DNA REPLICATION IN A POLYMERASE I DEFICIENT MUTANT AND THE IDENTIFICATION OF DNA POLYMERASES II AND III IN BACILLUS SUBTILIS

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Summary. A DNA polymerase I deficient mutant of Bacillus subtilis replicates DNA semiconservatively in an in vitro system. The hybrid density DNA, generated from a reaction, was biologically active and the DNA strands were separable. Two other polymerase activities present in the wild type, in addition to Polymerase I are identified in the mutant and are designated as DNA polymerases II and III.

Nonviable permeable cells of B. subtilis could promote synthesis of DNA from externally supplied deoxynucleoside triphosphates (dNTP) in a reaction that required ATP (1). This synthesis in the wild type cells was accompanied by extensive repair that obscured clear detection of semiconservative DNA synthesis. DNA polymerase I mutant (polA5) subjected to the same regimen (2), showed reduced repair and allowed a cleaner demonstration of DNA synthesis. The in vitro synthesized hybrid DNA was biologically active and the strands of template and product DNA were separable after denaturation. Wild type B. subtilis, like E. coli (3-6), possesses at least three DNA polymerases. The major activity, i.e., DNA polymerase I, was absent in the polA5 mutant. The two other activities, designated as polymerases II and III, are present in the polA5 mutant and their isolation is reported here.

MATERIALS AND METHODS

B. subtilis SB 1058 (trp_2 his_2 phe_1) and SB 1060 (trp_2 his_2 phe_1 polA5) were used in these studies. Permeable cells (1,2) were made and incorporation was followed as in legend of Fig. 1. DNA polymerase assays were performed using poly(d[A-T]), "activated" salmon sperm DNA and exonuclease III treated DNA templates (4,6). Labeled substrates in the reaction mixtures were ^{3}H-dTTP (4 x 10^4 cpm/nmole) or ^{3}H-dATP (1.2 x 10^5 cpm/nmole). Definition of unit and

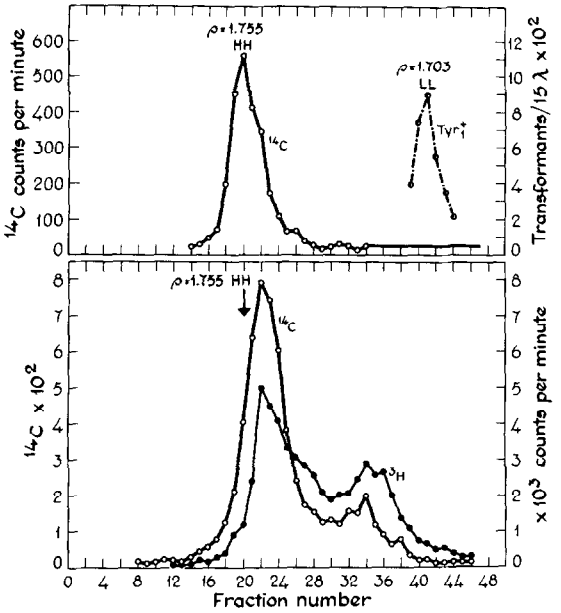

Figure 1. Pycnography of DNA synthesized by permeable cells of polA5 mutant. 3 x 10^9 cells of polA5 mutant of B. subtilis grown in ^{15}N, ^{2}H medium containing ^{14}C-thymidine were converted to permeable cells (1). The specific activity of the cellular DNA was 1.2 x 10^3 cpm/nmole. Synthesis of DNA by these cells was followed with dNTP + ATP in which the TTP was labeled with ^{3}H (2 x 10^4cpn/nmole). Each gradient contained 30 nmoles of DNA and 0.15 ml fractions were collected from an 8.0 ml gradient. 0.03 ml of each fraction was acid precipitated and counted for ^{3}H and ^{14}C. One microgram of light DNA from B. subtilis (ade_{16}, leu_2, and met_5) was added to each gradient. The light DNA position was determined by transformation for tyr_1^+ activity using tyr_1 competent cells. Top: Control heavy DNA from the permeable cells plus standard light DNA. Bottom: Profile of the template and product after synthesis with dNTP + ATP.

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method of assays were the same as in Ref. 12. The purified enzyme fractions from DEAE cellulose columns were subjected to sedimentation in linear sucrose gradients (2). The molecular weights of the enzymes were calculated using the formula of Martin and Ames (8).

Transformation assays were performed using a strain containing mutations for ade_{16}, leu_2 and $metB_5$ which are located at the origin, middle and terminus of the B. subtilis chromosome (1).

RESULTS

DNA Synthesis in Permeabilized Cells

It was pointed out (1) that 70% of the *in vitro* DNA synthesis observed in the presence of dNTP and ATP using wild type cells could be attributed to a mode of repair. Permeable cells were made of the polA5 mutant cells grown in a heavy medium (1). After 30 min synthesis, the DNA was purified and subjected to CsCl gradient centrifugation (1). There was a four-fold stimulation of DNA synthesis when the cofactor ATP was present compared to the assay with dNTP minus ATP. The profile (not shown) of the product synthesized without ATP was similar to the one reported earlier (1) suggesting that only repair, evidenced by covalent linkage of ^{3}H, light sequences with heavy template sequences, had occurred. The extent of this synthesis was 20% of that observed in wild type cells. From this data we concluded that 80% of the incorporation in the wild type permeable cells could be due to residual polymerase I.

In the presence of dNTP and ATP, the semiconservative DNA replication pattern (10) was very clear (Fig. 1) in the mutant. Using wild type cells (1), we observed no clear stepwise transition, but an overall movement of all the heavy molecules to lighter densities. Here control heavy DNA (Fig. 1, top) showed a unimodal distribution of radioactivity and of biological activity for the three markers. After synthesis with dNTP and ATP 15% of the template DNA moved to a hybrid density stratum (Fig. 1, bottom) due to the synthesis of light complementary strands. We found that 35% of the met_5^+ activity, which is located at the terminus of the chromosome, was transferred to a hybrid position whereas only 24% of the ade_{16}^+ activity was in this stratum (Fig. 2, top). We interpret this as being a continuation of synthesis to finish the replication cycle in a majority of the replicating chromosomes. Since the majority (74%) of the ade_{16}^+ activity remained in the heavy (HH) position, the presence of ade_{16}^+ in the hybrid strata could be due to either initiation of new rounds of replication or continuation of a growing point that existed ahead of this gene at the time permeable cells were prepared. These patterns of gene activity in concert with the leu_2^+ activity which is located in the middle of the chromosome led us to conclude that at least 15% of the DNA replicated semiconservatively.

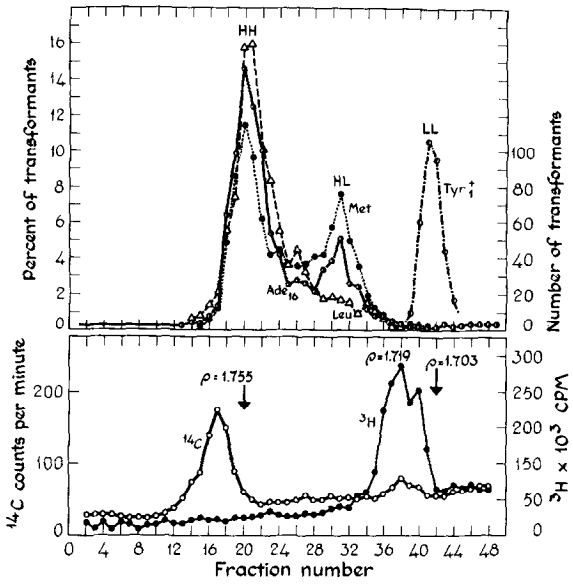

Figure 2. Biological activity profile of the gradient shown in Figure 1, bottom. The peak of activity in the HH position is located in fraction 22 in Figure 1, bottom. 0.015 ml of each fraction was incubated with 0.5 ml of competent B. subtilis cells carrying ade_{16}, leu_2 and met_5 (1.5×10^8 cells/ml). The three gene activities were scored by plating the transformed bacteria on appropriately supplemented plates (1). 100% activity represents 3.5×10^3 colonies for ade_{16}^+, 1.2×10^3 colonies for leu_2^+ and 2.4×10^3 colonies for met_5^+ (top). The remainder of each fraction from 30 to 37, corresponding to the hybrid position, was pooled and denatured in alkali at pH 12.5. The denatured DNA sample was centrifuged in a CsCl gradient at pH 8.0 after neutralization. Collected fractions were acid precipitated and counted for ^3H and ^{14}C. The denatured heavy DNA sediments at 1.769 g/cc density position (bottom).

The hybrid DNA molecules (fractions 30-37 of Fig.1, bottom) were denatured in alkali and run in a CsCl density gradient following neutralization. The results indicate (Fig. 2, bottom) that template (^{14}C, heavy) and product (^3H, light) strands are separable with little contamination of template atoms. Thus the polA5 mutant exhibits in vitro semiconservative replication and significantly reduced repair when compared with the wild type. Endonuclease activity found in this system caused loss of biological activity of DNA. This could be minimized by the inclusion of soluble RNA which inhibits this activity as with E. coli endonuclease I. It is true that several experiments

showed varying amounts of biological activity (as much as 8 fold less). This reduction is mainly due to nucleases of the recB type (7).

Identification of DNA Polymerases II and III

In the mutant as well as wild type cells DNA free lysates had a fast sedimenting polymerase activity (2) which, unlike DNA polymerase I, was inhibited by p-hydroxymercuribenzoate (pHMB). DNA polymerase activities were therefore isolated from wild type and polA5 mutant cells (Table I).

Table I

Purification of DNA Polymerases from B. subtilis

	Fraction	Specific Activity SB 1058	SB 1060
I.	Lysate	0.28	0.16
II.	Phase Partition	0.60	0.80
III.	DEAE Cellulose		
	Polymerase I	4.0	(0)
	Polymerase II	1.0	1.3
	Polymerase III	4.0	6.0
IV.	Sucrose Gradient		
	Polymerase I	24.0	(0)
	Polymerase II	18.0	16.0
	Polymerase III	11.0	12.2

Table 1. The various fractions and pooled polymerase peaks were assayed using "activated" salmon sperm DNA template. Specific activity is reported as units/mg protein. Polymerase I activity in the mutant fractions was less than 0.05 units/mg protein.

Enzyme Purification. 9 gms of cells grown in Penassay broth at 37C were suspended at twenty-fold concentration in 0.05 M Tris-HCl, pH 7.6 containing 0.01 M β-mercaptoethanol (TM) and lysed by lysozyme (50 ug/ml) at 37C. The lysate (Fraction I) was freed of nucleic acids by phase partition (11). The resulting upper phase (Fraction II) containing the polymerase activities was dialyzed extensively against TM, and was passed onto a DEAE cellulose column (5 cm^2 x 10 cm) previously equilibrated with TM, and eluted with a linear gradient of 0.1 to 0.9 M KCl. Collected fractions were assayed with "activated" salmon sperm DNA in the presence and absence of 2mM pHMB. An apparent unimodal

distribution for polymerase activity was observed for fractions from wild type cells (Fig. 3, top). The peak activity elutes at a concentration of 0.3 - 0.35

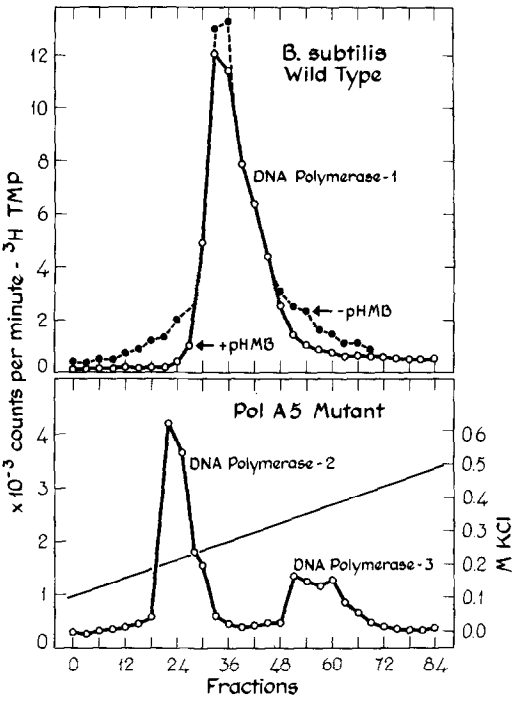

Figure 3. DEAE cellulose chromatography of B. subtilis DNA polymerases. Fractions II from the purification of wild type (top) and polA5 mutant (bottom) were fractionated on DEAE cellulose using a 500 ml linear gradient of KCl. 5ml fractions were collected and 0.025 ml of every third fraction was assayed for polymerase activity.

M KCl and was not inhibited by pHMB. However, both ends of the above distribution, activities eluting at salt concentrations of 0.2 - 0.3 M and 0.35 - 0.43 M KCl, were inhibited (dashed curve, Fig. 3, top). The major activity has been identified as polymerase I. The fractions inhibited by pHMB elute at the same salt concentrations upon refractionation on a second DEAE cellulose column. PolA5 cell extracts subjected to the same regimen (Fig. 3, bottom) showed the presence of only two pHMB inhibitable enzyme activities that eluted similar to the minor components observed in the wild type preparations. The three poly-

merase fractions from the columns were precipitated with $(NH_4)_2SO_4$ and kept in concentrated form in TM after dialysis (Fractions III). These were subjected to sucrose gradient sedimentation (Figure 4). DNA polymerase I was shown (2) to

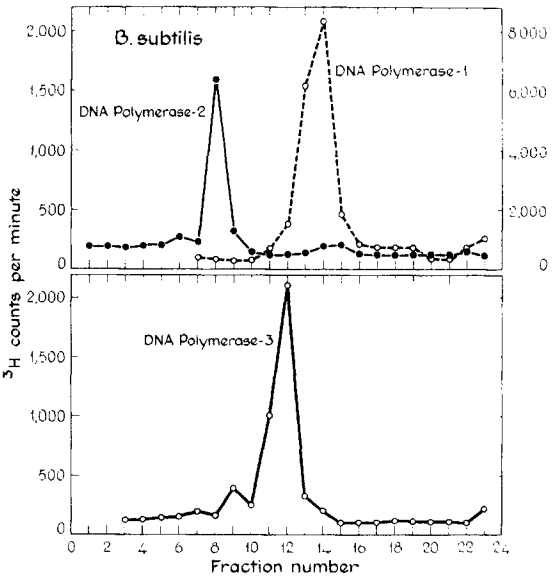

Figure 4. Sedimentation analysis of DNA polymerases from B. subtilis. Four units of DNA polymerases I and II and three units of DNA polymerase III from DEAE cellulose column fractions in 0.05 ml of Tris-HCl buffer (0.01 M) with 0.01 M β-mercaptoethanol were layered on top of separate sucrose gradients and run as described in Materials and Methods. After centrifugation, 23 fractions were collected and 0.025 ml of each fraction was assayed with a "activated" salmon sperm DNA as template for DNA polymerase activity.

cosediment with E. coli polymerase I under these conditions with a molecular weight of 110,000 daltons. Polymerases II and III have molecular weights of 160-180,000 and 140-150,000 daltons. Polymerase III is not as salt or heat sensitive as the enzyme from E. coli (4) (Table II). Both prefer exonuclease III treated templates and have pH optima of 8.0. Further purification of these activities and analysis for nuclease activity is in progress.

Table II

Properties of DNA Polymerases II and III of B. subtilis

Condition	Relative Activity	
	DNA polymerase II	DNA polymerase III
Control	100	100
+ 0.1 mM pHMB	4	10
+ 0.05 M $(NH_4)_2SO_4$	113	140
+ 0.15 M KCl	106	125
+ enzymes heated at 45 C for 10 min	35	62
+ template treated with exonuclease III	153	120

Table II. DNA polymerases II and III were purified 140- and 200-fold, respectively. Activities are expressed relative to those observed with "activated" salmon sperm DNA template as 100% (0.04 and 0.025 nmole of acid-precipitable ^{3}H-dTMP for polymerases II and III, respectively). E. coli exonuclease III treatment was sufficient to degrade 5-10% of the DNA template.

DISCUSSION

The polA5 mutant replicates DNA semiconservatively in the permeable cell system, with a minimum of repair synthesis compared to wild type cells (1). Replicating chromosomes appear to finish their cycles of replication. Two additional polymerases, polymerases II and III besides DNA polymerase I (12), are present in B. subtilis. The relative proportions of each activity could be roughly estimated from the fractionation profiles of the DEAE cellulose columns. In the linear range of incorporation we found that polymerase II and III activity levels are 10-12% and 1-2%, respectively, compared with that of polymerase I, as in E. coli (4,6). E. coli polymerase III is known to be involved in DNA synthesis (6) whereas the role of polymerase II is not clear (14). Present studies are directed toward determining the role of these enzymes in DNA synthesis in B. subtilis.

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