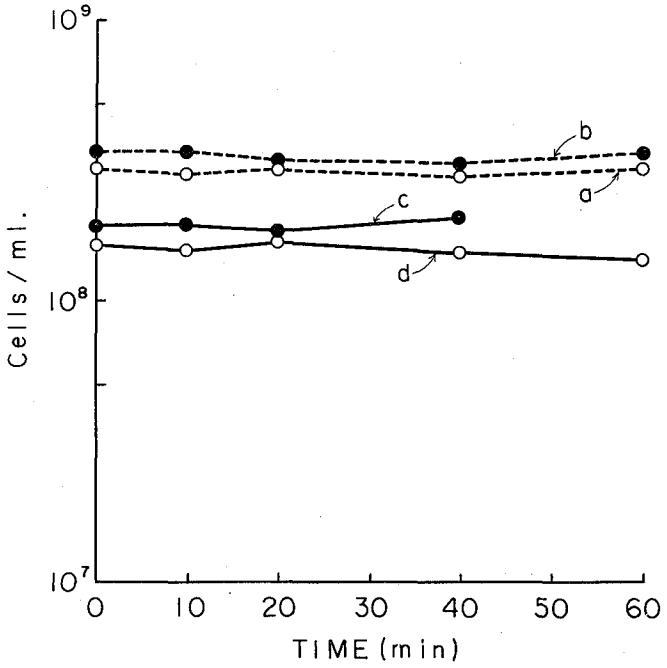

Fig. 2. Survivals of *B. subtilis* spores during incubation at 80°C.
(a) strain 168Tt; (b) strain HA101; (c) strain HA160; (d) strain 160C5.

this line are now in progress.

In order to confirm whether or not the spores of the mutant strains are biologically "normal", with the exception of the sensitivity of ionizing radiation, some physiological and morphological properties of the mutant spores were examined. As far as we observed, (i) Spores of the mutant strains 160C5 and HA160 could survive the heating at 80°C for 40 min (Fig. 2). (ii) The contents of DPA in spores were $4.1\times\times10^{-8}$ μg/spore for strain 160C5, 2.9×10^{-8} μg/spore for strain HA160, 2.3×10^{-8} μg/spore for strain HA101 and 2.1×10^{-8} μg/spore for strain 168Tt, respectively. These data indicate that the strains 160C5 and HA160 are not sensitive to the heat. (iii) During germination of the spores of strain 160C5 or 168Tt in the tryptophan and thymine supplemented Demain medium, the change in turbidity of the culture was chased (Fig. 3). The turbidity decreased to the lowest value in the first 1 hr of incubation and then increased after 3 hrs. (iv) The effect of postirradiation treatment of chloramphenicol (CM) on the survival of the spores of strains 160C5 or 168Tt is shown in Fig. 4. In this experiment the spores of strains 160C5 and 168Tt were irradiated by various doses of γ-ray and then germinated for 60 min at 37°C in the presence of CM (100 μg/ml). These treatment caused little or no reduction of the survival of spores of strain 168Tt, but reduced slightly the survival of spores of

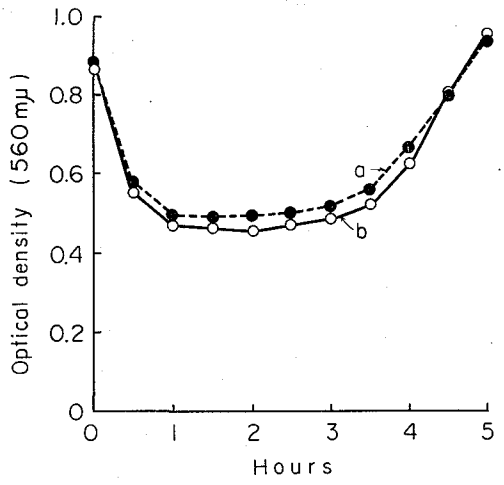

Fig. 3. Change of turbidity of suspension of *B. subtilis* spores during germination.
(a) strain 168Tt; (b) strain 160C5.

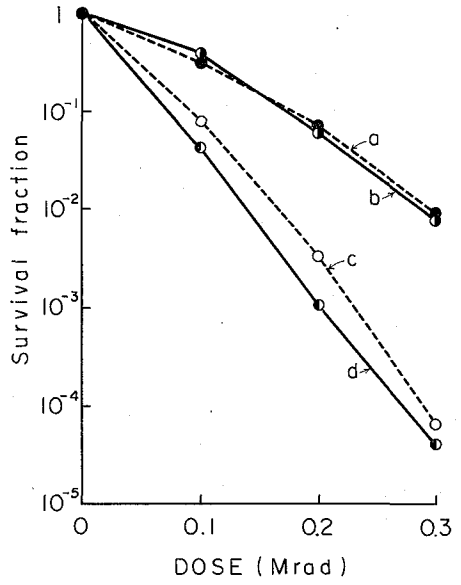

Fig. 4. Effect of chloramphenicol (CM) on survival of *B. subtilis* spores, irradiated at various doses.
(a) 168Tt spores, irradiated and incubated for 60 min; (b) 168Tt spores, irradiated and incubated for 60 min in the presence of CM (100 μg/ml); (c) 160C5 spores, irradiated and incubated for 60 min; (d) 160C5 spores, irradiated and incubated for 60 min in the presence of CM (100 μg/ml).

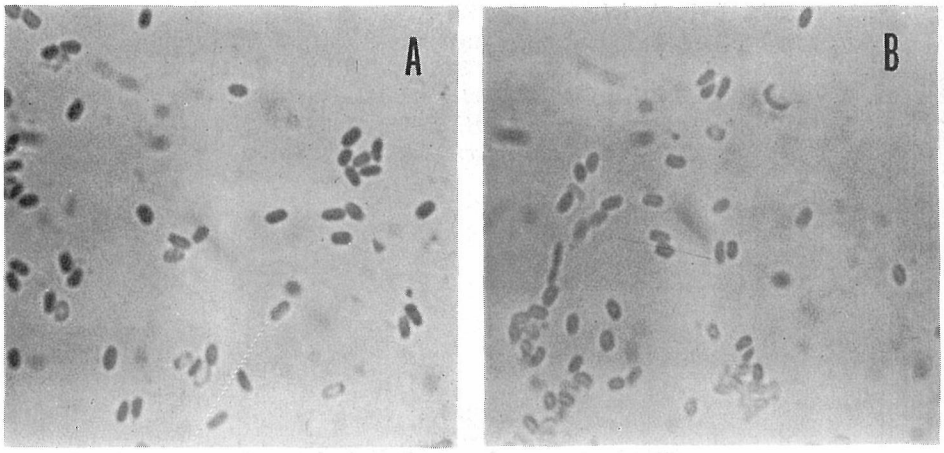

Fig. 5. Phase-contrast micrographs of *B. subtilis* spores.
(A) strain 160C5; (B) strain 168Tt.

strain 160C5. Survival during germination of the non-irradiated spores of both the strains 168Tt and 160C5 was not affected by the CM treatment. In the cases of several strains of *E. coli*, it has been reported that CM reduced the survival after X-irradiation.[13,14] (v) Phase microscopic photographs of spores of strains 160C5 and 168Tt are shown in Fig. 5. The average cell size of spores of strain 160C5 was about 1.5 times bigger than that of strain 168Tt. The fact that the ratio of viable count to optical density of the spore suspension of strain 160C5 was lower than that of strain 168Tt (see Fig. 2) may be due to the difference in cell size between strain 160C5 spores and strain 168Tt spores.

These results suggest that the spores of the mutant strains 160C5 and HA160 are biological "normal", though the sensitivity to CM and the cell size of the mutant spores were different from those of the parent and wild type strains.

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