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Serological Relatedness of Bacterial Deoxyribonucleic Acid Polymerases

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A number of bacterial species have been surveyed for serological activities with antiserum to *Escherichia coli* B deoxyribonucleic acid (DNA) polymerase I (EC 2.7.7.7.). The degree of serological cross-reaction is taken as a measure of relatedness of both the enzyme molecules from various species and the bacterial species themselves. Extracts were assayed by complement fixation only after treatment with deoxyribonuclease, since DNA bound to DNA polymerase alters the serological activity of the enzyme. Antiserum to *E. coli* DNA polymerase I did not react with either purified *E. coli* DNA polymerase II or the phage T4-induced DNA polymerase.

Complement fixation, which depends on complementarity between the antibody receptor sites and the several antigenic determinants on the surface of a protein molecule, has been used to analyze relationships among several mammalian proteins, including serum albumins (18), carbonic anhydrases (16), lysozymes (1), growth hormones (23), cytochromes (10), and hemoglobins (24), and bacterial proteins such as tryptophan synthetases (15), muconate lactonizing enzymes, muconolactone isomerases (22), and alkaline phosphatases (2). In this study we have surveyed the serological activities of deoxyribonucleic acid (DNA) polymerases from several bacterial species as well as of three different types of DNA polymerases.

DNA polymerase I has been purified to homogeneity from *Escherichia coli* B (8). The purified enzyme in vitro catalyzes several different reactions including polymerization of nucleotides and hydrolysis of DNA in either the 3′ → 5′ or 5′ → 3′ direction. The enzyme is thought to play an important role in the excision repair of lesions in DNA, although a non-essential role of the enzyme in replication and recombination has not been ruled out (3). The intact molecule appears to consist of not one but two distinct enzymes on a single polypeptide chain (21). *E. coli* B DNA polymerase I was the immunogen used for preparation of the antiserum. Thus, the gradations of cross-reactivities for the purified enzymes and the enzymes in the bacterial extracts reported here have been taken as their relationships to the *E. coli* B DNA polymerase I.

MATERIALS AND METHODS

Antigens and substrates. *E. coli* B DNA polymerase I was purified by the method of Jovin et al. (7). The purified enzyme showed only a single band of protein on sodium dodecyl sulfate-acrylamide gel electrophoresis. The large fragment of DNA polymerase I was produced and purified as described by Setlow, Brutlag, and Kornberg (20). The *E. coli* B DNA polymerase II (14) was a gift from Robb Moses, and the T4 DNA polymerase purified from extracts of T4 *am* N82 (gene 44)-infected *E. coli* B (6) was a gift of I. R. Lehman. Pancreatic deoxyribonuclease was purchased from Worthington Biochemicals.

Bacterial stocks. Wild-type stock cultures of *E. coli* B, *Bacillus subtilis*, *Aerobacter aerogenes*, *Proteus mirabilis*, and *E. coli* K-12 (JG 94) were furnished by members of the Departments of Biochemistry and Biology at Brandeis University. The remaining bacterial cultures were purchased from the American Type Culture Collection (Rockville, Md.).

Bacterial growth, harvesting, and rupture. All bacteria were grown in 1% tryptone (Difco), 1% NaCl in 1-liter batches (500 ml of medium in each of two 2-liter Erlenmeyer flasks) with continuous swirling. Batch cultures were inoculated with 2 to 5 ml of an overnight starter culture and grown to late log phase as measured turbidometrically. All cultures were grown at 37 C except *Erwinia carotovora*, *E. amylovora*, and *Serratia marcescens*, which were grown at 22 to 26 C.

Late log-phase cultures were chilled in a crushed ice-water bath and harvested by centrifugation at 6,000 × *g* for 10 min. The bacterial pellets were

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washed by suspension in 5 to 10 ml of 0.15 M NaCl and collected by centrifugation at 8,000 × *g* for 10 min. The washed bacteria were resuspended in 5 to 10 ml of 0.15 M NaCl.

The bacterial suspension was disrupted by treatment for 20 min in a Raytheon sonic oscillator with a transducer chamber kept cold by circulating ice water. The disrupted bacteria were centrifuged at 30,000 × *g* for 30 min, and the supernatant fluids were stored frozen at −20 C.

Deoxyribonuclease treatment of extracts. The crude extracts to be assayed for material reacting with anti-DNA polymerase I were diluted 1:2.5 to 1:5 in "isotris" buffer [0.14 M NaCl, 10 mM tris(hydroxymethyl)aminomethane (*p*H 7.4), 0.15 mM $CaCl_2$, and 0.5 mM $MgSO_4$]. The $MgSO_4$ concentration was adjusted to 20 mM, and pancreatic deoxyribonuclease was added to give a concentration of 20 μg/ml. This mixture was incubated at 37 C for 10 min and then chilled. The presence of the deoxyribonuclease did not interfere with the complement fixation assay.

Preparation of antisera. Antisera to DNA polymerase I were raised by immunizing a New Zealand albino rabbit with purified *E. coli* B DNA polymerase I. The enzyme (500 μg) in complete Freund adjuvant was injected into toepads and intramuscularly. A second injection of 500 μg in complete Freund adjuvant was given 1 week after the first, and a booster injection also of 500 μg in complete Freund adjuvant was given 3 months later. The rabbit was bled 1 week after both the second and third injections. All antisera were titered by complement fixation and examined by immunodiffusion. Antiserum 515C-3 was selected for this study.

Complement fixation. Purified antigens as well as crude bacterial extracts were assayed for material reacting with anti-DNA polymerase I serum by the technique of complement fixation (9). Gelatin at 0.1% (Atlantic Gelatin, pharmacological grade) was substituted for the 0.1% bovine serum albumin previously used in the assay buffer.

Calculation of the index of dissimilarity. In order to estimate quantitatively the immunological divergence among the DNA polymerases of various bacterial species, the maximum percent complement fixed at equivalence in a complement fixation assay (see arrows, Fig. 4) was plotted as a function of the concentration of the antiserum employed in the assay. A linear relationship exists between the logarithm of the antiserum concentration used and the value observed for *maximum complement fixation at equivalence*, when maximum fixation is in the range of 10 to 90%. The index of dissimilarity (ID) is calculated as the ratio of antiserum concentrations required to give the same percentage of maximum fixation at equivalence for the heterologous and homologous antigens, respectively (18). Since the ID is calculated from the maximum complement fixation at equivalence, it is independent of the amount of antigen released from the bacteria by sonic oscillation. We have chosen 50% fixation as the reference point for calculating ID. The greater the degree of immunological similarity between an enzyme of a particular bacterial species and *E. coli* B, the closer the ID will be to 1.0.

RESULTS

Homogeneity and specificity of the antiserum. The purified *E. coli* B DNA polymerase I was an effective antigen, and complement fixation assays with the purified antigen gave an equivalence region of 20 to 50 ng of antigen at a 1:10,000 dilution of antiserum (Fig. 1). The antiserum showed only a single component when tested with a crude *E. coli* B extract in either the complement fixation assay or by the double diffusion technique of Ouchterlony. Initial double diffusion experiments showed two precipitin bands between anti-*E. coli* B DNA polymerase I and a crude extract of *E. coli* B; however, this was due to the binding of DNA by some of the DNA polymerase I molecules. After treatment of the crude extract with pancreatic deoxyribonuclease, double diffusion experiments showed only a single component.

The antiserum to *E. coli* B DNA polymerase I did not fix complement with the purified DNA polymerase II from *E. coli* B (14), even at a 60-fold higher concentration of antiserum (Table 1). Similarly, the purified DNA polymerase induced by the bacteriophage T4 (6) did not react. In double diffusion experiments, only the homologous DNA polymerase I precipitated.

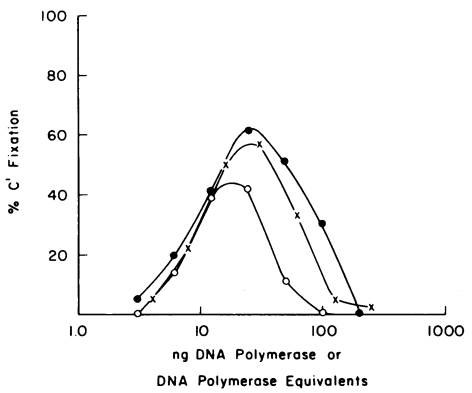

FIG. 1. *Complement fixation of purified E. coli B DNA polymerase I and sonic extracts of E. coli B with anti-E. coli B DNA polymerase I. Complement fixation, preparation of sonic extracts, and deoxyribonuclease treatment of sonic extracts are described in Materials and Methods. Antiserum was used at a 1:10,000 dilution with the purified enzyme (×), a sonic extract (○), and a sonic extract treated with deoxyribonuclease (●). The equivalence regions of the three curves have been aligned, and the relative concentrations of DNA polymerase in the sonic extracts are termed "DNA polymerase equivalents."*

TABLE 1. *Cross-reaction of purified DNA polymerases with anti-Escherichia coli B DNA polymerase I*[a]

Enzyme	Antiserum dilution for 50% maximum complement fixation at equivalence
E. coli B DNA polymerase I	1:12,000
E. coli B DNA polymerase II	<1:200[b]
T_4 DNA polymerase	<1:200[b]
Large fragment of *E. coli* B DNA polymerase I produced by proteolysis	1:9,500

[a] Complement fixation assays were carried out on the purified enzymes as described in Materials and Methods.

[b] No detectable cross-reaction at this dilution of antiserum.

DNA polymerase II and T4 DNA polymerase did not. The large fragment of *E. coli* B DNA polymerase I generated by proteolysis (20) reacted strongly with the antiserum toward the intact enzyme.

Effect of DNA on the serology of DNA polymerase. Initial experiments showed that the maximum complement fixation for a sonic extract of *E. coli* B was less than that of the purified enzyme at the same antiserum concentration (Fig. 1). Since it is known that DNA binds extremely tightly to DNA polymerase I (8), it is possible that the DNA in the crude extract was binding to the DNA polymerase I, thereby altering its serological activity. This does appear to be the case, because treatment of the crude extract with pancreatic deoxyribonuclease increased the maximum complement fixation of the extract to a level identical with that of the purified enzyme (Fig. 1).

The alteration in the serology of DNA polymerase I by DNA can be demonstrated directly by adding purified DNA to a complement fixation mixture containing the purified enzyme as the antigen (Fig. 2). Both native and denatured DNA from several sources have the same effect—a decrease in the maximum complement fixation and a shift in the equivalence region to a lower concentration of antigen. The differences between the effects of DNA from various sources undoubtedly reflect differences in structure and composition of the DNA, especially the number of nicks in the native double-strand DNA and the number of 3′-phosphoryl termini in the single-strand (denatured) DNA. The purified polymerase binds only at nicks (and ends) in doubled-strand DNA (8) and will degrade denatured DNA unless there is a 3′-phosphoryl terminus. This latter degradation is not inhibited by the antiserum to the enzyme, whereas other degrading activities are (P. Setlow, *unpublished results*).

In view of the effect of DNA on the serology of DNA polymerase I, sonic extracts intended for serological assay of DNA polymerase were pretreated with deoxyribonuclease. All bacterial species assayed showed an increase in the level of maximum complement fixation after deoxyribonuclease treatment, with the exception of extracts of *B. subtilis*. Complement fixation curves are shown in Fig. 3 for extracts of *Shigella dysenteriae* and *Proteus vulgaris* before and after deoxyribonuclease treatment. These curves are typical of those obtained with all bacteria except *B. subtilis*.

Cross-reaction of DNA polymerases from different bacteria. *E. coli* B, *E. coli* K-12, and the *Shigella* strains all required the same concentration of antiserum to give complement fixation curves with similar maxima at equivalence (Table 2). However, all other species required higher concentrations of antiserum. The complement fixation curves for extracts of *Klebsiella pneumoniae* and *Shigella alkalescens* are given as an example (Fig. 4).

As a quantitative estimation of immunological divergence among the DNA polymerases of bacteria, the ID (defined in Materials and Methods) was determined for each species; therefore, percent maximum complement fixation at equivalence was plotted as a function of the logarithm of the concentration of anti-*E. coli* B DNA polymerase I in the assay (Fig. 5). For the sake of visibility, the data for only seven species are included in the figure. The ID values for all species assayed are given in Table 2. An extract of *E. coli* B was assayed each day so as to have a reference point in calculating the ID values independent of daily variations in the complement fixation assay.

DISCUSSION

The *E. coli* B DNA polymerase I has been purified to homogeneity and is well characterized. DNA polymerases have also been isolated from other bacterial species (*B. subtilis*, *M. luteus*) and from bacteriophage-infected cells (5, 6, 17). Although very important to an understanding of enzyme function, protein purifications are not appropriate for extensive taxonomic work.

The absence of a detectable reaction between anti-*E. coli* B DNA polymerase I and either *E. coli* B DNA polymerase II or T4 DNA polymerase demonstrates the specificity of the an-

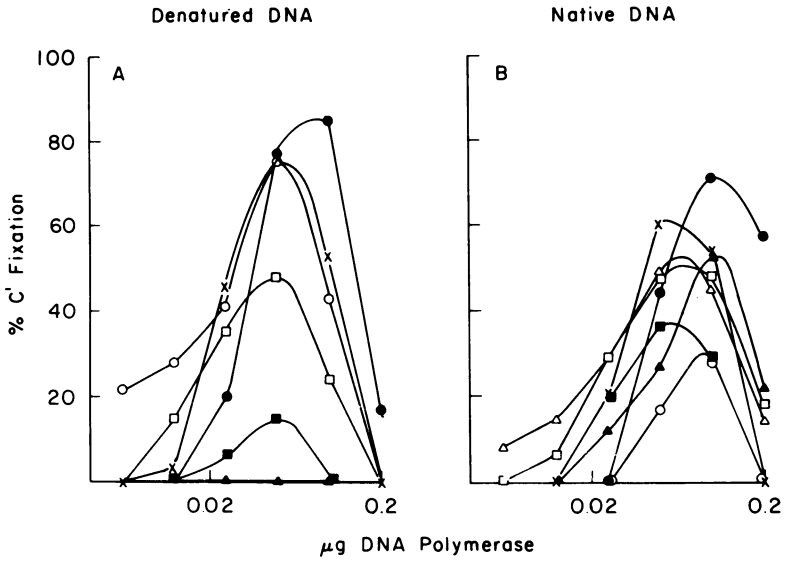

FIG. 2. *Effect of DNA on the serological activities of E. coli B DNA polymerase I. Complement fixation of anti-E. coli B DNA polymerase and purified E. coli B DNA polymerase in the absence of DNA (●) and in the presence of 1 μg of E. coli DNA (×); 1 μg of calf thymus DNA (○); 1 μg of Pseudomonas aeruginosa DNA (□); 1 μg of A. aerogenes DNA (■); 1 μg of salmon sperm DNA (△); 1 μg of bacteriophage T4 DNA (▲). A, DNA denatured by boiling for 10 min before addition to DNA polymerase. B, Native DNA. Antiserum was used at a 1:7,000 dilution.*

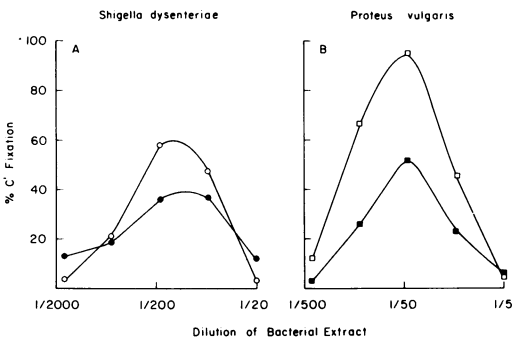

FIG. 3. *Effect of deoxyribonuclease treatment on complement fixation of anti-E. coli B DNA polymerase I with sonic extracts of two bacteria. A, Shigella dysenteriae. The antiserum was used at a 1:10,000 dilution with an untreated sonic extract (●) and a deoxyribonuclease-treated extract (○). B, Proteus vulgaris. Antiserum was used at a 1:6,000 dilution with an untreated sonic extract (■) and a deoxyribonuclease-treated sonic extract (□).*

tiserum for DNA polymerases of type I. Most of the antibodies in the antiserum are directed toward the large proteolytic fragment of *E. coli* B DNA polymerase I. Thus, proteolysis of the intact enzyme does not greatly alter its serological activity. This fact is important in assaying *extracts* of species such as *B. subtilis*, which contain proteases capable of cleaving DNA polymerase (20).

It was possible to detect reactions with anti-*E. coli* B DNA polymerase I in extracts of bacterial species with DNA base compositions as unlike *E. coli* (50% guanine plus cytosine [G + C]) as that of *Proteus vulgaris* (37% G + C). Serological reaction was also observed in both gram-negative and gram-positive organisms. All *Enterobacteriaceae* tested showed cross-reactions. The *Shigella* DNA polymerase was indistinguishable serologically from the *E. coli* enzyme; the *Salmonella* enzyme was closely related to that of *E. coli*, whereas *Klebsiella* and *Aerobacter* were only slightly less similar. DNA-DNA and ribonucleic acid (RNA)-DNA hybridization experiments have shown that *Salmonella* and *Shigella* are closely related to *E. coli* and that *Aerobacter* is somewhat less so (11, 19). This result has been further confirmed by experiments with transduction and conjugation (12).

Extracts of species within a given genus gave similar ID values: *Shigella dysenteriae* and *S. alkalescens* (0.97 and 0.95, respectively); *Salmonella typhimurium* and *S. chloreraesuis* (1.15 and 1.5, respectively); *Erwinia carotovora* and *E. amylovora* (5.3–7.4 and 7.0, respectively); extracts of *P. vulgaris*, *P. mirabilis*, and *P. morganii* gave fairly similar values for ID (7.3,

11.6–15.5, and 6.9–8.7, respectively), even though the DNA base composition of *P. vulgaris* and *P. mirabilis* is 37 to 39%, whereas that of *P. morganii* is 51 to 53% G + C. *Klebsiella pneumoniae* and *A. aerogenes*, which have been thought to be very similar by conventional criteria (13), have ID values close to each other (1.9 and 2.5, respectively).

B. subtilis, a gram-positive bacterium, gives reproducible cross-reaction with the anti-*E. coli* B DNA polymerase. A DNA polymerase has been purified from *B. subtilis*, but there is a possibility that this enzyme may have been altered by proteolysis during purification. Deoxyribonuclease treatment has no effect on

TABLE 2. *Serological relatedness of bacterial DNA polymerases*

Bacterial species	G + C content of DNA[a] (%)	Index of dissimilarity[b]
Escherichia coli B	51	1.0
E. coli K-12	51	1.0
Shigella alkalescens		0.95
S. dysenteriae	53	0.97
Salmonella typhimurium	50	1.15
S. chloreraesuis		1.5
Klebsiella pneumoniae		1.9
Aerobacter aerogenes	56	2.5
Erwinia carotovora	54.5	5.3–7.4
E. amylovora		7.0
Serratia marcescens	57.5	5.5–6.3
Proteus morganii	53	6.9–8.7
P. vulgaris	37	7.3
P. mirabilis	38	11.6–15.5
Bacillus subtilis	42	29

[a] The base compositions of bacterial DNAs (G + C) were taken from the literature (13).

[b] The ID was calculated as described in Material and Methods from the data shown in Fig. 4 and 5. The reference enzyme is *E. coli* DNA polymerase I. Indices were always calculated relative to complement fixation curves for *E. coli* B extracts that were prepared on the same day.

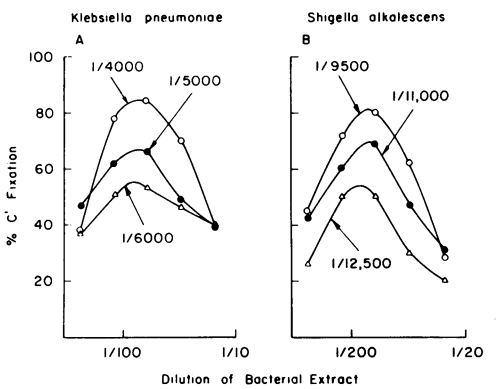

FIG. 4. *Effect of antiserum concentration on complement fixation of anti-E. coli B DNA polymerase I with extracts of two bacteria. Antiserum was used at the dilutions noted. A, Deoxyribonuclease-treated sonic extract of Klebsiella pneumoniae. B, Deoxyribonuclease-treated sonic extract of Shigella alkalescens.*

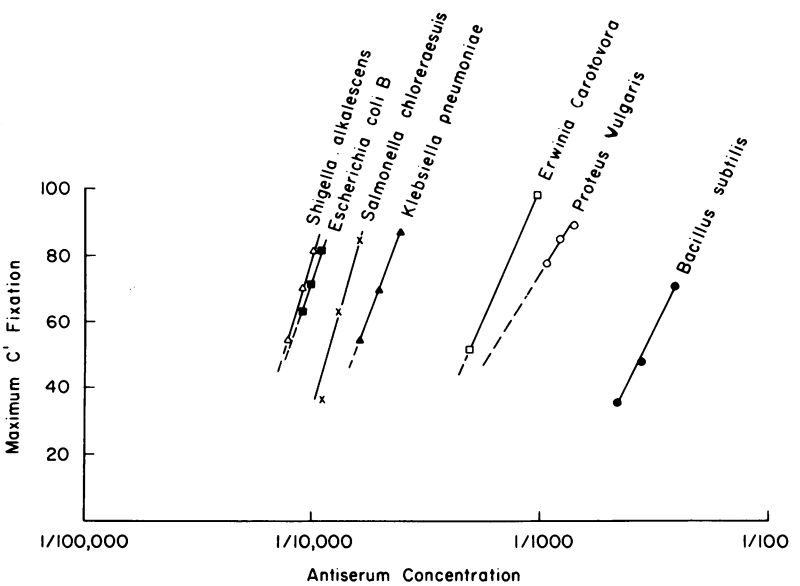

FIG. 5. *Maximum complement fixation at equivalence for deoxyribonuclease-treated extracts of several bacteria as a function of the concentration of anti-E. coli B DNA polymerase I in the assay. Individual points were determined as shown in Fig. 4.*

the complement-fixing activity of the *B. subtilis* extracts.

The implications of experiments showing the effect of DNA on the serology of DNA polymerase are several. Binding of DNA either changes the conformation of the enzyme in a fashion detectable by complement fixation or blocks interaction of antibodies with their antigenic determinants or both. It has been shown that DNA protects DNA polymerase against proteolytic inactivation (P. Setlow, D. L. Brutlag, and A. Kornberg, *unpublished results*). We have preliminary evidence that the binding of native DNA to purified DNA polymerase increases its heat stability, as measured serologically. There is evidence via centrifugation experiments that binding of short oligomers of deoxy(adenosine-thymidine) causes a structural change in the enzyme (4).

One might suspect that changes in DNA polymerase might be restricted in evolution, since its functional and binding properties are highly specific. If complete serological surveys could be made of several bacterial proteins, it would be very interesting to compare rates of divergence and to hypothesize on the effect of differential selective pressures on different enzymes.

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