RNA Polymerase from Clostridium acidi-urici

CHARACTERIZATION OF A NATURALLY OCCURRING RIFAMPICIN-RESISTANT BACTERIAL ENZYME*

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We report here the isolation of a prokaryotic RNA polymerase that shows pronounced template specificity. The enzyme from Clostridium acidi-urici is highly active on DNA templates isolated from phage that infect Gram-positive organisms and is essentially inactive at either high or low ionic strength on DNA from phage associated with Gram-negative bacteria. The enzyme is also unique among RNA polymerases isolated from wild type bacteria in being highly resistant to inhibition by rifampicin. These properties are characteristic of the enzyme present in several independently isolated strains of C. acidi-urici. However, RNA polymerase present in other clostridial species resembles the enzyme present in Bacillus subtilis in sensitivity to rifampicin and template specificity.

Analysis of the base sequence data of the 16 S rRNA of the prokaryotes has served as the basis for the reclassification of these organisms into two kingdoms (1). Most bacteria belong to the eubacterial kingdom (2), but it has been suggested that methanogens, extreme halophiles, and various thermoacidophiles comprise a separate line of descent among the prokaryotes to be called the Archaeabacteria (3). This reclassification is consistent with relationships based on comparative analyses of protein sequences and cell wall composition and suggests relationships not previously recognized. Thus, most of the Gram-negative nonphotosynthetic organisms, including the enterics, are classified in a subline of a major eubacterial group, the purple bacteria, while Gram-positive organisms, including the clostridia, bacillae, and lactobacillae, occur in another major group, the "clostridia."

The relatively distant relationship between Gram-negative and Gram-positive bacteria suggested by the comparative analysis of the 16 S rRNA sequences was of particular interest to us in relationship to the observation made in this laboratory (4) that ribosomal protein-synthesizing systems derived from Gram-positive organisms were unable to translate mRNAs derived from Gram-negative organisms or phages related to these organisms. It was subsequently reported that genes from Gram-negative organisms carried on hybrid plasmids were not expressed in vivo (5) by Gram-positive organisms. These observations both confirmed the earlier in vitro translational studies and raised the question of the possibility of transcriptional barriers to heterospecific gene expression.

RNA polymerase (EC 2.7.7.6) has been purified from relatively few Gram-positive bacterial species and not from any clostridial species (6). The subunit composition of the normal vegetative enzyme from Gram-positive species that have been examined resembles that of the enzyme from Gram-negative sources except for the fact that the σ subunit of the enzyme from four species of Bacillus and Lactobacillus curvatus has a molecular weight about one-half of that of the σ subunit of the Escherichia coli enzyme or any of the other enzymes isolated from Gram-negative species. The enzymes that have been isolated up to now from Gram-positive sources do not differ in any significant properties from those isolated from Gram-negative bacteria with respect to specificity of promoter utilization or antibiotic sensitivity, although differences in efficiency of promoter utilization have been noted as well as susceptibility to ionic conditions (7-9). RNA polymerases from the Archaeabacteria, however, may be distinguished from those derived from eubacteria on the basis of their subunit composition as well as by the rifampicin insensitivity of the enzyme (10, 11).

Because the clostridia, like the methanogens of the archaeabacteria, are strict anaerobes and are considered to closely resemble the first organisms in primeval evolution (2), and because of the observations described above, it appeared worthwhile to us to examine the properties of RNA polymerase from clostridia. The results obtained are described here and appear to justify our curiosity since it resulted in the discovery of an RNA polymerase with unique properties among those of bacterial origin.

MATERIALS AND METHODS

The sources of most materials, including bacteriophage DNAs, and methods have been previously cited (9). X4, a Myxococcus phage, was obtained from David R. Zusman of the Department of Microbiology and Immunology of this University. Other materials were obtained as follows. Rifampicin was obtained from Sigma; Bio-Gel A-1.5m (100–200 mesh) from Bio-Rad; uric acid from Pfaustehl; and poly(dA-dT) (alternating polymer) from Miles. Polym P (polyethyleneimine) was purchased from Eastman and a 10% (v/v) stock solution was prepared as described by Burgess (12). The restriction endonuclease Hae III was purchased from Bethesda Research Laboratories and T7 DNA was digested under conditions described by them.

DNAs from SP6 and both wild type T7 and T7 deletion mutant 3ΔD111 were obtained from Michael J. Chamberlin, University of California, Berkeley. Streptolydigin and streptovaricin were gifts of Dr. T. Leighton of the Department of Microbiology and Immunology of this University. Double-stranded DNA cellulose was prepared by the method of Litman (13). The adsorbent contained 1.2 mg of DNA/ml of wet packed cellulose as determined by the diphenylamine procedure using dAMP as standard (14).

Bacillus subtilis (W168) RNA polymerase was prepared by the heparin-agarose procedure of Davison et al. (9). E. coli (MRE-600) RNA polymerase was prepared by the method of Burgess (12).

Cells—Vegetative cells of Clostridium acidi-urici (strains 9a, AAM-2, and AC-1) (15) and Clostridium cylindrosorum (strains HC-1 and MJ-6 (15)) were grown on uric acid at 37 °C as previously described (16). Clostridium pasteurianum was grown as previously described (17), but glucose was substituted for sucrose and the growth temperature was 37 °C.

Activity Assay—RNA polymerase was assayed during purification as previously described (9) with the following modifications. [H]UTP
was present at 30 cpm/pmol and the reaction mixtures contained 160 mm KCl. For the accurate determination of specific activity on ϕ29 DNA, the assay (100 µl) contained 0.4 mm [α-35S]CTP (50 cpm/pmol) in place of the [3H]UTP. The extent of inhibition by rifampicin, streptolysin, and streptovaricin was determined by adding an aliquot of a stock solution of the inhibitor to the incubation mixture before the addition of enzyme. Synthesis of RNA for gel analysis was carried out as previously described (9) except that the reaction mixtures were incubated for 10 min at 37 °C. One unit of RNA polymerase activity corresponds to the incorporation of 1 nmol of CMP in 10 min at 37 °C.

Protein Determination—Protein concentrations were determined by a modified (18) Folin-phenol reagent method (19) on samples that had been precipitated with 7% trichloroacetic acid. Crystalline bovine serum albumin was used as a standard.

Buffers—Buffer I (breaking): 50 mm Tris-HCl (pH 8), 2 mm EDTA, 0.3 mm dithiothreitol, 5 mm 2-mercaptoethanol, 0.2 M NaCl, 10% glycerol, and 0.5 mm phenylmethylsulfonyl fluoride. Buffer II: 50 mm Tris-HCl (pH 8), 0.1 mm EDTA, 0.3 mm dithiothreitol, and 5% glycerol. Buffer III: 50 mm Tris-HCl (pH 8), 0.1 mm EDTA, 0.3 mm dithiothreitol, 0.6 M NaCl, and 20% glycerol. Buffer IV: 50 mm Tris-HCl (pH 8), 0.1 mm EDTA, 0.3 mm dithiothreitol, 0.1 M NaCl, and 20% glycerol.

Gene Electrophoresis—RNA was analyzed by electrophoresis either in 2.5% agarose slab gels (0.3 x 10 x 20 cm) containing 1% SDS, 50 mm Tris, 380 mm glycine, and 2 mm EDTA buffer, pH 8.3, or in 1.75% acrylamide and 0.7% agarose slab gels (20, 21) followed by autoradiography. RNA polymerase transcripts of ϕ29 DNA (9) were used as standards. For analysis of proteins, polyacrylamide gel electrophoresis in the presence of SDS was performed in slab gels (22) with a discontinuous buffer system (23) and an acrylamide/bisacrylamide ratio of 30:8.

Calculation of Subunit Molecular Weights—Molecular weights of subunits were determined on 7.5% SDS-polyacrylamide slab gels. The β′ (M, = 160,000), β (150,000), and α (36,500) subunits of E. coli RNA polymerase (24, 25), the α (M, = 56,000) and α (43,000) subunits of B. subtilis RNA polymerase (6), and C. acidi-urici RNA polymerase (16) were used as standards. Because of the large variation in reported values for the E. coli α subunit and the B. subtilis β and β′ subunits, the molecular weights were calculated from migration distances in our buffer system.

Purification Procedure—RNA polymerases from C. acidi-urici (strain 9a) and C. cylindrosporum (strain HC-1) were purified by a modification of the procedure of Burgess and Jendrisak (12). All steps were carried out at 4 °C.

Frozen cells (25 g) were suspended in 75 ml of Buffer I and sonicated for 1 min in a Branson Sonifier (model J-17A). The crude extract was then diluted with 100 ml of Buffer I and centrifuged at 15,000 rpm in the SS-34 rotor of a Sorval RC-5B centrifuge for 30 min. The supernatant solution was decanted and a 10% (v/v) solution of Polymin P was added dropwise with stirring to a final concentration of 0.2%. After stirring 10 min, the suspension was centrifuged 15 min at 6000 rpm and the clear supernatant solution was discarded. The pellet was resuspended with the aid of a Dounce homogenizer in 100 ml of Buffer II containing 0.5 M NaCl. The suspension was stirred for 10 min, centrifuged as before, and the supernatant fraction was discarded. The pellet was then extracted with 80 ml of Buffer II + 1.0 M NaCl. After stirring for 10 min, the mixture was centrifuged for 30 min at 6000 rpm and the precipitate was discarded.

The RNA polymerase in the supernatant solution was then precipitated by the addition of solid ammonium sulfate to a final concentration of 65% (39.8 g/100 ml), with the addition of 5 n NH4OH to maintain pH 7.5. The suspension was stirred for 30 min and centrifuged for 30 min at 18,000 rpm. The pellet was dissolved in a minimum volume of Buffer III (1 V, = 3 ml) and applied to a column (1.2 x 68 cm) of agarose A-1.5m equilibrated with the same buffer. Fractions containing polymerase activity were pooled and diluted with 20 mm Tris-HCl (pH 8), 0.1 mm EDTA, 0.3 mm dithiothreitol, and 20% glycerol until the conductivity was equal to that of Buffer IV.

The eluted enzyme (30 ml) was applied to a column (1.2 x 77 cm) of DNA cellulose equilibrated with Buffer IV and washed with 10 column volumes of buffer. The polymerase was then step eluted with Buffer IV + 1.0 M NaCl. The enzyme was stored in this buffer at 4 °C and was stable for at least 18 months. A summary of the purification is given in Table I. Purification of the enzyme from C. cylindrosporum (strain HC-1) was carried out by the same procedure and gave a similar yield of enzyme of the same specific activity.

Partial Purification of RNA Polymerases from Other Clostridial Strains—Enzymes from C. pasteurianum, C. acidi-urici (strains AAM-2 and AC-1), and C. cylindrosporum (strain MJ-6) were purified from 10 g of cells as outlined above through ammonium sulfate precipitation. The activity in the resuspended pellets (5 ml) was entirely DNA-dependent and proportional to the amount of enzyme added.

RESULTS

Subunit Composition—The subunit structure and purity of two preparations of RNA polymerase from C. acidi-urici are shown in Fig. 1 (lanes 2 and 3). For comparison, the enzymes purified from B. subtilis (lane 1) and E. coli (lane 4) are shown.

The enzyme obtained from C. acidi-urici after step 4 of the purification (lane 3) shows six components. If these are compared with the component subunits of RNA polymerase from E. coli, starting with the high molecular weight components and going to the lower molecular weight bands, one can identify the two largest components of the C. acidi-urici enzyme as the β and β′ subunits. We are designating the larger subunit β and the smaller one β′ by analogy to the findings reported for the other enzymes purified from Gram-positive sources (27). We have observed that the smaller of the two subunits of the C. acidi-urici enzyme binds to DNA, a characteristic of the β′ subunits (28). The next smaller component has a molecular weight very similar to that of the σ subunit of the E. coli enzyme. However, we do not believe that this is the normal σ subunit of the C. acidi-urici enzyme since it is removed following step 5 in the purification to yield an enzyme (lane 2) with even higher specific activity and no change in template specificity. The σ subunit of the C. acidi-urici enzyme appears to be the next component which is retained in the preparation even after step 5 of the purification and has a molecular weight similar to that of the σ subunit of the B. subtilis enzyme (lane 1). Finally, the α subunits of this enzyme, as well as those of the other bacterial polymerases examined here, appear as doublets. This behavior is not uncommon (29).

The molecular weights of the subunits of these enzymes are summarized in Table II. The molecular weights of the σ subunits are within the range of those reported for other Gram-positive bacteria (6). The β and β′ subunits of the

| Fraction | Protein Activity |
|----------|------------------|
|          | mg             | A₄₅₀/A₃₅₀ | Total Units | Units/mg % |
| 1.  S-30 | 3,290          | 0.54       | 9,800       | 3          |
| 2.  Ammonium sulfate, 65% ppt. | 160 | 1.10 | 9,000 | 56 |
| 3.  Agarose A-1.5m | 45 | 1.7 | 12,000 | 266 100 |
| 4.  Double-stranded DNA-cellulose column 1 | 3.8 | 1.7 | 6,450 | 1,730 | 54 |
| 5.  Double-stranded DNA-cellulose column 2 | 1.0 | 1.7 | 3,250 | 3,250 | 27 |

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
enzyme from all six of the clostridial strains that we have examined have identical mobilities on polyacrylamide gels (data not shown).

The RNA polymerase prepared from C. acidi-urici by the procedure described here, as well as by the method of Duffy and Geiduschek (30), consistently co-purified with a protein of $M_r = 95,000$ (the third protein component in lane 3, Fig. 1). This protein may be analogous to proteins of similar size that are present in preparations of RNA polymerase from a variety of bacterial species (25, 27, 31–34). Values reported for the molecular weights of these proteins vary from 84,000–110,000. In all cases, the migration of the protein in SDS-polyacrylamide gels closely resembles that of the E. coli $\sigma$ subunit. It has been found in other systems (25, 31–33) that this protein can be separated from polymerase either by zone centrifugation through a glycerol gradient or by chromatography on single-stranded DNA agarose. However, the clostridial enzyme was found to have a very loosely associated $\sigma$ subunit so that both chromatography on the single-stranded DNA analogue heparin agarose and zone centrifugation cause dissociation of the enzyme into the core and $\sigma$ subunits. We have been able to obtain a preparation of enzyme free of this protein only in the following manner. The DNA cellulose column employed in the final step of the purification was overloaded during one enzyme preparation. Although there was no RNA polymerase activity (or $\beta,\beta'$ material) in the early pass-through fractions, substantial amounts of polymerase were present in later fractions. Examination of these fractions on a polyacrylamide slab gel revealed that they contained none of the 95,000 component, all of which had bound to the DNA column. The pass-through fractions containing polymerase activity were then pooled and applied to a second column of DNA cellulose. The enzyme that eluted with 1 M NaCl was completely free of the 95,000 component. The presence of this protein does not appear to alter any of the properties of the polymerase, and we have used the enzyme obtained after step 4 of the purification for some of our studies. A summary of the purification is given in Table I. It should be noted that the total recovery of activity is roughly 80%.

**Resistance to Antibiotics**—Sensitivity to the antibiotic rifampicin is a general characteristic of RNA polymerases of eubacteria. Under assay conditions employed in these studies, the B. subtilis enzyme is inhibited 95% by 0.7 $\mu$g/ml of rifampicin (Fig. 2), which is typical of reports that 0.02 to 1 $\mu$g/ml of rifampicin causes complete inhibition of activity of the enzyme from bacterial sources (35–37). As shown in this figure, the enzyme from C. acidi-urici is extremely resistant to inhibition by this antibiotic. At the highest concentration of rifampicin tested, 400 $\mu$g/ml, the enzyme still shows 30% activity at low ionic strength.

Streptovaricin, like rifampicin, inhibits a step in the initiation of transcription (37). The C. acidi-urici RNA polymerase is inhibited 50% by 200 $\mu$g/ml of streptovaricin, while the enzyme from E. coli is inhibited 60% by 2 $\mu$g/ml.

Streptolydin, an antibiotic that has been found to inhibit phosphodiester bond formation (elongation) in the transcription process (38), was found to completely inhibit the clostridial enzyme at the lowest level tested, 10$^{-4}$ M.

**Template Specificity**—Three templates were used to quantitate the recovery of activity during the purification of the C. acidi-urici enzyme: B. subtilis phase $\phi 29$ DNA, E. coli phase T7 DNA, and poly(dA-dT). It was found at every stage of the purification that the enzyme was only 1–5% as active on T7 DNA as on $\phi 29$ DNA. The enzyme was highly active on poly(dA-dT). This apparent DNA template specificity is unique among bacterial RNA polymerases that have been described (8) and led us to determine the activity of this

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**Table II**

| Source | $\beta$ | $\beta'$ | $\sigma$ | $\alpha$ |
|--------|--------|--------|--------|--------|
| E. coli | 150$^a$ | 160$^b$ | 96$^c$ | 36.5$^c$ |
| E. coli | 145–155 | 150–165 | 82–95 | 36.5 |
| B. subtilis | 143$^d$ | 138$^d$ | 56$^d$ | 43$^d$ |
| B. subtilis | 154 | 155 | 56 | 43 |
| C. acidi-urici | 147 | 136 | 60 | 40 |
| C. cylindrosporum | 147 | 136 | 45 | 39 |

$^a$ Values reported in the literature used as standards (see text, “Materials and Methods”).
$^b$ Values determined in this investigation with 7.5% SDS-polyacrylamide slab gels as described under “Materials and Methods.”
$^c$ Values representing the range of values reported in the literature (6).
enzyme on a variety of DNAs. The results are summarized in Table III. The specific activity of the enzyme varied over a 100-fold range as a function of the template on which it was assayed. In general, much higher levels of activity were found on DNAs from bacteriophages that infect Gram-positive organisms. Consistently low levels of activity were found on DNAs from Gram-negative sources. The effect of salt on transcription will be discussed below.

Characterization of ϕ29 Transcripts—The in vitro transcription products made by B. subtilis RNA polymerase in response to ϕ29 DNA have been well characterized (39). It was, therefore, possible to determine whether the same promoter and termination sites are utilized by the C. acidii-urici enzyme as by the B. subtilis enzyme. As shown in Fig. 3, the

![Graph](image)

**Fig. 2. Inhibition of C. acidii-urici RNA polymerase on ϕ29 DNA by rifampicin.** Components of the reaction mixtures (including rifampicin where indicated) were assembled at 4 °C. A stock solution of rifampicin (20 mg/ml) was prepared in dimethylformamide and dilutions were made into dimethylformamide such that a 1-μl aliquot of the inhibitor was added to each 50 μl of incubation. In a control assay, it was shown that 1 μl of dimethylformamide does not affect the activity of the enzyme. Enzyme (2 μg of step 5 C. acidii-urici or B. subtilis enzyme) was then added and the reaction mixtures were incubated at 37 °C for 10 min. (L) Low salt, no added KCl; (H) high salt, 160 mM KCl. Inhibition of the B. subtilis enzyme (Δ) was the same at both salt concentrations.

**Table III**

| Source of DNA | KCl concentration | Specific activity (units/mg) |
|---------------|------------------|-----------------------------|
| Synthetic polymer |                 |                             |
| Poly (dA-dT)   | 0    | 1400                        |
|                | 160 mM | 720                         |
| Phages of Gram-positive cells |         |                             |
| ϕ29           | 0    | 960                         |
|                | 160 mM | 2100                        |
| ϕe            | 0    | 204                         |
|                | 160 mM | 640                         |
| SP01          | 0    | N.D.                        |
|                | 160 mM | 370                         |
| Phages of Gram-negative cells |         |                             |
| T7            | 0    | 37                          |
|                | 160 mM | 3                           |
| T7 AD111      | 0    | 12                          |
|                | 160 mM | N.D.                        |
| T4            | 0    | 73                          |
|                | 160 mM | 7                           |
| SP6           | 0    | 67                          |
|                | 160 mM | 7                           |
| X4            | 0    | 21                          |
|                | 160 mM | N.D.                        |

*Not determined.

**Fig. 3. Analysis of RNA transcripts from ϕ29 DNA.** The reaction mixtures (100 μl) contained 10 μg of ϕ29 DNA, approximately 2 μg of each RNA polymerase, [α-32P]CTP at 400 cpm/pmol, and 160 mM KCl unless otherwise indicated. Transcripts were analyzed by autoradiography of 1.75% polyacrylamide and 0.7% agarose slab gels. Lane a, E. coli enzyme; lane b, B. subtilis enzyme; lane c, C. acidii-urici (step 5) enzyme in the absence of KCl; lanes d and e, C. acidii-urici enzyme under standard assay conditions in the absence and presence of 20 μg/ml of rifampicin, respectively; lanes f and g, C. cylindrosorum enzyme (4 μg) in the absence of KCl and under standard conditions, respectively. The total amount of CMP incorporated in reactions a-g was 1.32, 1.47, 0.71, 1.3, 0.95, 1.45, and 2.3 nmol, respectively.

C. acidii-urici enzyme makes the same products on ϕ29 DNA (lane d) as does the B. subtilis enzyme (lane b), and these resemble the products formed on ϕ29 DNA by the E. coli enzyme (lane a), although there may be slight differences in efficiency of promoter site utilization.

It was also of interest to determine whether the products formed by C. acidii-urici RNA polymerase in the presence of rifampicin are the same as those formed by the enzyme in the absence of the inhibitor. The results shown in lane e of Fig. 3 demonstrate that the transcripts formed by the C. acidii-urici enzyme in the presence of 20 μg/ml of rifampicin are the same as those formed by the enzyme acting in the absence of the inhibitor (lane d) within the limits of resolution of this analysis.

Characterization of Interaction with T7 DNA—Although the C. acidii-urici RNA polymerase shows a very low level of activity on T7 DNA, some products can be detected (Fig. 4, lanes d and f). These products resemble those formed by the B. subtilis enzyme on T7 (Fig. 4, lane b) rather than those formed by the E. coli enzyme (Fig. 4, lane a). Transcription of T7 DNA by the B. subtilis enzyme is characterized by the
This endonuclease cleaves T7 DNA at a position 1450 base pairs from the left end of the T7 genome and transcription of the restricted DNA by E. coli RNA polymerase yields three RNA species that are easily separated by electrophoresis on a 2.5% agarose gel (Fig. 5, lane i). The three transcripts containing 948, 828, and 712 nucleotides result from initiation at the A1, A2, and A3 promoters, respectively. As shown in lane g of Fig. 5, the C. acidi-urici enzyme yields a single transcript that coincides in size with that formed from the A2 promoter. The efficiency of transcription from this promoter is low. In order to synthesize enough labeled RNA to be visible on the gel, it was necessary to double the concentrations of enzyme and DNA and to increase the specific activity of the [α-32P]ATP relative to that used for the E. coli enzyme.

**Salt Effect**—Since ionic strength affects every step in the transcription process (41), we have determined the effect of ionic strength on transcription of φ29 DNA by C. acidi-urici RNA polymerase as measured by the overall reaction (Fig. 6). Optimal activity is observed at 160 mM KCl, the same salt concentration at which the B. subtilis enzyme exhibits maximum activity (9). However, the extent of stimulation by salt is much higher for the clostridial enzyme (2.5×) than for the B. subtilis enzyme (1.3×). A comparison of transcripts produced at low and high ionic strength (Fig. 3, lanes c and d) suggests that high ionic strength stimulates termination of transcription. The only two RNA species made in large

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**Fig. 4. Analysis of RNA transcripts from T7 DNA.** The reaction mixtures (100 µl) contained 10 µg of T7 (lanes a–f) or T7 ΔD111 DNA (lane g), approximately 2 µg of RNA polymerase, [α-32P]CTP at 400 cpm/µmol, and no added KCl. Lane a, E. coli enzyme; lanes b and e, B. subtilis enzyme; lane c, C. cylindrosporum enzyme; lanes d and f, C. acidi-urici enzyme; lane g, C. acidi-urici (step 5) enzyme on T7 ΔD111 DNA. Total CMP incorporated in reactions a–d, and g were 3.5, 2.9, 1.2, 0.04, and 0.015 nmol, respectively. Lanes e and f are longer exposures of lanes b and d, respectively.

formation of significant amounts of C and D transcripts in addition to the A transcript, the main product synthesized by the E. coli enzyme. The A transcript is a mixture of products initiated at three strong A promoters that are located very near the end of the T7 genome (40). These products are similar in size and are not separated in the gel system used here. In order to determine whether the C. acidi-urici enzyme utilizes any of the three A promoters of T7 DNA or simply initiates at the end of the DNA to form the small amount of A-like product observed, transcription of the T7 deletion mutant AD111 by the C. acidi-urici enzyme was determined. This mutation deletes the region between 1.3% and 4.1% of the genome that includes the A2 and A3 promoters and only the A1 promoter is present (40). Only 30% as much acid-insoluble radioactivity was incorporated by the enzyme in response to the mutant DNA compared to the parent T7 DNA. Examination of the RNA synthesized from ΔD111 (Fig. 4, lane g) showed a single faint band corresponding in size to the C transcript. Since the efficiency of end initiation should be the same for both wild type T7 and the deletion mutant, it appears that the 8000 nucleotide transcript from wild type T7 DNA is a result of initiation at the A2 and/or A3 promoter. The absence of any higher molecular weight RNA indicates that the enzyme terminates transcription efficiently at the 18.9% terminator.

In order to substantiate this conclusion and to further characterize the site of interaction of the C. acidi-urici enzyme with T7 DNA, a Hae III digest of T7 DNA was transcribed.
amounts at low ionic strength (G1 and A1) terminate at the end of the DNA (39). As shown in Table III, transcription of ϕ29, φ6, or SP01 DNA by the clostridial enzyme is very efficient in the presence of 160 mM KCl. In contrast, the low level of activity on the templates related to Gram-negative organisms is essentially eliminated by high salt.

At low ionic strength, the specific activity of the purified clostridial enzyme is slightly higher on poly(dA-dT) than on ϕ29 DNA, while at high salt, the enzyme is only 37% as active on the synthetic template as on ϕ29 DNA.

Other Clostridial Enzymes—The unusual properties of the enzyme from C. acidi-urici (strain 9a) prompted us to investigate whether these properties were characteristic of all clostridial RNA polymerases. C. acidi-urici is classified in group V of the clostridia, those with special growth requirements (42).

As a representative of a more typical clostridial species, we chose C. pasteurianum, a member of group I of the genus Clostridium. The properties of the RNA polymerase from C. pasteurianum (Table IV) were very similar to those of the enzyme purified from B. subtilis. The C. pasteurianum enzyme was active on T7 DNA and showed the template-dependent salt effect that seems to be characteristic of polymerases isolated from Gram-positive organisms. The RNA polymerase from C. pasteurianum was also found to be sensitive to low levels of rifampicin.

C. cylindrosporum is another purine-fermenting organism that, like C. acidi-urici, can be classified as a group V Clostridium. The RNA polymerase from C. cylindrosporum (strain HC1) was purified to about 95% homogeneity. The specific activity of the purified enzyme on ϕ29 DNA was identical with that of the enzyme from C. acidi-urici and showed a similar response to increasing ionic strength. However, the RNA polymerase from C. cylindrosporum is inhibited 97% by 0.7 μg/ml of rifampicin and transcribed T7 DNA at low ionic strength 50% as efficiently as it did ϕ29 DNA. The products of the C. cylindrosporum enzyme on ϕ29 DNA (Fig. 3, lanes f and g) and on T7 DNA (Fig. 4, lane c) were identical with those formed by B. subtilis.

The difference in the effect of rifampicin on the RNA polymerases of C. cylindrosporum and C. acidi-urici was reflected very dramatically in the effect of the antibiotic on the growth of these two organisms. The effect of rifampicin on the 24-h growth of the two organisms was determined at 10-fold dilutions of the antibiotic from 200-0.00002 μg/ml. Growth of C. cylindrosporum, strain HC1, was completely inhibited by 0.0002 μg/ml, but was unaffected by the 10-fold lower dilution tested. Growth of C. acidi-urici, strain 9a, was completely unaffected by up to 2.0 μg/ml of rifampicin, but was finally inhibited at a concentration of 20 μg/ml.

Since spontaneous rifampicin mutations occur at a frequency of one in 10⁸ (37), we wished to determine whether C. acidi-urici strain 9a was simply the chance isolation of a rifampicin-resistant spontaneous mutant or was representative of a class of soil organisms. We, therefore, partially purified RNA polymerase from two different strains of clostridia, AAM-2 and AC-1, that had previously been classified as strains of C. acidi-urici (15) on the basis of spore morphology and fermentation products (43). As shown in Table IV, the enzymes from strains AAM-2 and AC-1 showed the same resistance to inhibition by rifampicin as did strain 9a. The enzymes also transcribed T7 DNA with very low efficiency. Strain MJ-6 had previously been classified as a strain of C. cylindrosporum (15), and the RNA polymerase from this organism showed the same sensitivity to rifampicin as did the enzyme from strain HC1 of C. cylindrosporum.

Transcription with Combinations of Heterologous Subunits—Previous experiments (39) based on the transcription of ϕ29 DNA and T7 DNA by E. coli core polymerase with either B. subtilis or E. coli φ subunit demonstrated that the transcription products were characteristic of the core component. Since C. acidi-urici RNA polymerase shows such a marked difference in its ability to transcribe T7 DNA compared to polymerase from E. coli, it appeared worthwhile to confirm the conclusion concerning this core function.

The experiments illustrated in Fig. 5 demonstrate that the transcription products formed by bacterial RNA polymerase composed of heterologous mixtures of core and α elements are characteristic of the core component rather than of the α subunit. Since the α subunit of C. acidi-urici RNA polymerase had not been purified to homogeneity, the holoenzyme was

Table IV

| DNA specificity and rifampicin sensitivity of clostridial RNA polymerases |
| DNA source | Inhibition by rifampicin on ϕ29 DNA |
| --- | --- |
| RNA polymerase source | Strain | KCl concentration (mM) | Rifampicin concentration (μg/ml) |
| --- | --- | --- | --- |
| T7 | ϕ29 | 0 | 160 | 0 | 160 | 0 | 160 | 7 | 70 |
| C. acidi-urici | 9a | 24 | 3 | 520 | 1320 | 1160 | 600 |
| AAM-2 | 14 | N.D. | N.D. | 400 | 440 | 218 |
| AC-1 | 10 | N.D. | N.D. | 540 | 480 | 258 |
| C. cylindrosporum | HC-1 | 500 | 28 | 440 | 960 | 2 | 1 |
| MJ-6 | 128 | N.D. | N.D. | 380 | 6 | 5 |
| C. pasteurianum | 6013 | 249 | 14 | 360 | 820 | 5 | 2 |

* Not determined.
used as a source of the σ subunit in an exchange reaction already demonstrated to occur between the E. coli core and the B. subtilis σ subunit present in the B. subtilis holoenzyme (39). The E. coli core enzyme had a significant level of activity on T7 DNA as measured by trichloroacetic acid-precipitable counts (see legend to Fig. 5), but the products of transcription (lane b, Fig. 5) are different from those made by E. coli holoenzyme (lane a, Fig. 5). The core products are probably the result of initiation at nicks or at the ends of the DNA (7).

The addition of purified E. coli σ subunit to the core polymerase stimulates activity 10-fold, and the products (lane d) are specific and characteristic of the E. coli holoenzyme (lane a). Transcription of T7 DNA by an equivalent amount of C. acidi-urici RNA polymerase alone does not yield any detectable products (lane c) under these reaction conditions. The addition of E. coli core polymerase to a transcription reaction containing the C. acidi-urici holoenzyme results in the formation of products (lane e) characteristic of the E. coli holoenzyme. The level of activity is comparable to that found with the homologous reconstituted E. coli enzyme. The addition of purified E. coli σ subunit to the C. acidi-urici polymerase (lane f) does not result in the formation of any detectable transcripts.

The role of the core components in determining the extent of promoter expression was also tested through the use of the Hae III digest of T7 DNA, since it has been demonstrated that the C. acidi-urici holoenzyme initiates transcription at the Aγ promoter only (lane g, Fig. 5), while the E. coli reconstituted enzyme also utilizes the Aα and Aγ promoters (lane i, Fig. 5). The addition of C. acidi-urici RNA polymerase as a source of the σ subunit to the E. coli core (lane h) results in the formation of transcripts of Hae III-digested T7 DNA that are characteristic of the E. coli enzyme (lane i). Transcription products from Hae III-digested T7 DNA with E. coli core polymerase alone are shown in lane j.

Although we have not prepared C. acidi-urici σ subunit in pure form, we have obtained pure C. acidi-urici core in the course of attempting to purify this enzyme by the heparin-agarose procedure (9). The enzyme in the crude extract bound to a column of heparin-agarose and was eluted with 0.5 M NaCl. Fractions containing the enzyme were then applied to a glycerol gradient in a zonal rotor. Following centrifugation, there was no ϕ29-dependent activity in any of the fractions of the gradient. However, by assaying mixtures of various fractions, it was discovered that the enzyme had dissociated into core and σ subunits. The core polymerase was near the bottom of the gradient and was essentially pure, although the σ subunit present in other fractions was still impure.

Since purified σ subunits of E. coli and B. subtilis were available (39), the activity of the heterologous RNA polymerase containing C. acidi-urici core subunits with σ subunits derived from other sources was tested (Table V). When assaysed on ϕ29 DNA, C. acidi-urici core or σ subunit alone was inactive. However, the C. acidi-urici core formed transcripts on ϕ29 DNA when mixed with σ subunits derived from C. acidi-urici or B. subtilis, but not from E. coli. The transcripts made by the two active reconstituted enzymes were identical with those made by C. acidi-urici holoenzyme (data not shown). When tested on T7 DNA, none of the heterologous mixtures described above showed any activity above that of the C. acidi-urici holoenzyme.

**DISCUSSION**

Clostridial RNA polymerase resembles the enzyme from other Gram-positive organisms in subunit structure (β, β', σ, αC) and in having a σ subunit roughly one-half the molecular weight of that present in E. coli or other Gram-negative organisms. This subunit structure is characteristic of the enzyme isolated from C. cylindrosporum as well as that from C. acidi-urici both classified as members of clostridial group V, species with special growth requirements (42). In this respect, these clostridial enzymes differ from those found in the Archaeabacteria, which appear to have subunit structures markedly different from that found in the organisms of the eu-bacterial kingdom.

Although the RNA polymerase of C. acidi-urici resembles the enzymes found in other eubacteria with respect to subunit composition, it differs significantly in two other respects: its apparent specificity in template recognition and its resistance to inhibition by rifampicin. The C. acidi-urici enzyme shows very low activity on DNAs derived from phages that infect Gram-negative organisms. We do not believe that this phenomenon is attributable to the presence of a high proportion of inactive enzyme molecules as has been demonstrated to explain the relatively poor efficiency of RNA polymerase from several bacterial orders in transcribing T7 DNA compared to the activity of the E. coli enzyme on that template (8, 44). This view was substantiated by the observation that the ratio of the activity of such enzymes to that of the E. coli enzyme on T7 DNA is the same as the ratio of the activities of the two enzymes on poly(dA-dT) (8). This is not true of the C. acidi-urici enzyme. Although it shows low activity on T7 DNA, it shows high activity on poly(dA-dT) as well as on DNAs derived from phages related to Gram-positive organisms. This is true whether the activity is determined at low or high salt concentrations.

In addition to possessing the unique template “specificity,” RNA polymerase from C. acidi-urici is also distinguished from all other RNA polymerases isolated from wild type prokaryotes in being resistant to rifampicin. In this respect, it resembles the enzyme from eukaryotes and from the Archaeabacteria. These unusual properties of the enzyme were associated with several other independently isolated strains of C. acidi-urici but not with the enzyme present in C. pasteurianum, a member of group I clostridia, or of another species of group V clostridia, C. cylindrosporum. Although C. cylindrosporum is very closely related to C. acidi-urici in its phenotypic characteristics in that both organisms can only utilize a limited number of purine bases as carbon, nitrogen, and energy sources (43), they have been shown to be genetically distantly related on the basis of several protein sequences (15). The classification of Woese (2) also suggests that C. acidi-urici is somewhat distantly related to other clostridia, but it has not been possible to include C. cylindrosporum in the studies of ribosomal 16 S base sequences needed for phylogeny determinations.

The extremely low level of activity of C. acidi-urici RNA polymerase on T7 compared to that of other eubacterial enzymes has prompted us to characterize this enzyme as

**TABLE V**

| Source of σ subunit | Cpm |
|---------------------|-----|
| None                | 123 |
| C. acidi-urici      | 1700|
| B. subtilis         | 2516|
| E. coli             | 150 |
| C. acidi-urici with no added core | 80  |
possessing template “specificity.” However, it must be recog-
nized that the C. acidi-urici enzyme is clearly related to the
other eubacterial enzymes. Thus, although the activity of C.
acidi-urici polymerase on T7 DNA is extremely low, the
enzyme does recognize three of the T7 early promoters, A, C,
and D. The ability to select promoter sites is a property of the
σ subunit (7, 32) and the C. acidi-urici σ subunit appears to
be homologous to other eubacterial σ subunits in this respect.
In addition, the C. acidi-urici σ subunit interacts with the E.
coli core polymerase to give a highly active heterologous
enzyme. However, strikingly different amounts of products
are synthesized on T7 DNA by the C. acidi-urici polymerase
than by any other eubacterial enzyme tested. We believe that
the results reported here related to the activity of heterologous
mixtures of σ subunits with core enzymes confirm those pre-
viously reported (39) and demonstrate that the yield of each
transcript formed by such enzymes is characteristic of the
core elements. The examples described in the current inves-
tigation are particularly striking since the transcripts of the C.
acidi-urici RNA polymerase on T7 DNA and Hae III-digested
T7 DNA are so easily distinguished from those of E. coli RNA
polymerase on these templates. However, these results were
obtained by measuring only productive transcription and con-
cusions based on binding or total initiation (including abortive
starts and paused products) might be somewhat different.

Although we have previously shown (39) that transcripts
formed by a heterologous mixture composed of E. coli core
and B. subtilis σ on φ29 DNA are characteristic of the core
element, it was not possible to carry out the experiment with
the reciprocal combination of E. subtilis core with E. coli σ
since this combination of subunits is inactive on any DNA
template tried (39). It was assumed that steric factors prevent
the interaction of these heterologous subunits since the σ
subunit of the E. coli enzyme is almost twice as large as that
of B. subtilis. Consistent with these findings, it has also been
found in the studies reported here that the heterologous
enzyme composed of C. acidi-urici core with E. coli σ is
inactive on any DNA template tested. However, the role of
the core elements in affecting the transcripts could be dem-
onstrated with a heterologous enzyme derived from Gram-
positive organisms. The heterologous enzyme composed of
C. acidi-urici core with B. subtilis σ subunit forms transcripts
on φ29 DNA indistinguishable from those formed by either
holoenzyme, which are indistinguishable from each other.
Although this experiment demonstrates that a heterologous
combination of subunits can generate an active enzyme, it
does not serve to distinguish unique functions of the subunit
components of the enzyme. However, this heterologous mix-
ture, although active on φ29 DNA, exhibits extremely low
activity on T7 DNA. This behavior is characteristic of the
core element rather than of the σ subunit of the heterologous
enzyme.

In a sense, the results reported here suggest that C. acidi-
urici RNA polymerase represents an extreme example among
the enzymes of Gram-positive bacteria with respect to appar-
ent promoter specificity. This concept depends on a recogni-
tion of the effect of ionic strength on the activity of RNA
polymerase. The activity of the enzyme from E. coli, represen-
tative of Gram-negative bacteria, is relatively unaffected by
ionic conditions whether transcribing DNA templates from
Gram-negative or Gram-positive sources. The RNA poly-
merase of Gram-positive bacteria, such as B. subtilis, is af-
fected by ionic conditions, and the effect is a function of the nature
of the DNA template. The enzymes are markedly inhibited at
high salt when transcribing DNA from Gram-negative sources,
like T7, but are not inhibited, or may be stimulated, by high
salt when transcribing DNA from Gram-positive sources such
as φ29 DNA. This effect is even more pronounced in the case
of the RNA polymerase of C. acidi-urici, where transcription of
T7 DNA at low salt concentrations is very poor, and the
enzyme is virtually inactive on this DNA at high ionic
strength, whereas it transcribes φ29 DNA most efficiently at
high ionic strength. These observations suggest that the tran-
scriptional process may play some role in the restriction of
genetic expression observed in vivo with systems derived from
Gram-positive organisms.

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