In Vitro Susceptibility and Resistance of Two Spiroplasmas to Antibiotics

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ABSTRACT

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More than 20 antibiotics that inhibit protein and nucleic acid synthesis in bacteria were examined for effectiveness against *Spiroplasma citri* and the honeybee spiroplasma. Rifampicin, nalidixic acid, actinomycin D, and streptomycin and three of its derivatives were ineffective at concentrations that ordinarily suppress bacterial growth. Aminoglycoside antibiotics that contain a deoxystreptamine moiety, such as kanamycin, neomycin, and gentamicin were highly effective; while those without a deoxystreptamine moiety, such as kasugamycin, hygromycin, and spectinomycin were not. Spiroplasma strains that permanently resist kanamycin (300 μg/ml), neomycin (300 μg/ml), gentamicin (300 μg/ml), chlortetracycline (300 μg/ml), tetracycline-HCl (20 μg/ml), oxytetracycline (10 μg/ml), and erythromycin (100 μg/ml) were selected at low frequencies (10^{-9}–10^{-10}). Resistance to kanamycin, neomycin, gentamicin, tobramycin, and paromomycin appeared to be linked, but a linked resistance to different forms of tetracycline was not found.

Spiroplasmas have been identified as the disease-causing agents of corn stunt (3,17), citrus stubborn (9), suckling mouse cataract (16), and an as yet unnamed disease of honeybee (4). Other yellows diseases of plants in a group of more than 100 also are suspected of being caused by mycoplasmas. In the past few years, antibiotic sensitivity of spiroplasmas has been examined (1,2,7) and antibiotics are presently used in the field for the control of these wall-less pathogens. Remission of symptoms following tetracycline treatments has been reported for citrus stubborn (6), peach X disease (12), pear decline (11), coconut lethal yellowing (10), and other yellows diseases (14). Recently, results of field observations indicate that tetracyclines gradually lose effectiveness after a long-term application to yellows-diseased plants (Y. P. Tsai and H. J. Su, *unpublished*). It is suspected that antibiotic-resistant strains of spiroplasmas or other mycoplasmas might emerge in diseased plants repeatedly treated with tetracycline. However, information as to whether spiroplasmas or other yellows disease agents mutate and become resistant to antibiotics in vivo or in vitro is as yet lacking.

The present investigation was undertaken to study the susceptibility of spiroplasmas to antibiotics that inhibit protein, DNA, or RNA synthesis in bacteria. Particular emphasis was placed on the resistance of spiroplasmas to various forms of tetracycline and to aminoglycoside antibiotics, catergories that comprise about 60% of the total antibiotics now identified.

MATERIALS AND METHODS

Organism and culture medium. *Spiroplasma citri* and the honeybee spiroplasma (HBS) were used for the experiment. The R8A2 strain (ATCC 27556) of *S. citri* (13) and the AS 576 strain (ATCC 29416) of HBS (5) were kindly provided by R. E. Davis (Plant Virology Laboratory, USDA, Beltsville, MD 20705) and were serologically characterized before use. Unless otherwise specified, both strains were grown at 30 C in a modified C-3G medium (8) containing PPLO broth 1.5%, agamma horse serum 15%, sucrose 10%, and phenol red (20 μg/ml). This modified formulation was designated as R-2 medium.

Antibiotics. Hygromycin, tobramycin, and derivatives of

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streptomycin (*N*-methyl dihydrostreptomycin, *N*-dimethyl dihydrostreptomycin, and mannsidostreptomycin) were generously furnished by M. Lechevalier (Waksman Institute of Microbiology, Rutgers University, New Brunswick, NJ 08903). The gentamicin and spectinomycin were, respectively, gifts of the Schering Co. (Bloomfield, NJ 07003) and J. E. Grandy of Upjohn Co. (Kalamazoo, MI 49001). All other antibiotics used were purchased from Sigma Chemical Co. (St. Louis, MO 63178).

Susceptibility of spiroplasmas to antibiotics. Approximately 10^6 helical cells per milliliter of late log-phase cultures of *S. citri* and HBS were used as inocula. Each antibiotic was serially diluted in twofold steps in broth medium to various concentrations ranging from 0.01 to 200 μg/ml. Spiroplasma growth in broth medium (pH 7.5) was indicated by the color change of the medium from red to yellow. The minimal inhibitory concentration (MIC) of each antibiotic (Table 1) was measured (1) as the lowest concentration of antibiotic that prevented color change of a broth culture after 4 days of incubation.

Selection of spiroplasma strains that resist aminoglycoside antibiotics. Medium R-2 amended with 1.7% agar (BBL, Division of Becton, Dickinson & Co., Cockeysville, MD 21030) was used. Kanamycin sulfate, neomycin sulfate, or gentamicin were added separately when the agar medium was relatively cool and about to solidify. Final concentrations of each antibiotic were 50, 100, and 200 μg/ml. Log-phase cultures of *S. citri* or HBS were centrifuged (16,000 *g*, 10 min) and the cell pellets were resuspended in 1/10 of the original volume of fresh medium. The 0.1-ml aliquots of this concentrated cell suspension were then spread on agar medium. Seeded plates were incubated at 30 C for 10–14 days. Colonies that became visible after incubation were isolated and triply cloned as previously described (15). The mutation frequency was calculated by dividing the number of colonies of apparently resistant organisms by the total cell number seeded onto the plate (normally 3×10^9 cfu [colony-forming units]).

Selection of erythromycin-resistant strains. Aliquots (0.1 ml) of concentrated cell suspensions (3×10^{10} cfu/ml) were seeded on agar plates that contained, respectively, 20, 50, and 100 μg of erythromycin per milliliter. Colonies that appeared after 2 wk of incubation (30 C) were isolated. To increase the level of resistance, resistant colonies that were initially isolated from medium containing 20 μg of erythromycin per milliliter were continuously subcultured in increasing concentrations of antibiotics (50, then 100 μg/ml). Single colonies were isolated and triply cloned in agar medium containing 100 μg/ml of erythromycin.

Selection of tetracycline-resistant strains. Three tetracycline antibiotics—tetracycline-HCl (achromycin), chlortetracycline (aureomycin), and oxytetracycline (terramycin)—were used. The final concentrations of each antibiotic in agar medium were, respectively, 2.5, 5.0, 10, and 20 μg/ml. The concentrated cell suspension was seeded as described above. Colonies observed after 3–4 wk of incubation were very small and isolation of single colonies was difficult. Usually, a piece of agar medium containing resistant colonies was removed and placed in broth medium that contained the same concentration of antibiotic. Strains that resisted high levels of aureomycin (200 μg/ml) and achromycin (20 μg/ml) were obtained by serial subculturing in broth medium with increasing concentrations of antibiotic.

RESULTS

Susceptibility of *S. citri* and HBS to various antibiotics. *S. citri* and HBS did not appear to differ in susceptibility to antibiotics that inhibit protein and nucleic acid synthesis in bacteria (Table 1). Both organisms were extremely sensitive to erythromycin, chloramphenicol, and tetracycline antibiotics. The MIC of these compounds was determined to be 0.1–15 μg/ml. Rifampicin, nalidixic acid, actinomycin D, and streptomycin sulfate were ineffective at concentrations far greater than those needed to kill bacteria. Kanamycin, neomycin, gentamicin, tobramycin, and paromomycin, antibiotics that shared a common deoxystreptamine moiety, were effective at concentrations lower than 20 μg/ml. Other aminoglycoside antibiotics, such as kasugamycin, spectinomycin, and hygromycin, had little antispiroplasmal activity even at concentrations higher than 200 μg/ml.

Resistance of *S. citri* and HBS to aminoglycoside antibiotics containing deoxystreptamine. Mutants of *S. citri* and HBS that resisted kanamycin, neomycin, gentamicin, and tobramycin were isolated separately. Rates of mutation of the spiroplasmas (Table 2) detected in agar plates containing different concentrations (50, 100, and 200 μg/ml) of antibiotics were estimated. Mutation rates inferred from plates containing 200 μg/ml of aminoglycoside antibiotics were the same as those from plates containing 50 μg/ml. Resistance to these antibiotics appears to be a one-step mutation. Resistant mutants isolated from plates that contained kanamycin (200 μg/ml) also were resistant to high concentrations of neomycin, gentamicin, and tobramycin. Similarly, resistant mutants isolated from plates that contained neomycin (200 μg/ml) were simultaneously resistant to other aminoglycosides that contained the deoxystreptamine moiety. Thus, resistance to kanamycin, neomycin, gentamicin, or tobramycin in spiroplasmas appears to be linked. Resistance patterns of mutants to different aminoglycoside antibiotics are summarized in Table 3.

Resistance of *S. citri* and HBS to erythromycin. The colonies of resistant organisms that appeared at the rate of $1.3–2.1 \times 10^{-10}$ were isolated from plates containing 20 μg of erythromycin per milliliter. No resistant colony was observed in plates supplemented with antibiotics at concentrations of 50 or 100 μg/ml. By subsequent subculturing in broth medium with increasing concentrations of erythromycin, strains that initially resisted 20 μg of erythromycin per milliliter gradually became resistant to 100 μg/ml. No loss of resistance was observed after 43 passages of *S. citri* and HBS in antibiotic-free erythromycin in R-2 medium. Colony and cell morphology of erythromycin-resistant mutants was similar to that of parent strains.

TABLE 1. Susceptibility of *Spiroplasma citri* and the honeybee spiroplasma (HBS) to antibiotics

Antibiotics	MIC[a] (μg/ml)		Antibiotics	MIC[a] (μg/ml)	
	S. citri	HBS		*S. citri*	HBS
Tetracycline-HCl	0.1	0.2	Spectinomycin	>200.0	>200.0
Chlortetracycline	10.0	15.0	Paromomycin	1.0	0.5
Oxytetracycline	0.1	0.1	Streptomycin	50.0	75.0
Erythromycin	0.1	0.1	*N*-methyldihydro streptomycin	75.0	75.0
Chloramphenicol	3.0	5.0			
Kanamycin	5.0	10.0	*N*-dimethyldihydro streptomycin	75.0	75.0
Neomycin	10.0	10.0	Rifampicin	100.0	100.0
Tobramycin	1.0	0.5	Nalidixic acid	25.0	50.0
Kasugamycin	>200.0	>200.0	Actinomycin-D	100.0	150.0
Hygromycin	>200.0	>200.0	Cycloheximide	>300.0	>300.0
Novobricin	40.0	80.0			

[a] MIC, minimal inhibitory concentration, measured as the lowest concentration of antibiotic which prevented color change of broth medium after 4 days of incubation at 30 C. The cell concentration of 10^6 helices per milliliter was used.

Resistance of *S. citri* and HBS to tetracyclines. Strains that resisted chlortetracycline, tetracycline-HCl, and oxytetracycline at 200, 20, and 10 μg/ml, respectively, were isolated (Table 4). Each strain maintained the same level of resistance after 35 passages in antibiotic-free medium. Resistant strains grew relatively slowly in the presence of antibiotics. Cells usually were shorter and an irregular head (or bleb) was often attached to the helical filament. Cells grew normally and exhibited the same morphology of parental strains shortly after the antibiotic was removed. Thus, the morphological change in the presence of antibiotic was temporary. Colonies of resistant strains in antibiotic-agar medium were smaller than those of parent strains in antibiotic-free medium. Linked resistance to different tetracyclines was not observed. In other words, chlortetracycline-resistant strains were not necessarily resistant to tetracycline-HCl and oxytetracycline or vice versa. Resistance to each form of tetracycline was probably derived from an independent mechanism (or mutation), although the chemical structures of the three tetracyclines are similar.

Resistance of *S. citri* and HBS to streptomycin, nalidixic acid, and rifampicin. The MICs of these three antibiotics were determined to be 50, 35, and 100 μg/ml. Strains that resisted higher levels (150–200 μg/ml) of each compound were isolated by serially subculturing the organisms in broth medium with increasing concentrations of antibiotic. The cell and colony morphologies of resistant strains were not affected by the presence of antibiotics.

DISCUSSION

To test the effectiveness of a large number of antibiotics on yellows diseases in the field is impractical. Study of in vitro susceptibility of cultivable spiroplasmas to various antibiotics as demonstrated here and reported previously (1,2,7) is useful for the selection of potent antimicrobial compounds before testing in vivo. Of more than 20 antibiotics examined in this study, nine inhibited spiroplasma growth at relatively low concentrations (25 μg/ml or less). These antibiotics may be useful for plant disease chemotherapy in the future. More surprisingly, we found that both *S. citri* and HBS resist rifampicin, nalidixic acid, actinomycin D, novobiocin, streptomycin, and their derivatives at concentrations far beyond those required to kill bacteria. It appears that spiroplasmas have a great tendency to adapt (or mutate) to and to grow in the presence of a wide spectrum of antibiotics.

Aminoglycoside antibiotics that contain the deoxystreptamine moiety, (eg, kanamycin, neomycin, gentamicin, tobramycin, and paromomycin) are highly effective against spiroplasmas. Those aminoglycosides without the deoxystreptamine moiety, which include hygromycin, kasugamycin, and spectinomycin, have little, if any, antispiroplasma activity. It seems that antispiroplasmal activity of aminoglycosides is directly or indirectly related to the presence of a deoxystreptamine moiety.

Mutants that permanently resist antibiotics have been isolated repeatedly from spiroplasma cultures, although at low frequencies. Our results further strengthen the speculation that tetracycline-resistant strains of yellows disease agents would emerge from diseased plants that have been repeatedly treated with these antibiotics. And it may in turn explain why tetracyclines gradually lose effectiveness after a long-term application (Y. P. Tsai and H. J. Su, *unpublished*). It may be proven more effective to treat diseased plants with two forms of tetracycline or a mixture of tetracycline and other antibiotics in the future.

TABLE 2. Frequencies of aminoglycoside antibiotic-resistant mutations in *Spiroplasma citri* and the honeybee spiroplasma (HBS)

	In vitro concentrations of antibiotics (μg/ml)					
	50		100		200	
Antibiotics	*S. citri*	HBS	*S. citri*	HBS	*S. citri*	HBS
Kanamycin	3.1×10^{-9}	1.6×10^{-9}	2.8×10^{-9}	1.5×10^{-9}	3.3×10^{-9}	1.9×10^{-9}
Neomycin	2.9×10^{-9}	1.1×10^{-9}	3.1×10^{-9}	2.5×10^{-9}	3.0×10^{-9}	1.5×10^{-9}
Gentamicin	8.3×10^{-9}	7.6×10^{-9}	10.1×10^{-9}	7.1×10^{-9}	6.9×10^{-9}	9.3×10^{-9}
Tobramycin	6.5×10^{-9}	3.2×10^{-9}	5.8×10^{-9}	3.1×10^{-9}	7.1×10^{-9}	2.7×10^{-9}

[a]Frequencies were estimated by dividing the number of resistant colonies by the total cell number seeded onto the plates (3×10^{9} colony-forming units per plate).

TABLE 3. Cross-resistance of *Spiroplasma citri* and the honeybee spiroplasma (HBS) to various aminoglycoside antibiotics

	In vitro resistance to various aminoglycosides at concentrations of (μg/ml)				
Resistant mutants[a]	Kanamycin	Neomycin	Gentamicin	Tobramycin	Paromomycin
S. citri (Km^r)	400	500	300	400	300
S. citri (Nm^r)	400	400	400	300	400
S. citri (Gm^r)	300	400	400	300	500
HBS (Km^r)	500	500	400	500	300
HBS (Nm^r)	500	500	300	400	300
HBS (Gm^r)	400	400	500	300	400
HBS (Tm^r)	300	300	400	400	300

[a]Km^r, Nm^r, Gm^r, Tm^r represent the strains originally selected from plates that contained 100 μg/ml of kanamycin, neomycin, gentamicin, and tobramycin, respectively.

TABLE 4. In vitro resistance of *Spiroplasma citri* and the honeybee spiroplasma (HBS) to different forms of tetracycline and erythromycin

	Concentrations of antibiotics that resistant mutants tolerated			
Resistant mutants[a]	Chlortetracycline	Tetracycline-HCl	Oxytetracycline	Erythromycin
S. citri (Ct^r)	200.0	0.1	0.1	0.1
S. citri (Am^r)	20.0	20.0	0.2	0.1
S. citri (Ot^r)	10.0	0.2	5.0	0.2
S. citri (Em^r)	20.0	0.1	0.1	100.0
HBS (Ct^r)	300.0	0.2	0.1	0.2
HBS (Am^r)	25.0	30.0	0.1	0.1
HBS (Otr^r)	20.0	0.1	10.0	0.2
HBS (Em^r)	10.0	0.2	0.1	150.0

[a]Ct^r, Am^r, Ot^r, Em^r represent the strains originally selected from plates that contained chlortetracycline, tetracycline-HCl, oxytetracycline, and erythromycin, respectively.

Few attempts have been made to elaborate how spiroplasmas acquire resistance to various antibiotics. Our findings that linked resistance exists to kanamycin, neomycin, gentamicin, tobramycin, and paromomycin are interesting and certainly warrant further investigations. Furthermore, stable antibiotic-resistant strains are now available. Such markers may be useful in genetic studies of spiroplasmas.

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