CHARACTERISTICS OF TWO BROAD SPECTRUM ANTIBIOTICS PRODUCED BY *MYCOPLASMA* SP. RP III*

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Two extracellular antibiotics produced by *Mycoplasma* sp. RP III growing in serum-containing media have been purified by solvent extraction and ion-exchange chromatography. Factor I inhibits Gram-positive and Gram-negative bacteria *in vitro* and a crude preparation protected mice from *Pseudomonas* and *Staphylococcus* infections; it is of low cytotoxicity. Factor II, a lipid, inhibits Gram-positive and Gram-negative bacteria, *Candida* species, and is as cytotoxic as the actinomycins.

Previous studies reported from these laboratories[2] have shown that *Mycoplasma* sp. PR III (isolated from a rat pituitary tissue culture) produced three types of antibiotics: One was extracellular; one was intracellular; and one was associated with the viable cells[2]. Further study of the extracellular antibiotic activity has shown that 2 substances (identified as Factor I and Factor II) are produced in fermentations of serum-containing media. Both inhibit the growth of Gram-positive and Gram-negative bacteria and have different biological properties. We wish to report in this communication on the fermentation process, the isolation techniques, and on biological and chemical properties of these antibiotics.

Materials and Methods

Culture maintenance. Stocks of *Mycoplasma* sp. RP III were grown in a medium based on Difco PPLO broth (without crystal violet) to which was added 0.5 % yeast extract and 1 % sterile calf serum. Aliquots were frozen at −20°C and stored until needed. Fermentations were started using inoculum grown on PPLO broth medium for 24 hours at 35°C in shaken flasks. Although 'the culture run down' was not studied in detail, it did seem that if the inoculum was not transferred very frequently the surviving cells lost their ability to produce the antibiotics. For practical purposes best results were obtained when the culture was transferred every 24 hours. Inoculum levels for the fermentations were usually 5 % of a 24-hour culture (these usually contained 1×10^9 cfu per ml).

Determination of antibiotic activity. As initial experiments had shown that the antibiotic activity in the fermented medium dropped rather rapidly even upon storage in a refrigerator, an effort was made to stabilize the activity. The following procedure was used routinely: The samples of fermented medium were adjusted to pH 4.0 (using 2 N HCl) and subsequently heated to 80°C for 3 minutes. The samples were then centrifuged. Five ml of the supernatant solutions were adjusted to pH 6.5 (with 2 N NaOH) and lyophilized. The recovered solids were dissolved in 0.25 ml of water. These concentrates were assayed for antibiotic activity using an agar diffusion assay with 24-hour-old cells of *Pseudomonas convexa* NRRL B-21 in 10 ml of Difco assay medium 1 (in a 100-mm Petri dish) 8-mm wells in the agar served as reservoirs. The assay

* The third paper in the series "Antibiotics from *Mycoplasma*". For papers I and II see references[1,2].

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plates were incubated at 26°C for 18 hours. Kanamycin solutions served as a temporary internal standard.

In studying the antimicrobial spectrum of the purified antibiotics, the test organisms (see Table 5) were grown in nutrient broth (except for the *Candida* cultures which were grown in the SABOURAUD's medium) for 24 hours and used to inoculate Difco assay agar no. 1. Paper discs (6.25 mm) were used as reservoirs, and zones of inhibition were measured after 18 hours at 37°C.

Determination of *Mycoplasma* growth. Growth of the *Mycoplasma* sp. RP III was determined using the method described by SMITH[3]. The results were recorded as colony-forming units (cfu) per ml.

Thin-layer chromatography. Analytical and preparative thin-layer chromatography was carried out by using the pre-coated plates (0.25 mm silica gel layer) obtained from Macherey-Nagel Company.

Results and Discussion

Fermentation Studies

Since during our previous work the production of the antibiotic by this culture was quite erratic, it was our first objective to improve this record. This was accomplished by use as inoculum for the fermentations of cells that had been growing in the log phase of growth (as mentioned above) and by using the shaken culture technique for the fermentation process. Some of the data showing that antibiotic activity was higher in shaken cultures than in static cultures are summarized in Table 1 and Fig. 1. We also found higher maxima for cell counts (colony-forming units) in the shaken cultures (Table 1 and Fig. 1) where lower volumes of media were present in the Erlenmeyer flasks.

A study of the effect of composition of the fermentation medium on antibiotic productivity led to standardization at 1 % calf serum and addition of yeast extract to Difco PPLO broth (without crystal violet) used as the basal medium. Data collected in an experiment where the serum content of the medium was varied is summarized in Fig. 2. The beneficial effects of

Table 1. Effect of fermentation conditions on antibiotic production by *Mycoplasma* sp. RP III.

Incubation temperature °C	Volume of medium per 250 ml Erlenmeyer* ml	Viable count: log of cfu	Diameter of zone of inhibition** mm	pH of fermentation at harvest (96 hours)
26	170	0.7	none	7.8
26	150	0.8	trace	7.7
26	100	1.3	13	7.7
26	50	2.8	14	7.7
31	170	9.0	none	7.8
31	150	7.6	none	7.5
31	100	8.3	17	7.2
31	50	8.8	16.5	7.4
35	170	9.0	none	7.8
35	150	8.8	14	7.7
35	100	12.0	18.5	7.5
35	50	15.0	17	7.3

* All flasks placed on rotary shaker, 400 rpm (1-inch circle diameter).
** Bioassay using *Pseudomonas convexa* as test organism and 6.25 mm paper discs.

the yeast extract supplement are shown in Table 2. Addition of an amino acid mixture (in the form of Difco Casamino Acids) resulted in some increase in antibiotic production (see Fig. 3), but only at the early stages of the fermentation period. Since inclusion of the amino

Fig. 1. Effect of aeration on growth and antibiotic production by *Mycoplasma* sp. RP III.

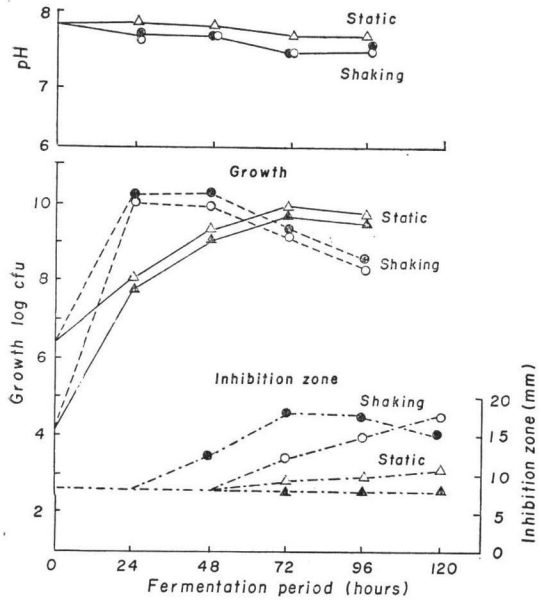


Fig. 2. Effect of serum concentration in medium on growth and antibiotic production by *Mycoplasma* sp. RP III.
Incubation period: 72 hours.

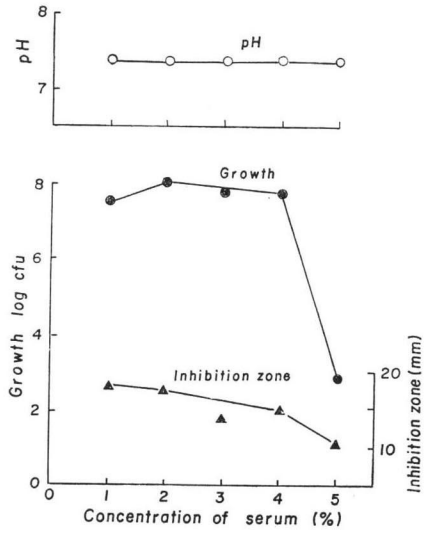


Table 2. Effect of yeast extract and other supplements to the fermentation medium on antibiotic production by *Mycoplasma* sp. RP III.

Combination	Supplement added	Diameter of inhibition zone (mm) after indicated fermentation periods	
		72 hours	96 hours
1	none	12	11.5
2	0.05 % Difco yeast extract	12	11.5
3	0.5 % Difco yeast extract	12.5	16
4	1.5 % Difco yeast extract	19.5	17.5
5	0.5 % Yeast extract +0.5 % Difco casamino acids*	19	17
6	0.5 % extract+vitamin mixture**	15.5	16
7	0.5 % Yeast extract+base mixture***	15	15
8	5+6	17	17.5
9	5+7	17	17
10	6+7	15	16

Note: Antibiotic activity determined using agar diffusion bioassay with *Pseudomonas convexa* as test organism and 8-mm wells in the agar as reservoirs.

* Supplemented with 20 mg/100 ml (of medium) of L-cysteine and L-tryptophan.

** Vitamin mixture contained 4 mg thiamine, 0.4 mg pyridoxal, 2 mg pyridoxamine-HCl, 2 mg *p*-aminobenzoic acid, 4 mg riboflavin, 2 mg calcium pantothenate, 20 mcg biotin, 20 mcg folic acid, 2 mg nicotinic acid, and 5 mg inositol per liter.

*** Base mixture contained 20 mg each of adenine·HCl, guanine·HCl, uracil, xanthine, and thymine per liter.

Fig. 3. Effect of concentration of Difco casamino acids supplement on antibiotic production by *Mycoplasma* sp. RP III.

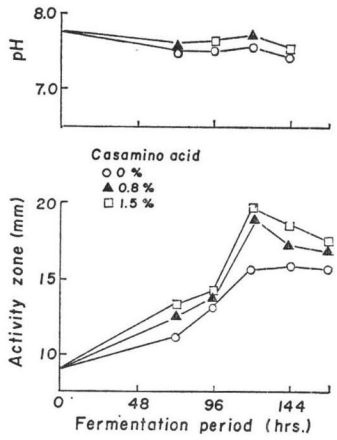
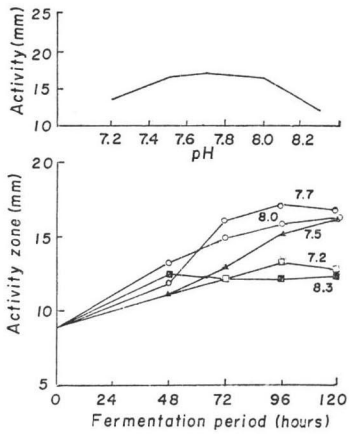


Fig. 4. Effect of initial pH of medium on antibiotic production by *Mycoplasma* sp. RP III. Antibiotic activity after 96-hour incubation.



acid mixture in the medium appeared to complicate the isolation of the antibiotics, this material was not routinely used as a component of the medium.

The initial pH of the fermentation medium was found to be an important determinant of antibiotic yield. Some of the data obtained in a series of experiments are summarized in Fig. 4. For routine antibiotic production all media were adjusted with NaOH to pH 7.7 after autoclaving (and after addition of sterile calf serum to the medium).

Purification of Antibiotics

Preliminary studies showed that in the 'active fermentations' there were acidic and basic antibiotics. The following procedure was used to isolate the basic antibiotic (Factor I): The fermented medium was adjusted to pH 4.5 with HCl and heated for 3 minutes in a 80°C water bath. After cooling to room temperature, the precipitate was removed by filtration through Whatman no. 1 paper. The filtrate was treated with Darco G-60 (30 g/liter filtrate) and the charcoal removed by filtration. The filtrate was evaporated to 1/20 volume by concentration *in vacuo* and extracted three times with equal volumes of ethyl acetate - methanol (9 : 1). The solvent layers were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in a minimum of distilled water and passed over a Dowex 1×4 column (OH^- form). The resulting effluent had a pH of 6.5. The antibiotic-containing solution was then applied to an Amberlite CG-50 (H^+ cycle) column and the antibiotic was eluted by using a 0.2 N formic acid solution. The bioactive fractions were combined and concentrated *in vacuo* to dryness. The residue was dissolved in a minimum of methanol and sufficient ethyl ether was added to give a 1 : 1 mixture. The precipitate, formed on addition of ether, was removed by centrifugation. The supernatant was evaporated to dryness, the residue dissolved in water and rechromatographed on CG-50 resin (H^+ cycle). The bioactive material was eluted with 0.2 N formic acid. The bioactive fractions were combined and passed through the Dowex 1 (OH^- cycle) to remove excess acid. The bioactive fractions were then combined and evaporated to dryness *in vacuo*. The solids were dissolved in a minimum of methanol and ethyl ether was added until the solution became cloudy. The mixture was then placed at 4°C and after 15 days at this temperature

about 1 mg of a hygroscopic semi-crystalline solid was obtained. This material, designated as Factor I, represented the yield from 30 liters of fermented medium. Under usual circumstances we recovered about 20 % of the material present in the fermented medium.

The acidic antibiotic (designated as Factor II) was recovered from the aqueous phase after the ethyl acetate - methanol extraction (mentioned above) by adjusting the solution to pH 3.0 with HCl and extracting it three times with an equal volume of ethyl ether. The ether extracts were combined and evaporated *in vacuo*. The residue was dissolved in a minimum of ethyl ether and further purified by preparative thin-layer chromatography with silica gel as support with the following solvent systems: $CHCl_3$ - acetone (1 : 1); $CHCl_3$ - MeOH (4 : 1); and $CHCl_3$ - EtOAc (1 : 1). The antibiotic containing areas of the silica gel chromatograms were eluted with $CHCl_3$ and the eluates concentrated to a small volume *in vacuo*. The material recovered as an oil amounted to approximately 10 mg per liter of fermented medium and usually was about 10 % pure.

Chemical Characteristics of Factors I and II

Factor I is a basic substance. In paper ionophoresis (Whatman no. 1 paper) with 5 volts/cm for 20 minutes it moved 3.3 cm toward the cathode at pH 1.9 (acetic acid - formic acid - water, 20 : 2 : 78, v/v/v). The R^- alanine as a reference was 0.16. The melting point of the compound was 120°C. There was no significant absorption in the ultraviolet. The infrared spectrum (KBr pellet) and the NMR spectrum (in D_2O) of a Factor I preparation which was a single component of TLC are presented in Figs. 5 and 6.

Fig. 5. Infrared absorption spectrum of Factor I (KBr).

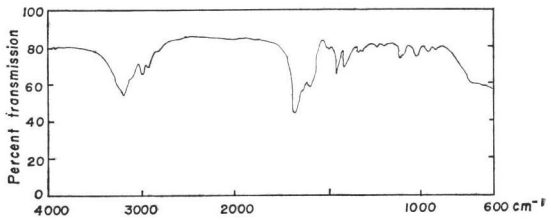


Fig. 6. NMR Spectrum of Factor I.

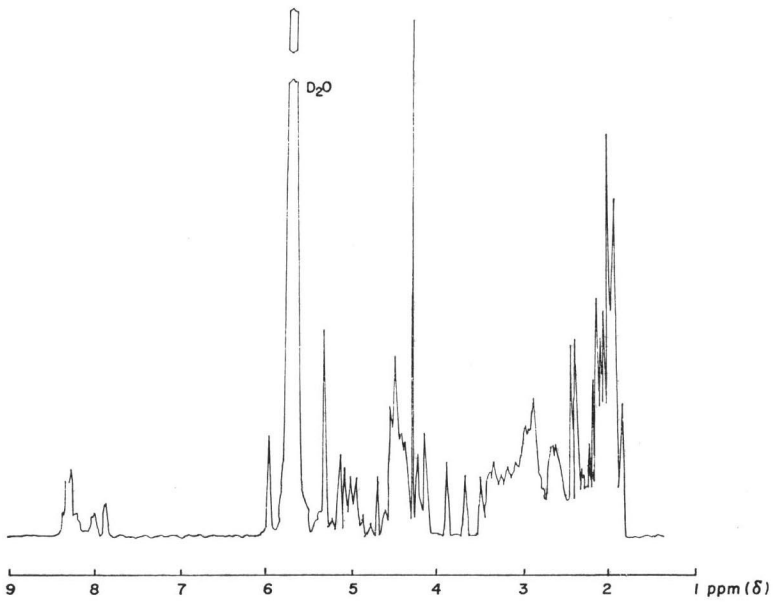


Table 3. Mobility of *Mycoplasma* sp. RP III antibiotics in paper partition chromatography.

Solvent system	Rf noted*	
	Factor I	Factor II
Water saturated *n*-BuOH	0.06	n.d.**
NH_4Cl, 3 % (w/v)	0.56	n.d.
n-BuOH - MeOH - H_2O (4 : 1 : 2)	0.44	n.d.
Water	0.53	n.d.
Benzene - MeOH (4 : 1)	0.02	0.63

* Position of antibiotic on paper chromatograms detected by bioautography using *Pseudomonas convexa* as test organism.

** Antibiotic could not be detected and we concluded it was inactivated by the solvent system.

Table 4. Mobility of *Mycoplasma* sp. RP III antibiotics in thin-layer chromatography on silica gel.

Solvent system	Rf noted*	
	Factor I	Factor II
$CHCl_3$ - MeOH (8 : 2)	0.44	0.88
$CHCl_3$ - MeOH (1 : 1)	0.49	0.25
$CHCl_3$ - Acetone (8 : 2)	inactivated	0.81
$CHCl_3$ - Acetone (1 : 1)	inactivated	0.42
$CHCl_3$ - *n*-Hexane (2 : 1)	0	0.31
$CHCl_3$ - Benzene (2 : 1)	0	0.47
EtOAC - AcOH - H_2O (6 : 2 : 1)	0.52	—

* Position of antibiotics on thin-layer chromatograms was detected by bioautography using *Pseudomonas convexa* as test organism.

The antibacterial potency of Factor I was lost when the solutions were stored at alkaline pH, or when the material was dissolved in acetone, or when it was lyophilized. Acidic solutions in water were stable for periods up to one month at 5°C. Spot tests showed positive reactions for the following reagents: $CeSO_4$; I_2; triphenyltetrazolium. Negative tests were found with the following reagents: phosphotungstic acid; aniline phthalate; ninhydrin; resorcinol-HCl; orcinol; and anthrone.

Factor II proved to be stable in aqueous solutions at acid pH, but was found to be labile at alkaline pH (pH 8). Spot tests showed positive reactions for the following reagents: $CeSO_4$; I_2; phosphotungstic acid; triphenyltetrazolium. Negative reactions were obtained with the following reagents: aniline phthalate; ninhydrin; resorcinol—HCl; orcinol; anthrone.

The mobilities of Factors I and II in paper chromatographic and thin-layer chromatographic systems are summarized in Tables 3 and 4.

Biological Characteristics of Factors I and II

The antimicrobial spectra of Factors I and II are summarized in Table 5 which also contains information on antibiotic activities of erythromycin, tetracycline, kanamycin, and gentamicin (complex). Most interesting is the potency of both Factors I and II against *Pseudomonas* strains.

Cytotoxicity tests using the inhibition of growth of KB cells (according to the protocols of the National Cancer Institute) showed that purified Factor I had an ID_{50} of 8 mcg/ml and Factor II an ID_{50} of 5.7 ng/ml. Thus, the cytotoxicity of Factor I is comparable with that of tetracycline or chloramphenicol, while the cytotoxicity of Factor II is of the order of the actinomycins in this test system.

In vivo tests using *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Gray) as test organisms in mice showed that when crude Factor I (perhaps 10 % pure) was injected subcutaneously at 600 mg/kg, two of the three mice survived, while those mice not receiving the antibiotic died. Further animal tests await production of more antibiotic material.

Studies of the mechanisms of action of these antibiotics have been limited so far by the lack of antibiotic material. In one study, using the agar diffusion assay with *Bacillus subtilis*

Table 5. Comparison of antibiotic activity of preparations from *Mycoplasma* sp. RP III with other antibiotics.

Assay organisms	Diameter of inhibition zone, mm*					
	Mycoplasma sp.		Erythromycin	Tetracycline	Kanamycin	Gentamicin
	Factor I	Factor II				
Bacillus subtilis Marburg	22	24.5	26	16	22	24
Bacillus cereus ATCC 14579	26	25	25.5	23	19	19
Staphylococcus aureus FDA 209P	14.5	25	10	10	19	18
Sarcina lutea	16.5	20	26	16	15.5	10
Corynebacterium sepedonicum ATCC 15391	17	26	14	13.5	22	21.5
Escherichia coli B	20.5	20	13	15	15	24.5
Erwinia carotovora ATCC 495	24	23	n.z.	16	21	22
Pseudomonas putida ATCC 21812	22	>40	n.z.	9	20	21
Pseudomonas convexa NRRL B-21	17	31	—	—	—	—
Pseudomonas aeruginosa IFO 3856	25	26	—	28	26.5	26.5
Pseudomonas chlororaphis NRRL B-502	25	32	n.z.	tr	15	10
Pseudomonas syringae NRRL B-865	21	29	tr	21.5	27	25.5
Pseudomonas stutzeri NRRL B-927	22	>30	12.5	13.5	18.5	17
Pseudomonas aureofaciens NRRL B-1543P	20	27.5	n.z.	tr	14	11
Candida utilis Y-421	n.z.	17.5	n.z.	n.z.	n.z.	n.z.
Candida albicans Y-4244	n.z.	14	n.z.	n.z.	n.z.	n.z.

* 6.25 mm paper discs used; discs with Factor I contained 200 mcg; discs with Factor II contained 20 mcg/disc; discs with erythromycin, kanamycin, tetracycline, and gentamicin all contained 5 mcg/disc.

Marburg as test organism we found that the antibacterial activity of Factor II could be reversed by sodium citrate, sodium fumarate, sodium succinate or sodium tartrate. Under the same test conditions KCl, $MgSO_4$, and Na_2SO_4 were much less effective as reversing agents than the sodium salts of the TCA cycle acids. Further experiments are needed to investigate the possibility of an impairment by Factor II of carbohydrate metabolism in sensitive cells.

We suppose from the above studies that the antibiotics produced by the *Mycoplasma* culture may not be related to antibiotics previously described in the literature.

Acknowledgements

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