DEPARTMENT OF THE INTERIOR

UNITED STATES GEOLOGICAL SURVEY

REACTIONS OF 12 STRAINS OF SOIL BACTERIA TO 15 METALS

By

John R. Watterson

Open-File Report 81-1082

1981

CONTENTS

ILLUSTRATIONS

TABLES

ABSTRACT

Reactions of 12 strains of common soil bacteria to 15 metals were tested in a laboratory experiment. Paper discs treated with metal ions were dried and placed on nutrient agar freshly inoculated with different varieties of bacteria. Growth patterns were observed at the end of 24 hours and at the end of 6 days. After 6 days, the overall toxicity of the metals to the bacteria followed the order: Hg>Ag>Cd>V>Co>Sb>Bi. Only one or two strains of bacteria were sensitive to Al, Se, Te, and Zn, and none were sensitive to As, Cu, Ni, or Sn at the test concentration. Each species of bacteria exhibited a unique resistance pattern, indicating that bacterial reaction to a suite of heavy metals is complex and strain-specific. Such strain-specific heavy-metal resistances may provide a taxonomic basis for the study of three-dimensional metal distribution patterns in nature.

INTRODUCTION

A number of recent reports, some of which are concerned with environmental pollution, have established the feasibility of extending mineral-exploration geobotany to include surveys of metal adaptation in soil and sediment microflora (Marques et al., 1979; Timoney et al., 1978; Austin et al., 1977; Nelson and Colwell, 1975; Koval'skii et al., 1968; Kendrick, 1962 Tuovinen et al., 1971). For a recent review of the phenomena of microbial heavy-metal resistance, see Summers and Silver (1978). The present study was undertaken as part of an investigation of procedures applicable to the detection of microbial resistance to one or more of the metallic elements commonly associated with economic mineral deposits.

A paper disc method (Bauer et al., 1966) is now commonly used to test microbial sensitivity to antibiotics. At least two previous studies report the use of this technique to test bacterial resistances to inorganic ions

(Novick and Roth, 1968; Groves and Young, 1975). The metal-binding capacity of cellulose (Belford et al., 1958; Ogiwara and Kubota, 1969) was considered negligible in these tests at seven micrograms (μg) of metal per disc, but might become a factor in applications of disc-held metals at lower concentrations.

Strains of bacteria tested for their natural resistance to metals in this experiment included Rhodospirillum rubrum, Bacillus megaterium, Sarcina flava, Bacillus subtilis, Bacillus subtilis var. niger, Flavobacterium denitrificans, Serratia marcescens, Serratia marcescens 933 (a colorless variant), Micrococcus coralinus, Sarcina aurantiaca, Micrococcus roseus, and Micrococcus luteus.

Materials and sources are listed in table 1.

PROCEDURE

A loose-leaf binder hole punch was used to punch out filter paper discs about 6 mm in diameter from Whatman 41 filter paper. Two or three dozen discs were placed in each of an appropriate number of screw-cap glass vials and moistened to saturation with one of the metal standard solutions. Three additional vials of discs were saturated with 10 percent solutions of concentrated HCl, HNO_3 and aqua regia (7:3 HCl:HNO_3) as carrier acid "blanks." The moistened discs were dried in the vials, with caps loosened, in a drying oven at approximately 90°C for 3 days prior to use. Discs containing Ag, Al, Sb, Sn, and Zn darkened to some extent during drying. Twenty discs absorbed 0.14 ml of distilled water; each disc therefore held about 7 μl of liquid prior to drying, or 7 μg of metal. All strains were subcultured from supply house cultures by evenly streaking the bacteria over 100 x 15 mm petri plates containing 45 ml of nutrient agar. After a week, one or two additional subcultures of each strain were prepared by repeating the process; ten days

Table 1

Materials and Sources

Bacteria	Carolina Biological Supply Company Burlington, North Carolina
Metals: "Certified Atomic Absorption Standard Reference Solution, 1,000 ppm" of Ag, Al, As, Bi, Cd, Co, Cu, Hg, Ni, Sb, Se, Sn, Zn, and V.	Fisher Scientific Company
Banco 1,000 ppm Te	Anderson Laboratories, Fort Worth Texas
"Lab-Tek" polystyrene petri dishes	Miles Laboratories
BBL nutrient agar	BBL Microbiology Systems Cockeysville, Maryland
Whatman 41 ashless filter paper	Whatman Ltd., England
Acids	Baker, analyzed reagent grade
"Swube" disposable cotton tip applicators	Becton-Dickinson

were allowed for growth. All innoculations were made using "Swube" disposable applicators. Nutrient agar plates poured two days previously were evenly swabbed from subcultures about an hour before applying the paper discs.

Eighteen metal and acid residue-containing paper discs were then arranged with tweezers as evenly as possible on each of the fresh bacterial lawns. All operations with bacteria were carried out through hand holes in a plastic glove box. Petri dish lids were then replaced and the cultures allowed to develop at room temperature, about 22°C.

RESULTS AND DISCUSSION

Results after 6-days' growth are presented in table 2. Two of the more colorful plates are shown in figure 1.

An attempt was made to distinguish between anion and cation effects upon the various microorganisms based on a comparison of growth patterns around discs containing metal and acid residues and discs containing acid residues alone. When acid blanks had a growth-inhibiting effect and various metals in that solvent had both inhibiting and noninhibiting effects, it was assumed that the cation contributed to the toxic effect and that the anion may have contributed to the toxic effect. See table 2. Allowance was made for the higher acid concentration in the blanks than in the standard solutions (10 percent as compared to less than one percent acid).

A table resembling table 2 was filled out at the end of 24-hours' growth. A few discrepancies between the early table and table 2 were later noted. The most striking difference was the response of Bacillus subtilis to the Bi, Cd, Co, and Sb discs. A faint shadow of a large (12-mm net radius) barren zone that was initially more pronounced around the Cd disc was still evident after 6 days, but initial barren zones evident at 24 hours around the Bi, Co, and Sb discs apparently had filled in without a trace by the end of 6-days' growth.

Table 2

Observed reactions of soil bacteria to metal and acid residues after 6-days' growth. ✓, toxic effect ascribable to cation; +, anion or acidity may contribute to toxicity; ?, effect marginal

Solvent and/or metal salt	water; as nitrate	dilute HCl	dilute HNO_3	dilute HNO_3	dilute HNO_3	dilute HNO_3	dilute HNO_3	water; as chloride	dilute HNO_3	dilute HNO_3	water; oxide	dilute HCl	dilute HNO_3	dilute HNO_3	dilute HCl	10% HCl	10% HNO_3	10% 7:3 aqua regia
Metal----------Bacteria	Ag	Al	As	Bi	Cd	Co	Cu	Hg	Ni	Sb	Se	Sn	Te	Zn	V			
Rhodospirillum rubrum	✓	✓+		?	?			✓+						✓	✓	?		+
Bacillus megaterium	✓			✓	✓			✓+			✓					+		+
B. subtilis var. niger	✓+					✓+		✓+							✓+	+	+	+
B. subtilis	✓				✓			✓							✓+	?		
Sarcina flava	✓+				✓+	✓+		✓+		✓+					✓+	+	+	+
S. aurantiaca	✓+			✓+	✓+	+?		✓+					+?		+?	+	+	+
Flavobacterium denitrificans	✓+			?	✓+	+?		✓+		✓+					✓+	+	?	+
Serratia marcescens 933	✓				✓	✓		✓		✓			✓		✓			
S. marcescens	✓				✓			✓		✓			✓	✓	✓			
Micrococcus coralinus	✓				✓	✓		✓										
M. roseus	✓				✓			✓+		✓+	✓				✓+	+		+
M. luteus	✓			✓	✓			✓		✓					✓			+

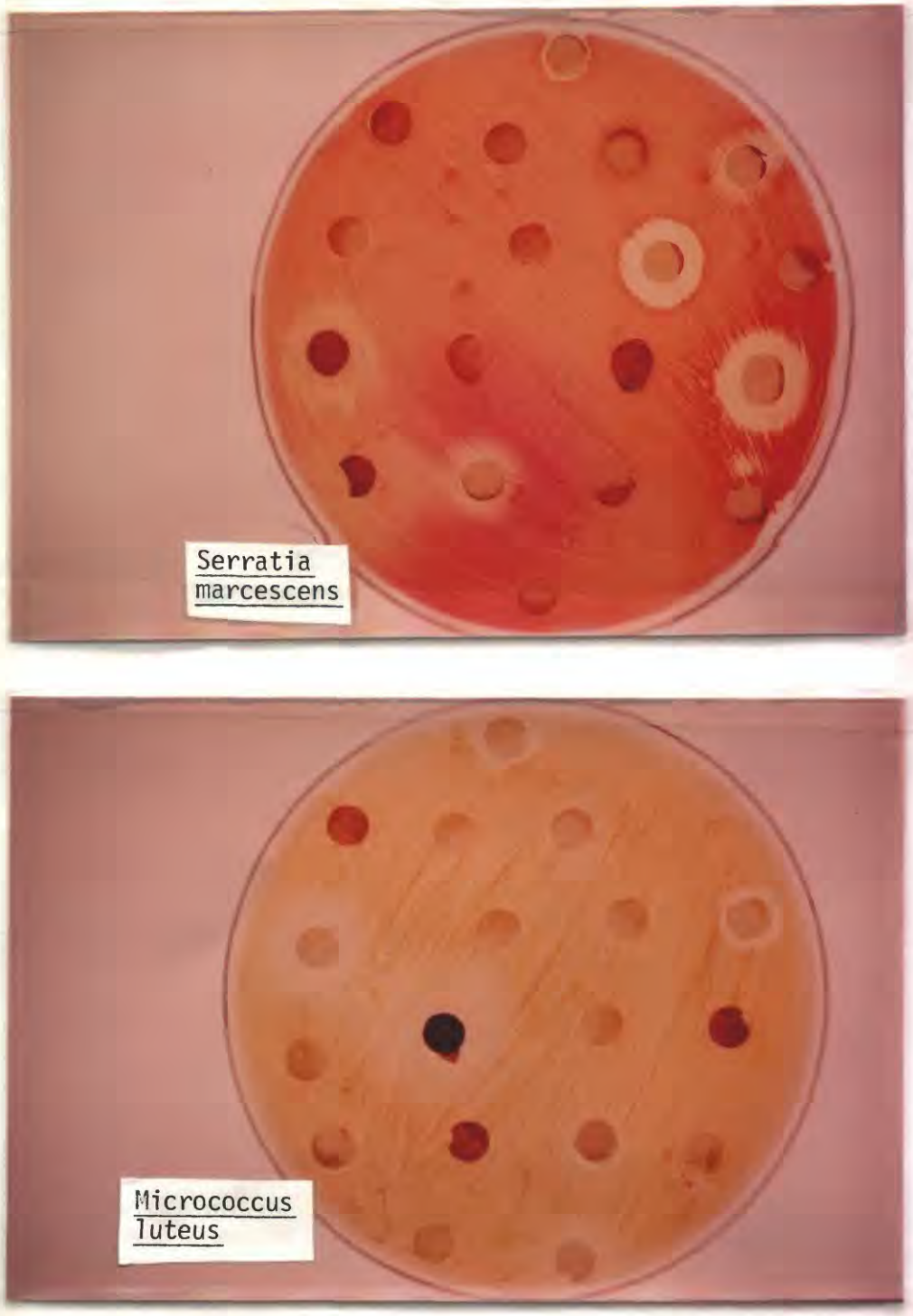

Figure 1. Photographs showing reactions of Serratia marcescens and Micrococcus luteus to test metal and acid residues after 6-days' growth. The order of the discs from top to bottom, left to right is: Ag, Al, As, Bi, Cd, Co, Cu, Hg, Ni, Sb, Se, Sn, Te, Zn V, 10 percent HCl, 10 percent HNO_3, 10 percent 7:3 HCl:HNO_3 aqua regia. Note the changes in the pigmentation of S. marcescens around the Te and V discs. Actual size.

Since the initial sensitivity of Bacillus subtilis to the acid discs also all but disappeared in 6 days, similar but unknown effects may be involved.

Sarcina flava also recovered area in which it was initially inhibited by Bi or perhaps by $Bi(NO_3)_3$. Since sensitivity to the HNO_3 on this lawn also disappeared after a few days, dissipation of the anion may have allowed the recovery of growth around the Bi disc. In other cases, however, growth inhibition around discs containing acid residues persisted without change for several weeks. Cobalt seems to have inhibited Micrococcus coralinus more as time progressed. Where sensitivity decreased, ion diffusion would appear to be the most likely cause although the possibility of resistant mutants cannot be excluded. (See also Den Dooren De Jong, 1971.) It is likely that such a diffusion gradient would exist because of the progressive binding of the metal to proteins and polysaccharides in the nutrient agar. The agar itself consists of anionic macromolecules with a cation-binding capacity. The persistence or increase of sensitivity, however, is not consistent with this interpretation and presents a puzzle.

Some kind of reciprocal relationship is suspected between Cd and Hg toxicity. Sb and Cd were equally as toxic as, if not more toxic than, Hg to Micrococcus luteus. Sarcina flava had the smallest of the Hg kill-rings. It was very sensitive to Cd, and was also sensitive to Co, Sb, and V. These two organisms thus have in common a minimal sensitivity to Hg, a large sensitivity to Cd, and a yellow pigment. Bacillus subtilis and Bacillus subtilis var. niger were, conversely, quite sensitive to Hg, but much less sensitive to Cd and Sb; both showed some sensitivity to V. Flavobacterium denitrificans was sensitive to Ag and very sensitive to Hg, but less so to Cd, Sb, and V.

Rhodospirillum rubrum was the slowest growing strain tested and had one of the blandest pigments. It apparently reduced Ag ions to metallic Ag on the disc-a phenomenon noted by Den Dooren De Jong (1971) and others. R. rubrum was the only strain apparently sensitive to the Al disc. R. rubrum and S. marcescens were the only bacteria exhibiting sensitivity to zinc.

Micrococcus roseus and Bacillus megaterium showed a distinct sensitivity to Se. Both strains of Serratia marcescens, but only these, showed a distinct sensitivity to the Te disc.

Serratia marcescens was perhaps the most interesting microbe tested. Besides registering its distinctive response to the test discs within 24 hours, it exhibited no sensitivity to any of the discs that had been treated with solvent acids either early or late in the test. It exhibited a striking sensitivity to cadmium, antimony, tellurium, zinc, and vanadium. Vanadium caused the rusty-colored pigment to assume a rosy-pink cast for a radius of about 15 mm. Because the kill-ring around the vanadium disc extended only 3 to 4 mm, it may be that vanadium migrates into the colony somewhat further than is indicated by the kill-ring. A change in pigmentation was also noted around the tellurium disc. Strain 933 was slightly more sensitive to cobalt than the pigmented strain.

The correspondence of higher-than-average Hg resistance with lower-than-average Cd and other metal resistance in the two strains of B. subtilis and in F. denitrificans is reminiscent of an increased Hg sensitivity noted by Norris and Kelly (1978) in an Ag-resistant strain of Thiobacillus ferrooxidans. The reduction of Ag uptake by fungal spores in the presence of mercury and copper (Sadler and Trudinger, 1961) would seem to be similar. The saturation of one or a few metal-resistance mechanisms would satisfy such observations.

Inspection of table 2 and a consideration of the sizes of the kill-rings produced indicates that overall toxicity for the bacteria tested follows the order: Hg>Ag>Cd>V>Co>Sb>Bi

Other metals, including Al, Cu, Ni, Sn, and As, are known to be toxic to microorganisms (Mowat, 1976; Malaney et.al., 1959; Den Dooren De Jong, 1977), but in the present qualitative test conditions, were not noticeable.

SUMMARY AND CONCLUSIONS

Twelve pure cultures of soil bacteria were grown in the presence of 15 metals and the inhibition of their growth noted. All of the strains of bacteria were inhibited by some of the metals, i.e. silver and mercury, and most of the bacteria were inhibited by others, i.e. cadmium and vanadium. Some of the bacteria were more sensitive to the generally inhibitory metals than others. Only one or a few of the tested strains were sensitive to some of the metals, i.e., aluminum, bismuth selenium, tellurium, and zinc. None of the strains of bacteria were sensitive to the same set of metals as any other strain, nor were any two strains sensitive in the same degree to the same metals. The conclusion drawn from this study is that inherent differences in sensitivity or resistance to the heavy metals exist in bacteria at the species and variety level.

IMPLICATIONS FOR MINERAL EXPLORATION

Other studies have shown that differences in specific heavy metal resistances are found among individuals of the same species in nature. If, as this study appears to show, dependable variations in heavy metal resistance are peculiar at the species level in soil bacteria, then it would appear likely that the heavy metal content of a given soil would be reflected in the bacterial taxonomy of that soil. A naive example from the present data might

be the following: in a high-antimony soil, one might have more reason to expect Bacillus megaterium than Serratia marcescens or, if bismuth is high, to expect the opposite case, more Serratia marcescens than Bacillus megaterium.

It is true that bacterial taxonomy is presently more laborious and perhaps subject to more variables than the direct analysis of the metal content of soils. It is also true that microorganisms are more difficult to see than the plants studied by geobotanical prospectors. New techniques, however, like immunofluorescence staining and pyrolysis mass spectrometry may now permit studies in bacterial taxonomy to compete, in terms of speed, with trace element analysis. Hence, the study of bacteria in the service of mineral exploration, with the additional insights their distribution may provide, is becoming more and more practical. It should be noted that the distribution of microorganisms is not confined to the two dimensions of the earth's surface, but can be studied in three dimensions coincident with the three dimensions of mineralized formations. Two interesting studies of microbial biocenoses in underground waters associated with mineral deposits have already been carried out by L. E. Kramarenko (1962) and A. K. Lisitsyn and E. C. Kuznetsova (1967); these are pioneering examples of three-dimensional microbial geobotany.

REFERENCES

Austin B., Allen, D.A., Mills, A.L. and Colwell, R.R., 1977. Numerical taxonomy of heavy metal-tolerant bacteria isolated from an estuary. Can. J. Microbiol., 23:1433-1477.

Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M., 1966. Antibiotic susceptibility testing by a standarized single disc method. Am. J. Clin. Path., 45:493-496.

Belford, D.S., Myers, A. and Preston, R.D., 1958. Electron diffraction study of adsorbed metal ions on the surface of cellulose microfibrils. Nature, 181:1516-1518.

Den Dooren De Jong, L.E., 1971. Tolerance of Azotobacter for metallic and nonmetallic ions. Antonie van Leeuwenhoek, 37:119-124.

Groves, D. J., and Young, F. E., 1975, Epidemiology of antibiotic and heavy metal resistance in bacteria: resistance patterns in Staphylococci isolated from populations not known to be exposed to heavy metals, Antimicrob. Agents Chemother., 7:614-621.

Kendrick, W.B., 1962. Soil fungi of a copper swamp. Can. J. Microbiol., 8:639-647.

Koval'skii, V., Ermakov, V., and Letunova, S. V., 1968. Geochemical ecology of microorganisms in soils with different selenium content. Translated from Mikrobiologiya, 37:122-130.

Kramarenko, L.E., 1962. Bacterial biocenoses in underground waters of some mineral fields and their geological importance. Translated from Mikrobiologiya, 31:694-701.

Lisitsyn, A.K. and Kuznetsova, E.C., 1967. Role of microorganisms in development of geochemical reduction barriers where limonitization bedded zones wedge-out. Translated from AN SSSR ser. geol., 1967. 1:31-44; Internat. Geol. Rev., 9:9:1180-1191.

Marques, A.M., Congregado, F. and Simon-Pujol, D.M., 1979, Antibiotic and heavy-metal resistance of Pseudomonas aeruginosa isolated from soils. J. Appl. Bacteriol., 47:347-350.

Melaney, G.W., Sheets, W.D. and Quillin, R., 1959. Toxic effects of metallic ions on sewage microorganisms. J. Water Pollution Control Fed., 31:1309-1315.

Mowat, A., 1976. Measurement of metal toxicity by biochemical oxygen demand. Water Pollution Control Fed. J., 48:853-866.

Nelson, J.D., Jr. and Colwell, R.R., 1975. The ecology of mercury-resistant bacteria in Chesapeake Bay. Microbial Ecol., 1:191-218.

Novick, R. P., and Roth, C., 1968, Plasmid-linked resistance to inorganic salts in Staphlococcus aureus, J.Bacteriol., 95:1335-1342

Norris, P.R. and Kelly, D.P., 1978. Toxic metals in leach systems, in Murr, L.E. et al., (Editors), Metallurgical applications of bacterial leaching and related microbial phenomena. Academic Press, 526 p., material p. 83-102.

Ogiwara, Y. and Kubota, H., 1969. Combination of cellulosic materials and metallic ions. J. Polymer Sci., 7:2087-2095.

Sadler, W.R. and Trudinger, P.A., 1967. The inhibition of microorganisms by heavy metals. Mineralium Deposita, 2:158-168.

Summers, A.O. and Silver, S., 1978. Microbial transformations of metals. Ann. Rev. Microbiol., 32:637-672.

Timoney, J.F., Port, J., Giles, J. and Spanier, J., 1978. Heavy metal and antibiotic resistance in the bacterial flora of sediments of New York bight. Appl. Environ. Microbiol., 36:465-472.

Tuovinen, O.H., Niemela, S.I. and Gyllenberg, H.G., 1971. Tolerance of Thiobacillus ferrooxidans to some metals. Antonie van Leeuwenhoek, 37:489-496.