Antibody to B. subtilis DNA polymerase III: use in enzyme purification and examination of homology among replication-specific DNA polymerases

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ABSTRACT
Bacillus subtilis DNA polymerase III (pol III), an arylhydrazinopyrimidine-sensitive, replication-specific enzyme, was used to generate a non-precipitating rabbit antibody which specifically inhibited pol III activity in vitro. The antibody was used to examine structural relationships among several DNA polymerases, and it was linked covalently to agarose; the antibody:agarose was employed to develop a rapid, selective method of purification of catalytically active B. subtilis pol III.

INTRODUCTION
Several 6-(arylazo)- and 6-(arylamino)-pyrimidines, exemplified by 6-(p-hydroxyphenylazo)uracil (HPUra), selectively inhibit the replication of DNA of Gram-positive bacteria by acting specifically on the replication-specific enzyme, DNA polymerase III (pol III) (1,2,3). Using B. subtilis pol III as a model, we have initiated an examination of the structure of Gram-positive pol III's and the basis for their unique sensitivity to the latter inhibitors. As part of this study, we have developed in rabbits an antibody specifically directed against B. subtilis pol III. This paper describes the generation of this antibody, its reaction with B. subtilis pol III, its reactivity towards several other HPUra-sensitive and HPUra-resistant DNA polymerases, and its use, via attachment to an insoluble agarose matrix, in the selective purification of catalytically active B. subtilis pol III. Part of these results have been presented briefly elsewhere (4).

MATERIALS AND METHODS
Source and Growth of Bacteria. Bacillus subtilis strains NB841 and the arylazopyrimidine-resistant derivative, NB841 azp-12, were from this laboratory (5). B. subtilis BD170 and B. licheniformis were obtained from Dr. D. Dubnau, B. pumilus L951 from Dr. P. Lovett, and B. megaterium KM from...
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Dr. M. Schaechter. *Streptococcus fecalis* (ATCC 8043), *Micrococcus luteus* (ATCC 4698), and *Lactobacillus acidophilus* (ATCC 11506) were purchased from the American Type Culture Collection.

Bacteria used for analytical scale preparation of polymerase were grown at 30° in Brain Heart Infusion Broth (Difco) containing 5% Yeast Extract (Difco). With the exception of *L. acidophilus*, which was cultured under nitrogen, all strains were grown with vigorous aeration. Growth was followed using a Klett-Summerson Photoelectric Colorimeter. One to two liter cultures were rapidly chilled in ice during the late exponential phase of growth, and cells were collected by centrifugation, washed in a tared tube by centrifugation from 10 volumes of 50 mM Tris-HCl (pH 7.6), weighed, and stored at -80°.

*B. subtilis* NB841 used for the large scale preparation of pol III was cultured with vigorous aeration at 37° in 100 liters of minimal salts medium (6) containing 0.5% glucose, 0.2% acid casein hydrolysate (Calbiochem), and thymine and tryptophan, 50 μg/ml each. During the late exponential phase of growth the cells were chilled to 4° with continuous aeration. The cells were harvested in a refrigerated Sharples centrifuge, and the cell pack resuspended in ten volumes of cold minimal salts, recovered by centrifugation in a tared vessel, weighed, and stored at -80°. One hundred liters of culture typically yielded 250 grams of packed cells.

Materials. DEAE-cellulose (DE-52) was purchased from Whatman, Sepharose 4B and Sephadex G-25 from Pharmacia, and Bio-Rex 70 from Bio-Rad. DNA-cellulose was prepared with Munktell's 410 cellulose (Bio-Rad) and heat-denatured calf thymus DNA (Worthington) according to the method of Alberts et al. (7). Unlabeled deoxyribonucleoside triphosphates were purchased from P-L Laboratories, [3H]-TTP from New England Nuclear, cyanogen bromide from Eastman Organic Chemicals, and phenylmethylsulfonyl fluoride, streptomycin sulfate, and Triton X-100 from Sigma. 6-(3',4'-dimethylanilino)uracil (DMAU) was a gift of Dr. G. Wright of this department.

HeLa cell DNA polymerase was provided by Dr. E. Baril, *E. coli* pol III by Dr. C. Richardson, and coliphage T4-specific DNA polymerase by Dr. P. Englund; the polymerases specified by *B. subtilis* phages SP01 and PBS2 were provided, respectively, by Dr. C. Yehle and Dr. R. Hitzeman. *B. subtilis* DNA polymerase II was prepared in this laboratory as a byproduct (0.2 M potassium phosphate eluate) of step IV of the purification scheme for pol III (Table 1 and text below). *E. coli* pol I was purchased from Boehringer.

DNA polymerase activity of crude extracts and purified preparations of
enzyme was assayed at 30°C in 0.05 ml of a mixture containing 50 mM Tris-HCl (pH 7.6), 20 mM potassium phosphate (pH 7.4), 0.2 mM disodium EDTA, 7 mM triethanolamine phosphate (pH 5.8), 20% glycerol, 0.1% Triton X-100, 1.3 mM activated calf thymus DNA, 5 mM dithiothreitol, 25 μM each of dATP, dCTP, and dGTP, 10 μM [3H]-TTP (specific activity 200 cpm/pmol), and 0.005-0.1 units of enzyme. One unit of polymerase activity is the amount of enzyme which catalyzes, during 5 minutes incubation in the above conditions, the incorporation of 1 nmol of [3H]-TMP into an acid-insoluble form. Preparation of the sample for determination of acid-insoluble radioactivity has been described (8).

Quantitation of pol III activity of crude extracts. Crude extracts of wild-type B. subtilis and other Bacilli display, in our conditions of assay, the activities of three distinct polymerases, pol I, pol II, and pol III. Of these, only pol III is sensitive to the action of the site-specific inhibitor, DMAU. Therefore, the pol III activity of crude extracts was estimated as that portion (approximately 70-80% in wild-type extracts) of the total polymerase activity susceptible to inhibition by DMAU at 300 μM, a concentration which, in our assay conditions, completely inhibited the polymerase activity of purified pol III. We assumed, in preparing crude extracts of arylazopyrimidine-sensitive, Gram-positive bacteria other than Bacilli, that their DNA polymerase composition and the conditions required for enzyme extraction and assay were not significantly different from those of B. subtilis.

Protein assay. Protein content of various fractions was determined by a micro technique exploiting precipitation and staining with amidoschwarz dye (9). Bovine serum albumin was used as a standard.

Conventional purification of B. subtilis pol III. Our procedure is outlined in Table I and detailed below. All procedures were performed at 4°C, and all buffers contained 20 mM β-mercaptoethanol. Absorbance was determined in a Gilford Model 2400-2 spectrophotometer, using a 1-cm light path.

Pressure lysate and Fraction I. 250 g of frozen B. subtilis N841 were thawed and suspended in 500 ml of extraction buffer (20 mM Tris-acetate, pH 8.2; 0.5 mM EDTA; 10 mM magnesium acetate; 2 mM phenylmethylsulfonyl fluoride) and ruptured by passage through an Aminco French Pressure Cell at 20,000 p.s.i. The lysate was sonicated briefly to reduce its viscosity, and 75 ml of a fresh 66% (w/v) streptomycin sulfate solution was added to it, with stirring, over a period of 30 minutes. After one hour's stirring the cloudy suspension was centrifuged for 30 minutes at
25,000 x g, yielding a clear amber supernatant, Fraction I.

**Fraction II.** Fraction I (approximately 625 ml) was rapidly mixed with ground ammonium sulfate (0.35 g per ml). After one hour's stirring, the cloudy suspension was centrifuged at 25,000 x g for 30 minutes, yielding a copious, well-packed precipitate; the precipitate was dissolved in approximately 100 ml of extraction buffer containing 20% (w/v) glycerol, yielding a slightly cloudy, amber solution, Fraction II.

**Fraction III.** Fraction II was adjusted to a volume of 300 ml, clarified by centrifugation at 20,000 x g for 10 minutes, and "desalted" by passage through a Sephadex G-25 column (1.5 liter bed volume) equilibrated in GAPE buffer (20% glycerol; 200 mM ammonium sulfate; 10 mM potassium phosphate, pH 7.4; 0.5 mM EDTA). The solution was adjusted with additional GAPE buffer to an absorbance (280 nm) of 30 and applied to a column containing an equal volume of DEAE-cellulose which had been packed and equilibrated in GAPE buffer. The sample was washed through the column with GAPE buffer, and the portion of the eluate containing visible color was collected and stirred for 60 minutes with ground ammonium sulfate (0.5 g per ml of eluate). The cloudy suspension was decanted from undissolved salt and clarified by centrifugation at 25,000 x g for 4 hours. The precipitate was dissolved in DE buffer (20% glycerol; 10 mM potassium phosphate, pH 6.5), and the solution was freed of debris by brief centrifugation and desalted on a Sephadex G-25 column equilibrated with DE buffer, yielding Fraction III. Fraction III could be stored at -80° for at least one month without significant loss of activity.
Fraction IV. The absorbance at 280 nm of Fraction III was determined, and the fraction was adsorbed to a 6.5-cm diameter column of DEAE-cellulose equilibrated with DE buffer (bed volume, 1 ml per 10 absorbance units of applied Fraction III). Following a brief wash with DE buffer, the column was eluted with two bed volumes of DE buffer supplemented with 0.2 M potassium phosphate (pH 6.5) to remove a family of proteins, including the DNAU-resistant pol II. The column was then eluted with two volumes of DE buffer supplemented with 0.4 M potassium phosphate (pH 6.5); the eluate was collected in 40 ml fractions, and those containing pol III activity were pooled and stirred for 60 minutes with ammonium sulfate (0.5 g/ml). The cloudy suspension was centrifuged at 60,000 x g for one hour, and the precipitate was dissolved in DE buffer, yielding Fraction IV.

Fraction V. Fraction IV was desalted on a Sephadex G-25 column into DE buffer supplemented with 50 mM potassium phosphate (pH 6.5) and diluted with DE buffer to an absorbance (280 nm) of 2. Magnesium acetate (M stock) was added slowly, with stirring, to a concentration of 10 mM. Triton X-100 was added to a concentration of 0.5% (v/v), and the sample was applied to a column of DNA-cellulose (bed volume, 800 ml; diameter, 6.5 cm) equilibrated with DNA buffer (20% glycerol; 10 mM potassium phosphate, pH 6.5; 10 mM magnesium acetate; 0.5% Triton X-100). The sample was washed on with 80 ml of DNA buffer and the column eluted with a linear gradient of potassium phosphate in DNA buffer (0.1 to 0.4 M, pH 6.5; total volume, 8 liters; 20 ml/min; 40 ml fractions). The bulk of the polymerase activity emerged as a single peak at a phosphate concentration of 0.23-0.30 M. The active fractions were pooled. The pool was diluted with three volumes of DNA buffer minus phosphate and passed, during a period of three hours, through a 20 ml column of DEAE-cellulose equilibrated with DNA buffer. The column was eluted with 30 ml of DNA buffer supplemented with 0.5 M potassium phosphate, yielding Fraction V; the concentrate contained more than 95% of the pol III activity of the diluted pool.

Fraction VI. Fraction V was desalted on a Sephadex G-25 column into TEA buffer (35 mM triethanolamine phosphate, pH 5.8; 1 mM EDTA; 0.5% Triton X-100) supplemented with 50 mM potassium phosphate (pH 7.4) and adsorbed to a column of Bio-Rex 70 (500 ml bed volume, 6.5 cm diameter) equilibrated with the same buffer. The column was washed with two volumes of the equilibration buffer and eluted with a linear gradient of potassium phosphate in TEA buffer (0.05-0.4 M, pH 7.4; total volume, 5 liters; 16 ml/min; 40 ml fractions). The polymerase activity emerged in a single peak at a phosphate concentration of 0.25-0.30 M.
concentration of 0.14-0.18 M. The active fractions were pooled; the pool was diluted with 1.6 volumes of TEA buffer and passed, during a period of 4 hours, through a 1 ml column of DEAE-cellulose equilibrated with TEA buffer. The column was eluted with 1.8 ml of TEA buffer containing 0.5 M potassium phosphate (pH 7.4), yielding Fraction VI; the concentrated fraction contained about 90% of the pol III activity present in the diluted pool.

Preparation of electrophoretically pure antigen. Fraction VI was subjected to analytical sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (10) to determine its purity; densitometric analysis of the fixed, stained gels indicated a single, well-segregated, 167,000-dalton pol III band which comprised approximately 25% of Coomassie blue-stained material. For preparation of antigen, the Laemmli method was scaled up, employing 6.5 x 1.5 cm diameter cylindrical resolving gels composed of 5% acrylamide:0.13% bis-acrylamide and a 1 cm stacking gel composed of 2.5% acrylamide:0.6% bis-acrylamide. Fraction VI was equilibrated in sample buffer (0.125 M Tris-HCl, pH 6.8; 20% glycerol; 10 mM β-mercaptoethanol), heated at 100° for two minutes in the presence of 1% β-mercaptoethanol and 0.1% SDS, and divided into samples containing approximately 250-400 μg of protein. A bank of several gels, each loaded with a sample of approximately 1 ml, was subjected to electrophoresis for 4 hours at 15 mA/gel. The gels were stained for 10 minutes in a 0.1% aqueous solution of Coomassie Brilliant Blue (Bio-Rad), and destained in water until bands were faintly visible. The 167,000-dalton pol III band was excised from each gel and air-dried. After desiccation, the gel slices were ground to a fine powder in a small mortar.

Immunization procedure. The protocol was modeled closely after that used to generate antibody to gel-purified actin (11) and to gel-purified components of B. subtilis RNA polymerase (12). Ground gel, containing approximately 500 μg of enzyme protein, was mixed with 1.6 ml Freund's complete adjuvant (Difco) and sufficient water (approximately 7 ml) to make a slurry which could be drawn through an 18 ga. hypodermic needle. One half of the mixture was injected subcutaneously into a white male New Zealand rabbit (approximately 4 kg) in six sites on the back. This procedure was repeated three days later with the remaining half of the immunization mixture. One week later, and, again, after one month, the animal was injected with a similar mix prepared with Freund's incomplete adjuvant (Difco). One week after the last injection, and two or three times weekly for the next six weeks, 20 ml of blood were removed from the ear vein. Subsequently, the
rabbit was boosted with a single injection of antigen in Freund's incomplete adjuvant, and after one week the bleeding regimen was repeated. The highest titer of anti-pol III was achieved in response to the booster dose of antigen.

**Antibody purification.** The immunoglobulin G (IgG) fraction of immune and non-immune serum was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography by the method of Livingston (13).

**Determination of anti-pol III activity.** Serum (or purified IgG) and Fraction IV (0.01-0.05 units), were equilibrated in TEA buffer supplemented with 0.05 M potassium phosphate (pH 7.4), mixed in equal volume, and incubated at 0° for at least 15 minutes. After appropriate dilution (10-50 x) in the same buffer, 10 μl samples were removed and added to 40 μl of concentrated (1.25 x) polymerase assay mixture (cf. assay conditions above) and incubated at 30° for 5 minutes to determine polymerase activity.

**Preparation of IgG-agarose.** The method of Wilchek et al. was used (14). Sepharose 4B was washed extensively with water and suspended at a concentration of 0.33 g/ml (dry weight). The pH of the slurry was adjusted to 11.2 with 1 M NaOH and ice was added to bring the temperature to 15°. Solid CNBr (33 mg/ml of slurry) was added with vigorous stirring, and for 10 minutes the temperature and pH were maintained in the ranges of 15-20° and 10.8-11.2, respectively, using ice and 1 M NaOH. The agarose was freed of CNBr by extensive laavage with ice cold distilled water, and suspended in a thick slurry with 0.1 M NaHCO₃ (pH 8). DEAE-cellulose-purified IgG from immune and non-immune rabbits was equilibrated in 0.1 M NaHCO₃ (pH 8) and adjusted to a protein concentration of 3 mg/ml. The IgG solution and agarose slurry were mixed in the proportion of 1 mg protein per 60 mg dry agarose and shaken gently at 4° for 18 hours. The suspension was filtered and washed with 0.1 M NaHCO₃ (pH 8) to remove unadsorbed protein. Absorbance measurements at 280 nm indicated that essentially all of the protein remained bound to the agarose matrix. The IgG-agarose was suspended in 0.1 M NaHCO₃:25 mM EDTA (pH 8) and stored at 4°.

**RESULTS**

Characterization of the antibody and its reaction with pol III. Within one week of the end of the primary series of antigen injections, the serum of the immunized rabbit developed a pronounced anti-pol III activity. The activity, which rose and fell in intensity over a four week period, clearly displayed the properties of an antibody; it was not present in the serum of the rabbit prior to immunization, it purified as a component of the immuno-
globulin G (IgG) fraction, and its inhibitory activity was specific for the polymerase and exonuclease activities of pol III. In addition, the inhibitory activity of the serum increased following a booster injection. The immune serum also mediated the binding of pol III protein to Staphylococcus A cells, whereas non-immune serum did not (method of Kessler [15,16]; results not shown).

Figure 1 depicts the results of assay of the anti-pol III activity of the antibody; the method involved the mixture of varying amounts of undiluted serum with a fixed amount of enzyme, incubation for a period of 1 or 20 hours, and subsequent dilution and assay of the mixture for polymerase activity. The titer of the inhibitory effect appeared low in relation to titers commonly observed for antigen-antibody reactions. Nevertheless, inhibition was specific for the immune serum, and it increased directly as a function of serum concentration in the serum-enzyme mixtures, reaching a maximum of 80% during a 20 hour incubation period. Low speed centrifugation (10 min. at 15,000 x g) of polymerase-serum mixtures containing saturating amounts of serum did not indicate the formation of an easily sedimented antigen-antibody precipitate.

Formation of a catalytically active pol III: antibody complex. The resistance of pol III to complete inhibition by high concentrations of

![Fig. 1. Inhibition of the polymerase activity of B. subtilis pol III by pol III-specific antiserum. Twelve units of Fraction IV and 1-50µl of immune or nonimmune serum (35µg protein/µl) were mixed in a volume of 250µl in TEA buffer supplemented with 0.1 M potassium phosphate (pH 7.4), and incubated for either 1 or 20 hours at 4°; 10µl of the mixture were diluted appropriately and assayed as described in Methods to determine pol III activity. Immune serum mix assayed after 1 (■) or 20 (■) hours; non-immune serum mix assayed after 1 (○) or 20 (□) hours. 100% activity at 1 and 20 hours was equivalent to the incorporation, respectively, of 600 and 425 pmol [³H]TMP per 10µl of serum:polymerase mixture.](image-url)
antiserum suggested that the polymerase retained partial activity while complexed to antibody. Direct examination of the sedimentation behavior of pol III:antibody mixtures during glycerol gradient sedimentation (Figure 2) supported this suggestion. In the absence of antibody, or in the presence of non-immune, "control" serum, the enzyme activity sedimented with its characteristic value of 7.6 S (17). Incubation mixtures containing less than saturating amounts of antiserum displayed a mixture of free pol III activity and a complex array of more rapidly sedimenting forms. When the

Fig. 2. Effect of pol III antiserum on the sedimentation of pol III.
12 units of Fraction IV and different amounts of serum were mixed in TEA buffer containing 2.5% glycerol and supplemented with 0.1M potassium phosphate (pH 7.4), and the mixtures were incubated at 0° for 1 hour in a final volume of 250μl. A small portion of each mixture was removed for determination of pol III activity (Fig. 1), and 200μl of the remainder was layered on a 5 ml, 5-30% glycerol gradient containing TEA buffer supplemented with 0.1 M potassium phosphate, pH 7.4. The gradients were centrifuged in a Spinco SW 50.1 rotor at 32,000 rpm for 18 hours at 4°. The tubes were punctured at the bottom, and 250μl fractions were collected and assayed for polymerase activity as described in the Methods section.

(●); either no serum or 50μl control serum. (△), (○), (▼), (□); 2μl, 4μl, 10μl, and 50μl of immune serum, respectively, in each mix. The activity remaining in the various incubation mixes at the time of layering was A, 100%; B, 100%; C, 80%; D, 59%; and E, 40%.
concentration of antibody was increased to saturating levels (more than 10\%, v/v), all detectable pol III activity appeared as a more rapidly sedimenting species.

**Antibody-agarose: use in pol III purification.** The capacity of antibody to bind pol III without completely abolishing its catalytic activity suggested that it could be incorporated into an immunoadsorbant useful in selective enzyme purification. We synthesized a reactive immunoadsorbant consisting of antibody covalently linked to agarose (IgG*-agarose; see Methods section for preparation), and we exploited it and the chaotropic salt, KSCN, to prepare highly purified pol III from crude extracts. The most successful protocol involved the stirring of Fraction II with IgG*-agarose overnight at 4°C and elution with a buffer containing KC1 and KSCN. Figure 3 summarizes a purification run at a relatively high extract:adsorbant ratio. In these conditions, approximately 25\% of the pol III activity of Fraction II was sufficient to saturate the IgG*-agarose. The affinity of the adsorbant for the pol III was relatively weak, permitting a slow release of pol III activity during rinsing with input buffer. Addition of KSCN and KC1 increased the rate of release of pol III and yielded a peak of pol III activity. The eluate, after rapid desalting on Sephadex G-25 to remove KSCN and KC1, contained approximately 20\% of the activity adsorbed to the IgG*-agarose. Comparison of the specific activity of pol III in the eluate with that of conventionally purified pol III (Fractions III, and VI, respectively, table 1), and the SDS gel electrophoretograms of each (not shown), suggested that the considerable loss of activity resulted from denaturation by KSCN and not the failure of KSCN to elute pol III from the IgG*-agarose.

The capacity of IgG*-agarose to bind pol III was related specifically to the use of immune IgG in its preparation. Treatment of Fraction II by the protocol of Figure 3 with IgG-agarose made from IgG derived from a non-immune rabbit (IgG\(c\)-agarose) did not decrease its content of pol III activity, nor did KSCN elution of the IgG\(c\)-agarose exposed to Fraction II yield detectable pol III activity or pol III protein (Figure 4, panel A).

**Purity and properties of the IgG*-agarose purified pol III.** The 167,000 dalton (17), pol III protein in the KSCN eluate of IgG*-agarose was contaminated with several other electrophoretically discrete *B. subtilis* proteins (see SDS gel electrophoretogram of Fig. 4A). Most of the contaminants were common to the eluates of both immune and non-immune IgG-agarose (compare panels A

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Fig. 3. Purification of B. subtilis pol III on IgG*-agarose. Fraction II was prepared and equilibrated in TEA buffer supplemented with 0.1 M potassium phosphate (pH 7.4) at a protein concentration of 20-30 mg/ml. 300 ml of the protein solution were stirred for 15 hours at 4° with 20 ml (packed volume) of IgG*-agarose. The suspension was poured into a 2.7 cm diam. column and allowed to settle, and the soluble effluent was collected. The column was rinsed with seven 20-ml portions of input buffer and the effluent of each rinse was collected separately (rinse 1-7). The column was eluted with 7, 5 ml portions of input buffer containing 0.4 M KSCN and 0.8 M KCl. Each fraction was desalted into input buffer on Sephadex G-25 to remove KSCN and KCl. All samples were diluted appropriately and assayed for polymerase activity in the absence and presence of DMAU (300 yM) to distinguish the drug-sensitive pol III activity (□) from drug-resistant pol II (□□).

and B, Fig. 4); however, three were IgG*-agarose specific, appearing in SDS gels as 3 discrete bands of Coomassie blue-staining material at the positions of 93,000, 82,000, and 69,000 daltons (see arrows of Fig. 4A).

The relation of the IgG*-agarose specific bands to pol III was not studied extensively. All three proteins could be removed without significantly altering the activity of pol III on the activated DNA template used in routine assay; using conditions similar to those described in the preparation of Fraction VI, we have routinely exploited Bio-Rex 70 to produce, in one step, a fully active 167,000-dalton protein with an electrophoretic purity of
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Fig. 4. SDS-polyacrylamide gel electrophoresis of the KSCN-KCl eluate of IgG-agarose. Panel A, control (IgG*) eluate; panel B, immune (IgG*) eluate. The desalted KSCN-KCl eluate of each agarose column was concentrated by adsorption to a small DEAE-cellulose column followed by elution with TEA buffer containing 0.5 M potassium phosphate (pH 7.4). 0.06 ml of each concentrated sample was desalted into SDS gel buffer, heated at 100°C for two minutes, and subjected to electrophoresis in 0.5 x 9 cm cylindrical gels. The gels were fixed in 15% trichloroacetic acid, stained with Coomassie Brilliant Blue dye (Bio-Rad) in 50% trichloroacetic acid, destained in 7% acetic acid, and scanned at a wavelength of 600 nm with a Gilford Model 2400-2 spectrophotometer fitted with a gel scanner.

more than 90%. (Fraction IVa, Table 1).

With the exception of a somewhat low specific activity, the properties of pol III purified by the IgG*-agarose method were indistinguishable from those of protein prepared conventionally. Several experiments, the results of which are not shown, indicated that the protein had normal stability, normal sensitivity to 6-(arylamino)- and 6-(arylhydrazino)-uracils, normal reactivity to the IgG fraction used in its preparation, and normal chromatographic properties on Bio-Rex 70. Pol III from cells bearing the polC mutation, dnaF (18), also purified normally on IgG*-agarose, retaining its characteristic temperature sensitivity.
Antigenic homology of polymerases from Gram-positive bacteria. Replicative DNA synthesis in all of the Gram-positive organisms which we have examined to date has been specifically sensitive to HPUra, suggesting that the pol III of each is similar with respect to the structure and conformation of its HPUra-binding site (19). We attempted to determine whether the homology about the drug-binding site of Gram-positive pol III's portended more extensive antigenic homology, and, therefore, we tested the reactivity of several enzymes with antibody directed against B. subtilis pol III. The experiments utilized the HPUra-sensitive, Gram-positive bacteria *Streptococcus faecalis*, *Lactobacillus acidophilus*, *Staphylococcus aureus*, *Microccocus luteus*, and the Bacilli, *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. megaterium*.

The results, which are summarized in Table II, allowed three generalizations. First, crude extracts of drug-sensitive organisms, as expected, contained

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<td><em>Lactobacillus acidophilus</em>&lt;sup&gt;5&lt;/sup&gt;</td>
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<sup>1</sup>300μM; see Methods section.
<sup>2</sup>Assayed as in Legend of Fig. 1; expressed as the fraction of the inhibition observed with B. subtilis NB841 pol III (Fraction IV).
<sup>3</sup>Determined by the method described in the legend of Fig. 3.
<sup>4</sup>Determined using enzyme purified to a stage corresponding to Fraction II (see Methods).
<sup>5</sup>Determined using enzyme purified to a stage corresponding to Fraction IV (see Methods).
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DMAU-sensitive "pol III" activity. Second, not all of the drug-sensitive polymerases exhibited antibody sensitivity; the only enzymes appearing to possess significant antigenic homology with B. subtilis pol III were those derived from the other Bacilli and from S. fecalis (purified pol III of S. aureus was slightly sensitive to inhibition by antiserum). Third, the susceptibility of a polymerase activity to inhibition by soluble antibody was accompanied by the ability of IgG*-agarose to bind the respective enzyme.

Reactivity of anti-B. subtilis pol III for polymerases insensitive to arylhydrazinopyrimidines. Table II also summarizes the results of examination of several replication-specific DNA polymerases derived from HPUrA-insensitive systems. As their origin suggested, the enzymes were insensitive to DMAU in vitro and, as expected, they were neither sensitive to the inhibitory activity of the B. subtilis-specific IgG, nor bound by IgG*-agarose.

DISCUSSION

Characteristics of antibody and its reaction with antigen. B. subtilis pol III is present in cells in very low concentration, and it is accompanied throughout purification by proteins which are difficult to eliminate. To ensure the purity of the pol III protein employed as antigen we used denatured material derived from preparative SDS-polyacrylamide gels, exploiting the technique developed for the generation of antibodies against actin (11) and components of B. subtilis RNA polymerase (12). The technique required relatively small amounts of pol III and provided an effective antigenic stimulus. The antibody generated against the denatured enzyme was not strongly reactive against native pol III; it neither precipitated pol III nor completely inhibited its catalytic activity. How pol III maintained partial activity in the presence of saturating levels of antibody was not entirely clear; the antigen:antibody complex either retained catalytic activity or released free enzyme under the conditions of assay. The results of glycerol gradient analysis of pol III: antibody mixtures are consistent with either mechanism.

Purification of pol III with IgG*-agarose. The relatively weak antigen: antibody reaction and its susceptibility to the dissociative effect of mild chaotropic salts were properties critical to the successful use of IgG for purification of catalytically active enzyme. KSCN, in the presence of a high concentration of KCl, was chosen as an eluant after trial of a number of other reagents. Strong denaturing agents such as SDS and urea were effective eluants; however, they irreversibly denatured the enzyme. Other agents, such as NaCl, LiCl, KBr, KI, and sodium deoxycholate were ineffective.
as eluting agents; sodium perchlorate was nearly as effective as KSCN. KCl itself was ineffective as an eluant but was used with KSCN because its presence increased slightly the yield of catalytically active enzyme. KSCN was by no means an ideal eluant; it slowly denatured pol III and, therefore, it had to be removed from eluates by desalting on Sephadex G-25 as soon as possible after collection. The KSCN did not appear to have a deleterious effect on the capacity of IgG*-agarose to bind pol III; we have washed IgG*-agarose repeatedly with buffer containing 1 M KSCN, and have found it to be indistinguishable from unused material as a pol III-specific immuno-adsorbent.

The IgG-agarose purification method, although capable of rendering a 1500-fold purification of pol III in a single step, did not yield a pure protein. The product of KSCN elution was contaminated with several other B. subtilis proteins, most of which bound to both immune or non-immune IgG-agarose. The identity of the non-core proteins which specifically bound to IgG*-agarose has not been resolved. We are certain that these proteins were not present as unique species in the purified antigen used for immunization. In SDS they are smaller than pol III (93, 82, and 69 kilodaltons, respectively), and, therefore, might represent antigenically reactive, digestion products of the pol III protein; they also could represent constituents of an holoenzyme complex similar to those which are associated in the formation of E. coli pol III holoenzyme (20). Alternatively, the IgG*-specific bands may represent proteins which are bound uniquely by IgG*-agarose in a manner entirely independent of the presence of pol III-specific antibody.

On the basis of the content of 167,000-dalton pol III protein, enzyme (i.e. Fraction IIIa) purified by the IgG*-agarose method had a specific activity consistently lower than that of conventionally purified material (Fraction VI). This property likely resulted from partial denaturation upon exposure to KSCN. Excluding its tendency to yield an enzyme with a slightly depressed specific activity, we find that the IgG-agarose method of purification has considerable merit. First, it is fast and economical, and provides enzyme protein in quantities comparable to the conventional method. Second, it yields an enzyme with normal $K_m$'s for deoxyribonucleoside triphosphates and $K_i$'s for 6-(arylamino)- uracils. Third, it provides a product free from contamination by detectable levels of non-specific nuclease activity.

Homology of DNA Polymerases. The failure of several arylhydrazinopyrimidine-sensitive polymerases to react with an antibody to the analogous
B. subtilis enzyme (Table II) indicated that these pol III's, despite the putative similarity of their active sites, were structurally diverse. Without directly characterizing the structure of these enzymes we can only speculate on the extent of their diversity, because even trivial differences in their primary structures could profoundly affect their capacity to react with a specific, heterologous antibody. Considering the strict structural requisites mediating the reaction of a polymerase with the 6-substituted pyrimidines, and the vital role of pol III in DNA metabolism (24,25), we doubt that the structure of this enzyme has changed radically during the evolutionary diversification of bacteria carrying the Gram-positive phenotype.

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