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Probable Insensitivity of Mollicutes to Rifampin and Characterization of Spiroplasmal DNA-Dependent RNA Polymerase

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The effect of rifampin on five mollicutes (*Spiroplasma citri*, *Spiroplasma melliferum*, *Spiroplasma apis*, *Acholeplasma laidlawii*, and *Mycoplasma mycoides*) was compared with that on *Escherichia coli*. We found that, in contrast to wild-type *E. coli*, mollicutes were insensitive to rifampin. DNA-dependent RNA polymerases from *S. melliferum* and *S. apis* were purified to the stage where the enzymes were dependent on the addition of exogenous templates for activity. The enzymes were then tested for their sensitivity to rifampin. Spiroplasmal enzymes were at least 1,000 times less sensitive to rifampin than the corresponding *E. coli* enzyme. This result provides a molecular basis for the resistance of mollicutes to rifampin. The RNA polymerase of *S. melliferum* was further purified and its subunit composition was investigated. The RNA polymerase has one small and two large subunits. The structure of *S. melliferum* RNA polymerase therefore resembles that of the eubacterial enzymes in spite of its insensitivity to rifampin.

Mollicutes are the smallest self-replicating cells known. By their genome size, sterol requirement, morphology, urea utilization, and other biochemical properties, they are classified into five genera: *Acholeplasma*, *Anaeroplasma*, *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* (12). It is believed that the mollicutes represent a coherent phylogenetic group derived by degenerative evolution from gram-positive bacteria (22). Loss of cell wall and genome reduction to 1,000 megadaltons (MDa) yielded the acholeplasmas, seen as the initial mollicutes. A splitting of the *Acholeplasma* branch led, without genome reduction, to the sterol-requiring spiroplasmas. Mycoplasmas and ureaplasmas, also sterol requiring, derived from the *Spiroplasma* branch with a further genome reduction to 500 MDa (15).

Rifampin sensitivity is a general property of most eubacteria (20). The target of the antibiotic is the DNA-dependent RNA polymerase (19). In eubacteria this enzyme has a similar structure (7) and is composed of two large subunits, β and β′, and one small subunit, α. The core enzyme contains two α subunits and one β and one β′ subunit ($\beta\beta'\alpha_2$). Rifampin combines with the β subunit to inhibit the initiation step of transcription (13). Promoter recognition is favored by an initiation factor (σ) which combines with the core enzyme (7).

Since the mollicutes are believed to be derived from eubacteria, it was of interest to investigate the effect of rifampin on mollicutes. There were indications in the literature that *Acholeplasma* (4) and *Ureaplasma* (17) spp. were unaffected by the antibiotic. In this paper we show that the growth of five different mollicutes is indeed insensitive to concentrations of rifampin much higher than those that fully inhibit the growth of *Escherichia coli*. In addition we demonstrate that the DNA-dependent RNA polymerases of *S. melliferum* and *S. apis* are insensitive to rifampin even though their subunit structure resembles that of eubacteria rather than archaebacteria.

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MATERIALS AND METHODS

Organisms and growth conditions. *S. citri* R8A2 (group I-1 of Whitcomb et al. [21]) (ATCC 27556) was originally isolated from a sweet orange tree affected by Stubborn disease (16). *S. melliferum* B88 (group I-2) was isolated from honey bees (3a). *S. apis* B31 (group IV) (ATCC 33834) came from May disease-affected honey bees in Southern France (10). *M. mycoides* PG50 was isolated from bovine articular fluid (18). *A. laidlawii* PG8 (ATCC 23206) came from sewage (8). Mollicutes were grown in BSR medium (2) at 32°C for *Spiroplasma* species and 37°C for *Mycoplasma* and *Acholeplasma* species. *E. coli* MRE600 was grown at 37°C in a medium containing (in grams per liter): tryptone, 10; NaCl, 5; yeast extract, 5; and glucose, 1 (pH 7.0).

In vivo rifampin sensitivity tests. For the antibiogram disk method, a culture plate (diameter, 5 cm) containing 1% agar medium was inoculated along a diameter of the plate with 30 μl of a late-log-phase broth culture of the organism to be tested. Filter paper disks containing the antibiotics to be tested were placed in the middle of the plates. After 8 to 10 days at 32°C for mollicutes or 20 h at 37°C for *E. coli*, the radius of the inhibition zone surrounding the disk was measured.

The MIC of rifampin was determined as described by Liao and Chen (9).

The number of CFU in broth culture was determined by plotting dilutions of the culture on agar plates as described by Rodwell and Whitcomb (14). Measurement of $^{32}P_i$ incorporation into the nucleic acids of organisms grown in broth cultures was done by the method of Rodwell and Whitcomb (14) with 5 μCi of $^{32}P_i$ per ml of medium.

Purification of spiroplasmal DNA-dependent RNA polymerase. All operations were carried out at 4°C. Cells from 1-liter late-log-phase cultures were harvested by centrifugation and kept frozen at −30°C. After thawing, they were suspended in TMED buffer (Tris hydrochloride, pH 7.4, 25 mM; $MgCl_2$, 5 mM; EDTA, 1 mM; dithiothreitol, 2 mM; glycerol, 10% [vol/vol]). Cells were lysed by adding 0.5% (vol/vol) Nonidet P-40 detergent. After standing on ice for 15 min, the suspen-

sion was treated in a Dounce homogenizer. The crude lysate was centrifuged at 20,000 × *g* for 20 min. Solid cold polyethylene glycol 6000 was added to the supernatant (0.3 g/ml). The resulting precipitate was recovered by centrifugation (20,000 × *g* for 20 min). The pellet was dissolved in 10 ml of TMED buffer and used as the partially purified RNA polymerase preparation. Further purification was obtained as follows. The crude lysate (see above) was centrifuged at 110,000 × *g* for 1 h, and the supernatant fluid was immediately applied to a DEAE-cellulose column (DE52; Whatman, Maidstone, Kent, United Kingdom). RNA polymerase was eluted by TMED buffer containing 0.3 M KCl. The active fractions were pooled, and their proteins were precipitated by polyethylene glycol 6000 (0.3 g/ml) and recovered by centrifugation. The pellet was solubilized in a small volume of TMED buffer, loaded on a 10 to 20% glycerol gradient, and subjected to 120,000 × *g* centrifugation for 13 h. The glycerol gradient was collected in 250-μl fractions, and those containing RNA polymerase activity were pooled and applied on a heparin-agarose column (IBF, Villeneuve la Garenne, France). The RNA polymerase was eluted by TMED buffer containing 0.3 M KCl, yielding the heparin enzyme.

Analysis of RNA polymerase preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (11).

DNA-dependent RNA polymerase assay. The reaction mixture contained (in a final volume of 200 μl): Tris hydrochloride (pH 9.0), 7.5 μmol; dithiothreitol, 0.5 μmol; $MgCl_2$, 15 μmol; ATP and CTP, 0.005 μmol; GTP, 0.01 μmol; KCl, 6 μmol; [^{3}H]UTP (40 Ci/mmol) (NET 380; New England Nuclear Corp., Aldrich, West Germany), 1 μCi; and calf thymus DNA, 25 μg. RNA synthesis was started by the addition of 25 μl of the enzyme preparation to the reaction mixture. The reaction was allowed to proceed for 25 min at 32°C. To test the effect of rifampin, the enzyme and the antibiotic were first incubated together for 10 min at 32°C and then added to the other constituents of the reaction mixture. RNA synthesis was stopped by adding 5 ml of cold 10% (wt/vol) trichloroacetic acid and 1 mg of bovine serum albumin as carrier. The acid-insoluble material was collected on glass fiber filters (GFA; diameter 2.5 cm; Whatman) by vacuum filtration and washed with 50 ml of 10% trichloroacetic acid and 5 ml of 95% ethanol. Filters were dried, and the radioactivity was measured in an Intertechnic liquid scintillation counter (SL30).

Antibiotics and enzyme. Rifampin was purchased from Sigma Chemical Co., St. Louis, Mo. Streptolydigin was obtained from Upjohn Co., Kalamazoo, Mich. Antibiogram disks were from Institut Pasteur Production, Marnes la Coquette, France. *E. coli* RNA polymerase was purchased from Boehringer Manheim, Meylan, France.

RESULTS

Growth of five different mollicutes is not affected by concentrations of rifampin that fully inhibit *E. coli*. The effect of rifampin on the growth of five mollicutes, *S. citri*, *S. apis*, *S. melliferum*, *M. mycoides*, and *A. laidlawii*, was investigated by four different techniques.

In the antibiogram disk method, 30 μg of rifampin per disk strongly inhibited the growth of *E. coli* (inhibition zone, 6 mm) but did not affect the mollicutes tested. The MIC of rifampin for the mollicutes was 100 times higher than for *E. coli*. The rifampin MIC was 100 μg/ml for all the mollicutes tested except *M. mycoides*, for which the MIC was 200 μg/ml; the MIC for *E. coli* was <2 μg/ml. In liquid broth the antibiotic at 10 μg/ml decreased the number of *E. coli* CFU by a factor of 10^4, whereas with *S. melliferum* and *M. mycoides* no inhibition was detected. With *A. laidlawii* some inhibitory effect was observed. We also examined the effect of rifampin on the growth of mollicutes and *E. coli* by determining the kinetics of ^{32}P incorporation into the nucleic acids of broth-grown organisms. Whereas *E. coli* growth was completely inhibited by 1 μg of rifampin per ml, 10 μg/ml had only slight effects on the growth of *M. mycoides* and *S. melliferum* (Fig. 1). There was some effect on *A. laidlawii* but much less than on *E. coli*.

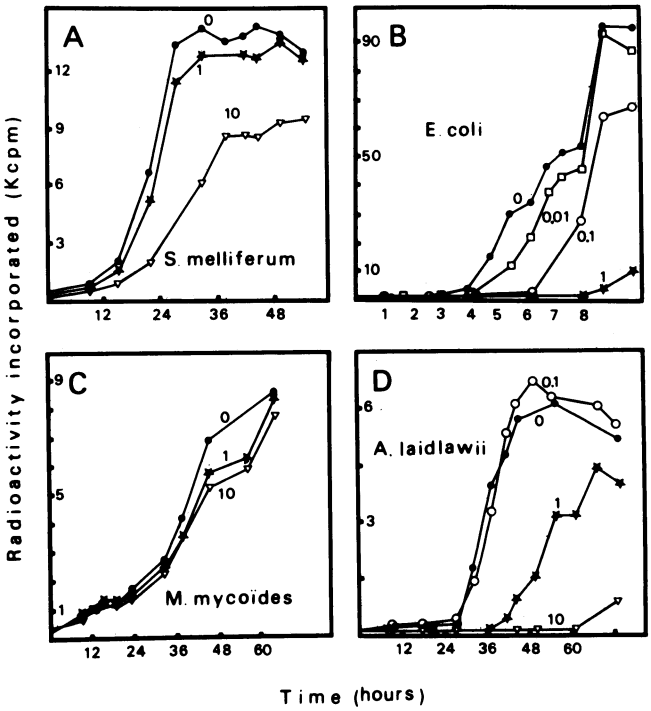

FIG. 1. Effect of rifampin on incorporation of $^{32}P_i$ by different mollicutes and *E. coli*. Organisms were grown in BSR medium containing 5 μCi of $^{32}P_i$ per ml. Samples of 90 μl were taken at indicated times and deposited on paper filters. The filters were washed three times in a 50-ml bath of 10% trichloracetic acid, a 50-ml bath of ether, and 20 ml of a mixture of ether and ethanol, and the radioactivity of the filter was measured in a scintillation spectrometer. Cultures contained no (●) or 0.01 (□), 0.1 (○), 1 (★), or 10 (▽) μg of rifampin per ml.

Our results, obtained with four different techniques, demonstrate unambiguously that three *Spiroplasma* species and a representative species of the genus *Mycoplasma* are not affected by concentrations of rifampin that fully inhibit *E. coli* growth. *A. laidlawii* was affected, but much less than *E. coli*.

DNA-dependent RNA polymerases of *S. melliferum* B88 and *S. apis* B31 are insensitive to rifampin. Rifampin is a specific inhibitor of bacterial DNA-dependent RNA polymerases; by binding to the β subunit of the enzyme it prevents the initiation of RNA synthesis (19). To study the effect of rifampin, a template-free enzyme is required since RNA polymerase already associated with DNA is not sensitive to the drug. We therefore purified the RNA polymerases of *S. melliferum* and *S. apis* to a stage where, in the absence of added DNA, the enzymes showed only 2% of the activity obtained in the presence of DNA. The four ribonucleoside 5′-triphosphates were necessary for RNA synthesis. Omis-

TABLE 1. Effect of rifampin on the activity of spiroplasmal and *E. coli* RNA polymerases[a]

Preincubation rifampin concn (ng/ml)	Relative RNA synthesis (% of control)[b] with RNA polymerase from:		
	S. melliferum	*S. apis*	*E. coli*
0	100	100	100
0.16	110	ND	85
1.6	99	77	69
16	99	86	23
160	101	74	2
1,600	92	87	3
16,000	55	75	1
160,000	37	69	1

[a] The RNA polymerase assay was carried out as described in Materials and Methods with reaction mixtures containing various amounts of rifampin.
[b] 100% activity represents 3,483, 7,292, and 4,789 cpm for *S. melliferum*, *S. apis*, and *E. coli*, respectively. ND, Not determined.

sion of Mg^{2+} resulted in a 10-fold decrease in activity. The enzymes were strongly inhibited by sodium PP_i. Addition of deoxyribonucleoside 5'-triphosphates to the reaction mixture had only a slight effect on RNA synthesis. These results clearly show that the enzyme involved in these studies is a DNA-dependent RNA polymerase.

The effect of rifampin on the DNA-dependent RNA polymerases of the spiroplasmas was compared with that on the *E. coli* RNA polymerase. For *E. coli*, two RNA polymerase preparations were used: one was a commercial enzyme (Boehringer Manheim), and the other was purified in the same way as the spiroplasmal enzymes. Both enzymes showed identical sensitivity to rifampin.

The *E. coli* enzyme was almost fully inhibited by as little as 160 ng of rifampin per ml of reaction mixture, while at a 1,000-fold-higher concentration, the spiroplasmal enzymes still showed about 50% activity (Table 1).

Structure of spiroplasmal RNA polymerase is similar to that of the eubacterial enzymes. The RNA polymerase of *S. melliferum* was further purified by DEAE-cellulose chromatography, glycerol gradient centrifugation, and heparin-agarose chromatography. Table 2 summarizes the purification scheme. A noticeable decrease in activity was observed after heparin-agarose chromatography.

In the glycerol gradient, the RNA polymerase activity was distributed through fractions 6 to 11, fraction 9 showing the highest activity (Fig. 2). All the fractions of the gradient were analyzed side by side on a slab gel by SDS-PAGE. The protein bands were stained with Coomassie blue, and their intensity was estimated from densitometer tracings of each track. The distribution of the various proteins through the glycerol gradient could thus be plotted as shown in Fig. 2. Proteins 1, 2, 7, 8, and 9 were associated with the enzyme activity peak (Fig. 2A), and proteins 3, 4, 5, 6, 10, and 11 were not (Fig. 2B).

The active fractions of the glycerol gradient were pooled and subjected to heparin-agarose chromatography to yield the heparin enzyme. Proteins present in the heparin enzyme (Fig. 3, track 4) were compared with those in the initial

TABLE 2. Purification of the DNA-dependent RNA polymerase of *S. melliferum*

Enzyme prepn	Vol (ml)	Protein (mg)	Activity[a] (pmol of [^{3}H]UMP)		Yield (%)
			Total	Sp act	
Lysate	40	600	545	0.91	
Supernatant (20,000 × *g*)	40	150	591	3.9	
Supernatant (110,000 × *g*)	40	140	659	4.7	100
DEAE enzyme	5	8	273	34.6	41
Glycerol gradient fractions (6 through 11)	6	ND[b]	45	ND	7
Heparin enzyme	0.25	0.13	3.2	25	0.5

[a] Expressed as picomoles of [^{3}H]UMP incorporated in 25 min (total) or picomoles of [^{3}H]UMP incorporated per milligram of protein in 25 min (specific).
[b] ND, Not determined.

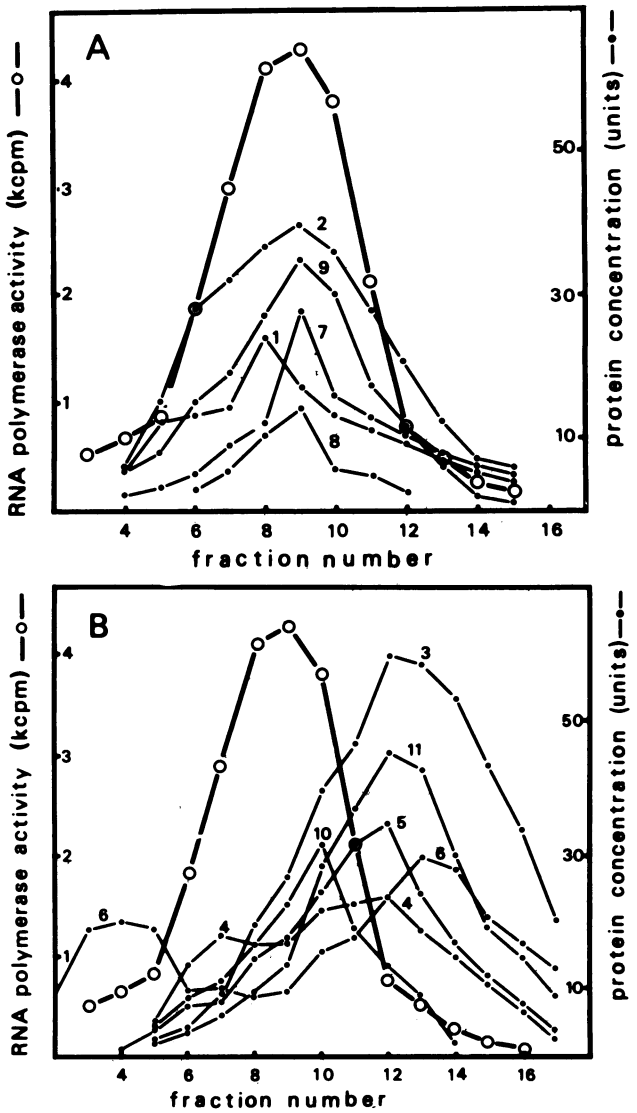

FIG. 2. Distribution of RNA polymerase activity and proteins through the glycerol gradient. The RNA polymerase activity (○) of each fraction of the gradient was determined as described in Materials and Methods. The protein content of each fraction was analyzed by SDS-PAGE. The proteins on the gel were stained with Coomassie blue, and their concentration (●) was roughly estimated from the densitometer tracings of each track. One unit of protein concentration corresponds to a peak surface of 2 mm^2 for protein 3 and 1 mm^2 for all other proteins. The numbers assigned to proteins are those shown in Fig. 3. (A) Protein comigrating with RNA polymerase activity; (B) protein not comigrating with RNA polymerase activity.

DEAE enzyme (Fig. 3, track 2) and those of fraction 9 of the glycerol gradient (Fig. 3, track 3). Peptide markers were *E. coli* RNA polymerase subunits $\beta\beta'\alpha$, β-galactosidase, and bovine serum albumin (track 1). The heparin enzyme (track 4) contained three major peptides: peptides 1 and 2 with relatively large molecular sizes, 140 and 130 kDa, respectively, and peptide 9 of smaller size, 38 kDa. These three peptides were among those found associated with RNA polymerase activity in the glycerol gradient fractions (Fig. 2A).

With two large peptides and a small one, the spiroplasmal heparin enzyme clearly resembled a eubacterial core RNA polymerase, peptides 1 and 2 being similar to peptides β and β′ and peptide 9 to peptide α. Furthermore, from the densitometer tracing of the heparin enzyme peptides, it appears that there was twice as much peptide 9 as peptides 1 and 2, suggesting a core structure of the type $\beta\beta'\alpha_2$.

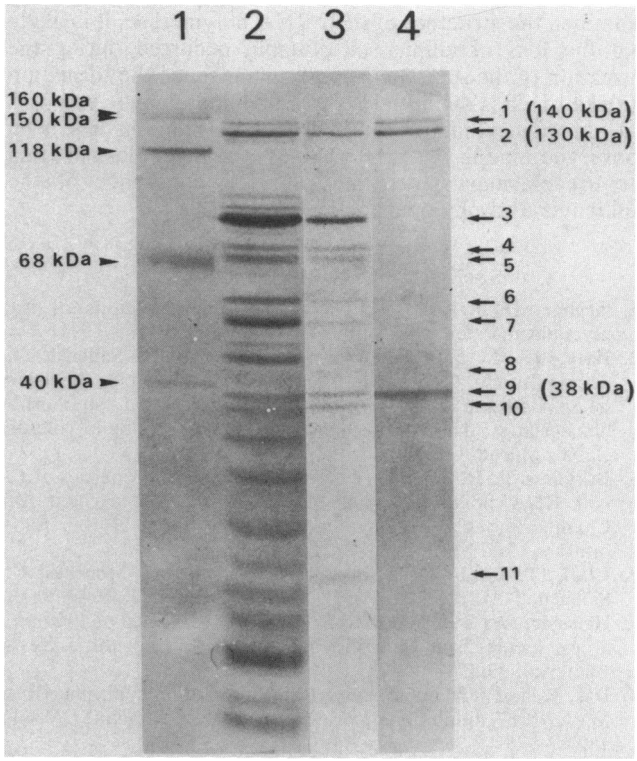

FIG. 3. SDS-PAGE of proteins from different *S. melliferum* RNA polymerase preparations. Electrophoresis was performed on a 15 to 20% gradient SDS-polyacrylamide slab gel, and the proteins were stained with Coomassie blue. Tracks: 1, molecular mass markers (160, 150, and 40 kDa are *E. coli* RNA polymerase subunits β′, β, and α, respectively; 118 kDa is β-galactosidase; 68 kDa is bovine serum albumin); 2, DEAE enzyme; 3, enzyme in glycerol gradient fraction 9; 4, heparin enzyme. Fraction numbers are indicated on the right.

DISCUSSION

We have shown by four different techniques that in vivo *S. apis*, *S. melliferum*, and *M. mycoides* are insensitive to concentrations of rifampin that totally inhibit the growth of *E. coli*. *A. laidlawii* exhibits some susceptibility to the antibiotic but at concentrations 10 times higher than those at which *E. coli* is fully affected. It has been reported that rifampin used at these concentrations (1 μg/ml and above) has a detergent effect (23). This effect would explain the inhibitory action of rifampin on *A. laidlawii*, which is known to be more sensitive to lytic agents than other mollicutes (1). Hence, the target of rifampin in *A. laidlawii* may not be RNA polymerase. Indeed, Das and Maniloff found that *A. laidlawii* RNA polymerase preparations were not affected by rifampin (4). In these studies we have not examined the effect of rifampin on ureaplasmas or anaeroplasmas. Ureaplasmas are known to be insensitive to the antibiotic (17); the sensitivity of anaeroplasmas to rifampin is still unknown. However, together these results suggest that the in vivo insensitivity to rifampin is a property widely distributed among mollicutes and may be a general characteristic of the division *Mollicutes*.

Rifampin inhibits eubacteria by combining with RNA polymerase and preventing initiation of RNA synthesis (7). The fact that the antibiotic did not inhibit spiroplasmas suggested that the RNA polymerase of these organisms was not inhibited by rifampin. Our data demonstrate that this was indeed the case. The RNA polymerases of *S. melliferum* and *S. apis* are insensitive to concentrations of rifampin 1,000 times higher than those inhibiting the *E. coli* RNA polymerase.

Among the procaryotes, the archaebacteria are also insensitive to rifampin. Their RNA polymerase has a structure totally different from that of the eubacterial enzyme and is unaffected by the antibiotic (23). It was thus of interest to examine the structure of spiroplasmal RNA polymerase. We have found that the *S. melliferum* enzyme has a structure of the type $\beta\beta'\alpha_2$, very similar to that of the eubacterial enzyme. During the purification of the spiroplasmal RNA polymerase, the enzyme activity decreased from 45 U for the glycerol enzyme to 3.2 U for the heparin enzyme (Table 2). This decrease can be associated with the loss of peptides 7 and 8 during the heparin-agarose chromatography step. The heparin enzyme, with only three peptides and a subunit structure of the type $\beta\beta'\alpha_2$, is characteristic of a core enzyme. The glycerol enzyme, with two additional peptides, peptides 7 and 8, and a much higher activity than the heparin enzyme, is probably of the holoenzyme type σ ($\beta\beta'\alpha_2$), peptide 7 or 8 being similar to σ factor. It is well known that σ factors can be lost during purification, with a resulting decrease in activity (3).

Rifampin combines with the β subunit of the eubacterial enzyme (6). The insensitivity of the spiroplasmal enzyme to rifampin suggests that the spiroplasmal equivalent of the eubacterial β subunit has no affinity for rifampin. The antibiotic streptolydigin is also known to inhibit the eubacterial RNA polymerases by combining with subunit β or β′ (5). We also examined the effect of streptolydigin on the RNA polymerase of *S. melliferum*. At a drug concentration of 100 μg/ml, the enzyme retained 65% of its original activity, while the *E. coli* RNA polymerase was totally inhibited. Hence, streptolydigin, like rifampin, does not seem to bind to the spiroplasmal equivalents of subunits β or β′.

It is now widely acknowledged that mollicutes form a coherent phylogenetic group that arose as a branch of the low-G+C, gram-positive tree and that the initial event in mollicute phylogeny was the formation of the *Acholeplasma* branch (15). An early splitting of this branch led to the spiroplasmas. Our finding that a spiroplasmal RNA polymerase has the same general structure as eubacterial enzymes is in agreement with the eubacterial origin of the spiroplasmas. However, during the degenerative evolution of the spiroplasmas from the eubacteria, the β or β′ subunit of the RNA polymerase probably lost the ability to bind antibiotics such as rifampin and streptolydigin. Acholeplasmas are also insensitive to rifampin (4). Even though we have not yet

examined the structure of their RNA polymerase, it is likely that the loss of affinity of rifampin occurred during the formation of the *Acholeplasma* branch. Since the formation of this branch is the initial event in mollicute phylogeny, it is not surprising that the insensitivity to rifampin has been conserved among the spiroplasmas, the mycoplasmas, and the ureaplasmas, stressing again the coherence of the mollicutes as a phylogenetic group.

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