Antibiotic Sensitivity In Vitro of the Mycoplasmalike Organism Associated with Citrus Stubborn Disease

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ABSTRACT

The mycoplasmalike organism associated with citrus stubborn disease was tested for susceptibility in vitro to 19 antibiotics, three systemic insecticides, and three systemic fungicides. Minimal inhibitory concn (MIC) and minimal biocidal concn (MBC) were determined for each substance by a dilution-broth method. The macrolide antibiotics, erythromycin and tylosin, were most active, each having MIC and MBC values of 0.2 and 0.8 μg/ml, respectively. Six members of the tetracycline group and carbomycin were inhibitory at low concns (0.1 to 0.4 μg/ml) but the biocidal levels were two to four times higher (1.6 to 3.1 μg/ml) than for erythromycin or tylosin. MIC values for oleandomycin, lincomycin, and filipin ranged from 1.6 to 3.1 μg/ml, and all were biocidal at 12.5 μg/ml. Chlortetracycline, chloramphenicol, and kanamycin were relatively ineffective, with MIC values of 12.5 to 25.0 μg/ml, and an MBC value of 50.0 μg/ml. Streptomycin and an antibiotic known as BP both had an MIC value of 50.0 μg/ml, and MBC values of 100 and 500 μg/ml, respectively. Penicillin and sulfanilamide were not inhibitory at 500 μg/ml, the highest concn tested.

Of the systemic insecticides (cygon, furadan, and lannate) and systemic fungicides (benomyl, thiabendazole, and thiophanate M), only thiabendazole in 5% (v/v) dimethylsulfoxide had an MIC value (10 μg/ml) similar to that of the effective antibiotics.

The results indicate that erythromycin, tylosin, and several members of the tetracycline group of antibiotics are potentially useful for control of stubborn disease. They contribute also to characterization of the mycoplasmalike organism associated with the disease.

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Evidence that citrus stubborn disease is caused by a mycoplasmalike organism consists of: (i) demonstration by thin-section electron microscopy of mycoplasmalike bodies in the phloem cells of infected, but not in healthy, citrus tissue; (ii) culture of a mycoplasmalike organism from infected, but not from healthy, tissue; and (iii) suppression of symptoms in greenhouse-grown sweet orange seedlings by tetracycline hydrochloride (7, 10, 11). There are indications that citrus greening disease, also believed to be caused by a mycoplasmalike organism, can be controlled to some extent by tetracycline treatment (19, 25). Because of the successful culture in vitro of a mycoplasmalike organism from stubborn-diseased citrus (7), susceptibility tests of the organism in vitro seemed to be a useful means of screening antibiotics of potential use in attempts to control the disease.

Following the initial work of Ishiie et al. (12), there have been numerous reports of suppression of symptoms in yellows-diseased plants by tetracycline treatment (3, 4, 6, 9, 14, 26), but this appears to be the first study in vitro of antibiotic sensitivity of a mycoplasmalike organism isolated from a yellows-diseased plant. The results contribute also to characterization of the organism.

MATERIALS AND METHODS.—*Organism.*—The mycoplasmalike organism was isolated from a greenhouse-grown 'Madam Vinous' sweet orange seedling infected with California 189 stubborn, as described by Fudl-Allah et al. (7). The organism was cloned four times, then subcultured 10 more times. After growth in broth at 30 C for 3 days, tubes of the 10th subculture were stored at −20 C until required. A culture has been deposited in the American Type Culture Collection, where the accession number is 27563. Cole et al. (5) have isolated and described a similar organism from stubborn-diseased citrus, and have proposed the name *Spiroplasma citri.*

Medium.—For routine culture and antibiotic sensitivity tests, the medium described by Fudl-Allah et al. (7) was used, except that the solid components were dissolved in 70 ml distilled water instead of 60 ml. Also, phenol red was added as a pH indicator to a concn of 0.0005% (w/v).

Antibiotics and other chemicals.—The following antibiotics were used: oxytetracycline hydrochloride, chloramphenicol, erythromycin, and kanamycin sulfate (Sigma Chemical Co., St. Louis, Mo. 63118); tetracycline hydrochloride, streptomycin sulfate, and sulfanilamide (Nutritional Biochemical Corp., Cleveland, Ohio 44128); potassium penicillin G (Calbiochem, Los Angeles, CA 90054); methacycline hydrochloride, doxycycline hyclate, oleandomycin phosphate, and carbomycin (Pfizer Inc., Brooklyn, NY 11206); chlortetracycline, demethylchlortetracycline, and minocycline, all as the hydrochloride salts (Lederle Laboratories, Pearl River, NY 10965); filipin (The Upjohn Company, Kalamazoo, MI 49001); tylosin and lincomycin hydrochloride (Grand Island Biological Company, Grand Island, NY 14072). An antibiotic known to us only as BP was obtained from Dr. M. J. Thirumalachar, Hindustan Antibiotics, Poona, India. The systemic insecticides cygon (American Cyanamid Company, Princeton, NJ), furadan (Niagara Chemical Division, Richmond, CA 94804) and lannate (E. I. duPont de Nemours Company, Princeton, NJ), and the systemic fungicides benomyl (E. I. duPont de

Nemours Company, Wilmington, DE), thiabendazole (Merck Chemical Division, Rahway, NJ) and thiophanate M (Pennwalt Company, Tacoma, Wash. 98401) were also used.

Stock solutions of erythromycin, chloramphenicol, and oleandomycin were prepared by dissolving 0.02 g in 1.0 ml methanol, and diluting this to 10 ml with distilled water. Filipin was prepared similarly, except that 1 ml dimethylformamide (DMF) was used as the initial solvent. BP was prepared by dissolving 0.1 g in 1.0 ml dimethylsulfoxide (DMSO), and diluting to 10 ml with water. Stock solutions of all other antibiotics were prepared in distilled water. All stock solutions were prepared on the day of use, and were sterilized by filtration through 0.22-μm Millipore filters. Serial two-fold dilutions were then prepared in sterile distilled water. Solutions of lannate and furadan were prepared by adding 0.1 g to 5.0 ml sterile distilled water, and an emulsion of cygon was prepared similarly. Suspensions of benomyl, thiabendazole, and topsin (0.1 g in 5.0 ml distilled water) were sterilized by autoclaving (121 C for 15 min). Solutions of these materials were prepared by dissolving 0.1 g in 5.0 ml DMSO. Serial 10-fold dilutions were made in sterile distilled water.

Tests for susceptibility.—The broth medium was dispensed in 1.8-ml amounts in sterile tubes, and 0.1 ml of the appropriate antibiotic dilution was added to produce final concns ranging from 100 to 0.1 μg/ml. Final concns of the insecticides and fungicides were 1,000, 100, and 10 μg/ml. To check the effects of solvents, tubes containing 0.5% (v/v) methanol or DMF, or 5.0% (v/v) DMSO were included. For standard tests, inoculum consisted of 0.1 ml of a 1:10 dilution of a culture grown for 3 days at 30 C. This gave approximately 5×10^5 colony-forming units (CFU) per ml inoculated broth. CFU were estimated by agar plate counts of 0.1-ml aliquots of serial 10-fold dilutions of the stock culture. The agar medium was prepared by adding 1.0% (w/v) Bacto Difco agar to the broth formulation. Plates were prepared on the day of use, and were dried at 36 C for 1 h before inoculation. Antibiotic-free cultures and noninoculated tubes were included as controls. All tests were conducted in duplicate, and repeated at least twice. After 3 days incubation at 30 C, tubes were examined for acid production resulting from fermentation of sugar. This caused a color change of the phenol red indicator from red (pH 7.5) to orange yellow (pH 7.1).

The minimal inhibitory concn (MIC) was regarded as the lowest concn of chemical which prevented color change of the medium after 3 days incubation, provided control antibiotic-free cultures had changed color. The minimal biocidal concn (MBC) was taken as the lowest concn of chemical which eliminated the organism, as indicated by sub-culture onto agar of all tubes showing no color change. These plates were incubated at 30 C for 7 days before assessment.

RESULTS.—*Effect of inoculum size.*—Serial 10-fold dilutions of a 3-day culture were prepared in sterile broth to give final levels of inocula of approximately 5×10^3, 5×10^4, and 5×10^5 CFU per ml. MIC values of tetracycline, chlortetracycline, and erythromycin were recorded after 3 days incubation at 30 C. Inoculum size had little effect for tetracycline or erythromycin, but caused an eight-fold difference in the MIC for chlortetracycline. The tubes were reincubated at 30 C for an additional 4 days, and the MIC values again recorded. The values for tetracycline and erythromycin were similar to those obtained after 3 days incubation, but for chlortetracycline there was a four- to eight-fold increase at the lower inoculum levels (Table 1). To minimize this effect, routine tests were based on an inoculum size of approximately 5×10^5 CFU per ml, and an incubation time of 3 days.

The MIC and MBC levels for the 19 antibiotics and six other chemicals were determined under these conditions. They are listed in Table 2. Plate counts showed that the number of organisms required to mediate a color change of the medium was approximately 10^6 CFU per ml after 3 days incubation. Thus, the test for MIC indicated the concn of chemical required to keep the CFU below this level.

When both inhibitory and biocidal effects are considered, the macrolides erythromycin and tylosin were the most active antibiotics tested. Six of the seven tetracyclines showed inhibitory effects similar to those of erythromycin and tylosin, but the biocidal concns were somewhat higher. Chlortetracycline was relatively inactive, with much higher concns (~30- to 60-fold) being necessary for both inhibitory and "cidal" effects. The MIC for oxytetracycline was similar to that of the other tetracyclines (except chlortetracycline) but the MBC was four to eight times higher. Carbomycin had activity similar to that of the tetracyclines. Oleandomycin, lincomycin, and filipin showed similar activity, at concns greatly in excess of those for most of the tetracyclines. Chloramphenicol and kanamycin, for which MIC and MBC values were similar, were much less active than oleandomycin, lincomycin, or filipin. Streptomycin and BP were relatively inactive, while penicillin and sulfanilamide were not inhibitory at 500 μg/ml.

None of the three systemic insecticides showed any activity. Of the three systemic fungicides, only thiabendazole in 5% DMSO caused inhibition at a concn (10 μg/ml) comparable with that of some of the antibiotics. Methanol, DMSO, and DMF, when tested at the levels used as solvents for some of the agents, had no apparent inhibitory effects.

DISCUSSION.—The sensitivity of the organism to tetracyclines and its resistance to penicillin and sulfanilamide parallels the general antibiotic susceptibility pattern of animal mycoplasmas (1, 13, 20,

TABLE 1. Effect of inoculum size and time of incubation (3 or 7 days) on minimal inhibitory concentration (MIC) of three antibiotics for the mycoplasmalike organism associated with citrus stubborn disease

Inoculum size (CFU/ml)[a]	MIC (μg/ml)					
	Tetracycline		Chlortetracycline		Erythromycin	
	3 days	7 days	3 days	7 days	3 days	7 days
5×10^3	0.1	0.2	1.6	12.5	<0.1	0.1
5×10^4	0.1	0.2	6.2	25.0	0.1	0.2
5×10^5	0.2	0.2	12.5	12.5	0.2	0.2

[a]CFU = colony-forming units.

TABLE 2. Susceptibility in vitro of the mycoplasmalike organism associated with citrus stubborn disease to antibiotics and other chemicals: Minimal inhibitory concentration (MIC) and minimal biocidal concentration (MBC) after 3 days incubation at 30 C

Antibiotic/Chemical	MIC (μg/ml)	MBC (μg/ml)
tetracycline	0.2	3.1
chlortetracycline	12.5	50.0
demethylchlortetracycline	0.4	1.6
oxytetracycline	0.2	12.5
doxycycline	0.1	1.6
methacycline	0.1	1.6
minocycline	0.1	1.6
erythromycin	0.2	0.8
tylosin	0.2	0.8
carbomycin	0.4	3.1
oleandomycin	3.1	12.5
lincomycin	1.6	12.5
filipin	3.1	12.5
chloramphenicol	12.5	50.0
kanamycin	25.0	50.0
streptomycin	50.0	100.0
BP	50.0	500.0
penicillin	> 500.0	>500.0
sulfanilamide	> 500.0	>500.0
cygon	>1,000.0	>1,000.0
furadan	>1,000.0	>1,000.0
lannate	>1,000.0	>1,000.0
benomyl/water	>1,000.0	>1,000.0
benomyl/5% DMSO[a]	100.0	>1,000.0
thiabendazole/water	100.0	1,000.0
thiabendazole/5% DMSO	10.0	100.0
thiophanate M/water	1,000.0	>1,000.0
thiophanate M/5% DMSO	100.0	1,000.0

[a]DMSO = dimethylsulfoxide.

27). Erythromycin and tylosin have also been found to be highly active against some animal mycoplasmas (13, 20, 27). The greater differences between the MIC and MBC values for the tetracyclines compared with those for erythromycin and tylosin may reflect a static effect of the tetracyclines at the lower concns, as has been found for animal mycoplasmas (16).

The marked effect of inoculum size and period of incubation on MIC and MBC values for chlortetracycline were probably due to a combination of interdependent factors: (i) longer time (i.e., greater than 3 days) required at lower inoculum levels for multiplication of the organism sufficient to effect a color change, as shown by control tubes containing no antibiotic; (ii) mycoplasmastatic action of the antibiotic; (iii) instability of chlortetracycline under prolonged incubation in alkaline media containing animal serum (17, 21). Use of a relatively large inoculum and short incubation period apparently minimized these effects.

The MIC and MBC values obtained in this study should not be considered definitive. Newnham and Chu (20) pointed out that factors such as susceptibility testing technique, medium composition, and use of solid or liquid medium may affect results of antibiotic susceptibility studies. Binding of antibiotics to animal sera in the medium, with consequent higher values for MIC and MBC, is well documented (8, 16). Presumably the results of the present study at least reflect the relative sensitivities of the organism, and thus suggest agents of potential value in chemotherapy of stubborn disease.

On the basis of low MBC values, the most promising antibiotics for this purpose would be erythromycin, tylosin, demethylchlortetracycline, doxycycline, methacycline, minocycline, tetracycline, and carbomycin. Suppression of symptoms in stubborn-diseased sweet orange seedlings (11) by tetracycline is consistent with the results of the present work. It must be recognized that factors such as stability and translocation in plants may greatly affect the performance of antibiotics in vivo compared with their activity in vitro.

The inhibitory concn of filipin is similar to that quoted for *Mycoplasma laidlawii,* which was inhibited only when grown in the presence of cholesterol or serum (28). In view of the evidence that the organism associated with stubborn might not require cholesterol (2), it would be interesting to study the effect of filipin both in the presence and absence of this sterol.

The material known as BP has been stated to be highly effective in the treatment of citrus greening disease in India (M. J. Thirumalachar, *personal communication*). The present results suggest that it is unlikely to be effective against stubborn. This may not be surprising in view of accumulating evidence that the two diseases are caused by different mycoplasmalike organisms (15, 24).

Materials such as systemic fungicides, if active against the stubborn agent, would offer obvious advantages. The results indicate that of the three compounds tested, only thiabendazole dissolved in 5% DMSO had an inhibitory effect comparable with that of certain of the antibiotics, but the MBC value was much higher than that of even the relatively ineffective antibiotics. Nevertheless, it is interesting to note recent reports of the response of sandal spike disease to benomyl treatment (22, 23).

The results contribute to characterization of the mycoplasmalike organism associated with stubborn. We recognize that the organism has not been proven to be the etiological agent of the disease, but all results to date indicate that it is primarily involved (7, 10, 11). We have now obtained primary cultures of the organism in a wholly autoclaved medium, and therefore there is no possibility that it originates from filtered horse serum. Thus, one of the "pitfalls encountered in attempts to isolate MLO", as reiterated recently by Maramorosch (18), has been avoided.

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