

NOTES

In Vitro Susceptibility of Human and Environmental Isolates of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* to Heavy-Metal Salts and Oxyanions

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Because of the widespread distribution of *Mycobacterium intracellulare* and *M. scrofulaceum* in southeastern U.S. waters, the susceptibility of members of these species to heavy-metal salts and oxyanions was investigated. Isolates with abnormally high tolerance to mercuric chloride or cadmium chloride were identified.

The *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum* group includes pathogens whose source for human infection appears to be environmental (18). In fact, the great abundance of these mycobacteria in water (5, 6) correlates with the high proportion of residents in counties of the southeastern states who show evidence of prior infection (3, 4).

Because of the widespread distribution of *M. intracellulare* and *M. scrofulaceum* (*M. avium* is rarely found) in southeastern U.S. waters (5, 6), including the heavy-metal-polluted Chesapeake and Delaware Bays (8, 15), we investigated the susceptibility of members of these species to heavy-metal salts and oxyanions, to identify highly resistant strains that will serve as subjects for studies of the genetic and physiological basis for resistance. The rationale of the work was based on the observation of Nelson and Colwell (10) who inferred a "possible causal relationship between ambient mercury levels and numbers of mercury-resistant bacteria" in the Chesapeake Bay. Further, resistance in a variety of bacteria to high concentrations of mercury salts (11, 12, 14), as well as to other heavy metals such as cadmium (16), is due to plasmid-encoded gene functions. Though plasmid DNA in *M. avium* and *M. intracellulare* has been shown to be involved in restriction and modification (2) and in colonial variation (9), none of these characteristics are useful genetic markers, unlike metal resistance. Previous studies of the influence of heavy-metal salts on mycobacteria have used only very high concentrations of salts, and these studies have either focused on the effect of these concentrations on colonial characteristics such as pigmentation (13) or have been limited to a few isolates of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* (1). That work demonstrated the relative susceptibility of *M. tuberculosis* strains to metals at concentrations that are tolerated by atypical mycobacteria, though such data were not useful for the identification of mycobacterial isolates (1, 13).

Eighty-eight *M. avium*, *M. intracellulare*, and *M. scrofulaceum* isolates were used in this study, 21 from cases of human infection and 67 from fresh, brackish, or saline

waters of the eastern United States (5). The basal medium was Middlebrook and Cohn 7H10 agar medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.5% (vol/vol) glycerol (M7H10), to which was added HgCl₂, NiSO₄ · 6H₂O, Co(NO₃)₂ · 6H₂O, CdCl₂ · 2½H₂O, AgNO₃, CuSO₄ · 5H₂O, K₂CrO₄, Na₂SeO₃, Na₂SeO₄, K₂TeO₃, or K₂TeO₄ to achieve final metal salt concentrations of 10⁻⁶ to 10⁻³ M. Basal medium was prepared and sterilized by autoclaving, and appropriate amounts of filter-sterilized stock solutions of the metal salts were added before pouring plates, with the pH adjusted to that of M7H10 (pH 6.6) if necessary. For susceptibility tests, the isolates were grown to the mid-log phase (ca. 2 × 10⁸ cells per ml) in Middlebrook and Cohn liquid medium 7H9 (BBL) containing 0.5% (vol/vol) glycerol and 10% (vol/vol) OADC enrichment (BBL) in screw-capped tubes. A loop containing 0.01 ml of each culture was streaked on the surface of the metal-containing and metal-free (control) 7H10 agar medium. The plates were incubated at 30°C in candle jars for 5 weeks in darkness. Heavy-metal susceptibility was determined by comparing isolate growth on heavy-metal-containing medium with growth on metal-free medium. Minimal growth only at the point of inoculum with no isolated single colonies was interpreted as no growth. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of heavy-metal salt used which inhibited visible growth.

The results are presented as the number and percentage of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* isolates with a particular MIC for each of the heavy-metal salts tested. In three repetitions of the experiment, none of the isolates showed any variation, which is similar to the value for differences in a comparable study of spiroplasmas (17). This low variation is most likely due to the 10-fold differences in concentrations tested (17), chosen to simplify the identification of highly resistant strains. The failure of a particular isolate to grow was not due to changes in pH after the addition of metal salts, because the pH of the medium was adjusted after salts were added. Possibly, the long period of incubation could result in a loss of heavy metals from the agar medium and hence in the growth of an isolate. This did not appear to explain the appearance of mercury-

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TABLE 1. In vitro susceptibilities of 88 human and environmental *M. avium*, *M. intracellulare*, and *M. scrofulaceum* isolates to heavy-metal salts and oxyanions^a

Heavy-metal salt or oxyanion	No. (%) of isolates with MIC of:			
	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M
CuSO ₄ · 5H ₂ O	0 (0)	0 (0)	5 (6)	6 (7)
NiSO ₄ · 6H ₂ O	0 (0)	2 (2)	8 (9)	14 (16)
Co(NO ₃) ₂ · 6H ₂ O	0 (0)	4 (5)	1 (1)	27 (31)
AgNO ₃	0 (0)	15 (17)	17 (19)	38 (43)
CdCl ₂ · 2½H ₂ O	26 (30)	34 (39)	4 (5)	12 (14)
HgCl ₂	43 (49)	32 (36)	1 (1)	12 (14)
K ₂ CrO ₄	0 (0)	0 (0)	0 (0)	88 (100)
Na ₂ SeO ₃	2 (2)	8 (9)	78 (89)	0 (0)
Na ₂ SeO ₄	0 (0)	0 (0)	2 (2)	2 (2)
K ₂ TeO ₃	0 (0)	1 (1)	35 (40)	52 (59)
K ₂ TeO ₄	0 (0)	0 (0)	0 (0)	4 (5)

^a The sum of percentages of isolates inhibited by a salt is less than 100 because some grew at 10⁻³ M.

resistant colonies, because an *Escherichia coli* K-12 strain carrying plasmid R100-1 had the same MIC toward HgCl₂ when grown either on plates that were incubated for 30 days at 30°C before inoculation or on plates that were freshly prepared. Incidentally, the MIC of that strain and the MIC of its R100-1-free derivative were the same on the medium employed here as on the tryptone broth agar medium used by others (14, 16). Concentrations higher than 10⁻³ M were not tested because of the appearance of precipitates and because the occurrence of such concentrations in nature is uncommon. In some cases mycobacterial growth appeared to be stimulated by low concentrations of certain heavy-metal salts [e.g., 10⁻⁵ M Co(NO₃)₂ · 6H₂O]. Though stimulation of growth was not noted in other studies of the influence of metal salts on nontuberculous mycobacteria, Jaquess et al. (7) have demonstrated that sodium selenate stimulates growth of *M. tuberculosis* at a concentration of 2.5 × 10⁻⁵ M.

In general, the isolates were less susceptible to sodium selenate, potassium tellurate, copper sulfate, nickel sulfate, and cobalt nitrate than to sodium selenite, potassium tellurite, cadmium chloride, and mercuric chloride. Susceptibility to silver nitrate was intermediate (Table 1). The percentage of isolates susceptible to these heavy-metal salts changed gradually with the 10-fold increments in concentration. Susceptibility to potassium chromate was unique in that all strains failed to grow at 10⁻³ M, yet they did grow at 10⁻⁴ M. Statistical (chi-square) analysis indicated that, although the interspecies differences in mercuric chloride and cadmium chloride susceptibilities were not significant, *M. avium* and *M. intracellulare* isolates were significantly more tolerant to 10⁻³ M cobalt nitrate or silver nitrate than were *M. scrofulaceum* isolates at the *P* = 0.05 level. Differences in heavy-metal susceptibilities between isolates of human and of environmental origin were small and not statistically significant at the *P* = 0.05 level.

Because of the apparently bimodal distribution of isolates susceptible to mercuric chloride and cadmium chloride (Table 1), those isolates tolerant to 10⁻⁴ M mercuric chloride or to 10⁻⁴ and 10⁻³ M cadmium chloride or to all of these concentrations may carry unique genetic determinants encoding for heavy-metal resistance. Of the seven environmental isolates which were able to grow on medium containing 10⁻⁴ M mercuric chloride, five were isolated from water samples collected in either the Chesapeake or the Delaware

Bay (5, 6) where others have found a variety of bacteria harboring plasmids encoding for mercury resistance (11). These isolates will serve as subjects for studies of the genetic and physiological basis for heavy-metal tolerance of mycobacteria.

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