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Characterization of DNA Topoisomerase Activity in Two Strains of *Mycoplasma fermentans* and in *Mycoplasma pirum*

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DNA topoisomerases (topos) are essential enzymes that participate in many cellular processes involving DNA. The presence of the DNA-gyrase genes in various mycoplasmas has been reported elsewhere. However, the characterization of DNA topo activity in mycoplasmas has not been previously undertaken. In this study, we characterized the topo activity in extracts of *Mycoplasma fermentans* K7 and incognitus and in *Mycoplasma pirum*, as well as in partially purified extract of *M. fermentans* K7. The topo activity in these microorganisms had the following properties. (i) The relaxation of supercoiled DNA was ATP dependent. (ii) ATP independent relaxation activity was not detected. (iii) Supercoiling of relaxed topoisomers was not observed. (iv) The relaxation activity was inhibited by DNA gyrase and topo IV antagonists (novobiocin and oxolinic acid) and by eukaryotic topo II (*m*-AMSA [4′-(9-acridylamino)methanesulfon-*m*-anisidide]) and topo I antagonists (camptothecin). Other eukaryotic topo II antagonists (teniposide and etoposide) did not affect the topo relaxation activity. (v) Two polypeptides of 66 and 180 kDa were found to be associated with the mycoplasma topo activity. These results suggest that the properties of the topo enzyme in these mycoplasma species resemble those of the bacterial topo IV and the eukaryotic and the bacteriophage T4 topo II. The findings that mycoplasma topo is inhibited by both eukaryotic topo II and topo I antagonists and that *m*-AMSA and camptothecin inhibited the growth of *M. fermentans* K7 in culture support our conclusion that these mycoplasma species have topo with unique properties.

Topoisomerases (topos) catalyze the interconversion of topological isomers of DNA molecules and have been identified and purified from both prokaryotic and eukaryotic organisms. These enzymes introduce a transient break in the phosphodiester backbone through formation of a covalent protein-DNA intermediate and allow the DNA strands to pass through one another. They participate in many cellular metabolic processes which are associated with DNA, such as replication, transcription, recombination, and repair (14, 32, 33). Topos are classified into three evolutionarily independent types, based primarily on their mode of cleaving DNA: type I-5′, type I-3′, and type II (24). Type I-5′ and I-3′ DNA topos act by making a transient single-stranded nick, passing another strand through the nick, and changing the linking number by one unit (14, 24, 32). Type I-5′ topos were characterized mainly in prokaryotic cells while type I-3′ topos were identified in eukaryotic cells. Type II topos (topo II), found in both prokaryotic and eukaryotic cells, act by transiently nicking both strands of the DNA, passing another double-stranded DNA segment through the gap, and changing the linking number by two (5, 25). In prokaryotes, DNA gyrase and topo IV are type II enzymes, and both enzymes are composed of two subunits: GyrA and GyrB (19) and ParC and ParE (10), respectively. In an ATP-dependent fashion, gyrase can supercoil relaxed DNA, catenate and decatenate DNA rings, knot and unknot circular DNA, and convert supercoils directly to negative ones. Gyrase will relax negatively supercoiled DNA in the absence of ATP (5, 34). All topo IV-catalyzed reactions require ATP, and it relaxes both positive and negative supercoils, knots and unknots DNA, and decatenates DNA rings (21, 22).

Mycoplasmas, the smallest free-living wall-less bacteria, which have a minimal essential genome (577 to 1,380 kbp), are parasites in humans, animals, and plants (23). Some mycoplasmas have been shown to be pathogens in their hosts, others have been shown to be associated with diseases, and some species are commensals only. In addition, mycoplasmas present a major problem in biological research, being the most common contaminant in cell and tissue cultures, and have been found to alter many cellular functions (18). The identification of new genes and proteins that participate in mycoplasma DNA replication is important for the understanding of the mycoplasma cell cycle and may shed light on the role of mycoplasmas in pathogenic processes. Moreover, it may contribute to the identification of new targets for antimycoplasma agents. The genes, *gyrA* and *gyrB*, that encode both subunits of DNA gyrase were identified in *Mycoplasma pneumoniae* (3). The *gyrB* gene was identified in *Mycoplasma hominis* (13), *Mycoplasma genitalium* (1), *Mycoplasma capricolum* (26), and *Mycoplasma gallisepticum* (4). In addition, the genes encoding topo IV were identified in *M. genitalium* (2). However, the characterization of topo activity in these mycoplasma strains or other mycoplasmas has not yet been reported. Recently, certain mycoplasmas have generated increased interest as putative cofactors in AIDS development (i.e., *Mycoplasma fermentans* and *Mycoplasma pirum*) (15, 20). Since topos participate in many vital processes and are the target of antibacterial and anticancer drugs (34), we examined and characterized the topo activity in these mycoplasmas. We report the presence of topo activity in *M. fermentans* and *M. pirum* which differs from those of the known prokaryotic topos. The mycoplasma topo is a topo IV-like enzyme which is inhibited by eukaryotic topo II and topo I antagonists. The inhibition of mycoplasma growth by eukaryotic topo antagonists is demonstrated.

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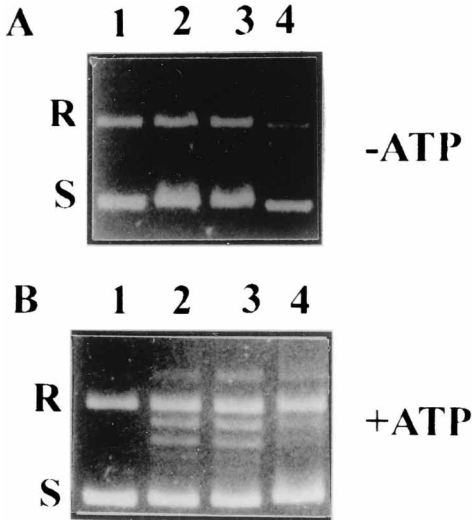

FIG. 1. Topo relaxation activity in *M. fermentans* K7. Increasing concentrations of *M. fermentans* protein lysate, 3 (lane 2), 4.2 (lane 3), and 7 (lane 4) μg, were added to a topo reaction mixture in the absence (A) or presence (B) of 1 mM ATP. The supercoiled plasmid is shown in lane 1. The reaction products were analyzed by 1% agarose gel electrophoresis, and the gel was stained with ethidium bromide and photographed with a UV table. The symbols S and R represent the supercoiled and relaxed forms of the plasmid DNA, respectively.

MATERIALS AND METHODS

Strains. *M. fermentans* K7 was originally obtained from J. G. Tully (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.); *M. fermentans* (strain incognitus) and *M. pirum* were kindly donated by C. Bébear (University of Bordeaux, Bordeaux, France).

Propagation of mycoplasmas. *M. fermentans* strains and *M. pirum* were cultivated in SP4 broth as previously described (11), harvested by centrifugation at 25,000 × *g* for 30 min, and washed twice with phosphate-buffered saline. The cell pellets were resuspended in 0.5 ml of phosphate-buffered saline, and quantification of the mycoplasmas was performed by determination of CFU (30).

Preparation of protein extracts for topo assay. Mycoplasma pellets, prepared as described above, were lysed by the freezing-thawing technique as follows. Cells (10^9 CFU/ml) were resuspended in hypotonic buffer A containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 1 mM EDTA. Cells were immediately frozen in liquid nitrogen followed by thawing in a hot water bath. This procedure was repeated three times, and then 1 M NaCl (final concentration) was added. The extract was centrifuged at 13,000 rpm for 10 min (Microfuge; Eppendorf) to remove cell debris, and the protein concentration was determined by the Bio-Rad protein assay kit (Richmond, Calif.).

Topo assay. (i) Relaxation. Mycoplasma proteins were added to a specific topo reaction mixture containing at a final volume of 25 μl, 20 mM Tris-HCl (pH 8.1), 1 mM dithiothreitol, 20 mM KCl, 20 μg of bovine serum albumin per ml, 10 mM $MgCl_2$, 0.5 mM EDTA, 1 mM ATP, and 250 ng of pUC-19 supercoiled plasmid DNA as a substrate. In some experiments, prokaryotic and eukaryotic topo inhibitors at different concentrations were added. Following incubation at 37°C for 30 min, the reaction was terminated by adding 5 μl of stopping buffer (final concentrations: 1% sodium dodecyl sulfate [SDS], 15% glycerol, 0.5% bromophenol blue, 50 mM EDTA [pH 8]). The reaction products were analyzed by electrophoresis on a 1% agarose gel with a TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 62 mM EDTA) at 1 V/cm, stained with ethidium bromide (1 μg/ml), and photographed with a short-wavelength UV lamp.

(ii) Supercoiling. To prepare relaxed topoisomers, the supercoiled pUC-19 plasmid (250 ng/sample) was relaxed with 10 U of purified calf thymus topo I (MBI). The relaxed molecules were isolated by phenol-chloroform extraction and precipitated with ethanol. The pellet was resuspended in a TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA). Mycoplasma proteins (4 μg) or 15 U of *Micrococcus luteus* DNA gyrase (Bethesda Research Laboratories, Gaithersburg, Md.) were added to the topo reaction mixture followed by incubation at 37°C for 30 min. The reaction was terminated by adding 5 μl of stopping buffer and analyzed by electrophoresis on a 1% agarose gel as described above.

Partial purification of topo activity from mycoplasma protein extract. Protein extract (80 mg) from *M. fermentans* K7 was chromatographed on columns of DEAE-cellulose at high (0.2 M NaCl) and low (0.05 M NaCl) ionic strength as described elsewhere (16, 27). Fractions were collected and assayed for topo activity, and the fractions that contained this activity were pooled, diluted in 4 volumes of column buffer (50 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8], 2.5 mM dithiothreitol, 10% [vol/vol] glycerol, 0.01% Nonidet P-40), and applied to a 6-ml column of single-stranded DNA agarose (Bethesda Research Laboratories). This column was equilibrated with 0.1 M NaCl in column buffer and developed with an 80-ml linear gradient of 0.1 to 1.2 M NaCl in column buffer, and fractions (1 ml) were collected and assayed for topo activity. The fractions that contain this activity were concentrated with Centricon-10 microconcentrators (Amicon, Beverly, Mass.) that contained an anisotropic membrane (pore size, 10,000-molecular-weight cutoff). By using centrifugal force (2,987× *g* for 20 min), the solvents and low-molecular-weight solutes were driven through the membrane into a filtrate cup. Macrosolutes above the membrane cutoff were transferred into a retention cup after the device was inverted and were centrifuged (119 × *g*, 5 min). Samples from the filtrate cup and from the retention cup were examined for topo activity.

Silver staining. Proteins (40 μg) from the fractions containing topo activity were analyzed on an SDS–10% polyacrylamide gel. The protein bands were visualized with the Bio-Rad silver stain kit.

Formation of a DNA-protein cleavable complex. The labeling of pUC-19 DNA plasmid was performed with a DNA labeling kit (Ready To Go; Pharmacia). The labeled pUC-19 DNA fragments were purified from the free-labeled nucleotide with a Sephadex G-50 column and purified from the enzymes used for DNA labeling by phenol-chloroform extraction. Samples (10 μl) from the DNA agarose column fractions that contained topo activity were added to a topo reaction mixture containing 10 ng (6×10^5 cpm) of labeled DNA followed by incubation at 37°C for 30 min. The reaction products were boiled at 100°C; 30 U of P1 nuclease and 0.1 M sodium acetate (pH 5.6) were added, followed by incubation for 2 h at 37°C. Where indicated, 400 μg of proteinase K was added and the reaction mixture was further incubated for 1 h at 50°C. The reaction was stopped by adding 5 μl of protein sample buffer (0.5 M Tris-HCl [pH 6.8], 4% SDS, 0.1% bromophenol blue, 20% glycerol, 4% [vol/vol] β-mercaptoethanol), the reaction mixture was boiled and analyzed on an SDS–10% polyacrylamide gel, and autoradiography was performed. Prestained protein molecular weight markers were purchased from BRL-GIBCO.

Inhibition of *M. fermentans* growth by eukaryotic topo inhibitors. Equal amounts of *M. fermentans* K7 (5×10^3 CFU) were cultured either in the presence or in the absence of topo inhibitors: camptothecin (CPT) or *m*-AMSA [4′-(9-acridylamino)methanesulfon-*m*-anisidide] at a concentration of 2 or 20 μM each. Samples were taken from these cultures at different times (24, 48, and 72 h), and cell viability was monitored by determination of CFU.

RESULTS

Topo activity in *M. fermentans* and *M. pirum*. Topo activity was examined by the relaxation of a supercoiled DNA plasmid with the protein lysate obtained from these mycoplasmas. The lysates were diluted (1:20) in buffer A, and various concentrations of mycoplasma proteins were added to the topo I reaction

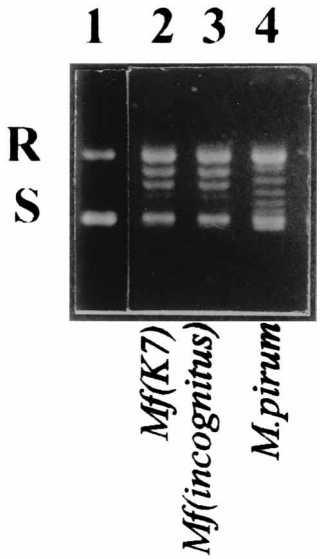

FIG. 2. Topo relaxation activity in *M. fermentans* incognitus and in *M. pirum*. Protein lysate (4 μg) from *M. fermentans* K7 (lane 2) or incognitus (lane 3) and from *M. pirum* (lane 4) was added to the topo reaction mixture in the presence of 1 mM ATP. The supercoiled plasmid is shown in lane 1. The reaction products were analyzed by 1% agarose gel electrophoresis as described in the legend to Fig. 1. S and R, supercoiled and relaxed forms of plasmid DNA, respectively.

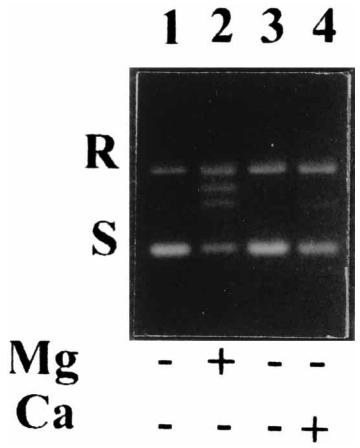

FIG. 3. The effect of cofactors on the topo relaxation activity in *M. fermentans* K7. Four micrograms of protein lysate from *M. fermentans* K7 was added to the topo reaction mixture in the presence (lane 2) or absence (lane 3) of Mg^{2+} ions or in the presence of Ca^{2+} ions instead of Mg^{2+} ions (lane 4). The supercoiled plasmid is shown in lane 1. The reaction products were analyzed as described in the legend to Fig. 1. S and R, supercoiled and relaxed forms of plasmid DNA, respectively.

mixture. The topo activity of *M. fermentans* K7 is shown in Fig. 1. The specific ladder of topoisomers was detected, at the various protein concentrations (lanes 2 to 4), only in the presence of ATP in the reaction mixture (Fig. 1B) and not in the absence of ATP (Fig. 1A). As shown in Fig. 2, an ATP dependent relaxation activity was also detected in *M. fermentans* incognitus (lane 3) and in *M. pirum* (lane 4). These results suggest that the mycoplasma-associated topo has an ATP dependent relaxation activity.

The effect of cofactors on the topo relaxation activity. To further characterize this relaxation activity, we examined the effect of divalent cations Mg^{2+} and Ca^{2+} on this activity. As shown in Fig. 3, the relaxation activity in *M. fermentans* K7 was strictly dependent on the presence of Mg^{2+} ions (compare lane 2 to lane 3). In addition Ca^{2+} ions could not replace Mg^{2+} ions since no relaxation was observed when Mg^{2+} ions were replaced by Ca^{2+} ions (lane 4). Similar results were obtained with protein extracts from *M. fermentans* incognitus and *M. pirum* (data not shown).

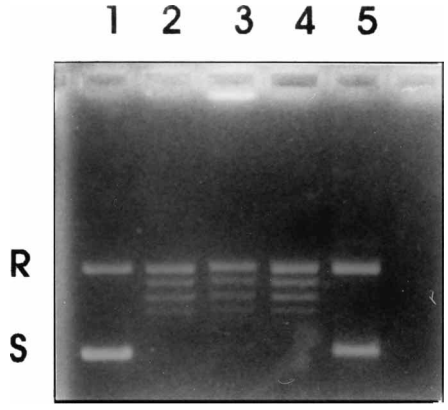

FIG. 4. Determination of DNA supercoiling activity in mycoplasma extract. Four micrograms of protein lysate from *M. fermentans* K7 (lane 3) or *M. pirum* (lane 4) or 15 U of *Micrococcus luteus* DNA gyrase (lane 5) was added to the topo reaction mixture containing 250 ng of the relaxed DNA topoisomers. Lane 1, supercoiled plasmid only. Lane 2, partially relaxed DNA topoisomers only. S and R, supercoiled and relaxed forms of plasmid DNA, respectively.

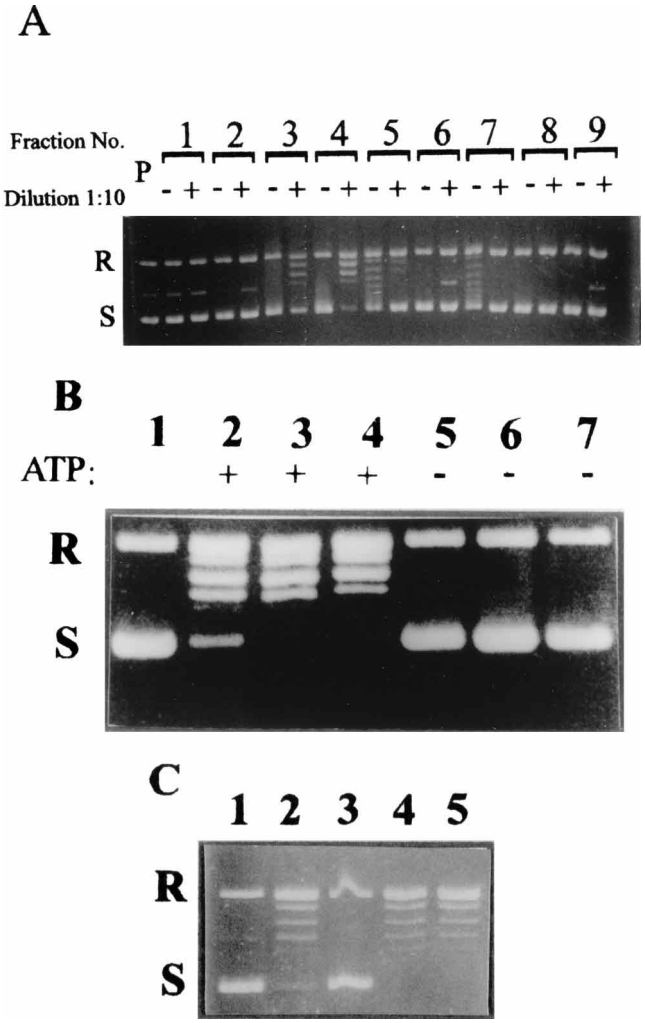

FIG. 5. Partial purification of topo activity from *M. fermentans* K7. (A) Samples (2 μl) from the fractions obtained from the DNA-agarose column, undiluted (−), or diluted 1:10 (+), were added to the topo reaction mixture in the presence of 1 mM ATP. The peak of topo relaxation activity in fractions 3, 4, 5, and 7 is shown. P, pUC-19 DNA. (B) Topo relaxation activity was measured in samples (1 μl) from concentrated fractions 3 (lanes 2 and 5), 5 (lanes 3 and 6) and 7 (lanes 4 and 7), in the presence (lanes 2 to 4) or absence (lanes 5 to 7) of 1 mM ATP. (C) Topo supercoiling activity was measured in samples from the concentrated fraction 3 (lane 4) or fraction 7 (lane 5), in comparison to 15 U of DNA gyrase (lane 3). Supercoiled pUC-19 DNA (lanes 1 in panels B and C) and relaxed topoisomers (lane 2) are also shown. S and R, supercoiled and relaxed forms of plasmid DNA, respectively.

Determination of DNA supercoiling activity in mycoplasma extract. DNA gyrase converts partially relaxed DNA to a negative supercoiled form. To examine whether the mycoplasma topo displays this type of activity, mycoplasma extract was added to a reaction mixture containing pUC-19 partially relaxed DNA molecules. As shown in Fig. 4, conversion of the partially relaxed DNA molecules to the supercoiled form was not observed (compare lane 3 to lane 2). In comparison, under the same assay conditions, a purified DNA gyrase converted most of the relaxed molecules to the supercoiled forms (compare lane 5 to lane 2). These results suggest that the mycoplasma topo does not possess supercoiling activity.

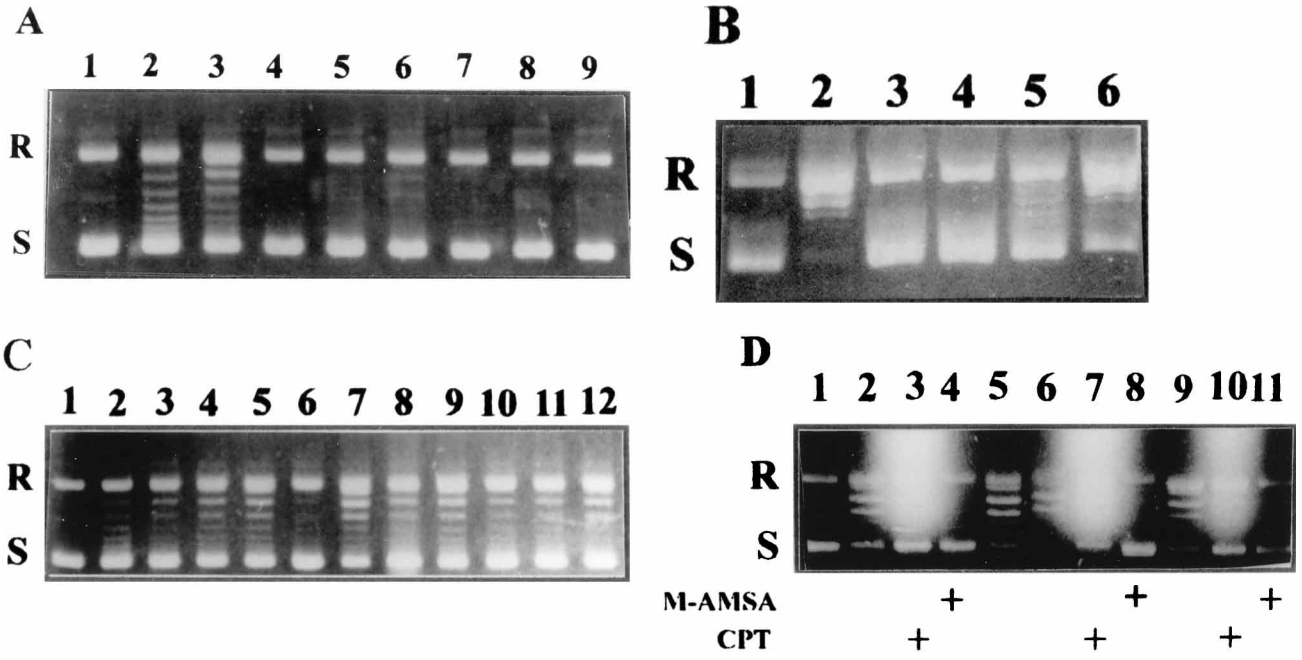

FIG. 6. The effect of prokaryotic and eukaryotic topo inhibitors on mycoplasma topo activity. (A) The inhibitory effect of novobiocin (lanes 3 to 5) and oxolinic acid (lanes 6 to 9) was measured in samples (1 μl) from the topo-active fraction. Also shown is topo relaxation activity in the absence (lane 2) or in the presence of increasing concentrations of novobiocin (1 μM [lane 3], 5 μM [lane 4], and 10 μM [lane 5]) or oxolinic acid (0.1 mM [lane 6], 0.5 mM [lane 7], 2 mM [lane 8], and 4 mM [lane 9]). Lane 1, pUC-19 DNA. (B) The inhibitory effect of *m*-AMSA was measured in samples (5 μl) from the topo-active fraction. The topo relaxation activity is shown in the absence (lane 2) or presence of *m*-AMSA: 200 (lane 3), 100 (lane 4), 50 (lane 5), and 10 (lane 6), μM. Lane 1, pUC-19 DNA. (C) The effect of increasing concentrations of VP-16 (lanes 3 to 7) or VM-26 (lanes 8 to 12) on the topo relaxation activity was measured in 1 μl of the topo-active fractions. The concentrations used for each of the compounds are 10 (lanes 3 and 8), 50 (lanes 4 and 9), 100 (lanes 5 and 10), 200 (lanes 6 and 11), and 400 (lanes 7 and 12) μM. Lanes: 1, pUC-19 DNA; 2, topo activity in the absence of drugs. (D) The effect of *m*-AMSA and CPT on the topo relaxation activity in crude extracts of 4 μg from *M. fermentans* K7 (lanes 2 to 4), 4 μg (lanes 5, 7, and 8) or 6 μg (lane 6) from *M. fermentans* incognitus, and 4 μg from *M. pirum* (lanes 9 to 11). The reaction was carried out in the absence (lanes 2, 5, 6, and 9) or presence (lanes 3, 7, and 10) of 200 μM CPT or in the presence of 200 μM *m*-AMSA (lanes 4, 8, and 11). Lane 1, supercoiled plasmid only. S and R, supercoiled and relaxed forms of plasmid DNA, respectively.

Partial purification of topo activity from *M. fermentans* K7. To examine the possibility that the mycoplasma lysate contains more than one type of topo we partially purified the topo activity from the lysate by affinity chromatography. The results, depicted in Fig. 5A, demonstrate two peaks of topo relaxation activity in fractions 3, 4, and 5 (the first peak) and fraction 7 (the second peak) obtained from the DNA agarose column. Fractions 3, 5, and 7 were separately concentrated and assayed for topo relaxation activity in the presence or absence of ATP. The results shown in Fig. 5B, indicate that topo activity assayed by the relaxation of supercoiled plasmid DNA is ATP dependent (lanes 2, 3, and 4), and no relaxation activity could be observed in the absence of ATP (lanes 5, 6, and 7). In addition, supercoiling activity was examined in these fractions, but no such activity could be observed (Fig. 5C, lanes 4 and 5). These results are compatible with the data obtained when a crude lysate of mycoplasma was examined (Fig. 1). It should be noted that all fractions obtained from the DNA agarose column were assayed for an ATP-independent relaxation activity, but no such activity could be detected (data not shown).

The effect of prokaryotic and eukaryotic topo antagonists on mycoplasma topo. Our data suggest that, similar to the eukaryotic topo II and to the bacterial topo IV, the mycoplasma relaxation activity is ATP dependent and differs from that of DNA gyrase. Therefore, the effect of various prokaryotic and eukaryotic topo inhibitors on the partially purified *M. fermentans* K7 topo was examined. Each topo-active fraction was tested separately, and since identical results were obtained, the data depicted in Fig. 6 present a typical dose response to the indicated inhibitors observed with each topo-active fraction. The DNA gyrase antagonists, novobiocin at concentrations of 1, 5, and 10 μM (Fig. 6A, lanes 3 to 5), and oxolinic acid at concentrations of 0.1 to 4 mM (Fig. 6A, lanes 6 to 9), inhibited the relaxation activity of mycoplasma topo. Among the eukaryotic topo II antagonists, *m*-AMSA inhibited the relaxation activity of the mycoplasma topo in a dose-dependent manner as shown in Fig. 6B. In contrast, neither etoposide (VP-16) nor teniposide (VM-26) had any inhibitory effect, even at a concentration of 400 μM (Fig. 6C, lanes 3 to 7 and lanes 8 to 12, respectively). The inhibitory effect of *m*-AMSA on topo activity in other mycoplasmas was also examined. As demonstrated in Fig. 6D, *m*-AMSA at a concentration of 200 μM totally inhibited the topo relaxation activity in crude extracts of *M. fermentans* incognitus (lane 8) and in *M. pirum* (lane 11). Surprisingly, CPT, a specific inhibitor of eukaryotic topo I, also had an inhibitory effect on this relaxation activity, although to a lesser degree than the observed effect of *m*-AMSA. This inhibitory effect is demonstrated by the remaining supercoiled form (Fig. 6D, lanes 3, 7, and 10). The prominent fluorescence in lanes 3, 7, and 10 is the result of the fluorescence property of CPT.

The formation of a DNA-topo cleavable complex. To examine the polypeptide composition of the topo-active fractions obtained after chromatography on a DNA agarose column, samples were analyzed on an SDS–10% polyacrylamide gel, with subsequent visualization of protein bands by silver staining (Fig. 7A). Six polypeptides in the range of 35 to 68 kDa and one faint polypeptide band of 180 kDa were observed in fraction 3 (lane 1) and fraction 7 (lane 2). It has been shown that

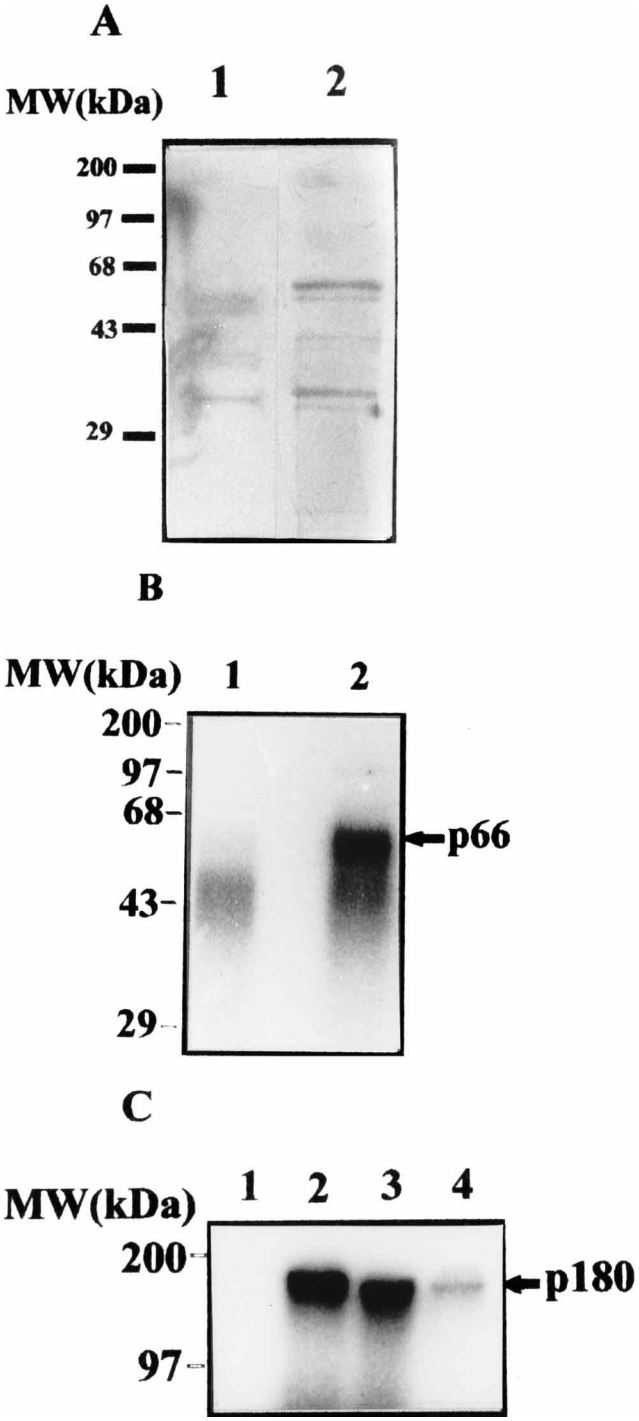

FIG. 7. The polypeptide composition and the formation of DNA-enzyme cleavable complex in the topo-active fractions. (A) Silver staining of proteins (40 μg) from the topo-active fractions analyzed by polyacrylamide gel electrophoresis. Shown are fraction 3 (lane 1) and fraction 7 (lane 2). (B and C) DNA-enzyme cleavable complex formation in a sample of 40 μg of proteins from fractions 3 (B, lane 2), 5 (C, lane 2), and 7 (C, lanes 3 and 4). The labeled DNA digested with P1 nuclease is shown in lanes 1. Lane 4 in panel C is fraction 7 treated with proteinase K after the formation of the DNA-enzyme cleavable complex. MW, molecular mass.

for DNA-relaxing enzymes, such as DNA topos, the relaxation activity involves formation of a DNA-enzyme cleavable complex as an intermediate product, in which a covalent bond is formed between the active site of the enzyme and the phosphate moiety from the DNA backbone (16, 27). To determine which of the proteins present in the topo-active fractions may form such a DNA-enzyme cleavable complex, samples from these fractions were reacted with ^{32}P-labeled pUC-19 DNA fragments, heated, and then digested with P1 nuclease and analyzed by SDS-polyacrylamide gel electrophoresis. When a complex of protein-DNA is formed, the labeled phosphate residues of the DNA remain bound to the protein after P1 nuclease digestion and boiling in the presence of SDS. Analysis of the topo-active fractions 3, 5, and 7 is shown in Fig. 7B and C. A major protein band of 66 kDa was labeled in fraction 3 (Fig. 7B, lane 2), and a 180-kDa labeled protein was observed in fraction 5 (Fig. 7C, lane 2) and in fraction 7 (Fig. 7C, lane 3). The labeled band was sensitive to digestion by proteinase K (Fig. 7C, lane 4).

The effect of eukaryotic topo antagonists on *M. fermentans* K7 growth. Topos are known as essential nuclear enzymes, and it was previously shown that mycoplasma growth is inhibited by quinolone derivatives, some of which are DNA gyrase inhibitors (31). However, since the mycoplasma topo activity was inhibited by eukaryotic topo antagonists, we tested the effect of these drugs on the growth of mycoplasma in culture. The results, depicted in Fig. 8, show that 24 h after the addition of the drugs, both *m*-AMSA and CPT significantly inhibited the growth of *M. fermentans* K7. At low doses (2 μM), the inhibitory effect was reversible after 48 and 72 h, while at higher doses (20 μM) almost 100% inhibition was observed at all times.

DISCUSSION

This is the first study to demonstrate and characterize a DNA topo activity in two different strains of *M. fermentans* (K7 and incognitus) and in *M. pirum*. Topo activity was examined in the crude extracts of these mycoplasmas and in the partially purified lysate of *M. fermentans* K7. This topo relaxation activity was ATP dependent and as such resembles that of the bacterial topo IV (21, 22), the eukaryotic DNA topo II (32, 33), and the bacteriophage T4 topo II (7, 8, 12). ATP-independent relaxation activity was not detected in these mycoplasma extracts or in the partially purified fractions of *M. fermentans* K7, and thus it differs from the relaxation activity of DNA gyrase, which is ATP independent (17), and from the known prokaryotic and eukaryotic DNA topo I that relaxes supercoiled DNA in the absence of ATP (32, 33).

In addition, we found that the mycoplasma topo could not convert relaxed, closed circular duplex DNA to the negatively superhelical form, which is a typical activity of DNA gyrase. In this respect, it again resembles the bacterial topo IV (21, 22) and the eukaryotic (32–34) and the T4 (9) topo II enzymes. The mycoplasma topo relaxation activity was inhibited by novobiocin and oxolinic acid at concentrations that inhibited DNA gyrase and the *Escherichia coli* topo IV (21), suggesting that the mycoplasma topo resembles the prokaryotic type II topo. However, our data also demonstrate that unlike the bacterial topos and similar to T4 topo II (9), mycoplasmal topo activity was totally inhibited by *m*-AMSA, which is a specific eukaryotic topo II antagonist. However, teniposide (VM-26) and etoposide (VP-16), which are also eukaryotic topo II antagonists, did not inhibit the mycoplasma topo even at high doses (400 μM). These findings can be explained by the different properties of these inhibitors: *m*-AMSA is an acridine derivative and a DNA intercalator, while VP-16 and VM-26 are two nonintercalative glycosidic derivatives of podophyllotoxins (14, 29). Therefore, it is possible that due to its DNA-intercalating action, *m*-AMSA inhibited the mycoplasma topo.

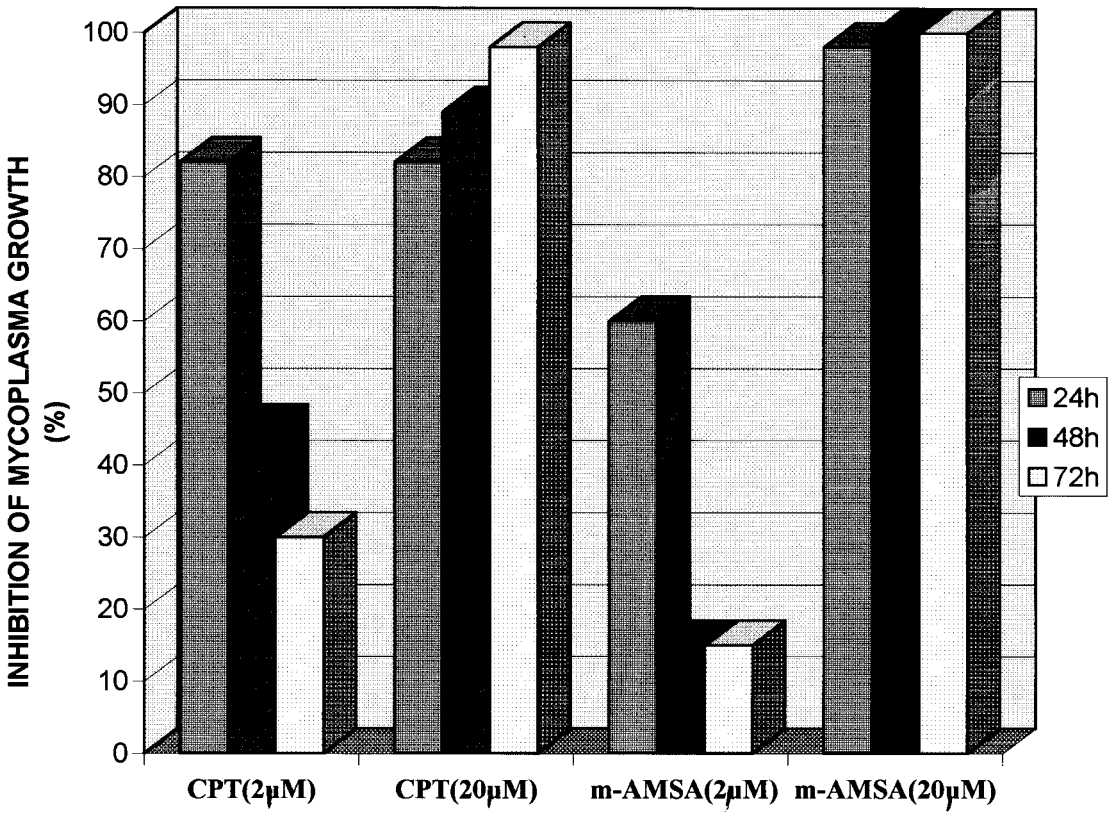

FIG. 8. The effect of CPT and *m*-AMSA on the growth of *M. fermentans* K7. Equal amounts (5×10^3 CFU/ml) of *M. fermentans* K7 were cultured either in the presence or in the absence of CPT (2 and 20 μM) or *m*-AMSA (2 and 20 μM). Samples were taken from the cultures at 24, 48, and 72 h after treatment, and cell viability was determined by counting CFU. Inhibition values were calculated as percentages of the control untreated cells, and the results are the means of two separate experiments with standard deviations of ±1%.

Moreover, this drug also inhibited the growth of mycoplasma in vivo, and at high doses (20 μM), it was cytotoxic. Thus, as was demonstrated in eukaryotic cells (14, 29), we suggest that this effect was probably due to the inhibition of mycoplasma topo activity and to the stimulation of topo-mediated DNA cleavage. Surprisingly, CPT, which is a specific eukaryotic topo I antagonist, also inhibited the topo relaxation activity in mycoplasmas, although to a lesser extent, and similar to *m*-AMSA, it was cytotoxic to mycoplasma growth. CPT does not affect the prokaryotic topo I activity or the growth of other bacteria such as *E. coli* (22a); therefore, our finding that CPT has an inhibitory effect on the mycoplasma topo activity and on the mycoplasma growth is novel and unique. It was previously shown that CPT is a specific antagonist of eukaryotic topo I; it does not bind or react with purified DNA or inhibit other DNA-binding enzymes (14, 29). It is cytotoxic to eukaryotic cells due to the formation of topo I-mediated DNA cleavage (14). Therefore, it is likely that CPT exhibited its cytotoxic effect on mycoplasma due to its ability to inhibit the mycoplasma topo activity.

To further identify the proteins that are associated with topo activity in the partially purified fractions, we used the DNA-enzyme cleavable complex formation assay (16, 27). Two polypeptides of 66 kDa (in fraction 3) and 180 kDa (in fractions 5 and 7) were radioactively labeled, probably due to their ability to form a DNA-enzyme cleavable complex. Since the mycoplasma topo activity resembles the topo IV relaxation activity, it is possible to assume that the 66-kDa protein resembles the ParC subunit of topo IV (10) and that the 180-kDa protein is a different topo with the same properties. Alternatively, since the activities in fractions 3, 5, and 7 had the same catalytic properties and were inhibited by the same inhibitors, it is possible that the 66-kDa protein observed in fraction 3 is a degradation product of the 180-kDa protein present in fractions 5 and 7. However, to exactly identify the mycoplasma proteins that possess the topo activity, it is necessary to purify them to homogeneity.

In the genome of some mycoplasmas, the *gyrA* and *gyrB* genes were identified (1, 3, 4, 13, 26), and *parC* and *parE*, the topo IV genes, were identified in *M. genitalium* (2). Therefore, it can be assumed that these genes are also present in the species examined in this study. However, since the typical DNA gyrase activity was not observed in these species, it is possible that this enzyme is present in a very small amount and is undetectable by the usual DNA gyrase assay conditions (Fig. 4 and 5C). It should be noted that in addition to the four main classes of topos (prokaryotic type I and type II and eukaryotic type I and type II), other topos with different properties have been isolated from viral (27), plasmid (6), and organelle (28) sources. However, the mycoplasma topo is unique in its characteristics, since it is a topo IV-like enzyme which can be inhibited by eukaryotic topo II and topo I antagonists.

Our findings that *m*-AMSA and CPT inhibited the mycoplasma DNA topo activity and the growth of *M. fermentans* K7 in culture suggest that the mycoplasmal topo is essential for the replication of mycoplasmas. Moreover, since topos participate in most of the DNA transactions and their inhibitors have been previously used as antibacterial and anticancer drugs, it will be

useful to further examine the effect of these compounds as antimycoplasma agents and to investigate the role of topo in the mycoplasma life cycle.

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