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Effects of Metals on a Range of *Streptomyces* Species

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A plate diffusion method was used to assess the tolerance of 28 mesophilic and 4 thermophilic species of streptomycetes to eight metals. This method allowed a qualitative screen of the effects of the metals on growth, on sporulation, and in some cases, on secondary metabolite production. Tolerant and sensitive species were identified, some of which exhibited the same response (i.e., tolerance or sensitivity) to a number of metals. Experiments in liquid culture were used to quantify the precise concentration ranges of the metals at which the most tolerant or sensitive species could grow. Distribution profiles of the sensitivities of all species examined toward different metals revealed that the order of toxicity was Hg > Cd > Co > Zn > Ni > Cu > Cr > Mn.

Heavy metals are generally toxic to microorganisms (9), especially if they exist at high concentrations (7, 8, 22). A few, such as mercury and cadmium, are considered to be highly toxic (4). Environmental pollution (particularly in soil) with heavy metals can stem from industrial activities or sewage discharges (1, 2, 5, 7, 18, 22). Their toxicity can be affected by abiotic factors such as pH, E_h, temperature, organic materials, or clay minerals, factors which also influence their speciation and bioavailability (3, 8, 15, 16). The tolerance of soil bacteria to heavy metals has been proposed as an indicator of potential toxicity to other forms of life (13), and, generally, gram-negative species appear to be more tolerant than gram-positive ones in soils that contain comparatively low levels of metal pollution (6).

The genus *Streptomyces* comprises a large group of soil bacteria that are important in the recycling of carbon in polymeric macromolecules. They may be exposed to heavy metals in a variety of ways, especially when agricultural soils are subjected to treatment with sewage sludges (5). The ways in which they interact with heavy metals are unknown. It has been reported, however, that actinomycetes as a group are more tolerant to cadmium than other species are and that streptomycete species that are able to detoxify Hg^{2+} to volatile Hg^0 by means of a mercuric reductase enzyme have been isolated (14, 17).

This study reports a rapid method for assessing the effects of heavy metals on a number of streptomycete species and discusses the range of tolerances found to the toxic metals tested in representative type species of a range of taxonomic cluster groups according to the scheme of Williams et al. (21).

MATERIALS AND METHODS

Bacterial strains and maintenance. The *Streptomyces* species used in this work are listed in Table 1. Streptomycetes were maintained on solid medium (L agar) that contained (g $liter^{-1}$) tryptone (Difco Laboratories), 5; yeast extract, 5; NaCl, 5; glucose, 1; and agar, 10 (pH 7.2). When spores had developed, they were suspended in 15% (vol/vol) glycerol by gentle aspiration from the agar surface and were then stored at −20°C until required.

Plate diffusion method. Qualitative assessments of the effects of heavy metals on growth, sporulation, and any pigment production were determined as follows. By using a hot wire, a trough, 0.2 by 90 mm, was cut into agar contained in a square dish which measured 10 by 10 cm so that it ran parallel to one side approximately 1 cm from the wall of the plate. Into the trough was added 0.2 ml of stock solution of the appropriate metal chloride with stock solutions of 0.25 M Hg^{2+}, 0.89 M Cd^{2+}, 1.7 M Co^{2+}, 1.53 M Zn^{2+}, 1.7 M Ni^{2+}, 1.6 M Cu^{2+}, 1.92 M Cr^{2+}, and 1.82 M Mn^{2+}. Metal-amended plates were then incubated at 30°C for 24 h to allow diffusion of the metal into the agar, by which time a concentration gradient of the metal had been formed. Spore suspensions of the test streptomycete species were streaked heavily in a line at right angles to the trough and right up to its edge. Plates were then incubated at either 30°C (mesophilic species) or 50°C (thermophilic species) for up to 7 days. After incubation, the distance of growth inhibition (in mm) was taken as that from the edge of the trough to the leading edge of the growing mycelium. This was used to determine the metal tolerance of each strain and was expressed as a percentage of the total measured distance on the agar available for growth (90 mm). Therefore, the greater the distance of the colony from the trough edge, the greater the inhibition exerted by the metal.

Assessment of metal toxicity in liquid media. In order to assess quantitatively the effects of heavy metals, streptomycete species were grown in a liquid salts medium that contained the following (g $liter^{-1}$): K_2HPO_4, 6; KH_2PO_4, 2; $(NH_4)_2SO_4$, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4$, 0.01; and glucose, 10, and that was amended with 0.1% yeast extract, after which the pH was adjusted to 7.4. The appropriate metal, as a sterilized stock solution of the chloride salt, was added to the desired concentration after autoclaving. Liquid media were inoculated with 0.1 ml of spore suspension and were incubated at 30°C (50°C for thermophilic species) with shaking at 200 rpm.

RESULTS

Qualitative analysis of metal tolerance. The plate diffusion method described in Materials and Methods was used to give a rapid but qualitative estimation of the tolerance of the streptomycetes listed in Table 1 to a range of heavy metals. The effects of zinc and nickel on selected species are shown in Fig. 1A and B in order to illustrate this method. In addition to enabling a qualitative assessment of metal tolerance, the effect of metals on sporulation and production of colored secondary metabolites either within the mycelium or which had diffused out into the agar could also be determined. For example, higher concentrations of zinc-inhibited

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TABLE 1. *Streptomyces* strains used in the study

Strain	ISP no.[a]	Cluster group[b]
S. coelicolor A3(2)		
S. lividans TK-64		
S. albidoflavus	5445	A-1
S. scabies	5078	A-3
S. atroolivaceus	5137	A-3
S. exfoliatus	5060	A-5
S. californicus	5058	A-9
S. fulvissimus	5593	A-10
S. rochei	5231	A-12
S. cellulosae	5362	A-13
S. chromofuscus	5273	A-15
S. albus	5313	A-16
S. griseoviridis	5229	A-17
S. cyaneus	5108	A-18
S. diastaticus	5496	A-19
S. canarius	5528	A-20
S. ambofaciens	5053	A-23
S. flaveolus	5061	A-24
S. viridochromogenes	5110	A-27
S. lydicus	5461	A-29
S. filipinensis	5112	A-30
S. antibioticus	5234	A-31
S. violaceoniger	5563	A-32
S. chromogenus	5384	A-33
S. nogalater	5546	A-34
S. griseoflavus	5456	A-37
S. griseoluteus	5392	C-43
S. lavendulae	5069	F-61
S. thermoviolaceus[c]		
S. thermonitrificans[c]		
S. thermoflavus[c]		
S. thermovulgaris[c]		

[a] ISP no. is the International *Streptomyces* Project Number of relevant strains.

[b] Cluster group to which each species has been assigned according to the scheme of Williams et al. (21).

[c] Thermotolerant species.

sporulation in *Streptomyces californicus* and *S. griseoviridis* were found (Fig. 1A). Tolerance of 32 species of streptomycetes to eight different metals is presented in Table 2. Mercury was by far the most toxic metal, and 18 out of 32 species tested failed to grow on the diffusion plates. Cadmium, cobalt, zinc, and nickel all yielded a range of sensitivities, but no species was completely inhibited by these metals. Chromium and manganese showed the least growth inhibition, and the range of inhibition for chromium was restricted to between 12 and 26%. Some striking features emerged (Table 2). Some species exhibited sensitivity, and others tolerance, to a number of metals. For example, the species showing tolerance to the most metals (the numbers of metals are given in parentheses) were *S. albidoflavus* (3), *S. lavendulae* (3), *S. californicus* (5), *S. cellulosae* (3), *S. canarius* (6), and *S. thermovulgaris* (3). The species that were the most sensitive to a number of metals (exact number in parentheses) were *S. cyaneus* (3), *S. violaceoniger* (6), and *S. thermonitrificans* (7).

Assessment of the sensitivity of the plate diffusion method. The results described in Fig. 1 and Table 2 yielded qualitative data on metal tolerance with solid medium and, as such, allowed the screening of large numbers of species to give a rapid indication of those species worthy of further investigation. To validate the sensitivity of this procedure, we measured the effects of heavy metals on species representative of the most susceptible or tolerant to a given metal. These experiments involved growing the appropriate species in liquid media that contained a range of metal concentrations, a culture grown in the absence of metal serving as the control. Figure 2A shows the growth of *S. cellulosae* (tolerant) and *S. atroolivaceus* and *S. diastaticus* (sensitive) in the presence of increasing concentrations of mercury. The IC_{50} (concentration of mercury resulting in 50% reduction in biomass) values for each were 3.4, 0.40, and 0.36 $\mu g\ ml^{-1}$, respectively, indicating that the most tolerant species as shown by the results from the plate diffusion experiments (Table 2) was approximately 10 times more resistant to the metal than the most sensitive species. Similarly, for copper the IC_{50} values were 11 $\mu g\ ml^{-1}$ for *S. californicus* (most

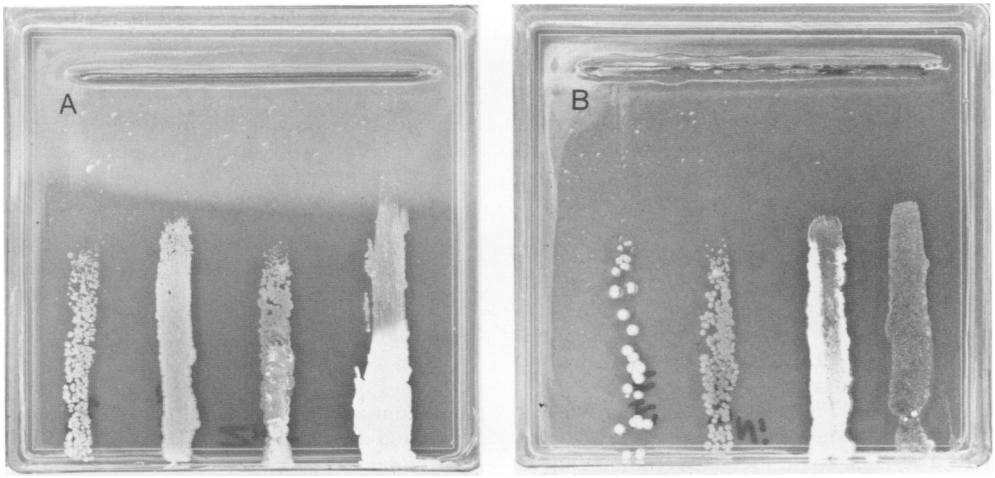


FIG. 1. Growth of streptomycetes on gradients of heavy metals in solid medium. Growth inhibition was taken as the distance (mm) from the leading edge of the colony up to the trough and was expressed as a percentage of the total distance on the agar available for growth (90 mm). (A) Effects of zinc on growth of (from left to right) *S. atroolivaceus*, *S. lydicus*, *S. griseoviridis*, and *S. californicus*. (B) Effects of nickel on growth of (from left to right) *S. lydicus*, *S. flaveolus*, *S. californicus*, and *S. albidoflavus*.

TABLE 2. Metal tolerance of selected *Streptomyces* species assessed by the plate diffusion method[a]

Streptomyces sp.	Distance of growth inhibition (%)[b]							
	Hg (38–100)	Cd (39–64)	Co (30–61)	Zn (27–66)	Ni (29–42)	Cu (29–71)	Cr (12–27)	Mn (0–39)
S. coelicolor	85	44	41	40	34	40	23	13
S. lividans	66	**40**	36	35	32	31	21	**0**
S. albidoflavus	100	48	42	32	**30**	**28**	21	**0**
S. scabies	100	44	38	**28**	34	**28**	23	**0**
S. atroolivaceus	100	52	40	43	34	40	24	24
S. exfoliatus	100	52	37	36	32	34	24	20
S. californicus	**50**	45	40	**26**	**30**	**28**	20	**4**
S. fulvissimus	100	44	43	36	32	**30**	20	11
S. rochei	100	44	36	35	33	**30**	21	21
S. cellulosae	**37**	**38**	43	36	38	**28**	22	26
S. chromofuscus	100	52	44	37	35	37	23	25
S. albus	88	55	42	40	36	37	25	21
S. griseoviridis	100	44	37	53	33	36	21	20
S. cyaneus	58	52	51	47	36	40	26	27
S. diastaticus	100	52	45	36	34	31	23	22
S. canarius	100	43	**30**	**27**	**30**	**30**	**13**	**3**
S. ambofaciens	73	41	**31**	32	31	31	18	8
S. flaveolus	100	43	35	38	31	33	20	26
S. viridochromogenes	88	46	45	33	33	33	21	22
S. lydicus	100	45	42	34	38	32	22	16
S. filipinensis	100	47	46	37	34	33	24	10
S. antibioticus	**46**	42	42	**27**	33	31	23	20
S. violaceoniger	100	61	45	43	41	41	24	30
S. chromogenus	100	46	43	38	36	32	17	16
S. nogalater	77	43	33	35	32	32	22	18
S. griseoflavus	100	47	40	32	33	34	20	23
S. griseoluteus	**50**	46	38	33	32	33	23	8
S. lavendulae	**50**	54	48	**28**	36	32	22	**0**
S. thermoviolaceus	100	47	48	44	31	40	14	30
S. thermonitrificans	100	64	61	55	42	71	16	38
S. thermoflavus	70	53	40	42	33	37	**12**	34
S. thermovulgaris	**53**	47	36	34	**28**	32	**13**	23

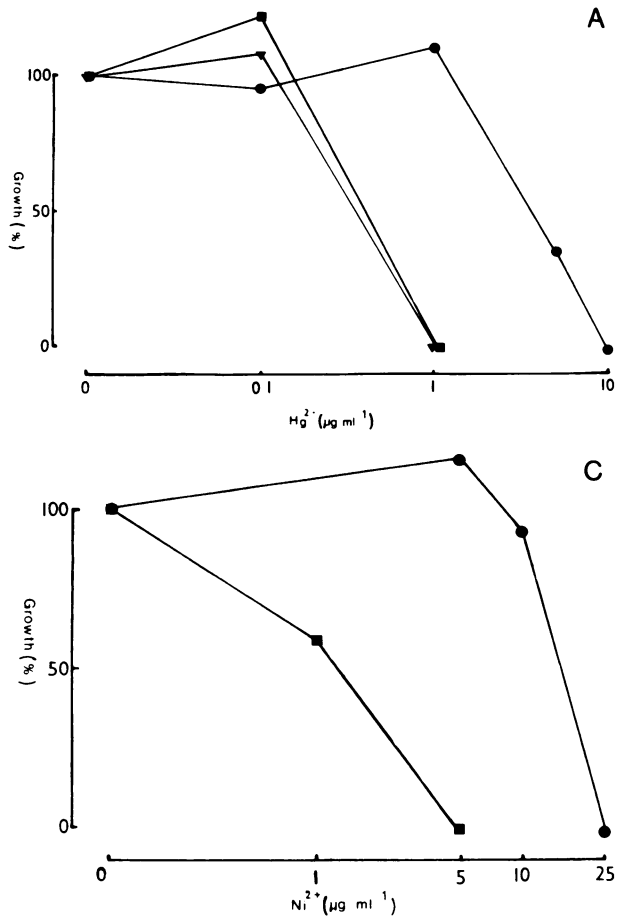
[a] All figures represent the mean of two separate experiments; the difference between each was never more than 1%. Numbers in boldface indicate strains regarded as the most tolerant for a given metal; underscored numbers indicate the strains most sensitive for a given metal.
[b] Figures below metal symbols represent the ranges of growth inhibition (%).

tolerant) and 2 and 0.45 μg ml^{-1} for *S. atroolivaceus* and *S. violaceoniger* (most sensitive), respectively (Fig. 2B). Finally, for nickel the IC_{50} values for *S. canarius* (tolerant) and *S. violaceoniger* (nontolerant) were 15 and 4 μg ml^{-1}, respectively (Fig. 2C). The sequence of metal toxicity for the streptomycete species listed in Table 2 was as follows: mercury > cadmium > cobalt > zinc > nickel > copper > chromium > manganese. Accordingly, two species that showed tolerance to most metals and two that showed the least tolerance (Table 2) were examined for their ability to grow in the presence of increasing concentrations of the four most toxic metals (Hg^{2+}, Cd^{2+}, Co^{2+}, and Zn^{2+}) and the results are shown in Table 3. These show (i) decreasing toxicity of the metals in the range mercury to zinc; i.e., greater amounts of zinc were required to inhibit the growth of both tolerant and sensitive species compared with mercury, and (ii) the tolerant species, as identified by the plate diffusion method, generally grew at much higher metal concentrations than the more sensitive ones did.

Profiles of metal resistance. Figure 3 shows the profiles of growth inhibition for the 32 species of streptomycetes and eight metals tested. The least toxic metals were manganese (0 to 39%) and chromium (12 to 27%). Nickel and copper gave similar inhibition profiles, with the majority of species exhibiting growth inhibition in the range 30 to 40%; for nickel, 29 out of the 32 species tested fell into this range. Zinc gave inhibition ranging from 26 to 56%, with most species falling into the 30 to 40% range. Cobalt and cadmium appeared to give similar inhibition profiles and, apart from mercury, were generally the most toxic metals. Mercury was the only metal capable of total inhibition of growth, and this occurred for 18 of the species tested. Some species grew to some extent on mercury-containing plates, and *S. cellulosae* (38% inhibition) and *S. californicus* (50%), in particular, appeared to show some tolerance to this metal. In general, the inhibition profiles for each metal were narrow (except for mercury), indicating, at least for the species tested here, that there were few highly tolerant or highly sensitive species.

DISCUSSION

The toxic effects of heavy metals in soil environments are a matter of increasing concern. Within soil are numerous species that are important in a number of biogeochemical cycles. For example, a study on Chesapeake Bay water and sediment samples revealed that nitrification could be totally inhibited by all metals tested (11). Many soil species, in particular the actinomycetes, are responsible for the recycling of carbon locked in polymeric materials such as plant matter, and their activity is crucial to soil fertility. Therefore, an understanding of the response of these bacteria to heavy metals is timely. An additional reason for the work is



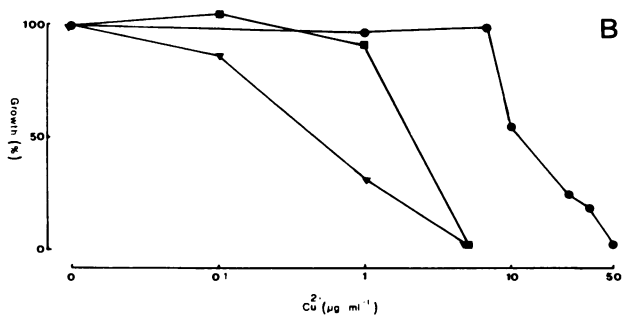


FIG. 2. Growth in liquid medium of streptomycetes that in Table 2 are indicated as being tolerant or sensitive to different metals. In all cases biomass was measured after 5 days of growth in liquid medium that contained different metal concentrations and was expressed as a percentage of the growth yield in unamended medium. (A) Effects of different concentrations of mercury on growth of tolerant *S. cellulosae* (●) and sensitive *S. atroolivaceus* (■) and *S. diastaticus* (▼). (B) Effects of copper on growth of tolerant *S. californicus* (●) and sensitive *S. atroolivaceus* (■) or *S. violaceoniger* (▼). (C) Effects of nickel on growth of tolerant *S. canarius* (●) or sensitive *S. violaceoniger* (■).

the emerging evidence that metal resistance and antibiotic resistance are often found together in many clinical isolates. Since it is thought that in many cases antibiotic resistance genes originate from the producing organisms, of which the streptomycetes are predominant, it should be of interest to monitor their tolerance to heavy metals (10, 12).

By using a simple plate diffusion assay system, we have been able to identify the effects of heavy metals on a number of streptomycete species representative of different taxonomic cluster groups (see reference 21). Other potential applications of this method include a rapid visual indication of the effects of different metal concentrations on sporulation and on any pigments produced as secondary metabolites. It should also be possible to adapt the method to screen the effects of metals on antibiotic production by treating the fully grown metal-inhibited mycelium with an agar overlay that contains a susceptible test organism. It has been known for some time that the concentration of some metals can grossly affect secondary metabolism, particularly in actinomycetes (20). The method described here should allow the rapid screening of the effects of metals on production of secondary metabolites by commercially important species.

An interesting finding was that in general species that appeared to be the most tolerant or sensitive to one metal also exhibited a similar response to other metals. Therefore, in Table 2 a number of species that are either tolerant or sensitive to three or more different metals can be recognized. For example, *S. canarius* was one of the most tolerant species for six of the eight metals tested. At the other end of the scale, the thermophile *S. thermonitrificans* was one of

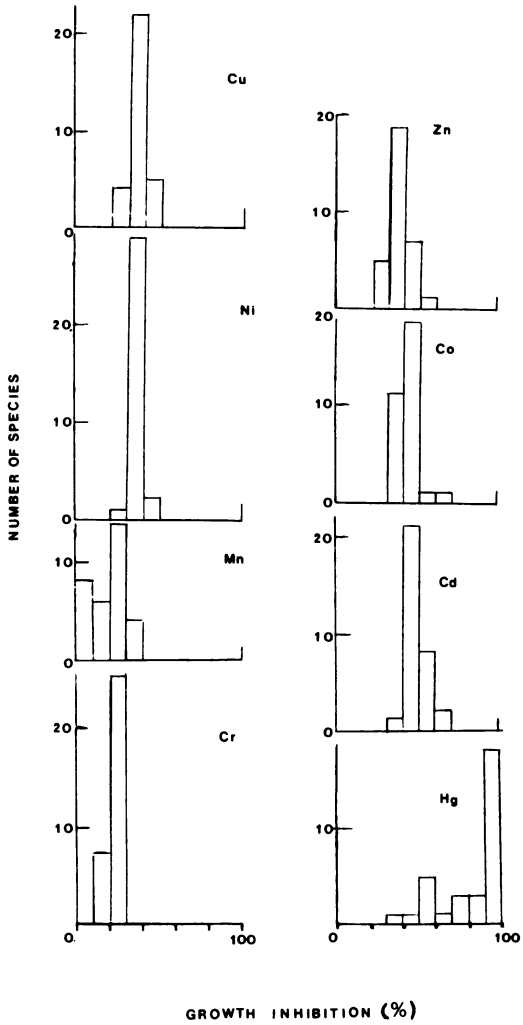


FIG. 3. Distribution of tolerance to different heavy metals of the 32 species of streptomycetes listed in Tables 1 and 2.

TABLE 3. The metal concentrations at which selected tolerant and nontolerant strains were able to grow[a]

Strain	% Growth[a] with the following metal (μg/ml^{-1}):																			
	Hg^{2+}			Cd^{2+}				Co^{2+}					Zn^{2+}							
	0.1	1	5	0.1	1	5	10	10	25	50	100	10	25	50	100	250	500	1,000	2,500	5,000
Tolerant																				
S. californicus	67 ± 7	23 ± 4.2	0	119.3 ± 4.7	94.4 ± 9	0	0	86.6 ± 10.2	82.4 ± 1.8	0	0	113 ± 1.3	101.5 ± 0.6	83.2 ± 1.3	57.8 ± 9.3	0	0	0	0	0
S. canarius	93.3 ± 2.4	0	0	96.8 ± 3	101.8 ± 1.4	62.4 ± 8.1	0	101.7 ± 4.7	72.1 ± 9.2	55.3 ± 10	0	95 ± 2.5	89.4 ± 0.8	78 ± 9.6	51.2 ± 5.1	0	0	0	0	0
Nontolerant																				
S. violaceo-niger	56 ± 6.6	0	0	54.3 ± 10.2	0	0	0	51.1 ± 6.5	37 ± 5	0	0	104 ± 10.2	56.8 ± 7.2	5.5 ± 3.1	0	0	0	0	0	0
S. thermoni-trificans	81.9 ± 5.6	0	0	38.3 ± 3.3	0	0	0	49.7 ± 2.8	5.5 ± 4	0	0	60 ± 7.3	0	0	0	0	0	0	0	0

[a] All figures represent the percentage of mycelial growth in the presence of different metal concentrations, compared with that measured in the absence of metal (calculated as 100%). Mean ± standard error for three determinations.

the most sensitive species for seven different metals. As pointed out by Trevors et al. (19), there is a problem in defining exactly what is meant by resistance to heavy metals. We have adopted the term tolerance throughout this work, whereby tolerance is a relative term within the 32 species examined. Duxbury (6) proposed an equation whereby resistance could be defined more precisely; for example, concentrations of mercury, cadmium, and zinc at which resistance was defined were 4 to 10, 30, and 110 μg per ml, respectively. For copper, nickel, and cobalt, work cited by Trevors et al. (19) quoted concentrations of 30 to 60, 60, and 50 to 100 μg per ml, respectively, as being the concentrations at which resistance could be defined. From these considerations, it would appear from this work that few of the streptomycete species examined exhibited any true resistance to heavy metals. *S. cellulosae* could be said to show a degree of resistance to mercury (IC_{50} for growth at 3.4 μg ml^{-1}). However, it is interesting to note that the descending order for heavy metal toxicity in streptomycetes identified in this work as Hg, Cd, Co, Zn, Ni, Cu, Cr, and Mn contrasts with that reported by Duxbury (6) for soil bacteria, which is ordered as Hg > Cd > Cu > Ni ≥ Zn. The major difference would appear to be the relatively low position of copper as a toxic metal for streptomycetes and the correspondingly higher toxicity of cobalt.

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