## NOTES

# ANTIBIOTICS FROM *MYCOPLASMA*
## II. CHARACTERIZATION OF ANTIBIOTICS PRODUCED BY *MYCOPLASMA* SP. RPIII

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Several of the *Mycoplasmatales*, including members of both the genera *Acholeplasma*[1] and *Mycoplasma*,[2] have already been shown to produce antimicrobial components, bacteriolytic[3] or proteolytic[4] factors. Even though none of these antimicrobials have yet been fully characterized, it is not unlikely that new, interesting antibiotics could be produced in mycoplasmal fermentations. As a result of systematic screening for antibiotic-producing mycoplasmas, a strain designated as *Mycoplasma* sp. RPIII was selected for further study in our laboratory.

This culture is an unidentified serum-requiring strain of mycoplasma isolated ten years ago from a contaminated rat pituitary tissue culture. It produces at least three types of antibacterial activity: Component I was observed when whole broth cultures of *Mycoplasma* sp. RPIII were tested for their inhibitory activity against bacterial cultures; Component II is associated with the viable *Mycoplasma* cells; and Component III is released from the sonicated cells of *Mycoplasma* sp. RPIII.

When *Mycoplasma* sp. RPIII was grown in stationary culture at 37°C in Bacto® PPLO broth supplemented with 0.1% sodium acetate, 0.5% Bacto® yeast extract and 5% calf serum, the growth rate in log phase was 0.37 (corresponding to a generation time of 2.8 hours) and the maximum colony-forming units of the culture, determined as described by SMITH,[5] reached values above $1\times10^9$. Under these conditions, 1 of 34 fermentations produced antibiotic activity inhibiting the growth of *Bacillus subtilis* Marburg (both spores and vegetative cells), *Staphylococcus aureus* FDA 209 P, *Escherichia coli* B and *Pseudomonas aeruginosa* Bristol Laboratories No. A9843A. This antibiotic referred to as component I is a dialysable, basic molecule resistant to heat at 100°C in acidic or basic solutions. The antibiotic activity in agar diffusion assay is reversed by organic and inorganic salts, including ammonium sulfate, sodium chloride, sodium acetate and sodium citrate. No direct relationship was observed between the production of component I and the growth of *Mycoplasma* sp. RPIII.

Component II was found when a suspension of *Mycoplasma* sp. RPIII cells in log phase of growth containing at least $5\times10^9$ colony-forming units per ml was studied in agar diffusion assay with *B. subtilis* (spores or vegetative cells) as test organism. Component II activity was bound to the mycoplasma cells. For the convenience of this study, since large amounts of cells were required, the *Mycoplasma* was grown in a dialysis bag culture apparatus. The basal medium used in the reservoir and the bag had the same composition as the one used for the production of component I, but the calf serum concentration was adjusted to 10% inside the bag and 1% in the reservoir. The maximum number of viable cells per ml in the dialysis culture apparatus was ten times higher than the maximun viable count in the stationary culture technique used for production of component I.

Component II is active only against some members of the genus *Bacillus*: *B. subtilis* Marburg, *B. cereus* ATCC 14579, *B. megaterium* USDA 234 and *B. mycoides*. No direct relationship between the CFU/ml and the inhibitory potency of the mycoplasma culture was observed. It was not possible to release component II from the viable mycoplasma cells, and any treatment affecting the viability of the mycoplasma cells (heating, ultraviolet irradiation, high salt concentration, freezing and thawing, sonication) also destroyed the inhibitory activity of component II.

The dialysis bag culture apparatus was also used for production of component III. The optimal condition for the release of the active component from the cells included the collection of the cells by centrifugation at 12,000

×*g* for 10 minutes at 4°C. They were then washed in 0.15 M sucrose and suspended in pH 5.6 McIlvaine buffer (0.048 M). The cell suspension was then sonicated for 3 minutes in a Raytheon sonic oscillator at a frequency of 10 kHz, killing 99% of the initial population. The sonicated suspension was centrifuged at 28,000×*g* for 20 minutes and the supernatant dialysed against water. The active component III (detected in agar diffusion assay by inhibition against *B. subtilis* spores) was found in the dialysate of the sonicated cells.

The crude component III solution was desalted by mixing 1 volume of the aqueous solution adjusted to pH 9.0 with 4 volume of absolute methanol. The precipitate was collected by centrifugation (and discarded), and the methanol-soluble fraction was evaporated *in vacuo* and redissolved in water. The methanol-treated component III was purified further by filtration through a Millipore® Pellicon® filter followed by chromatography over an Amberlite® CG-50 column ($NH_4^+$ cycle) and eluted with ammonium hydroxide.

The purified component III inhibited the growth of *B. subtilis* Marburg (spores and vegetative cells), *B. cereus* ATCC 14597, *B. mycoides*, *B. megaterium* USDA 234, but was inactive against *E. coli* B, *Ps. aeruginosa* Bristol A-9843A, *S. aureus* FDA 209P or *Candida albicans* Roche 1343-Y. At its MIC, the antibiotic was bacteriostatic for the growth of *B. subtilis* spores.

The antibiotic activity moved as a single entity (as detected by bioautography) in paper chromatography using *n*-propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12) system ($R_f$= 0.6) and in *n*-propanol - acetic acid - water (50 : 5 : 40) system ($R_f$=1.0). When the active component III was analyzed by ionophoresis at pH 1.8 using the acetic acid - formic acid - water buffer (20 : 2 : 78) for 20 minutes with an electric field of 34 volts/cm, the antibiotic activity moved 1.5 cm toward the cathode. (Under the same conditions, the L-lysine standard moved 12 cm toward the cathode.) Component III gave positive anthrone and $KMnO_4$-$KIO_4$ tests and a negative ninhydrin test. The active molecule has a molecular weight lower than 1,000, since it is filterable through a Pellicon® membrane. The ultraviolet spectrum of the purified component III showed end absorption.

Mutants of *B. subtilis* resistant to component III remained sensitive to penicillin G, spectinomycin, lincomycin, erythromycin, bacitracin and mikamycin A+B. On the basis of these chemical and biological results, we conclude that component III is distinct from these antibiotics.

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