Changes in Deoxyribonucleic Acid Polymerase Activities and Synthesis of Deoxyribonucleic Acid During Sporulation of *Bacillus subtilis*

MASARU HONJO, YUJI SHIBANO, AND TOHRU KOMANO*

Laboratory of Biochemistry, Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan

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The deoxyribonucleic acid (DNA) polymerase activities in *Bacillus subtilis* strains Marburg 168 (*thy⁻ trp₂⁻*) and D22, a DNA polymerase I-deficient mutant, were measured at various stages of sporulation. The DNA polymerase I activity, which had decreased after the exponential growth, began to increase at the early stage of sporulation, reached a maximum and then again decreased. The activity of neither DNA polymerase II nor III was observed to change so drastically as that of DNA polymerase I during sporulation. The incorporation of [³H]deoxythymidine 5'-triphosphate ([³H]dTTP) into Brij 58-treated permeable cells increased during sporulation. The stimulation of [³H]dTTP incorporation into the cells by irradiation with ultraviolet light was also observed to coincide with DNA polymerase I activity. In strain D22, the activities of DNA polymerase II and III were almost constant with time. Neither change of [³H]dTTP incorporation into Brij 58-treated cells nor stimulation of incorporation by irradiation with ultraviolet light was observed.

It is known that bacterial sporulation is a process associated with changes in activities of many different kinds of enzymes such as protease (20), alkaline phosphatase (17), and glucose-phosphoenolpyruvate-transferase (7). As for deoxyribonucleic acid (DNA) polymerase in *Bacillus subtilis*, it has been reported by Falaschi and Kornberg (5) that the bulk of activity disappears during sporulation. Ryter and Aubert have observed that DNA replication of sporulating *B. subtilis* ceases around t_1 , i.e., before the last nuclear division (22). According to Mandelstam, Sterlini, and Kay (18), occurrence of DNA replication is essential for spore induction when cells are transferred to a poor medium. It is also known that the configuration and location of DNA alter at an early stage of sporulation in *B. subtilis* (14, 18). Spore-specific photoproducts are produced by irradiation of ultraviolet light as sporulation proceeds (13, 26). It is considered, therefore, that in spore formation there are some relationships between the changes of DNA configuration, such as filamentation, separation, and functions of DNA polymerases.

An attempt has been made to elucidate the detailed relation between DNA synthesis and sporulation. In this paper, it is reported that there are changes in levels of DNA polymerase activities and capacity of DNA synthesis during sporulation of *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strain Marburg 168 (*thy⁻ trp₂⁻*) (6) was used as a wild-type strain. Strain D22 (*thy⁻ trp₂⁻*), lacking a detectable level of DNA polymerase I activity (24), isolated by Munakata from the Marburg 168 (*thy⁻ trp₂⁻*) strain, was kindly supplied by H. Terano.

Growth and sporulation. Vegetative cells of bacteria were transferred into Schaeffer medium and the culture was incubated at 37°C with vigorous shaking. The composition of this medium was nutrient agar (Difco), 8 g/liter; MgSO₄·7H₂O, 0.25 g/liter; KCl, 1.0 g/liter; 10⁻⁶ M FeSO₄; 10⁻³ M Ca(NO₃)₂; and 10⁻⁵ M MnCl₂; pH 7.0. The growth was monitored by absorbance at 650 nm by a Hitachi model 101 spectrometer. The number of spores was determined by counting viable colony-forming units after heating the cell suspension, which was diluted 10⁴-fold with saline, at 80°C for 10 min.

Chemicals. *p*-Chloromercuriphenylsulfonate (pCMS) was purchased from Sigma Chemical Co. *N*-ethylmaleimide was obtained from Nakarai Chemicals Co. Nucleoside triphosphates were purchased from Sigma Chemical Co. and Boehringer Mannheim Co. Brij 58 was the product of Kao-Atlas Industries. [methyl-³H]deoxythymidine 5'-triphosphate ([³H]dTTP) (30 Ci/mmol) and [methyl-³H]thymidine (22.3 Ci/mmol) were obtained from the Radiochemical Centre, Amersham/Searle. 6-(*p*-Hydroxyphenylazo)-2,4-dihydropyrimidine (HPUra) was generously supplied by B. Langley of Imperial Chemical Industries, Ltd. Lysozyme was purchased from Sigma Chemical Co.

Assay of DNA polymerase activity. DNA polym-

erase was measured by the modified method (8) of Okazaki and Kornberg (21). The assay mixture (total volume of 0.25 ml) contained 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, 5 mM $MgCl_2$, 10 μM deoxythymidine 5'-triphosphate (dTTP), 50 μM each deoxyadenosine 5'-triphosphate, deoxycytidine 5'-triphosphate, and deoxyguanosine 5'-triphosphate, 0.5 μCi of [3H]dTTP, 25 μg of activated calf thymus DNA per ml as a template, and 0.025 ml of the cell lysate. Preparation of the cell lysate was described in the legend to Fig. 2. After incubation at 37°C for 30 min, the reaction mixture was chilled in ice. A 0.25-ml amount of chilled yeast ribonucleic acid solution (1 mg/ml) as carrier and 0.5 ml of chilled 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate were added to the mixture. The precipitates were collected on Whatman GF/C glass paper disks and washed with a total volume of 40 ml of chilled 5% trichloroacetic acid containing 0.05 M sodium pyrophosphate. The disks were dried and immersed in 6.0 ml of a toluene-based scintillator. The radioactivity was measured by a Beckman liquid scintillation counter. To distinguish each DNA polymerase activity, 0.4 mM pCMS or 2.0 mM *N*-ethylmaleimide was added to the mixture. Under these concentrations *N*-ethylmaleimide inhibits only DNA polymerase III activity, whereas pCMS inhibits DNA polymerase II and III activities (11). The protein concentration of the cell lysate was measured by the method of Lowry et al. (16).

Assay of alkaline phosphatase. Alkaline phosphatase activity of the cell lysate (0.4 ml) was assayed by the method of Sterlini and Mandelstam (23), in which *p*-nitrophenylphosphate was used as substrate and the liberated color was measured at an absorbancy at 410 nm.

Brij 58 treatment and assay of [3H]dTTP incorporation. The methods of Brij 58 treatment and [3H]dTTP incorporation were essentially the same as those described in the previous paper (8). Cells were collected from 10 ml of sporulating culture and suspended in 0.9 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 6.6 mM $MgCl_2$. After addition of 0.1 ml of 5% Brij 58, the suspension was incubated for 15 min at 37°C. The cells were collected and suspended in 0.5 ml of the same buffer without detergent. The assay mixture (total volume of 0.5 ml) contained 50 mM Tris-hydrochloride buffer, pH 8.0; 5 mM $MgCl_2$; 2 mM 2-mercaptoethanol; 10 μM dTTP; 0.5 μCi of [3H]dTTP; 50 μM each deoxyadenosine 5'-triphosphate (dATP), deoxycytidine 5'-triphosphate and deoxyguanosine 5'-triphosphate; 0.2 ml of the cell suspension; and 0.4 mM ATP when needed. The mixture was incubated at 37°C for 30 min and chilled. A 0.2-ml amount of yeast ribonucleic acid (1 mg/ml) was added to the mixture as carrier, and the product was precipitated by adding 0.5 ml of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate. The filtration method of acid-insoluble materials and radioactivity measurement were the same as described above.

Incorporation of [3H]thymidine into sporulating cells. A 0.5-ml sample was withdrawn from a sporu-

lating culture and transferred into a small test tube (12 by 100 mm) that contained 0.5 μCi of [3H]thymidine (in 0.02 ml). The culture was then incubated at 37°C with vigorous shaking. Ten minutes later, the culture was chilled in ice and 1 ml of 10% trichloroacetic acid was added. The filtration method of the acid-insoluble materials and radioactivity measurement were the same as described above.

Irradiation of ultraviolet light. Throughout this study, the irradiation of ultraviolet light was carried out at a dose rate of 20 ergs/mm²·sec. As the source of ultraviolet light, a 20 W germicidal lamp (Matsushita Electric Co.) was used.

RESULTS

Levels of DNA polymerase activity during sporulation. When *B. subtilis* strain Marburg 168 (*thy*⁻ *trp*₂⁻) growing in nutrient broth culture was transferred into Schaeffer medium, heat-resistant spores started to appear after about 12 h. The sporulation level increased gradually up to 20 h and was 90% at 24 h after transferral into Schaeffer medium. Essentially the same growth curve was obtained with strain D22 (Fig. 1).

In the Marburg 168 (*thy*⁻ *trp*₂⁻) strain, total activity of DNA polymerases in the lysate, which had decreased after the exponential growth, was observed to increase at the early stage of sporulation and reached the maximum

FIG. 1. Bacterial growth and sporulation. Each strain was precultured overnight in nutrient broth medium; then 3 ml of the culture was withdrawn, inoculated into 150 ml of Schaeffer medium in a 500-ml flask, and incubated at 37°C with vigorous shaking. The bacterial growth was measured by absorbance at 650 nm. The percent of sporulation was calculated from a sample of 0.1 ml that was withdrawn at indicated times, diluted to 10⁴-fold, and divided into equal portions. One portion was treated with heat and the other was not. A portion was plated, and the number of colonies formed were compared. Symbols: ●, Bacterial growth; ○, sporulation; —, Marburg 168 (*thy*⁻ *trp*₂⁻); - - -, strain D22.

FIG. 2. Changes in DNA polymerase and alkaline phosphatase activities during sporulation. Samples of 8 ml were withdrawn from sporulating culture at indicated times. Cells were collected by centrifugation at $6,000 \times g$ for 10 min, washed once with saline, and suspended in 0.8 ml of chilled 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.8, containing 0.14 M sodium chloride, 1 mM 2-mercaptoethanol and 1 mM ethylenediamine-tetraacetic acid. Then 0.2 ml of lysozyme solution (1 mg/ml in the same buffer) was added to the suspension. After incubation at 37°C for 30 min, the resultant clear solution was centrifuged at $10,000 \times g$ for 10 min, and the supernatant fluid was used as the cell lysate. The detailed assay method is described in Materials and Methods. Symbols: ●, DNA polymerase activity; ○, alkaline phosphatase activity; ×, bacterial growth.

at the middle stage of sporulation (Fig. 2). The increased activity in the Marburg 168 (*thy⁻ trp₂⁻*) strain then decreased rapidly with progress of sporulation. In strain D22, DNA polymerase activity was low throughout sporulation.

To examine whether the cell lysate preparative method was satisfactory or not, alkaline phosphatase activity was also assayed. As shown in Fig. 2, change in activity of alkaline phosphatase in the lysate showed the well-known pattern with progress of sporulation as reported by Warren (27).

It is known that *B. subtilis*, like *Escherichia coli*, has three distinct DNA polymerases (DNA polymerase I, II, and III) (11). The DNA polym-

erases can be distinguished from one another by their sensitivity to thiol-blocking agents (11, 25). It was considered that the DNA polymerase activity remaining in the presence of pCMS was due to DNA polymerase I, and that the activity that was inhibited by pCMS, but not by *N*-ethylmaleimide, reflected that of DNA polymerase II. The activity blocked by only *N*-ethylmaleimide was assigned to DNA polymerase III (11).

As shown in Fig. 3, the increase in DNA polymerase in the Marburg 168 (*thy⁻ trp₂⁻*) strain during sporulation was mainly due to DNA polymerase I. DNA polymerase II activity in this strain changed slightly and reached maximum at the time when DNA polymerase I reached maximum. DNA polymerase III activity was very low and changed only very

FIG. 3. Changes in cell-free DNA polymerase activities. DNA polymerase activity on the cell lysate, prepared as described in the legend to Fig. 2, was measured in the presence of 2.0 mM *N*-ethylmaleimide (NEM) or 0.4 mM pCMS. The activity that was not inhibited in the presence of pCMS was considered to be DNA polymerase I. The activity that was blocked by pCMS, but not by NEM, was considered to be DNA polymerase II, and that inhibited by only NEM was considered to be DNA polymerase III. One hundred percent represents the maximum activity observed in the Marburg 168 (*thy⁻ trp₂⁻*) strain DNA polymerase I during sporulation and is equivalent to 50,437 cpm per mg of protein for 30 min. This value was also used for the calculation of relative rates in strain D22. Symbols: ○, DNA polymerase I; ●, DNA polymerase II; ●, DNA polymerase III; ×, bacterial growth.

slightly. DNA polymerase II and III activities in strain D22 were low and almost constant throughout sporulation (Fig. 3). The formation of heat-resistant spores in strain D22 was, however, normal (Fig. 1).

[³H]dTTP incorporation into Brij 58-treated cells during sporulation. Sporulating cells were treated with Brij 58 and [³H]dTTP was incorporated in the system containing ATP. In the Marburg 168 (*thy*⁻ *trp*₂⁻) strain, the incorporation increased at the early stage of sporulation, which coincided with the increase in DNA polymerase I activity (Fig. 4). The pattern of [³H]dTTP incorporation activity into Brij 58-treated cells of Marburg 168 (*thy*⁻ *trp*₂⁻) strain during sporulation was nearly the same as that of DNA polymerase I activity. In strain D22, the level of [³H]dTTP incorporation was low and almost constant during sporulation.

When ATP was omitted from the assay system the level of the incorporation in the Mar-

burg 168 (*thy*⁻ *trp*₂⁻) strain was depressed remarkably (Fig. 4). When Brij 58-treated cells of the Marburg 168 (*thy*⁻ *trp*₂⁻) strain were irradiated by ultraviolet light, the incorporation was higher in the absence of ATP (Fig. 5). In strain D22, such a marked change of the [³H]dTTP incorporation was not observed, although the level of the incorporation with ATP was slightly higher than that without ATP (Fig. 4, 5).

If it is assumed that the stimulation of the incorporation in the Marburg 168 (*thy*⁻ *trp*₂⁻) strain by addition of ATP (Fig. 4) is the result of the repair of DNA damaged by an ATP-dependent deoxyribonuclease, the stimulation both by ATP and by ultraviolet light irradiation can be assigned to the repair synthesis by DNA polymerase I. An ATP-dependent deoxyribonuclease in *B. subtilis* has been reported by Chestukhin et al. (4).

[³H]thymidine incorporation into sporulating cells. Ryter and Aubert (22) reported that replicative DNA synthesis in *B. subtilis* ceases at the early stage of sporulation. Figure 6 shows that the incorporation of [³H]thymidine into sporulating cells decreased rapidly in both strains. This might reflect the cessation of DNA replication for cell division.

To confirm further that replicative DNA synthesis still continued at this stage, HPURA was added to the incorporation system. HPURA is a specific inhibitor of DNA polymerase III in *B. subtilis* and other gram-positive bacteria (1, 2, 15), and DNA polymerase III is known to be necessary for semiconservative replication of DNA (2). It was observed that the incorporation in both strains was at the same low level during sporulation (Fig. 6).

Thus, it seemed that the activity of DNA polymerase I that increased during sporulation did not participate significantly in the [³H]-thymidine incorporation. Therefore, this result might suggest that the increase in DNA polymerase I activity was due not to replicative DNA synthesis but to potential for repair synthesis. In the *B. subtilis* Marburg 168 (*thy*⁻ *trp*₂⁻) strain, we also obtained results to support the assumption that the efficiency of repair synthesis increased in vivo during the early stage of sporulation (data not shown).

DISCUSSION

The vegetative cells of *B. subtilis* have three distinct DNA polymerases, DNA polymerase I, II, and III (11), like *E. coli*. In the previous investigation (25), it is reported that the three DNA polymerases in the spores of *B. subtilis* cannot be distinguished from those in vegeta-

FIG. 4. [³H]dTTP incorporation into sporulation cells after Brij 58 treatment. A 10-ml amount was withdrawn from sporulating cultures at indicated times. [³H]dTTP was incorporated into Brij 58-treated cells with (●) or without (○) ATP as described in Materials and Methods. The final concentration of ATP was 0.4 mM. Symbol: ×, Bacterial growth.

FIG. 5. $[^3\text{H}]\text{dTTP}$ incorporation into Brij 58-treated cells after ultraviolet light irradiation. Harvested cells, as described in the legend to Fig. 3, were treated with Brij 58. The treated cells were suspended in 1 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 6.6 mM MgCl_2 ; the suspension was then placed in a petri dish (9 cm in diameter) and exposed to ultraviolet light at a dose rate of 20 ergs/mm 2 ·s for 75 s. To 0.2 ml of the irradiated suspension was added the $[^3\text{H}]\text{dTTP}$ incorporation assay mixture. In this assay, ATP was omitted. Symbols: $\bigcirc$ — $\bigcirc$, $[^3\text{H}]\text{dTTP}$ incorporation in the Marburg 168 ($\text{thy}^- \text{trp}_2^-$) strain; $\bigcirc$ — $\bigcirc$, $[^3\text{H}]\text{dTTP}$ incorporation in strain D22; $\times$ — $\times$, growth of Marburg 168 ($\text{thy}^- \text{trp}_2^-$); $\times$ — $\times$, growth of D22.

tive cells and that only about 20% of DNA polymerase activity in vegetative cells is recovered in the spores.

Present results show that the DNA polymerase I activity decreased at the stage of late exponential growth and then began to increase and reach a maximum during sporulation of *B. subtilis* (Fig. 3). It is known that DNA polymerase I functions in the repair of DNA damage (9, 10, 24). Therefore, the repair capacity is considered to increase during sporulation coinciding with the increase of DNA polymerase I activity. This is supported by the following results. (i) The $[^3\text{H}]\text{dTTP}$ incorporation into Brij 58-treated cells of the Marburg 168 ($\text{thy}^- \text{trp}_2^-$) strain increased as sporulation proceeded. The time of increase in $[^3\text{H}]\text{dTTP}$ incorporation coincided with that in DNA polymerase I activity. In strain D22, the $[^3\text{H}]\text{dTTP}$ incorporation into Brij 58-treated cells was not observed (Fig.

3, 4). (ii) The $[^3\text{H}]\text{dTTP}$ incorporation into Brij 58-treated cells of the Marburg 168 ($\text{thy}^- \text{trp}_2^-$) strain was stimulated by ultraviolet light irradiation in the absence of ATP. The time of this stimulation coincided with that of increase in DNA polymerase I activity (Fig. 5). The $[^3\text{H}]\text{dTTP}$ incorporation into permeable cells of strain D22 was not stimulated by the irradiation of ultraviolet light.

DNA polymerase II and III activities are considered to be almost unchanged. However, DNA polymerase II activity in the Marburg 168 ($\text{thy}^- \text{trp}_2^-$) strain shows rather high activity level and changes slightly. As described above, the activity of DNA polymerase II is calculated indirectly from the DNA polymerase activities measured in the presence of specific inhibitors. It is reported that about 20% of DNA polymerase I activity is reduced in the presence of 0.4

FIG. 6. $[^3\text{H}]\text{thymidine}$ incorporation into sporulating cells. Samples of 10 ml were withdrawn from sporulating cultures and were transferred into two 50-ml flasks at indicated times. To one of them was added HPURa at a final concentration of 80 μM ($\bullet$); to the other no HPURa was added ($\bigcirc$). Both samples were incubated at 37°C with vigorous shaking. After 30 min, 0.5 ml of each culture was transferred into a chilled small test tube, which contained 0.5 μCi of $[^3\text{H}]\text{thymidine}$ (0.02 ml). The incorporation was carried out during 10 min of incubation at 37°C with vigorous shaking. Symbol: $\times$, Bacterial growth.

mM pCMS (11). Therefore, in this assay, it must be noted that a part of DNA polymerase I activity might be estimated to be DNA polymerase II.

It was observed that the incorporation of [³H]dTTP into Brij 58-treated cells of the Marburg 168 (*thy⁻ trp₂⁻*) strain was lower when ATP was removed from the incorporation system. In this semi-in vitro system, semiconservative replication of DNA occurs and requires ATP (3, 19). But it has been reported that the replication is already completed at about this stage of sporulation in *B. subtilis* (22). Therefore, this DNA synthesis does not reflect the replicative DNA synthesis but repair-type synthesis that might arise from the DNA damage caused by ATP-dependent deoxyribonuclease. This type of repair has been described by Henneberry and Freese in *B. subtilis* (12).

The essential function of the observed increase in DNA polymerase I during sporulation is not clear. Because sporulation in strain D22 can proceed normally as judged by the formation of heat-resistant spores, the increase in DNA polymerase I activity apparently is not required in the process of sporulation. Nevertheless, it is possible that during sporulation some conformational change in the DNA molecule might have occurred. The DNA molecule in this state could be susceptible to environmental conditions so as to be damaged easily, so that the increase in activity of DNA polymerase I might enhance the capacity of DNA repair. The change in [³H]dTTP incorporation into Brij 58-treated cells in the sporulating cells under the condition of ultraviolet light irradiation may support this assumption.

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