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Differentiation of Sterol-Requiring from Sterol-Nonrequiring Mycoplasmas by Amphotericin B

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Growth inhibition of mycoplasmas by amphotericin B in a liquid medium was found to be a simple means of differentiating sterol-requiring from sterol-nonrequiring species.

Growth requirement for sterols is a major taxonomic criterion for mycoplasma classification. Its importance is evident from the recent division of the Mycoplasma tales into two families, *Mycoplasmataceae* [genus *Mycoplasma*] and *Acholeplasmataceae* [genus *Acholeplasma*], the first requiring and the second not requiring sterols (3). Thus, it seems of importance to devise a simple method for establishing the sterol requirement of new mycoplasma isolates. The method described here stems from a previous observation that the polyene antibiotic amphotericin B inhibits growth of the sterol-requiring T-strain mycoplasma but not of the sterol-nonrequiring *A. laidlawii* (11). The indirect determination of the sterol requirement of mycoplasmas through their sensitivity to amphotericin B has the advantage of simplicity over tests based on the stimulation of growth by cholesterol (10).

The experiments were carried out in test tubes containing 4 ml of liquid Edward medium (9) supplemented with 2% PPLO serum fraction or various concentrations of horse serum and with L-arginine to a final concentration of 20 mM. The *p*H of the medium was adjusted to 7.5 with 0.01 N HCl. Amphotericin B (E. R. Squibb & Sons, New York, N.Y.) containing sodium deoxycholate (0.8 mg/mg of amphotericin B) was dissolved in water to give a final concentration of 10 mg/ml. This solution was kept for a few months at −20 C without decrease in activity and was added to the medium to a final concentration of 5, 10, and 20 μg/ml. At a concentration greater than 20 μg/ml, the drug precipitated out during incubation. Media containing the drug had to be used immediately since, when stored at 4 C for more than 3 days, the inhibitory activity of the drug was considerably reduced. The media were inoculated with young mycoplasma cultures at an inoculum level of 1% (v/v) for fast growing-strains and of 2 to 4% (v/v) for slow-growing strains. Growth was determined by measuring the absorbance of the cultures at 500 nm. The results obtained are presented in Fig. 1. At 20 μg of amphotericin B per ml, growth of the sterol-nonrequiring strains was not affected, whereas the growth of the sterol-requiring strains was almost completely inhibited. The inhibition was somewhat less with *M. mycoides* var *capri* and *M. anatis*. Their lower sensitivity to the drug is in accordance with their ability to grow for several subcultures in the absence of serum (2, 10).

Growth inhibition by amphotericin B decreased upon prolonged incubation, either because of the decomposition of the drug or because of its binding to cholesterol in the medium. Hence daily growth check is essential. The effect of the serum content of the medium on the growth inhibition of *M. hominis* by amphotericin B is shown in Table 1. With 20 μg of amphotericin B per ml, growth was almost completely inhibited even in a medium containing 20% horse serum, such as is widely used for the primary isolation of mycoplasmas or for cultivating poorly growing strains. Attempts to perform the test on agar medium by using paper discs impregnated with amphotericin B failed, apparently because of the low diffusibility of the drug in the agar.

It seems that the inhibitory action of amphotericin B is due to its combining with sterols in the cell membrane (5). The sterol-requiring mycoplasmas have a much higher concentration of cholesterol in their cell membranes than the sterol-nonrequiring strains (1). This may explain the higher sensitivity of the sterol-requiring mycoplasma to amphotericin B. For use in growth inhibition experiments, amphotericin B is preferable to other polyene antibiotics which are known to vary in their activity (4, 6). Thus, nystatin, though bound to

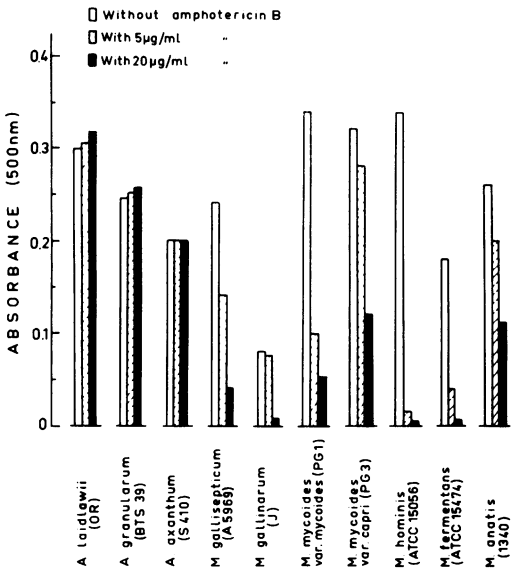

FIG. 1. *Effect of amphotericin B on growth of mycoplasmas in Edward medium (9) containing 2% serum fraction. Cells were grown for 48 hr at 37 C, and the absorbance of the cultures was measured at 500 nm.*

TABLE 1. *Growth inhibition of Mycoplasma hominis by amphotericin B in the presence of various serum concentrations*[a]

Amphotericin B (µg/ml)	Per cent inhibition[b]		
	2% Serum[c] fraction	10% Horse serum[c]	20% Horse serum[c]
5	78	76	62
20	96	98	98

[a] Cells were grown in media containing 2% PPLO serum fraction or various concentrations of horse serum. Growth was tested after 24 hr of incubation at 37 C.

[b] Compared to growth with no inhibitor.

[c] Serum supplement in the medium.

sterol-requiring mycoplasmas, did not affect their growth (8), whereas filipin inhibited the growth of both sterol-requiring (7) and sterol-nonrequiring strains when grown in a serum-containing medium (12).

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