ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1983, p. 22-25
0066-4804/83/010022-04$02.00/0

Vol. 23, No. 1

# In Vitro Susceptibility of Spiroplasmas to Heavy-Metal Salts†

SUSAN C. WHITMORE,[1] JANE F. RISSLER,[1]* AND ROBERT E. DAVIS[2]

*Department of Botany, University of Maryland, College Park, Maryland 20742,[1] and Plant Virology Laboratory, Plant Protection Institute, Science and Education Administration-U.S. Department of Agriculture, Beltsville, Maryland 20705[2]*

Received 21 July 1982/Accepted 15 October 1982

The susceptibility of six spiroplasma strains to heavy-metal salt was characterized in terms of minimal inhibitory concentrations and minimal biocidal concentrations in broth tube dilution tests. The strains were most susceptible to mercuric chloride and silver nitrate; less susceptible to copper sulfate, cobalt chloride, lead nitrate, and cadmium sulfate; and least susceptible to nickel chloride and zinc sulfate. *Spiroplasma citri* strains Maroc R8A2 and C189 were the most susceptible to five of eight heavy-metal salts, and honeybee spiroplasma strain AS576 and *Spiroplasma floricola* strain 23-6 were generally the least susceptible. The difference between the minimal biocidal concentrations and the minimal inhibitory concentrations was greater for certain heavy-metal salts than for others.

Spiroplasmas are motile, helical, cell wall-free procaryotes which have been classified as Mollicutes (14). Subsequent to their discovery in 1972 in association with stunted corn plants (12), they have been shown to induce some diseases of plants and insects, to induce disease experimentally in suckling rodents, and to be associated with apparently healthy plants, insects, and ticks (23, 27, 29). The ability to cultivate these organisms in vitro permitted a dramatic increase in research on taxonomy, habitats and distribution, growth conditions, vector relations, antibiotic sensitivities, and morphological, biochemical, and physiological properties (26-28). Taxonomic studies have shown that spiroplasma strains can be separated into distinct groups and subgroups (6, 10, 16, 17), and it has been suggested (9) that major groups as well as some distinct subgroups could be designated as separate species of the genus *Spiroplasma*. Three species, *Spiroplasma citri*, *Spiroplasma floricola*, and *Spiroplasma mirum*, of this genus have been described thus far (11, 22, 25).

Nothing is known about the nature of inheritance in spiroplasmas, and little is known about the genome except for guanosine + cytosine contents (3, 5, 16, 17, 22), molecular weight (3, 17, 22), and the presence of plasmids in some strains (1, 21). Basic knowledge of spiroplasma genetics necessitates the development of a genetic system, which in turn requires a stock of mutant strains that provide genomic markers to follow and quantify movement and recombination of the genome.

† Scientific Article A-3246, Contribution 6317 of the Maryland Agricultural Experiment Station.

At the time this study was begun, no defined medium was available for the growth of spiroplasmas and for modification for selection of auxotrophic mutants. It was therefore desirable to obtain other genetic markers in addition to antibiotic resistance markers already obtained by ourselves (unpublished data) and others (18; S. A. Field, 68th Annu. Rep. John Innes Inst. 1977, p. 106). In the present study, we determined the susceptibilities of spiroplasmas to heavy-metal salts (HMSs) as a prelude to attempts to select heavy-metal-resistant spiroplasma mutants in six spiroplasma strains representing four major serogroups. Because the concentration of HMS to be used for the selection of resistant mutants is in the range between the lethal and the inhibitory concentrations of the HMS, we determined the minimal biocidal concentrations (MBCs) and minimal inhibitory concentrations (MICs) of the HMSs for these six spiroplasmas.

## MATERIALS AND METHODS

**Microorganisms.** Information on the strains used in this investigation is given in Table 1. Strains were triply cloned (7) before these experiments and were stored in medium at −80°C.

**Medium.** A modified DSM4 medium (pH 7.2) (11) containing 1.5% (wt/vol) PPLO broth (Difco Laboratories, Detroit, Mich.), 6.5% (wt/vol) sucrose, 1.5% (wt/vol) HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Calbiochem-Behring Corp., La Jolla, Calif.), and 10% (vol/vol) horse serum (GIBCO Laboratories, Grand Island, N.Y.) was used. The agar medium contained 1.5% (wt/vol) PPLO broth, 8.0% (wt/vol) sucrose, 0.5% (vol/vol) phenol red solution (GIBCO), 10% (vol/vol) horse serum, and 1.0% (wt/vol) agar.

TABLE 1. Spiroplasma strains

| Strain | Serogroup[a] | Source of original isolation | ATCC no.[b] | Reference |
|---|---|---|---|---|
| Maroc R8A2 (*S. citri*) | I (A) | Diseased citrus | 27556 | 22 |
| C189 (*S. citri*) | I (A) | Diseased citrus | 27665 | 22 |
| AS576[c] | I (B) | Diseased honey bee | 29416 | |
| 23-6 (*S. floricola*) | II | Tulip tree flowers | 29989 | 11 |
| PPS1 | III | Powder puff flowers | 33450 | 19, 20 |
| brevi[d] | V | Tulip tree flowers | 33474 | |

[a] According to Davis and Lee (9). Serological subgroups are in parentheses.
[b] ATCC, American Type Culture Collection, Rockville, Md.
[c] R. E. Davis, J. F. Worley, T. B. Clark, and M. Moseley, Abstr. Proc. Am. Phytopathol. Soc. 1976, p. 304.
[d] Strain brevi was isolated from the surface of a tulip tree flower during work reported previously (7, 8) and represents a distinct major serogroup of flower-inhabiting spiroplasmas (R. E. Davis and I.-M. Lee, unpublished data).

**Susceptibility tests.** The HMSs employed were mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), copper sulfate ($CuSO_4$), lead nitrate [$Pb(NO_3)_2$], nickel chloride ($NiCl_2 \cdot 6H_2O$), cobalt chloride ($CoCl_2 \cdot 6H_2O$), cadmium sulfate ($3CdSO_4 \cdot 8H_2O$), and zinc sulfate ($ZnSO_4 \cdot 7H_2O$). Susceptibility was determined by broth tube dilution tests. Reaction tubes contained 1.8 ml of liquid medium to which 0.1 ml of the appropriate HMS dilution and 0.1 ml of diluted spiroplasma cultures were added. HMSs were weighed, dissolved in sterile distilled water, and diluted in a twofold series. The highest concentration of HMS tested was determined from preliminary experiments on strain susceptibility to each HMS or by the limits of solubility of the HMS. All stock HMS solutions were prepared on the day of use. Spiroplasmas from 1- to 2-day-old late-log-phase cultures were diluted in 10 mM sodium phosphate buffer (pH 7.5) containing 10% (wt/vol) sucrose and were added to the reaction tube solution to provide a final concentration in the reaction tubes of approximately $10^7$ colony-forming units per ml. The generation time of the slowest-growing spiroplasma strain used (Maroc R8A2) necessitated the relatively high inoculum concentration to give a susceptibility test that could be completed within 3 days. In each experiment, the inoculum concentration was estimated by counting colonies growing from 0.1-ml samples of serial 100-fold dilutions of the inoculum placed on agar plates. The plates were incubated at 31°C for 7 days, and the developing colonies were stained with Dienes' stain (13) before counting. HMS-free cultures of the strains and non-inoculated tubes containing HMS served as controls. Each strain was incubated at 31°C for a period of time equal to that required for the particular strain to reach late log phase in the HMS-free control tube. At the end of the incubation period, the titers of the spiroplasmas in the reaction tubes were determined (as described above), and the pH of each culture was determined. The MBCs and MICs of HMSs were determined from a comparison of the final titer in the reaction tubes with the initial titer. The MBC was defined as the lowest concentration of HMS which killed 99.9% of the spiroplasma population. The MIC was defined as the lowest concentration of HMS in which viable (i.e., able to produce colonies on agar medium) but inhibited (i.e., no increase in number over the initial inoculum level) spiroplasmas were present after incubation in the presence of HMS. These definitions are based on those used in antimicrobial susceptibility testing (2). All tests were conducted in duplicate and repeated at least twice.

## RESULTS AND DISCUSSION

The broth dilution test method was used because determination of the MBC required that, after incubation, the spiroplasma suspension in the reaction tube be diluted sufficiently to achieve a HMS concentration below the inhibitory level. This dilution was necessary to estimate titers of surviving spiroplasmas.

The results are presented as ranges which encompass those values obtained in two to three repetitions of the experiments (Table 2). The MBC and MIC for each HMS varied somewhat from experiment to experiment. Of the MICs or MIC ranges reported for the 48 strain-HMS combinations, 90% differed from experiment to experiment by three steps in the twofold dilution series, 8% differed by four dilution steps, and 2% differed by six steps. All of the 48 MBCs or MBC ranges differed from experiment to experiment by three dilution steps. Addition of certain concentrations of acidic HMS lowered the pH of the culture medium. For lead nitrate and copper sulfate only, addition of a high concentration ([Pb] = $80 \times 10^{-4}$ M; [Cu] = $64 \times 10^{-4}$ M) of HMS lowered the pH to 6.5 to 7.0 such that part, but certainly not all, of the inhibition in five MICs could possibly be attributed to this nonoptimal growth condition. The problem of heavy-metal precipitation at certain pH's (15) precluded in some cases adjustment of the pH of the medium to 7.2.

Generally, the spiroplasma strains were most susceptible to mercuric chloride, silver nitrate, and cadmium sulfate and were least susceptible to nickel chloride and zinc sulfate. Although no single spiroplasma strain was found to be the most or least susceptible to all eight HMSs, a least- or most-susceptible strain was determined for most of the HMSs. For five HMSs, strains

TABLE 2. In vitro susceptibilities of six spiroplasma strains representing five distinct serological groupings to eight HMSs

| HMS | Spiroplasma strain (serological grouping)[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Maroc (IA) | | C189 (IA) | | AS576 (IB) | | 23-6 (IIA) | | PPS1 (III) | | brevi (V) | |
| | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC |
| Mercuric chloride | 0.1–0.2 | 0.025 | 0.2–0.4 | 0.006–0.025 | 0.4 | 0.1 | 0.8–3.2 | 0.8 | 0.8 | 0.2 | 0.8–1.6 | 0.4–0.8 |
| Silver nitrate | 0.5–1.0 | 0.25–0.5 | 1.0 | 0.125–0.25 | 0.5–1.0 | 0.25 | 1.0 | 0.5 | 0.5–2.0 | 0.25 | 0.5–2.0 | 0.125 |
| Cadmium sulfate | 12.8–51.2 | 0.4–3.2 | 25.6–51.2 | 0.4–3.2 | ≥102.4 | 0.4 | 51.2–102.4 | 0.8–6.4 | 6.4–25.6 | 0.4–0.8 | 25.6–51.2 | 0.2–0.8 |
| Copper sulfate | 4–16 | 4–8 | 4–8 | 4–8 | 16–64 | 8–16 | 64 | 64 | 8–16 | 8 | 64–256 | 64 |
| Cobalt chloride | 10–40 | 2.5 | 10–40 | 2.5–5.0 | 160–320 | 20 | 160–320 | 10–40 | 80–320 | 10 | 80–160 | 10 |
| Lead nitrate | 80–160 | 40–80 | 80 | 40–80 | 80–160 | 40 | 80 | 40–80 | 80 | 40 | 80 | 40 |
| Nickel chloride | 187.6 | 5.86 | 187.6–375 | 5.86–187.6 | >1,500 | 11.7–23.4 | 375 | 93.8 | 375 | 46.9–93.8 | 375 | 23.4–46.9 |
| Zinc sulfate | 32–64 | 2–16 | 32–128 | 2 | 128–512 | 4 | 256–512 | 64–128 | 64–256 | 16–32 | 256–512 | 16–64 |

[a] MBCs and MICs are expressed as $\times 10^{-4}$ M.

Maroc and C189, members of the same serogroup and subgroup, were the most susceptible. Strains AS576 and 23-6 were the least susceptible, even though strain AS576 is in the same serogroup as strains C189 and Maroc. All strains were nearly equally susceptible to silver nitrate and lead nitrate. A greater difference between the MBCs and MICs was found for certain HMSs than for others independent of the spiroplasma strain tested. For example, for cadmium sulfate, the MBCs were on the average 35 times as great as the MICs, whereas for copper sulfate, the MBCs were on the average 1.9 times as great as the MICs.

For some HMS-spiroplasma strain combinations, the MIC equaled the MBC (e.g., for $CuSO_4$ and *S. floricola* 23-6, the MBC and MIC equaled $64 \times 10^{-4}$ M). Strains for which this phenomenon is observed could be tested at HMS concentrations differing by smaller increments than twofold dilution steps; this experiment might reveal that the MIC is actually lower and not equal to the MBC. On the other hand, the similarities between some MICs and MBCs observed here may reflect the nature of the HMS inhibitory and biocidal effects. The concentration of HMS necessary to inhibit the spiroplasmas of a particular strain may also kill a majority of the organisms.

To provide a perspective for considering the MIC of certain HMSs for spiroplasmas (Table 2) with those for other procaryotes, we note that the reported MIC of mercuric chloride for a strain of *Escherichia coli* is $0.1 \times 10^{-4}$ M (24), compared with $0.0006 \times 10^{-4}$ to $0.8 \times 10^{-4}$ M for spiroplasmas in our work, and that the reported MIC of cadmium chloride for a strain of *Staphylococcus aureus* is $0.1 \times 10^{-4}$ M (4), compared with the MIC of cadmium sulfate of $0.2 \times 10^{-4}$ to $6.4 \times 10^{-4}$ M for spiroplasmas. Because the MICs for these different organisms were obtained under different test conditions, direct comparisons of the data may not be appropriate, but it is interesting that the MICs for spiroplasmas were in the range of the MIC observed previously for some bacteria.

Susceptibilities are reported for the salts of heavy metals rather than for the heavy-metal cation alone because the anions may have an inhibitory effect (unpublished data). For example, we found that the salts caused varying degrees of inhibition of spiroplasma growth depending on the anion and on the strain of the spiroplasma tested. However, it is apparent that differences in activity observed among HMSs with the same anions in equimolar concentrations (see Table 2) can be due to the heavy-metal cations and not anions. For example, since the anion was the same in mercuric chloride, cobalt chloride, and nickel chloride and was used in

equimolar concentrations in tests with these salts, the differences in effect on spiroplasmas among these salts are presumed to be due to the heavy-metal cations themselves.

The present work is the first study of the susceptibility of spiroplasmas to HMS. The results contribute to the basic knowledge of spiroplasmas and provide MBCs and MICs that can be used as guides in future attempts to select heavy-metal-resistant spiroplasma mutants.

## ACKNOWLEDGMENTS

We gratefully acknowledge J. Zangara for technical assistance.

This research was supported in part by U.S. Department of Agriculture Competitive Research grant 59-2241-1-1-466-1 and by Biomedical Research Supports grant RR-07042 to the University of Maryland from the Division of Research Resources, National Institutes of Health.

## LITERATURE CITED

1. **Archer, D. B., J. Best, and C. Barber.** 1981. Isolation and restriction mapping of a spiroplasma plasmid. J. Gen. Microbiol. **126**:511–514.
2. **Barry, A. L.** 1976. The antimicrobic susceptibility test: principles and practices. Lea & Febiger, Philadelphia, Pa.
3. **Bové, J. M., P. Saglio, J. G. Tully, A. C. Freundt, Z. Lund, J. Pillet, and D. Taylor-Robinson.** 1973. Characterization of the mycoplasma-like organisms associated with 'stubborn' disease of citrus. Ann. N.Y. Acad. Sci. **225**:462–470.
4. **Chopra, I.** 1975. Mechanisms of plasmid-mediated resistance to cadmium in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **7**:8–14.
5. **Christiansen, C., G. Askaa, E. A. Freundt, and R. F. Whitcomb.** 1979. Nucleic acid hybridization experiments with *Spiroplasma citri* and the corn stunt and suckling mouse cataract spiroplasmas. Curr. Microbiol. **2**:323–326.
6. **Daniels, M. J., D. B. Archer, M. A. Stephens, R. Townsend, J. M. Longland, and J. Best.** 1980. Comparison of spiroplasmas by polyacrylamide gel electrophoresis of cell proteins. Curr. Microbiol. **4**:377–380.
7. **Davis, R. E.** 1978. Spiroplasma associated with flowers of the tulip tree (*Liriodendron tulipifera* L.). Can. J. Microbiol. **24**:949–959.
8. **Davis, R. E.** 1981. The enigma of the flower spiroplasmas, p. 259–279. *In* K. Maramorosch and S. P. Raychauduri (ed.), Mycoplasma diseases of trees and shrubs. Academic Press, Inc., New York.
9. **Davis, R. E., and I.-M. Lee.** 1982. Comparative properties of spiroplasmas and emerging taxonomic concepts: a proposal. Rev. Infect. Dis. **4**(Suppl.):S122–S128.
10. **Davis, R. E., I.-M. Lee, and L. K. Basciano.** 1979. Spiroplasmas: serological grouping of strains associated with plants and insects. Can. J. Microbiol. **25**:861–866.
11. **Davis, R. E., I.-M. Lee, and J. F. Worley.** 1981. *Spiroplasma floricola*, a new species isolated from surfaces of flowers of the tulip tree, *Liriodendron tulipifera* L. Int. J. Syst. Bacteriol. **31**:456–464.
12. **Davis, R. E., J. F. Worley, R. F. Whitcomb, T. Ishijima, and R. Steere.** 1972. Helical filaments produced by a mycoplasmalike organism associated with corn stunt disease. Science **176**:521–523.
13. **Deeley, J., W. A. Stevens, and R. T. V. Fox.** 1979. Use of Dienes' stain to detect plant disease induced by mycoplasmalike organisms. Phytopathology **69**:1169–1171.
14. **Freundt, E. A.** 1974. The mycoplasmas, p. 929–955. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
15. **Gadd, G. M., and A. J. Griffiths.** 1978. Microorganisms and heavy metal toxicity. Microb. Ecol. **4**:303–317.
16. **Junca, P., C. Saillard, J. Tully, O. Garcia-Jurado, J. R. Degorge-Dumas, C. Mouches, J. C. Vignault, R. Vogel, R. McCoy, R. Whitcomb, D. Williamson, J. Latrille, and J. M. Bové.** 1980. Caracterisation de spiroplasmes isolés d'insectes et de fleurs de France continentale, de Corse et du Maroc. Proposition pour une classification des spiroplasmes. C. R. Acad. Sci. D. **290**:1209–1212.
17. **Lee, I.-M., and R. E. Davis.** 1980. DNA homology among diverse spiroplasma strains representing several serological groups. Can. J. Microbiol. **26**:1356–1363.
18. **Liao, C. H., and T. A. Chen.** 1981. In vitro susceptibility and resistance of two spiroplasmas to antibiotics. Phytopathology **71**:442–445.
19. **McCoy, R. E., M. J. Davis, and R. V. Powell.** 1981. In vivo cultivation of spiroplasmas in larvae of the greater wax moth. Phytopathology **71**:408–411.
20. **McCoy, R. E., D. S. Williams, and D. L. Thomas.** 1979. Isolation of mycoplasmas from flowers, p. 75–81. *In* H. J. Su and R. E. McCoy (ed.), Proceedings of the Republic of China-United States Cooperative Science Seminar on Mycoplasma Diseases of Plants. National Science Council Symposium series no. 1. National Science Council, Taipei, Taiwan.
21. **Ranhand, R. M., W. O. Mitchell, T. J. Popkin, and R. M. Cole.** 1980. Covalently closed circular deoxyribonucleic acids in spiroplasmas. J. Bacteriol. **143**:1194–1199.
22. **Saglio, P., M. Lhospital, D. Laflèche, G. Dupont, J. M. Bové, J. G. Tully, and E. A. Freundt.** 1973. *Spiroplasma citri* gen. and sp. n.: a mycoplasma-like organism associated with "stubborn" disease of citrus. Int. J. Syst. Bacteriol. **23**:191–204.
23. **Saglio, P. H. M., and R. F. Whitcomb.** 1979. Diversity of wall-less prokaryotes in plant vascular tissue, fungi, and invertebrate animals, p. 1–36. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. 3. Plant and insect mycoplasmas. Academic Press, Inc., New York.
24. **Smith, D. H.** 1967. R factors mediate resistance to mercury, nickel, and cobalt. Science **156**:1114–1115.
25. **Tully, J. G., R. F. Whitcomb, D. L. Rose, and J. M. Bové.** 1982. *Spiroplasma mirum*, a new species from the rabbit tick (*Haemaphysalis leporispalustris*). Int. J. Syst. Bacteriol. **32**:92–100.
26. **Whitcomb, R. F.** 1980. The genus *Spiroplasma*. Annu. Rev. Microbiol. **34**:677–709.
27. **Whitcomb, R. F.** 1981. The biology of spiroplasmas. Annu. Rev. Entomol. **26**:397–425.
28. **Whitcomb, R. F., and J. G. Tully (ed.).** 1979. The mycoplasmas, vol. 3. Plant and insect mycoplasmas. Academic Press, Inc., New York.
29. **Whitcomb, R. F., and D. L. Williamson.** 1975. Helical wall-free prokaryotes in insects: multiplication and pathogenicity. Ann. N.Y. Acad. Sci. **266**:260–275.