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Purification and Partial Characterization of the Principal Deoxyribonucleic Acid Polymerase from *Mycoplasmatales*

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In this report we present the first description of the isolation and partial characterization of the deoxyribonucleic acid (DNA) polymerase activity from two species of *Mycoplasmatales*, *Mycoplasma orale* type 1 and *M. hyorhinis*. We have identified only a single DNA polymerase species in the mycoplasma crude extracts, and the enzymes from the two organisms are very similar in their structural and enzymatic properties. The purified polymerase from each source has a specific activity of >50,000 U/mg of protein, a sedimentation coefficient of 5.6*s*, and an estimated molecular weight by gel filtration of 130,000. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the most highly purified *M. orale* fraction contains a single major protein band of 130,000 daltons, which we believe may represent the polymerase protein. The enzymes are most reactive with gapped (activated) DNA and show a marked preference for this primer template over oligodeoxyribonucleotide-initiated homoribo- or homodeoxyribopolymers. The most purified preparations are devoid of contaminating endonuclease activity and also appear to lack associated 5′ → 3′- or 3′ → 5′-exonuclease activities, as determined by highly sensitive assays. The absence of the 3′ → 5′-exonuclease is particularly remarkable in that this activity is essentially ubiquitous among the DNA polymerases that have thus far been characterized from procaryotes.

The *Mycoplasmatales* comprise a group of microorganisms that are the smallest known free-living procaryotes. They are characterized by their minute size (0.25 to 1.0 μm in diameter), the absence of a cell wall, and a relatively small genome (5×10^8 to 10×10^8 daltons) of presumably limited complexity that has been shown to be a single, circular, double-stranded deoxyribonucleic acid (DNA) molecule (14, 19, 24). Limited studies to date have indicated that mycoplasma DNA replication appears to be semiconservative and to proceed unidirectionally from one or a few growing-point regions (21) that may be in a membrane-associated complex (22). The capacity of at least one *Acholeplasma* species to carry out light and dark repair of ultraviolet-irradiated DNA has also been described (23). Apart from these few published reports, however, almost nothing is yet known about the biochemical basis of DNA replication in these organisms.

Aside from their intrinsic interest, mycoplasmas have increasingly been recognized as common and troublesome contaminants of cell cultures, difficult to detect and difficult to eradicate (19, 24). Moreover, there is now abundant evidence that such contamination, often unrecognized, can have profound effects on cultured cell properties, including alterations of metabolic properties, induction of chromosomal aberrations, and modifications of cellular morphology that can be mistaken for virus-provoked cytopathic effects (19, 24).

In view of the intensive effort that is currently being expended in many laboratories on the identification and characterization of DNA polymerase activities from a variety of cultured eucaryotic cells, we felt it was important to document the properties of the DNA polymerases of representative *Mycoplasmatales*. In this paper, we present the purification and partial characterization of the principal DNA polymerase activity that we have isolated from crude extracts of two *Mycoplasma* species that are well-recognized cell culture contaminants, *Mycoplasma orale* type 1 and *M. hyorhinis*. In corroboration of the potential value of this undertaking, we note that at least one reported species of eucaryotic DNA polymerase, mitochondrial D-DNA polymerase from HeLa cells (7, 28), has recently been suggested to be a possible mycoplasma activity resulting from

culture contamination (1a). The properties of that enzyme are very similar to those of the highly purified mycoplasma polymerases that we describe in this report.

MATERIALS AND METHODS

Materials. Unlabeled deoxyribonucleotides were purchased from P-L Biochemicals; [^{3}H]deoxythymidine 5'-triphosphate (dTTP) was from New England Nuclear Corp.; calf thymus DNA was from Calbiochem; Triton X-100 was from Beckman Instruments, Inc.; Sephadex G-25 and G-200 were from Pharmacia Fine Chemicals, Inc.; diethylaminoethyl (DEAE)-cellulose (DE-52) and phosphocellulose (P-11) resins were from Whatman; acrylamide, bismethylene acrylamide, and N_1,N_1,N_1',N'-tetramethylenediamine were from Bio-Rad Laboratories; BDH sodium dodecyl sulfate (SDS) was from Gallard-Schlesinger; bovine serum albumin and 7S immunoglobulin were from Sigma Chemical Co.; ovalbumin was from Pharmacia; and β-galactosidase was from Worthington Biochemical Corp. Coomassie brilliant blue was from Schwarz/Mann, and amido black 10B was from Baker. LKB ampholytes were used for isoelectric focusing. Polydeoxyadenylate [poly(dA)] was from Collaborative Research, and polyadenylate [poly(A)] and poly(deoxyadenylate-deoxythymidylate) [poly(dA-dT)] were from Miles Laboratories, Inc. Heat-denatured calf thymus DNA-cellulose (Cellex-N-1; Bio-Rad) was prepared (1) by D. C. Eichler, Stanford University. Oligo(dT)$_{\overline{31}}$ and oligo(dT)$_{\overline{50}}$-[^{3}H]deoxycytidylate (dC)$_{\overline{0.7}}$ (specific activity, 14,000 cpm/pmol) were prepared (26) by T. S-F. Wang, Stanford University, using d(pT)$_4$ (Collaborative Research) as initiator. Terminal deoxynucleotidyl-transferase (13,000 U/mg), purified from calf thymus, was a gift from R. L. Ratliff, Los Alamos Scientific Laboratory; bacteriophage T4 DNA polymerase, phosphocellulose fraction (15) (~15,000 U/mg), was from N. G. Nossal, National Institutes of Health; ColE1 form I [^{14}C]DNA (4,000 cpm/μg) and PM2 form I [^{3}H]DNA (1,600 cpm/μg) were the gift of D. A. Clayton, Stanford University; and specifically incised, ultraviolet-irradiated *E. coli* [^{3}H]DNA (1.7×10^5 cpm/μg) was provided by K. H. Cook, Stanford University.

Growth and harvest of mycoplasmas. *M. hyorhinis* and *M. orale* type I were grown in a modified Hayflick medium (9) consisting of 85 volumes of beef heart infusion broth (Difco), 10 volumes of unheated horse serum (Irvine Scientific), 5 volumes of a 25% stock solution of freshly prepared yeast extract (Standard Brands, Inc.), and glucose and arginine at a final concentration of 1% (wt/vol) each. Penicillin G at a final concentration of 100 U/ml was added to prevent bacterial contamination. Flasks containing 500 ml of prewarmed growth medium were inoculated with 5 ml of an actively growing culture at a density of 10^6 to 10^7 organisms per ml and incubated 3 to 4 days at 37°C. Growth of organisms was monitored by assay of colony-forming units (CFU) on agar (19). Two hours before harvest, 50 to 100 ml of prewarmed growth medium was added to each culture. The cultures were centrifuged at 10,000 rpm for 20 min at 4°C. The pellet, which contained organisms and particulate constituents from the growth medium, was washed twice with phosphate-buffered saline (PBS) and then stored frozen at −70°C as an unpurified mycoplasma pellet. Initial efforts to isolate the DNA polymerase activity from such pellets revealed unpredictable variations in column chromatographic behavior, which we attributed to the presence of undefined medium components in the crude extract. We therefore introduced an additional procedure to purify the mycoplasma organisms. The crude pellets were suspended in 20 ml of PBS, and 5-ml portions were overlayered on 30-ml discontinuous 30 to 60% (wt/wt) sucrose gradients in PBS and centrifuged in an SW25.1 rotor at 10,000 rpm for 60 min. The visible bands of mycoplasma were collected, pooled, suspended in PBS, and pelleted. The washing and centrifugation steps were repeated, and the final pellet was stored at −70°C as a purified mycoplasma preparation. Just before the last centrifugation, the PBS suspension was assayed for mycoplasma CFU. The majority of harvests yielded a total of 1×10^{11} to 5×10^{12} CFU.

Standard DNA polymerase assay. The standard assay was run for 10 min at 37°C and contained the following in a total volume of 0.25 ml: tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.5), 10 mM; β-mercaptoethanol, 2.0 mM; bovine serum albumin, 50 μg; $MgCl_2$, 20 mM; KCl, 100 mM; activated calf thymus DNA (20), 200 μg/ml; deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, deoxycytidine 5'-triphosphate, and dTTP, 100 μM each; [^{3}H]dTTP, 40 mCi/mmol; and enzyme. In addition, 0.1% Triton X-100 was incorporated into the reaction with the most highly purified enzyme fractions, since it appeared to enhance the reproducibility of the assays. The reactions were terminated and processed as previously described (20). One unit of DNA polymerase activity is defined as the amount catalyzing the incorporation of 1 nmol of labeled deoxythymidine 5'-monophosphate into acid-insoluble product in 60 min at 37°C under standard reaction conditions. Specific activity is expressed as units per milligram of protein. All enzymatic properties were determined under conditions of linearity with respect to time and protein.

Glycerol gradient centrifugation. Linear 20 to 40% (vol/vol) glycerol gradients were prepared in 50 mM potassium phosphate (pH 7.5) and 1 mM each ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol, with or without KCl at 0.5 M. Enzyme sample (0.25 ml) was layered onto the top of the gradient and overlayered with mineral oil. Sedimentation was carried out at 50,000 rpm for 24 h at 5°C in an SW50.1 rotor. Fractions of 0.25 ml were collected dropwise from the bottom of the tube, and portions were tested in the standard polymerase assay.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gels, 3 mm in diameter and containing 7% acrylamide, were prepared and run according to Laemmli (11), without stacking gel. Samples were prepared for electrophoresis by alternate concentra-

tion against dry Sephadex G-200 beads and dialysis versus 0.05 M Tris-hydrochloride (pH 7.2), 1% SDS, 1% β-mercaptoethanol, and 20% (vol/vol) glycerol, until the sample volume had been reduced to <0.2 ml. Samples were then boiled for 2 min, and 5 μl of 0.125% bromophenol blue marker in 50% (wt/vol) glycerol was added to each. The standard proteins were handled similarly. Electrophoresis was carried out at 3 mA/gel for 4 to 5 h. The gels were stained for 2 h at 37°C with Coomassie brilliant blue and diffusion destained in 7.5% glacial acetic acid.

Isoelectric focusing. Isoelectric focusing was performed as previously described (2), using carrier ampholytes (pH 3 to 10) at a concentration of 2.5%.

DNA polymerase assays with synthetic primer templates. Homopolymer primer templates were prepared as described previously (26). The incubation mixture contained the following in a total volume of 0.05 ml: Tris-hydrochloride (pH 7.5), 50 mM; bovine serum albumin, 10 μg; [^{3}H]dTTP, 100 μM (1 μCi/nmol); poly(dA) or poly(A), 0.1 mM; oligo(dT), 0.01 mM (polymer concentrations are expressed as moles of total nucleotide); and optimum concentrations of $MgCl_2$, $MnCl_2$, and KCl, as determined separately for each polymerase with both ribo- and deoxyribohomopolymer primer templates. Incubations were carried out for 15 min at 30°C, and reaction products were analyzed on DE-81 paper disks (27). Assays with poly(dA-dT) as primer template were performed exactly as above, except that poly(dA-dT) was present at 0.1 mM (total nucleotide) and deoxyadenosine 5′-triphosphate was present at 100 μM. Incubations were carried out for 10 min at 37°C.

3′ → 5′-Exonuclease assays. 3′-terminally labeled oligonucleotide substrates were prepared with terminal transferase as before (26) and were used either alone or after annealing to poly(dA) to produce double-stranded polymers (polymer nucleotide to oligomer nucleotide was 10:1). The following incubation mixes were used: (i) in a total volume of 0.25 ml—Tris-hydrochloride (pH 7.5), 50 mM; bovine serum albumin, 50 μg; $MgCl_2$, 5 mM; $(dT)_{\overline{31}}$-[^{3}H]-$(dT)_{\overline{5}}$, 0.02 mM (732 cpm/pmol); ±poly(dA), 0.2 mM; and enzyme; (ii) in a total volume of 50 μl—Tris-hydrochloride (pH 7.5), 50 mM; bovine serum albumin, 10 μg; $MgCl_2$, 5 mM; $(dT)_{\overline{59}}$-[^{3}H]$(dC)_{\overline{0.7}}$, 0.01 mM; poly(dA), 0.1 mM; and enzyme. Incubations were carried out for 30 min at 35°C and were processed as previously described (26, 27). As a control, highly purified T4 DNA polymerase (15) was incubated under its optimum exonuclease conditions (16) with the substrate $(dT)_{\overline{31}}$-[^{3}H]$(dT)_{\overline{5}}$. Hydrolysis of the labeled residues was complete within 2 min and yielded a ratio of nuclease activity to polymerase activity for this enzyme fraction of 0.60. The rapid and complete digestion of the terminally mismatched [^{3}H]deoxycytidine 5′-monophosphate residues from poly(dA)·$(dT)_{\overline{59}}$-[^{3}H]$(dC)_{\overline{0.7}}$ by the 3′-exonuclease activity of *E. coli* DNA polymerase I has been previously documented (26).

Assay for excision of thymine dimers. The reaction mix contained the following in a total volume of 0.25 ml: Tris-hydrochloride (pH 8.7), 10 mM; bovine serum ablumin, 100 μg; β-mercaptoethanol, 2 mM; $MgCl_2$, 2 mM; specifically incised, ultraviolet-irradiated *E. coli* [^{3}H]DNA, 12 μg (6); and enzyme. The incubation was carried out for 30 min at 37°C and was terminated and processed as described by Cook and Friedberg (4). This assay measures the specific 5′-exonucleolytic excision of thymine dimers from irradiated, incised duplex DNA (6).

Assay for endonuclease activity. The incubation mixture contained the following in a final volume of 0.25 ml: Tris-hydrochloride (pH 7.5), 55 mM; $MgCl_2$, 5 mM; bovine serum albumin, 50 μg; NaCl, 10 mM; EDTA, 1 mM; ColE1 form I [^{14}C]DNA and PM2 form I [^{3}H]DNA, 0.25 μg of each; and enzyme. After incubation at 37°C for 30 min, the reaction products were analyzed (17) by ethidium bromide-cesium chloride buoyant density centrifugation to detect conversion of covalently closed, circular form I DNA molecules to open circular form II species. This conversion is effected by the incision of a single phosphodiester bond (25).

Other methods. The pH and ionic strength of buffers were determined at room temperature with a Corning digital pH meter and a radiometer conductivity meter, respectively. Protein was estimated spectrophotometrically by measuring absorbance at 260 and 280 nm, or was assayed by the technique of Schaffner and Weissmann (18) with bovine serum albumin as standard. Coomassie brilliant blue-stained polyacrylamide gels were scanned at 600 nm with a Transidyne RFT densitometer.

RESULTS

Purification of enzymes. All operations were carried out at 0 to 4°C. Buffer A was 50 mM potassium phosphate (pH 7.2), and buffer B was 50 mM potassium phosphate (pH 7.5). Buffers contained, in addition, 30% (vol/vol) glycerol and 1 mM each EDTA and β-mercaptoethanol, unless otherwise indicated. The purification protocols are summarized in Tables 1 and 2.

Preparation of crude extracts. Purified or unpurified pellets of *M. hyorhinis* and *M. orale* were thawed and suspended in 20 ml of 10 mM potassium phosphate (pH 7.2; without glycerol). Solid NaCl was added to a final concentration of 1 M. The suspension was kept on ice for 45 min and was then sonically disrupted with an MSE sonicator with 10 15-s bursts interspersed with 15-s intervals of cooling at 4°C. The suspension was centrifuged at 15,000 rpm for 20 min and the supernatant was collected (fraction I).

Gel filtration. Crude extracts were desalted on Sephadex G25 columns (2.5 by 35 cm) that had been equilibrated with buffer A. Elution of protein was carried out with buffer A and was monitored by following the absorbance at 280 nm (fraction II).

DEAE-cellulose chromatography. The Sephadex eluates were loaded directly onto a

TABLE 1. *Purification of M. orale DNA polymerase*

Source[a]	Fraction	Protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)
Unpurified pellet	I. A, Crude extract	243	24,900	103	100
	II. A, Sephadex G-25	165	25,000	150	100
	III. A, DEAE-cellulose	17	15,900	930	64
	IV. A, First phosphocellulose	15	17,800	1,190	72
	V. A, Second phosphocellulose	1.8	10,000	5,600	40
	VI. A, DNA-cellulose	<0.2	10,300	>50,000	41
Purified pellet	I. B, Crude extract	15	12,900	880	100
	II. B, Sephadex G-25	15	11,200	750	87
	III. B, DEAE-cellulose	8	8,000	1,000	62
	IV. B, Phosphocellulose	0.3	5,900	19,600	46
	V. B, DNA-cellulose	<0.07	3,900	>55,000	31

[a] Each purification was initiated with a mycoplasma pellet harvested from a single 5-liter culture containing between 1×10^{11} and 5×10^{12} CFU.

TABLE 2. *Purification of M. hyorhinis DNA polymerase*

Source[a]	Fraction	Protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)
Unpurified pellet	I. A, Crude extract	215	1,600	7	100
	II. A, Sephadex G-25	145	1,350	9	84
	III. A, DEAE-cellulose	32	1,300	42	81
	IV. A, Phosphocellulose	1.0	1,200	1,230	75
	V. A, DNA-cellulose	<0.07	820	>12,000	51
Purified pellet	I. B, Crude extract	80	12,700	160	100
	II. B, Sephadex G-25	62	11,800	190	93
	III. B, DEAE-cellulose	4	3,500	870	27
	IV. B, Phosphocellulose	0.4	3,800	9,400	29
	V. B, DNA-cellulose	<0.03	1,600	>50,000	12

[a] Each purification was initiated with a mycoplasma pellet harvested from a single 5-liter culture containing between 1×10^{11} and 5×10^{12} CFU.

column (2.5 by 13 cm) of DEAE-cellulose equilibrated with buffer A. The column was washed with buffer A until all unadsorbed protein was removed and was then developed with a single step of 0.3 M potassium phosphate (pH 7.2). The majority of the DNA polymerase activity does not adsorb to the column under these conditions; a minor fraction is recovered in the step eluate and usually represents ≤5% of the total recovery. In a single instance, a sufficient amount of polymerase activity (1,020 U; 23% of the total) was recovered in the step fraction to permit its further purification as described below for the major activity. In its subsequent chromatographic behavior, sedimentation in glycerol gradients and reactivity with activated DNA, this fraction was indistinguishable from the majority species (fraction III).

Phosphocellulose chromatography. Fraction III was loaded onto a phosphocellulose column (1.5 by 8.5 cm) equilibrated with buffer A. After it was washed with 2 column volumes of buffer A, the chromatogram was developed with a 120-ml linear gradient of 50 to 300 mM potassium phosphate in buffer A. The DNA polymerase activity from *M. hyorhinis* eluted as a single sharp peak between 130 and 150 mM (fraction IV), while the activity from *M. orale* was recovered in a single sharp peak between 65 and 85 mM (fraction IV B). The polymerase activity from the unpurified *M. orale* pellet did not adsorb to the column under these conditions. In this case, the flow-through material (fraction IV A) was dialyzed against buffer A adjusted to 25 mM potassium phosphate and reloaded on the same column that had been re-equilibrated in 25 mM potassium phosphate. The column was washed with buffer until all unadsorbed protein had been removed and was then developed with a 120-ml linear gradient of 25 to 300 mM potassium phosphate in buffer A. The polymerase activity eluted at 85 mM (*M. orale*, fraction V A).

DNA-cellulose chromatography. The phosphocellulose fraction of *M. hyorhinis* DNA polymerase was either dialyzed overnight versus buffer B or diluted with 30% (vol/vol) glycerol, 1 mM EDTA, and 1 mM β-mercaptoethanol to

a final concentration of 70 mM potassium phosphate (pH 7.2). The enzyme fraction was loaded onto a 2-ml column of DNA-cellulose equilibrated with buffer B and eluted with a 30-ml linear gradient from 50 to 300 mM potassium phosphate in buffer B. The polymerase was recovered in a single peak between 125 and 150 mM (fraction V). The phosphocellulose fraction of *M. orale* DNA polymerase (fraction V A or IV B) was loaded directly onto a 2-ml column of DNA-cellulose equilibrated with buffer B and eluted with a 30-ml linear gradient from 0 to 0.5 M KCl in buffer B. The polymerase was recovered in a single peak at 0.18 M KCl (fraction VI A or V B). The DNA-cellulose peaks were pooled and stored at −70°C for up to 6 months without appreciable loss of activity. Once thawed, however, enzyme portions were highly unstable and lost ~50% of their activity in 24 h. In most instances, enzyme purification was taken from preparation of crude extract to completion within 56 h.

Physical properties of mycoplasma DNA polymerases. (i) **Gel filtration.** The results of Sephadex G-200 gel filtration of the *M. hyorhinis* and *M. orale* DNA polymerases are presented in Fig. 1A. Under conditions of high ionic strength, both enzymes eluted identically as single, homogeneous peaks behind the bovine immunoglobulin marker at a position corresponding to an apparent molecular weight of 130,000 (Fig. 1B).

(ii) **Glycerol gradient sedimentation.** The purified DNA polymerase from each organism sedimented similarly at both low ionic strength and in 0.5 M NaCl as a single symmetrical peak of activity with a sedimentation coefficient of 5.6*s* (Fig. 2). This sedimentation value is in reasonable agreement with the molecular weight estimate obtained from gel filtration.

(iii) **Isoelectric focusing.** The isoelectric point of *M. orale* DNA polymerase fraction V A is at pH 7.0 (Fig. 3). Attempts to isoelectric focus the most highly purified preparations (*M. orale* fractions VI A and V B and *M. hyorhinis* fraction V B) have not yet been successful. The purified enzymes appear to be highly unstable to this procedure, and recoveries of activity on the order of 10% preclude the confident assignment of an isoelectric point value to these fractions.

(iv) **Polyacrylamide gel electrophoresis.** SDS-gel analysis of the most highly purified polymerase fraction (V B) from *M. orale* revealed a major band of protein of 130,000 daltons, as well as one or two minor bands that were not visible to the eye but were resolved by gel scan (data not shown). On nondenaturing polyacrylamide gels, enzyme activity

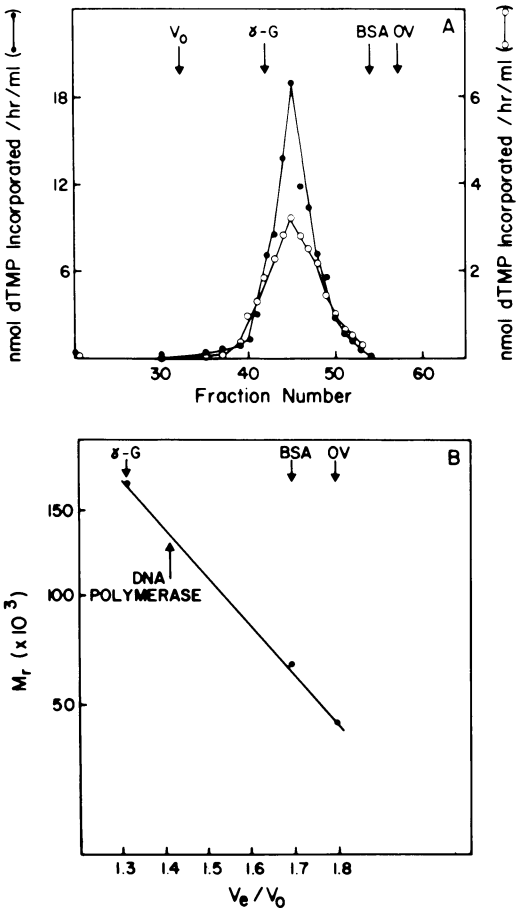

FIG. 1. *Gel filtration of mycoplasma DNA polymerases. (A) M. orale fraction V B, 125 U (●), and M. hyorhinis fraction V B, 37 U (○), were separately analyzed on a column (1.5 by 57 cm) of Sephadex G-200 equilibrated and developed in 10 mM Tris-hydrochloride (pH 7.5), 0.5 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% (vol/vol) glycerol. Fractions of 1.3 ml were collected and portions were assayed under standard conditions. Recovery of initial M. orale activity was 97%; recovery of M. hyorhinis activity was 82%. V_0 was determined with blue dextran. The elution positions of the marker proteins (↓) were determined by absorbance at 280 nm. γ-G, Bovine gamma globulin (160,000); BSA, bovine serum albumin (67,000); Ov, ovalbumin (43,000). (B) Standard semilog plot of M_r versus elution position.*

from *M. orale* fractions VI A and V B and *M. hyorhinis* fractions V A and B in each case migrated as a single peak with an R_f (relative to the dye marker) of 0.40. Due to the very limited quantities of highly purified enzyme that have thus far been available, we have not yet been able to load sufficient material on nondenaturing gels to visualize protein, nor

has denaturing gel analysis of the *M. hyorhinis* polymerase been completed. In spite of these limitations, the data suggest that the most highly purified polymerase fractions from both organisms are essentially identical in their physical properties.

Enzymatic properties of mycoplasma DNA polymerase. (i) General requirements. The activity of the purified mycoplasma polymerases is absolutely dependent upon added primer template. The enzymes are minimally active with native or heat-denatured salmon sperm DNA and show a marked preference for activated DNA, suggesting that, like *E. coli* DNA polymerases II and III (10) and most or all eucaryotic DNA polymerases (2), the mycoplasma polymerases are most effective in filling in small gaps (20). The ratio of polymerase activity with activated, native, and heat-denatured DNA is ~100:5:3. With activated DNA, the *M. orale* and *M. hyorhinis* enzymes have very similar reaction requirements (Table 3). In addition, they are essentially identical in their partial sensitivity to inhibitors that block sulfhydryl groups. Thus, in the absence of β-mercaptoethanol, the activity of both polymerases was reduced by 10 to 30%, while in the presence of 1 mM *N*-ethylmaleimide or 30 μM *p*-chloromercuribenzoate, the polymerase activities were inhibited by 25 to 35% and 20 to 50%, respectively. Both enzymes show only

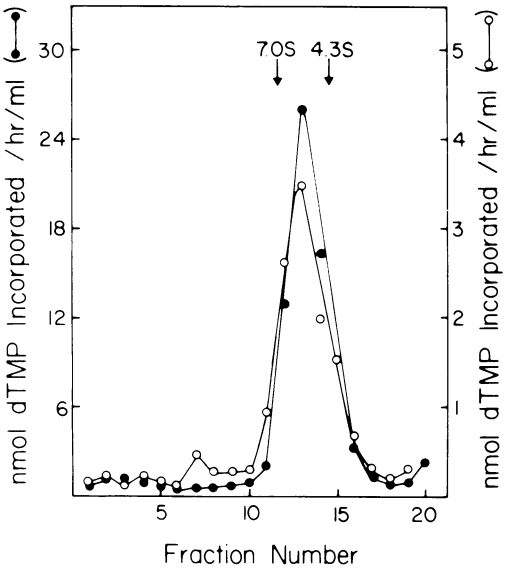

FIG. 2. *Glycerol gradient sedimentation of mycoplasma DNA polymerases. M. orale fraction V B, 21 U (●), and M. hyorhinis fraction V B, 7 U (○), were separately analyzed on 20 to 40% (vol/vol) glycerol gradients in 0.5 M NaCl. Fractions (~0.25 ml) were collected and portions were assayed under standard conditions. The marker proteins bovine gamma globulin (7.0s) and ovalbumin (4.3s) were run in a separate tube, and their positions were determined by measuring absorbance at 280 nm. About 40% of initial enzyme activity was recovered in each instance.*

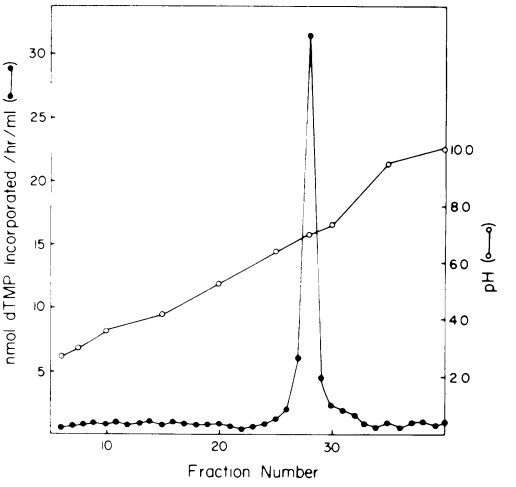

FIG. 3. *Isoelectric focusing of mycoplasma DNA polymerase. M. orale fraction V A, 18 U, was isoelectric focused for 50 h at 2°C. Fractions (0.3 ml) were collected and portions were assayed under standard conditions. Recovery of enzyme activity was 75%. The pH values (○) were measured at 4°C.*

TABLE 3. *General properties of mycoplasma DNA polymerases with activated DNA*

Mycoplasma species	pH optimum[a]	Mg^{2+} optimum (mM)	KCl optimum[b] (mM)	K_m for dNTP (μM)	K_m for DNA (μg/ml)	$[PO_4^{2-}]$ optimum[c] (mM)	$[PO_4^{2-}]$ optimum[d] (mM)
M. orale[e]	7.5 to 8.5	20	80	14	45	2	6
M. hyorhinis[f]	7.5 to 8.5	30	100	17	40	0	4

[a] The buffer was Tris-hydrochloride.
[b] Reactions contained 20 mM $MgCl_2$.
[c] Reactions contained 100 mM KCl.
[d] Reactions contained no KCl.
[e] *M. orale* fraction VI A (0.7 U) was used in each reaction.
[f] *M. hyorhinis* fraction V B (0.8 U) was used in each reaction.

slight salt stimulation (1.5- to 2-fold) at optimum ionic strength.

(ii) **Utilization of synthetic primer templates.** The *M. orale* and *M. hyorhinis* enzymes have similar requirements for optimum utilization of synthetic primer templates (Table 4). Even under the best conditions, however, both polymerases demonstrated a strong preference for activated DNA in comparison with oligo(dT)-primed poly(dA) or poly(A) templates (Table 5), while the alternating copolymer, $(dA\text{-}dT)_n$, was used at an intermediate but appreciable rate. With respect to the homopolymers, both enzymes copied the deoxynucleotide template significantly better than the ribonucleotide template irrespective of which divalent cation was available, although in the presence of Mn^{2+}, the relative preference for the deoxypolymer was considerably reduced (Table 5).

TABLE 4. *Properties of mycoplasma DNA polymerases with synthetic primer templates*

Primer template	Optimum	*M. orale* (mM)	*M. hyorhinis* (mM)
$(dA)n \cdot (dT)_{\overline{34}}$[a]	Mg^{2+}	5	7
	KCl[b]	25	0
	Mn^{2+}	0.6	0.6
	KCl	25–50	25–50
$(A)n \cdot (dT)_{\overline{34}}$[a]	Mg^{2+}	5	7
	KCl	0	0
	Mn^{2+}	0.4	0.4
	KCl	50	100
$(dA\text{-}dT)n$[c]	Mg^{2+}	2	
	Mn^{2+}	0.4–0.8	

[a] *M. orale* fraction VI A (1.1 U) or *M. hyorhinis* fraction V A (0.3 U) was used in each assay.

[b] KCl reactions contained Mg^{2+} or Mn^{2+} at optimum concentration.

[c] *M. orale* fraction VI A (1.4 U) was used.

(iii) **3′ → 5′-Exonuclease activity.** Examination of the mycoplasma polymerases for the presence of associated or contaminating nuclease activities has thus far been carried out extensively only with the *M. orale* enzyme. Using the synthetic oligonucleotide substrates listed in Table 6, which were very highly labeled at their 3′ termini, we could not detect 3′ → 5′-exonuclease activity in *M. orale* fraction V B and could exclude it at a level of ~0.03% of the polymerizing activity. Preliminary examination of *M. hyorhinis* fraction V suggests that it is similarly devoid of 3′-exonuclease activity (L. Minium and D. Korn, unpublished data).

(iv) **Thymine-dimer excising activity.** *M. orale* fraction VI A was tested for its ability to perform 5′-exonucleolytic excision of thymine dimers from specifically incised, ultraviolet-irradiated DNA, as described under Materials and Methods. Fourteen units of DNA polymerase activity excised 1.3 pmol of dimers during a 30-min incubation, yielding a ratio of dimer-excising activity to polymerase activity of

TABLE 6. *3′ → 5′-Exonuclease activity of M. orale DNA polymerase*

Substrate	Polymerase[a] activity (nmol of total dNMP incorp./h per ml)	Exonuclease activity (nmol of dNMP released/h per ml)	Nuclease/polymerase (%)
$(dT)_{\overline{31}} - [^3H](dT)_{\overline{5}}$	340	<0.1	<0.03
$(dA)_n \cdot (dT)_{\overline{31}} - [^3H](dT)_{\overline{5}}$	760	<0.2	<0.03
$(dA)_n \cdot (dT)_{\overline{59}} - [^3H](dC)_{\overline{0.7}}$	1,030	<0.4	<0.04

[a] *M. orale* fraction V B was used. Polymerase activity was determined with activated DNA under standard conditions.

TABLE 5. *Relative utilization of synthetic primer templates by mycoplasma DNA polymerases*

Primer template		*M. orale*[a]		*M. hyorhinis*[a]	
		Activity	%	Activity	%
Activated DNA		4,700[b]	100	2,700[b]	100
$(dA)n \cdot (dT)_{\overline{34}}$[c]		940[d]	20	470[d]	17
$(A)n \cdot (dT)_{\overline{34}}$[c]		120[d]	3	50[d]	2
$(dA\text{-}dT)n$[c]		3,130[b]	67		
$\frac{(dA)n \cdot (dT)_{\overline{34}}}{(A)n \cdot (dT)_{\overline{34}}}$[e]	Mg^{2+}	45		10	
	Mn^{2+}	6		1.5	

[a] Reactions were performed as described under Materials and Methods with *M. orale* fraction VI A or *M. hyorhinis* fraction V A, using the optimized conditions defined in Table 4.

[b] Picomoles of total dNMP incorporated per hour at 37°C.

[c] *M. orale* polymerase (1.1. U) or *M. hyorhinis* polymerase (0.3 U) was used in each assay.

[d] Picomoles of deoxythymidine 5′-monophosphate incorporated per hour at 30°C.

[e] *M. orale* polymerase (1.4 U) was used.

~0.02%. We believe that this trace activity is most likely a contaminant.

(v) **Endonuclease activity.** *M. orale* fraction VI A and *M. hyorhinis* fraction V B were tested for their ability to convert covalently closed, circular form I DNA molecules to open circular form II species, as described under Materials and Methods. By this method, which in principle can detect single endonucleolytic incisions, we could exclude endonuclease activity from these enzyme fractions at levels of <0.005% (*M. orale*) and <0.05% (*M. hyorhinis*).

DISCUSSION

With the relatively rapid and simple purification protocol that is presented in this paper, we have been able to obtain highly purified preparations of the major DNA polymerase activity from two different species of *Mycoplasma*. Although the two polymerases demonstrate some minor differences in their column chromatographic behavior, the most purified fractions of each are essentially identical in their physical and catalytic properties, suggesting that the enzymes described in this report may be prototypic of the mycoplasma DNA polymerases. The polymerase has a sedimentation coefficient of 5.6*s* in high salt and an apparent molecular weight of 130,000, a size that falls comfortably within the range of those reported for near homogeneous preparations of *E. coli* DNA polymerases I, II, and III (8, 10). The specific activity of our most purified polymerase fractions, >50,000 U/mg of protein, is considerably higher than those reported for the eubacterial DNA polymerases II and III (8, 12, 13, 29) and compares favorably with the best values recorded for *E. coli* DNA polymerase I (8). Although detailed analysis of the molecular structure of the mycoplasma enzymes is still incomplete, preliminary study by denaturing gel electrophoresis suggests that the polymerase protein may be a single polypeptide chain of 130,000 daltons.

In our studies to date, we have obtained no evidence for the existence of more than a single DNA polymerase species in *M. orale* and *M. hyorhinis*. Given the small size and limited complexity of the mycoplasma genome, this result may not be considered surprising. It should be recalled, however, that it was only after the isolation of *polA* mutants of *E. coli* (5) that polymerases II and III were recognized (8), and thus it would be premature to conclude that additional DNA polymerases may not await discovery in the *Mycoplasmatales*.

In their general enzymatic properties, the purified mycoplasma polymerases do not precisely resemble any of the characteristic eubacterial DNA polymerases that have been described (8, 10). The strong preference of the mycoplasma enzymes for activated DNA primer templates suggests that these enzymes, like bacterial polymerases II and III (8, 10), carry out a repair type of gap-filling synthesis; but, in their ionic-strength response and their only moderate sensitivity to sulfhydryl reagents, the mycoplasma enzymes differ from polymerases II and III. Most surprising is the apparently complete absence from the most highly purified mycoplasma fractions of associated exonuclease activities, particularly of the 3′ → 5′-exonuclease function. The latter, with perhaps the single exception of *Bacillus subtilis* DNA polymerase II (13), has been found to be present invariably in appropriately characterized eubacterial DNA polymerases (10). By contrast, these exonuclease activities are clearly not associated with highly purified eucaryotic DNA polymerases (2), with the only exception to date being the report of two distinct high-molecular-weight polymerases in yeast, one of which has, and one of which lacks, the 3′ → 5′-exonuclease function (3).

Of all the DNA polymerase activities that have been described thus far, the properties of the mycoplasma enzymes most closely resemble those of the mitochondrial D-DNA polymerase from HeLa cells (7, 28), an enzyme that has recently been proposed to be a possible artifact resulting from mycoplasma contamination of the cell cultures (1a). The similarities include chromatographic behavior, response to ionic strength and sulfhydryl reagents, and relative preference for DNA and synthetic homopolymer primer templates. The mitochondrial enzyme was reported to have a molecular weight of 106,000, as estimated by gel filtration (7), but, given the limitations of the methodology, we believe the difference between that size estimate and our own of 130,000 for the mycoplasma enzymes is probably not significant. Based on the data that we have presented here, together with the finding by Bolden et al. that the D-DNA polymerase-mt species is not detected in mitochondria prepared from rat liver cells or mycoplasma-free HeLa cells, it is reasonable to conclude that the mitochondrial polymerase in question was in fact the mycoplasma enzyme.

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LITERATURE CITED

1. **Alberts, B. M., and G. Herrick.** 1971. DNA-cellulose chromatography. Methods Enzymol. 21:198-217.
1a. **Bolden, A., G. Pedrali Noy, and A. Weissbach.** 1977. DNA polymerase of mitochondria is a γ-polymerase. J. Biol. Chem. 252:3351-3356.
2. **Bollum, F. J.** 1975. Mammalian DNA polymerases. Prog. Nucleic Acid Res. Mol. Biol. 15:109-144.
3. **Chang, L. M. S.** 1977. DNA polymerase from bakers' yeast. J. Biol. Chem. 252:1873-1880.
4. **Cook, K. H., and E. C. Friedberg.** 1976. Measurement of thymine dimers in DNA by thin-layer chromatography. II. The use of one-dimensional systems. Anal. Biochem. 73:411-418.
5. **DeLucia, P., and J. Cairns.** 1969. Isolation of an E. coli strain with a mutation affecting DNA polymerase. Nature (London) 224:1164-1166.
6. **Friedberg, E. C., and I. R. Lehman.** 1974. Excision of thymine dimers by proteolytic and amber fragments of E. coli DNA polymerase I. Biochem. Biophys. Res. Commun. 58:132-139.
7. **Fry, M., and A. Weissbach.** 1973. A new deoxyribonucleic acid dependent deoxyribonucleic acid polymerase from HeLa cell mitochondria. Biochemistry 12:3602-3608.
8. **Gefter, M. L.** 1974. DNA polymerases II and III of Escherichia coli. Prog. Nucleic Acid Res. Mol. Biol. 14:101-115.
9. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. 23 (Suppl. 1):285-303.
10. **Kornberg, T., and A. Kornberg.** 1974. Bacterial DNA polymerases, p. 119-144. *In* P. D. Boyer (ed.), The enzymes, vol. 10. Academic Press Inc., New York.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
12. **Livingston, D. M., D. C. Hinkle, and C. C. Richardson.** 1975. Deoxyribonucleic acid polymerase III of Escherichia coli. J. Biol. Chem. 250:461-469.
13. **Low, R. L., S. A. Rashbaum, and N. R. Cozzarelli.** 1976. Purification and characterization of DNA polymerase III from Bacillus subtilis. J. Biol. Chem. 251:1311-1325.
14. **Maniloff, J., and H. J. Morowitz.** 1972. Cell biology of the mycoplasmas. Bacteriol. Rev. 36:263-290.
15. **Nossal, N. G.** 1974. DNA synthesis on a double-stranded DNA template by the T4 bacteriophage DNA polymerase and the T4 gene 32 DNA unwinding protein. J. Biol. Chem. 249:5668-5676.
16. **Nossal, N. G., and M. S. Hershfield.** 1971. Nuclease activity in a fragment of bacteriophage T4 DNA polymerase induced by the amber mutant am B22. J. Biol. Chem. 246:5414-5426.
17. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. 57:1514-1521.
18. **Schaffner, W., and C. Weissmann.** 1973. A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem. 56:502-514.
19. **Schneider, E. L., and E. J. Stanbridge.** 1975. Comparison of methods for the detection of mycoplasmal contamination of cell cultures: a review. In Vitro 11:20-34.
20. **Sedwick, W. D., T. S-F. Wang, and D. Korn.** 1972. Purification and properties of nuclear and cytoplasmic deoxyribonucleic acid polymerases from human KB cells. J. Biol. Chem. 247:5026-5033.
21. **Smith, D. W.** 1969. DNA replication in Mycoplasma laidlawii B. Biochim. Biophys. Acta 179:408-421.
22. **Smith, D. W., and P. C. Hanawalt.** 1967. Properties of the growing point region in the bacterial chromosome. Biochim. Biophys. Acta 149:519-531.
23. **Smith, D. W., and P. C. Hanawalt.** 1969. Repair replication of DNA in ultraviolet irradiated Mycoplasma laidlawii B. J. Mol. Biol. 46:57-72.
24. **Stanbridge, E. J.** 1971. Mycoplasmas and cell cultures. Bacteriol. Rev. 35:206-227.
25. **Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis.** 1965. The twisted circular form of polyoma viral DNA. Proc. Natl. Acad. Sci. U.S.A. 53:1104-1111.
26. **Wang, T. S-F., W. D. Sedwick, and D. Korn.** 1974. Nuclear deoxyribonucleic acid polymerase: purification and properties of the homogeneous enzyme from human KB cells. J. Biol. Chem. 249:841-850.
27. **Wang, T. S-F., W. D. Sedwick, and D. Korn.** 1975. Nuclear deoxyribonucleic acid polymerase. Further observations on the structure and properties of the enzyme from human KB cells. J. Biol. Chem. 250:7040-7044.
28. **Weissbach, A., D. Baltimore, F. J. Bollum, R. Gallo, and D. Korn.** 1975. Nomenclature of eukaryotic DNA polymerases. Science 190:401-402. Eur. J. Biochem. 59:1-2.
29. **Wickner, R. B., B. Ginsberg, I. Berkower, and J. Hurwitz.** 1972. Deoxyribonucleic acid polymerase II of Escherichia coli. I. The purification and characterization of the enzyme. J. Biol. Chem. 247:489-497.