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Penicillin resistance in soil bacteria is an index of soil metal content near a porphyry copper deposit and near a concealed massive sulfide deposit.

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ABSTRACT

Geochemical and microbiological tests have been conducted on soils collected above the Poorman Creek porphyry copper deposit, Lewis and Clark County, Montana, and above the Keystone massive sulfide deposit, Shasta County, California, to determine the possible influence of natural metal enrichments on the penicillin resistance of a normally penicillin-sensitive group of soil bacteria.

Over both deposits, penicillin resistance in Bacillus spp. correlates significantly with soil metal content. At the Poorman Creek deposit, the metalliferous soils contain increased numbers of one naturally metal- and antibiotic-resistant organism, Bacillus cereus. Statistical analysis reveals a close correlation between percent B. cereus and percent penicillin-resistant Bacillus spp. Whether the metals, or increased amounts of soil penicillin, or both, are selecting for B. cereus (and thus for the observed penicillin resistance of the Bacillus group) we cannot say. These findings, however, supported by recent information on the metal binding capacity of penicillamine, a penicillin hydrolysate, suggest that the penicillin resistance trait itself may confer an ecological advantage on bacteria in metalliferous soils.

Either penicillin resistance in Bacillus spp. or increased numbers of Bacillus cereus in soils or stream sediments may characterize similar ore deposits. One or the other may be an aid in prospecting.

INTRODUCTION

Known mineral deposits studied by the U.S. Geological Survey over the past 25 years have been found to display some surface indication of their presence, commonly the dispersion in water, vegetation, or surface materials of ore-associated "indicator elements." One of the tasks of the U.S. Geological Survey has long been to try new methods of detecting known, but concealed, mineral deposits. The reason for this effort is that new economic mineral deposits are increasingly difficult to locate. The intimate association of soil bacteria with their geochemical environment and their ability to rapidly adapt to one or more of the generally toxic ore indicator elements have led us to begin some investigations of microbial heavy metal and antibiotic resistance as it may relate to ore-associated geochemical dispersion patterns in soil.

Evidence is steadily accumulating that the genetic resistance features of soil microorganisms faithfully reflect the chemical constituents, including the concentrations of heavy metals, in their soil environment. The monograph of Letunova and Koval'sky (1978), summarizing their research since 1954, presented a great deal of evidence that microbial adaptation to natural heavy metal accumulations is a predictable phenomenon. The work of other researchers (Marques and others, 1979; Timoney and others, 1978; Austin and others, 1977; Nelson and Colwell, 1975; Kendrick, 1962; Olson and Thornton, 1981; Troyer and others, 1980; Troyer and others, 1981; Ehrlich, Colwell, and Olson et al., this volume) validates the conclusion that microorganisms reproducing in a metal-enriched environment carry genetic traits suited to that environment. Our investigations of microorganisms associated with naturally metalliferous soils in the vicinity of metal deposits are based on

the apparent trustworthiness of this phenomenon, which we have come to refer to as the predictability of adaptation.

An obvious means of examining the utility of microorganisms in geochemical studies is to determine the response of soil or stream sediment microflora to growth media containing different test concentrations of the various metals of interest. This approach, which is being taken by Olson and her group at the University of California at Irvine, has much to recommend it, especially if a rapid, multi-element metal-resistance test perhaps similar to that of Bauer and others (1966) can be developed. The potential advantage of such a test is that microbial resistance to any toxic metal or other agent can, in theory, be tested with equal facility. In some cases this might circumvent analytical difficulties involved in direct chemical determination.

The other approach to using microorganisms as metal sensors, which we believe may have equal if not more merit in mineral exploration, is that of using a single test to indicate the presence of any of a number of toxic metals. Evidence that such a test is feasible is presented here.

Although numerous studies have noted the coincidence of various antibiotic and heavy metal resistances in bacteria, there has been little interest in discovering whether ecological reasons exist for these dual resistances. Timoney and others (1978), in a pioneering study, were the first to forcefully conclude that heavy metals can result in a selection pressure for antibiotic resistance in bacteria in that system. Unfortunately for our purposes, the conclusion of Timoney and others (1978) was compromised by the unknown origin of the New York bight dumping ground sediments tested in their study. It could be argued that ampicillin or other semi-synthetic penicillins in hospital sewage may have provided a direct selective pressure for ampicillin resistance in the Bacillus spp. examined. We thus became interested in learning if antibiotic resistance in soil bacteria could be found to increase with increasing soil metal content. We therefore began to look for variations in antibiotic resistance in soil bacteria in pristine environments affected only by variations in the soil geochemistry. Some preliminary disk assay tests of Bacillus spp. in heat-treated samples of soil collected adjacent to and over a vein deposit in Montana indicated that large variations in ampicillin resistance in soil bacteria coincided with and were apparently conditioned by the soil geochemistry. Bacteria in soils with high metal content were far more ampicillin resistant than those in control soils.

The genus Bacillus, we should note, is of interest because their durable endospores are present in most soils (Holding and others, 1965; Mishustin and Mirsoeva, 1968; Gray and Williams, 1971) and stream sediments (Watterson, unpublished data) and they are ideally suited, because of their longevity, for use in potential geochemical assays. Because penicillin (a natural product of many common soil molds) is twice as active against Bacillus spp. as ampicillin (Davis and others, 1980), penicillin was chosen for this study.

MATERIALS AND METHODS

Ore Deposits

The Poorman Creek deposit. The Poorman Creek deposit is marked by an oval treeless zone about 80 meters at its maximum (north-south) diameter. The

deposit is located on a 20° to 30° south-facing slope just north of Poorman Creek about 11 miles southeast of Lincoln, Montana, and can be seen from the road. It lies at the common boundary of the Swede Gulch and Nevada Mountain 7.5-minute quadrangles (USGS topographic series, 1968). The soils in the treeless zone contain substantially higher values of copper, lead, silver, and other metals than do soils in the surrounding forest. The predominant vegetation consists of some sparse, presumably metal-tolerant grasses and a luxuriant field of the recognized copper-indicator plant, Eriogonum ovalifolium (Cannon, 1960). The deposit is of a porphyry copper type, in chloritized quartz monzonite. This and neighboring deposits are related to the Tertiary Silver Bell stock which intrudes Precambrian igneous and metamorphic rocks, and these deposits occur at the southwest edge of the Big Belt Mountain thrust fault zone (Kleinkopf and Mudge, 1972). Components of Tertiary gravels have been interpreted (R. G. Schmidt, oral communication) as indicating that the Silver Bell stock was exposed as far back as the Eocene. Accordingly, deposits in the Poorman Creek area may have been weathering since Eocene time (50 m.y.). Individual deposits in this area may have been continuously exposed since the end of Pliocene volcanism (2 m.y.). The Poorman Creek deposit may or may not have been exposed prior to valley glaciation approximately 10,000 years ago. The bacteria colonizing this deposit, however, have had at least 2,000,000 years to adapt to its conditions.

The Keystone deposit

The Keystone mine is in sec. 14, T. 33 N., R. 6 W., Shasta County, California. The Keystone deposit is one of nine massive pyrite sulfide deposits in the West Shasta copper-zinc district that have produced approximately half of California's copper through 1946. Kinkel and others (1956) interpreted the base metal mineralization in this district to be Late Jurassic or Early Cretaceous (150 m.y.). Later investigations have suggested this is a typical massive sulfide deposit formed as a submarine exhalative deposit and of the same age as the surrounding Devonian rocks (360 m.y.). All of the massive pyrite deposits in the district occur in the Balaklala Rhyolite of Middle Devonian age, and contain chalcopyrite, sphalerite, and minor quantities of gold and silver minerals. The most striking features of the ore are its uniform, dense, pyritic character, and its sharp boundary with barren or weakly pyritized wall rocks. Most of the ore in the mine stopes averages about 15 ft in thickness with a maximum thickness of 50 ft. The lenticular ore bodies are within the middle unit of the Balaklala Rhyolite. A total of 200 ft of flat-lying middle and upper units of the Balaklala Rhyolite presently overlies the ore bodies. Most of the ore bodies dip 5°-10° more steeply than the contact between the middle and upper units of the Balaklala Rhyolite. The ore bodies are intersected in various places by vertical faults.

The Balaklala Rhyolite capping this ore deposit, judging by exposures in other places, is broken and faulted and therefore probably not impervious to solutions or gases from the weathering sulfide minerals. The thickness of the rhyolite, however, despite its probable permeability, apparently serves as a significant barrier to the surface expression of the target minerals. If, as has been suggested, this is a typical massive sulfide deposit, then this deposit and the Poorman Creek copper porphyry deposit have a fundamental difference in origin which could explain the difference in the soil expression

of the two deposits. The difference in time of formation may also have a strong influence. Indeed, it can be plausibly argued that the minor variations in soil geochemistry at the Keystone are not directly attributable to the presence of subjacent ore.

Sampling procedures

Sampling and soil-handling procedures at the Poorman Creek and Keystone deposits differed. Undifferentiated samples were collected at the Keystone deposit, refrigerated, and the -1 mm fraction ground to about -80 mesh prior to chemical and microbiological study. A more satisfactory procedure of sieving to -30 mesh at the sampling site was adopted for the Poorman Creek study. The latter procedure has the advantage that no further preparation is required prior to microbiological and chemical analysis. In addition, the -30 mesh samples require little or no centrifuging after the shaking step and thus are easier to work with than ground samples in the microbiological procedure.

Poorman Creek Deposit. Sampling was done under dry conditions, August 21, 1982. Twenty-six samples were taken along a north-south line at 10-ft intervals. The first 11 sample sites were in the forest, which consisted of both deciduous and coniferous trees. Samples 12 through 26 were in the Eriogonum zone. A control sample was taken several hundred yards west of the deposit. At each sample site any loose duff was removed, and soil from an area of about 100 cm^2 to a depth of about 10 cm was dug up with a stainless steel garden trowel, sieved through a 30-mesh Tyler equivalent stainless steel sieve and transferred to four factory-closed plastic ziplock bags. A total of about 1.5 kilograms of sieved soil was taken from each site. No sterile precautions were taken during sampling, but visible soil was brushed from the equipment between sites. The sieved material was a light grayish tan in color that did not change appreciably between the wooded and the barren zone. Samples were stored in the dark at ambient temperatures for about 10 days prior to division into two sample sets using a cross channel sample splitter. At that time, one-half of each sample was stored at about -15°C. Bulk samples for chemical and microbiological testing were kept at room temperature in the dark; tests were conducted on 150 g subsamples kept at room temperature in sterile, wide-mouth glass jars.

Keystone Deposit. Samples were collected under dry conditions September 21, 1981, at 50 ft intervals in two parallel traverses directly above mapped underground workings of the Keystone mine in mixed coniferous and deciduous forest. After loose forest litter was removed, organic-rich soil was collected within 5 to 10 cm of the surface. Unsieved samples were taken. Soils for microbiological study were kept as cool as possible for about a week until they could be refrigerated at about 6°C.

As needed for chemical and microbiological study, several grams of sample were sieved through a 1-mm sieve and ground to approximately -80 mesh in a porcelain mortar. All instruments were cleaned in 70% ethanol prior to use. The 80 mesh sample was stored in sterile sputum jars at 6°C between operations.

Analysis of soil. All chemical analyses on Poorman Creek soils were carried out at least three times; all chemical analyses on Keystone soils were carried out at least five times. Cd, As, and Ag were determined by a modification of

the method of Viets (1978). Al, Ca, Fe, Mg, Ba, Be, Ce, Co, Cu, La, Mn, Mo, Nb, Ni, P, Pb, Sr, Ti, V, Y, and Zn were determined by inductively coupled argon plasma-atomic emission spectrometry (ICP) (Motooka and Sutley, 1982) following overnight digestion in concentrated nitric acid, evaporation to dryness, and dissolution in 20% HCl. Hg was determined according to an unpublished in-house procedure as follows: 0.1-g samples were placed in fritted pyrex tubes connected to a Jerome mercury vapor collection unit and heated to red heat with a propane torch. Collected mercury was then revolatilized and passed through a glass chamber in the light path of a Perkin Elmer 303 atomic absorption instrument with a chart recorder. Rare earth elements in the Keystone soils were determined individually by the procedure of Crock and Lichte (1982); pH values were obtained by mixing 2.5 g of either -1 mm (Keystone) or -30 mesh (Poorman) soils with 5 ml of deionized water and testing with an Orion model 201 digital pH meter after about 10 minutes equilibration. Percent moisture was calculated from the weight loss occurring in a 3.00 g sample after heating at 90°C overnight and temperature equilibration in a desiccator containing $CaCl_2$. Percent organic matter was calculated from the weight loss occurring in desiccated, approximately 3 g samples after heating at 525°C overnight and temperature equilibration in a 90°C drying oven and a desiccator over $CaCl_2$.

Microbiological tests

Test medium. A minimal medium similar to one suggested by Stanier and others (1976) was used alone or amended with one-tenth of its volume of appropriate concentrations of filter-sterilized potassium penicillin G (United States Biochemical). The minimal medium consisted of 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $Fe_2SO_4 \cdot 7H_2O$, .01 g $CaCl_2$, 1 g glucose, 1 g NH_4Cl, 0.1 g yeast extract, 0.07 g cycloheximide, and 15 g agar per liter of deionized water. In the Poorman Creek study, appropriate volumes of fresh penicillin solution were filter sterilized and added to sterilized medium at 47°C to make media containing 0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10., 20., 50., 100, 200, and 500 µg/ml penicillin immediately prior to use. Final test concentrations of penicillin in the Keystone study were 0, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10., 20., 50., and 100 µg/ml.
Procedure. One gram of soil was added to 9 ml sterile 33 mM K_2HPO_4 in 16x150 mm screw cap culture tubes, shaken 10 min. in a mechanical shaker, diluted to three successive 1/10 dilutions in 33 mM K_2HPO_4, submerged in circulating 68°C water for 12 min., and cooled in cold tap water. Prior to the dilution step, Keystone samples were centrifuged twice for one min. at 1200 rpm and the supernatant cloudy suspension immediately transferred by pasteur pipette to sterile 16x150 mm tubes; this served to separate most of the spores from soil debris that interfered with colony counting. This step was unnecessary with the -30 mesh Poorman Creek samples, as they settled adequately without centrifuging. Within a few hours of heat treatment, 0.5-ml portions of the four dilutions of each sample were used to make standard 10-ml pour-plates with unamended or penicillin-amended medium in the above concentrations. Plates were incubated inverted at 30°C for 72 hours and counted at 9x with a stereo binocular microscope. Only typical bacterial colonies were counted. Randomly selected colonies from the Keystone soils were transferred by loop from the surface of spread plates made with unamended medium and isolated with three successive streakings. Approximately 400 Keystone isolates were tested for Gram reaction and slides prepared for phase-contrast microscopic examination; approximately 80% were clearly gram

positive. The presence of rod-shaped vegetative cells, spores, and sporangia (typical of virtually all isolates) was considered adequate to classify the bacteria as Bacillus spp. All sterile operations were carried out in a laminar flow hood.

B. cereus enumeration procedure. The B. cereus enumeration procedure is based on the development of a circular, white, opaque zone around B. cereus colonies in overnight pour plates made by amending our medium with 2.5% (volume/volume) egg yolk as a 1:1 dilution in deionized water and 0.5% (weight/volume) tri-sodium citrate as recommended by Donovan (1958). According to McGaughey and Chu (1948), only two Bacillus species give positive results with this test, Bacillus cereus and Bacillus anthracis. The anthrax bacillus, apart from being rare enough to be dismissed a priori at most sites, gives a much weaker reaction than B. cereus with egg yolk agar (the opaque zone not extending beyond the colony in the case of B. anthracis). McGaughey and Chu (1948) tested 80 different strains of B. cereus and obtained positive results with all 80 strains.

Statistical analysis. The percent of Bacillus spp. resistant to each test concentration of penicillin was calculated according to the formula:

$$\text{percent of organisms resistant to penicillin} = \frac{\text{(no. colony forming units in medium amended with penicillin)} \times 100}{\text{no. colony forming units in control plate (medium not amended with penicillin)}}$$

Data were standardized by the following procedure. All percent values were plotted on the y (4-cycle) axis against test concentrations of penicillin on the x (6-cycle) axis of 4x6 cycle log paper for each soil site. Points for each site were then grouped into either two or three sets of points forming approximately straight lines. Linear regression lines were then fitted to each set of points after converting coordinates to their logarithms. The antilogs of values read off the regression lines were used for statistical analysis. The U.S. Geological Survey STATPAC program library (VanTrump and Miesch, 1977) was used to generate additional statistics.

RESULTS

Poorman Creek deposit. Figure 1 shows the relationship between the percent Bacillus spp. resistant to the seven lowest test concentrations of penicillin and the location of the soil sample sites with respect to the Poorman Creek deposit. Sample sites 0 through 10 are in the forested zone, and sites 11 through 26 are in the Eriogonum or "barren" zone directly over the deposit. A general increase of penicillin resistance in Bacillus spp. at all test concentrations of penicillin was observed in the soils over the barren zone.

Statistical analyses of the Poorman Creek soil data are summarized in Table 1. Scatter diagrams were printed and inspected for all significant

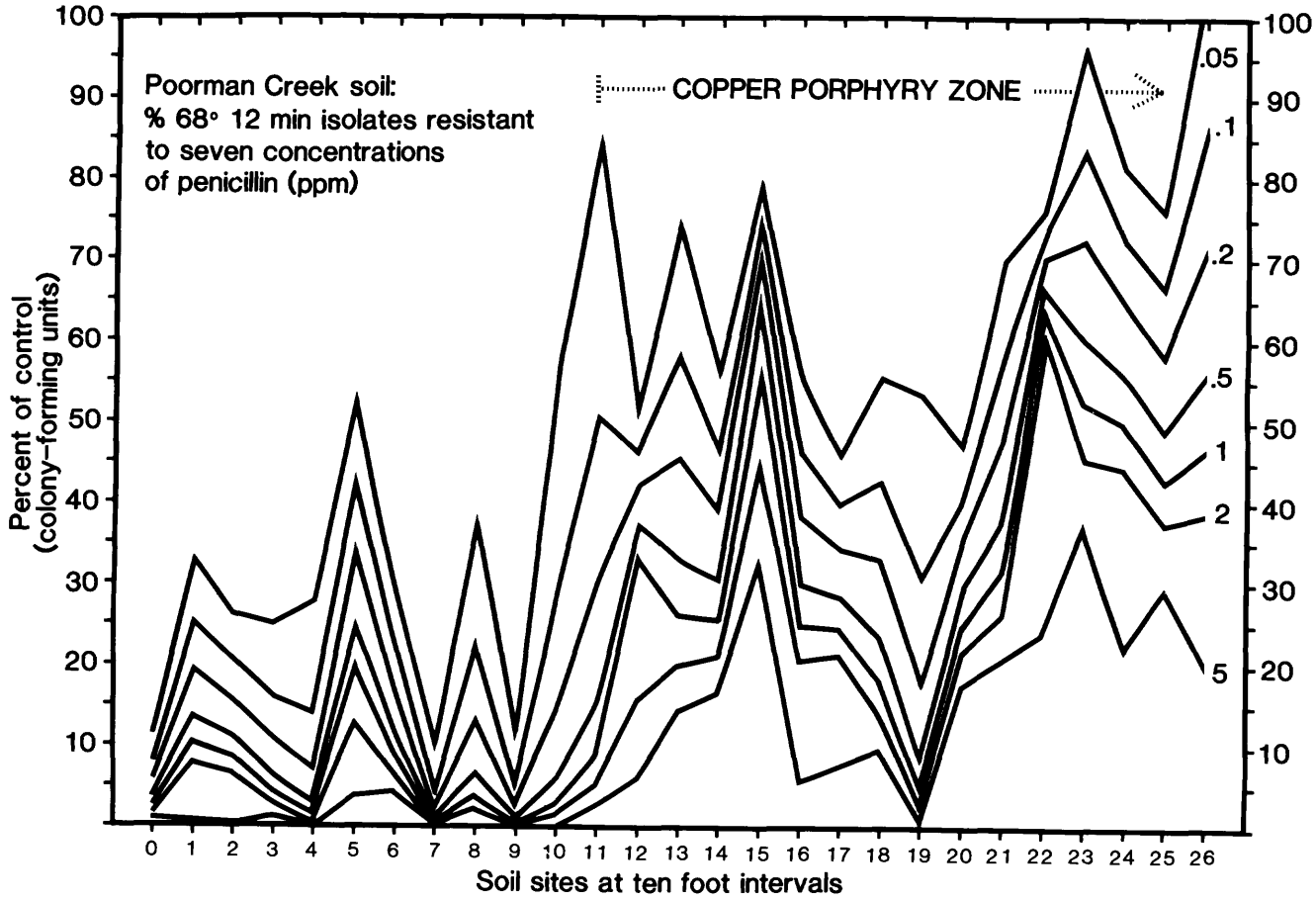


Figure 1. Percent Bacillus spp. (68° 12-minute isolates) resistant to the seven lowest test concentrations of penicillin. A general increase in percent penicillin resistant Bacillus spp. coincides with the treeless zone over the porphyry copper deposit.

TABLE 1. Correlation coefficients, Poorman Creek soils.
N = 27. $P \leq .05$ for $\pm r \geq .37$, $P \leq .01$ for $\pm r \geq .47$

	Cu	% *Bacillus* spp. resistant to 2 ppm pen	% *Bacillus* spp. resistant to 5 ppm pen	Total *B. cereus*	% *B. cereus*
Total *B. cereus*	.77	.80	.78	1.00	.86
% *B. cereus*	.74	.91	.89	.86	1.00
% organic matter	.68	.55	.62	.61	.60
Al	.75	.41	.43	.63	.54
Fe	.64	.25	.26	.50	.44
As	.81	.58	.50	.66	.65
Ba	.64	.38	.42	.50	.47
Be	.94	.57	.60	.74	.73
Ce	.99	.67	.68	.78	.76
Co	.69	.56	.60	.67	.69
Cu	1.00	.66	.67	.77	.74
La	.98	.61	.62	.75	.70
Mo	.63	.37	.39	.41	.51
Nb	.74	.46	.49	.61	.60
Ni	.87	.49	.50	.65	.64
P	.92	.57	.50	.60	.56
Sr	.87	.49	.55	.71	.64
V	.84	.56	.56	.72	.71
Y	.97	.69	.69	.77	.76

TABLE 2.

Test concentrations of penicillin	Correlation coefficients (n = 27) between % penicillin-resistant bacteria and: Total *B. cereus*	% *B. cereus*
.05	.69	.72
.1	.77	.82
.2	.79	.86
.5	.79	.88
1.0	.80	.89
2.0	.80	.91
5.0	.78	.89
10.0	.64	.76
20.0	.58	.70

Table 2. Correlations between numbers of *Bacillus* spp. ($N = 27$, $P \leq .05$ for $\pm r \geq .37$, $P \leq .01$ for $\pm r \geq .47$) resistant to various test concentrations of penicillin and total numbers of *B. cereus* per gram of soil or percent of total *Bacillus* spp. consisting of *B. cereus*. The correlations indicate that the majority of penicillin-resistant soil *Bacillus* spp. at these sites are *Bacillus cereus*. The correlations indicate that *B. cereus* may be selected for in these soils by a penicillin resistance trait.

correlations shown to ensure that the underlying assumptions of correlation analysis (using Pearson's r) were not violated. Although many significant correlations between various metals and percent penicillin-resistant bacteria occur at other test concentrations of penicillin in the Poorman Creek set, the correlations become maximum at either 2 or 5 g/ml penicillin, with most occurring at 5 g/ml penicillin. Only soil samples 7 and 9 contained no spores able to form colonies in 5 g/ml penicillin. We conclude from this that the maximum penicillin test concentrations that permit the growth of some Bacillus spp. from all, or nearly all, sample sites would be the most diagnostic of soil metal content.

Keystone deposit. Figure 2 shows the relationship between Bacillus spp. at the Keystone sample sites resistant to various test concentrations of penicillin in an early trial. The extreme penicillin resistance recorded at sites 14 and 15 was due to one organism (not B. cereus), which was an unidentified, small, highly motile, aerobic, spore-forming rod that formed tiny amber-colored, spindle-shaped subsurface colonies. In two separate tries we were not able to repeat the data at sites 14 and 15 six months after the first resistance trials, although some tiny amber colonies were still present. The correlations recorded in Table 3 are based on a more exhaustive range of penicillin test concentrations in a repeat experiment conducted about 6 months after the first one.

Correlation and stepforward regression analysis of the Keystone chemical and penicillin resistance data, based on 24 samples, are summarized in Table 3. Site number six, which was suspected of having been disturbed, was eliminated from the data set. Scatter diagrams for all single correlations shown in Table 3 were printed and inspected to ensure that assumptions about regression and correlation analyses were not violated. Question marks in two cases indicate that the correlation is based on too few points.

DISCUSSION

Poorman Creek deposit interelement correlations. Of the elements listed in Table 1, copper correlates internally most strongly with the other elements for which we have analyses. Of the transition metals, copper has the highest correlation with penicillin resistance and B. cereus counts, with a maximum r = .77 for total B. cereus. The high correlations between copper and a number of other elements caused concern about the possibility of a spectral interference in the ICP procedure. Repeat ICP runs with similar concentrations of copper, however, revealed no spectral interferences with any of the elements correlating with copper in Table 1. A somewhat bimodal distribution of copper and other metal values in the soil traverse, in conjunction with the substantial spread of copper values (141 to 2,253 ppm) appears to adequately explain the rather high correlations between copper and other elements. The high positive correlations between Ce and Y and the bioparameters are probably to be ascribed to mutual correlations with copper rather than to any biological effects caused by Ce and Y.

Metal-bioparameter correlations. An apparent trend exists between the magnitude and sign of the copper-metal correlation coefficients and that of bioparameter-metal correlation coefficients. That is, the decrease in correlation between the bioparameters and the elements can be seen to parallel the decrease in correlation between copper and those elements. This is

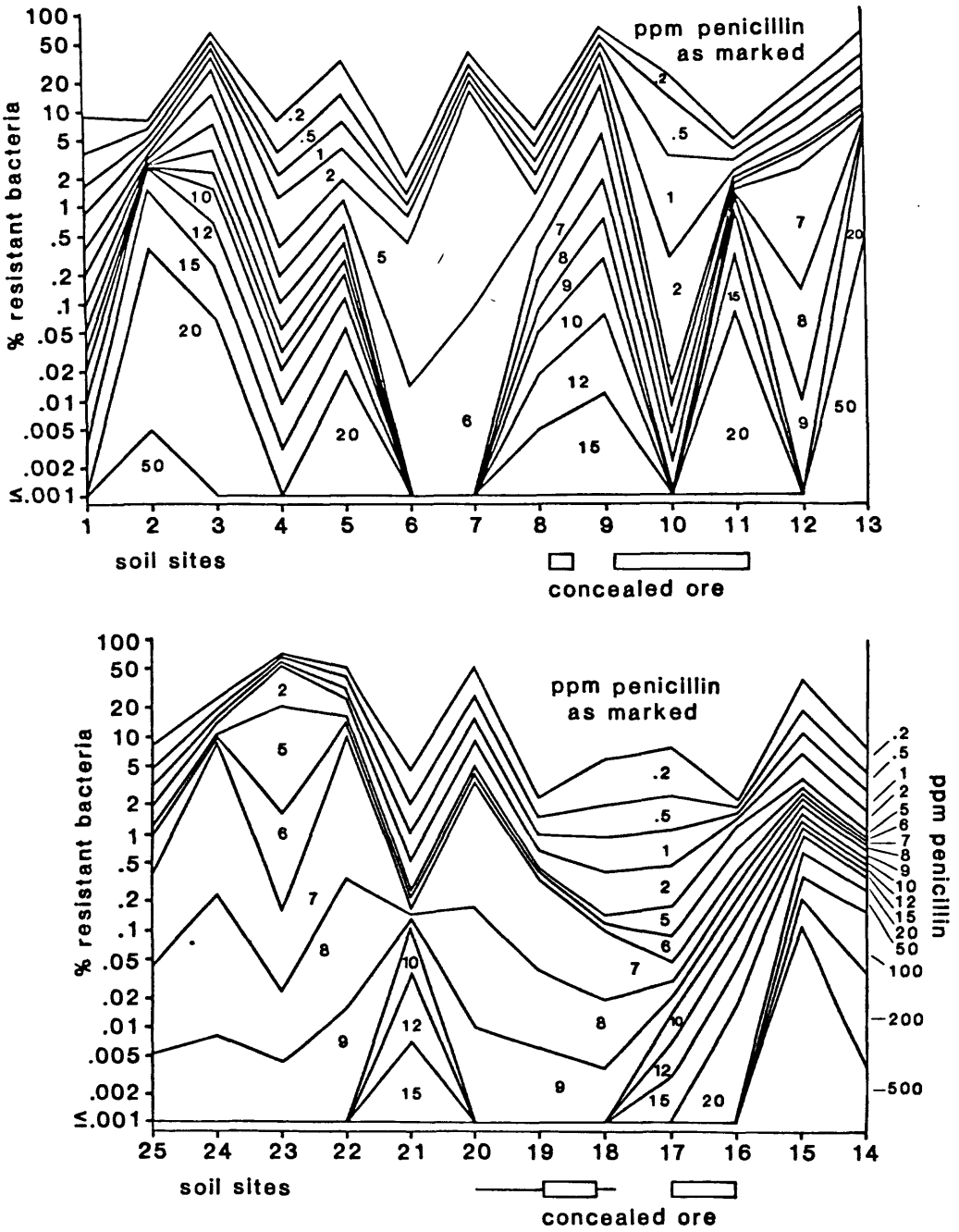


Figure 2. Early Keystone data composites showing the regular collapse of penicillin resistance in Bacillus spp. with increased penicillin through both traverses. Each line traces the percent of Bacillus organisms (from site to site) able to form colonies in minimal agar amended with the indicated test concentrations of penicillin. Note difference in scale between Figures 1 and 2.

TABLE 3. % Penicillin resistant Bacillis spp. at various test concentrations of penicillin vs. element content of soil at the Keystone deposit

Dependent variable	Correlation analysis: indep. var. (soil conc.)	cor. coef. (range)		Stepforward regression analysis: indep. var.	mult. coef.
	(N = 24; P ≤ .05 for r ≥ .39, P ≤ .01 for r ≥ .50)				
(ppm penicillin):					
.001	Al Fe	.63	.50	Al Be	.75 (57%)
	Ca	-.44			
.002	Bact.	-.49		----	
.005	Be Ni Bact.	-.56	-.52	Be	-.56 (31%)
	V Ba Nb Ti	-.51	-.46		
	Al Mn	-.42	.39		
.01	Be Nb Al	-.67	-.59	Be	-.67 (45%)
	Ni Ba Bact.	-.58	-.49		
	La V?	-.48	-.46		
	Ca/La	.49			
.02	Be Al La Ni	-.65	-.52	Be	-.65 (42%)
	Nb Ba Bact.	-.51	-.47		
	Ce Tb Co	-.45	-.42		
	Ca/La	.50			
.05	Be La Ce Ba	-.64	-.52	Be Fe	.74 (54%)
	Al Co Tb	-.50	-.49		
	Ni Pr Nb	-.47	-.41		
	Nd Bact.	-.40			
	Ca/La	.47			
.1	Be La Ce Ba	-.60	-.50	Be Tb	.70 (49%)
	Tb Co Al Ni	-.48	-.44		
	Pr Nd Y	-.43	-.40		
.2	Be La Ce Ba Tb	-.54	-.44	Be	-.54 (29%)
	Al Pr Co Ni?	-.40	-.39		
.5	Be La Ba Tb	-.49	-.41	Be	-.49 (24%)
1	Be La Ba	-.46	-.40	Be	-.46 (21%)
2	Be La Ba	-.44	-.39	Be	-.44 (20%)
5	Be	-.39		----	

? = poor scattergram

consistent with our hypothesis that more than one metal is involved in bringing about penicillin resistance in these soils. However, with the possible exception of lead, the correlation of the various toxic metals other than copper with penicillin resistance or B. cereus counts appears to be satisfactorily explained by their correlation with copper. This picture may change with additional chemical data. We note that the soil content of the rare earth elements (REE) correlates positively with % penicillin resistance (pen^r) and B. cereus counts at this deposit.

B. cereus versus metals and penicillin resistance versus metals. B. cereus counts and % B. cereus (i.e. B. cereus x 100/total Bacillus spp.) both correlate substantially better with all the metals than do the percentages of penicillin-resistant Bacillus spp. (Table 1). This, in concert with the agreement between B. cereus counts and penicillin resistance at all test concentrations (Table 2) leads us to believe that B. cereus is an intervening variable by which the penicillin resistance data can be explained. That is, the percent B. cereus accounts for the percent penicillin resistant Bacillus spp. This idea is supported by the work of Curran and Evans (1945) (summarized in Table 4) which shows that B. cereus is the least sensitive of the Bacillus species to penicillin. The fact that B. cereus counts correlate better with the metals than do the percentages of penicillin resistant Bacillus spp. may indicate that the metals are the primary selective determinants and that penicillin is a secondary or derivative selective determinant.

It is also a possibility that the metals alone (and not penicillin) are selecting for penicillin resistance and/or a penicillin resistant organism (B. cereus) in these soils. That, however, is contrary to recent theories of gene selection and maintenance in bacterial populations (Koch, 1981). We think it more probable that metal and penicillin resistance are simultaneously selected for, as thought by Timoney and others (1979), but by both metal and penicillin.

Percent organic matter versus copper. It is interesting and perhaps paradoxical in view of the apparent toxicity of the soils for trees at the Poorman Creek deposit that copper should correlate significantly with percent organic matter in the soils. The primary explanation for this may be a kind of "reverse-rain forest" effect. Copper is a well-known inhibitor of fungi--the principal agents that decompose soil organic matter. It may thus be that copper and other available metals in the more metalliferous soils act as preservatives of soil organic matter. A minor, contributing explanation may lie in a well-known experimental phenomenon. Powell (1950), Levinson and Sevag (1953), and Krishna Murty and Halvorson (1957) have noted the ability of the cations of mercury, copper, chromium, and iron to completely inhibit the germination of Bacillus spores. The experiments of Krishna Murty and Halvorson (1957) indicate that this inhibition occurs through the binding of the metal cations to the spore coat, similar to the effect observed with fungal spores. It seems probable that in soils with substantial concentrations of these cations, a large percentage of Bacillus spores and fungal spores would be inhibited. Since these soils apparently have active spore-forming populations more or less continuously forming spores, it appears possible that organic matter in the form of metal-inhibited spores may accumulate in proportion to the presence of the inhibitory cations and contribute to the organic matter present. The correlation (Table 1) between

TABLE 4. Relative sensitivity of *Bacillus* spp. to penicillin

		# strains
Most sensitive	*B. alvei*	4
	B. brevis	3
	B. circulans	1
	B. firmus	1
	B. laterosporus	2
	B. mascerans	1
	B. megaterium	5
	B. polymyxa	1
	B. pumilus	1
	B. sphaericus	2
	B. subtilis	5
Intermediately sensitive	*B. alvei*	2
	B. subtilis	4
	B. subtilis (anaerobic)	1
	B. subtilis var. *aterrimus*	1
	B. subtilis-niger	1
Least sensitive	*B. albolactis*	1
	B. cereus	7
	*B. metiens**	1
	*B. mycoides**	2

data from
Curran and Evans, 1946, *J. Bact.* 52: 89.

*now classified as *Bacillus cereus*

percent organic matter and the bioparameters may be explained by their common correlation with soil copper.

Keystone deposit. The correlations shown in Table 3 are more complex . An explanation for the significant negative correlations between penicillin resistance and the rare-earth elements may lie in the quite different effects rare-earth elements are known to have on the calcium-transport systems of Bacillus spp. and organisms (such as fungi) that contain mitochondria. Lanthanum and all other rare-earth cations are potent inhibitors of respiration-dependent calcium transport in mitochondria, extrapolating to complete inhibition at less than 0.1 mole of lanthanide/milligram of protein (Mela, 1969, Vainio and others, 1970). The experiments of Eisenstadt and Silver (1972) with sporulating cells of Bacillus subtilis, on the other hand, show that lanthanum does not inhibit calcium accumulation in these prokaryotes (which lack mitochondria), but, in fact, stimulates it. This markedly different effect may explain our negative correlations. Where the rare-earth elements increase in the traverse, the penicillin production of the molds would suffer; as a consequence there may be fewer penicillin-resistant Bacillus spp. Conversely, penicillin production and percent penicillin-resistant Bacillus spp. may increase where the rare-earth elements decrease. That this effect occurs despite the ordinary content of rare-earth elements in Keystone soils (La varies between 4 and 22 ppm, Y between 17 and 120 ppm) suggests to us that the ecology of molds and Bacillus spp. may be quite sensitive to low-level variations in the rare-earth elements. In a geologic setting similar to that of the Keystone deposit (the Kuroko massive sulfide deposit in Japan), the rare-earth elements, which may normally be tied up in biologically unavailable rutile, are thought to be present as phosphate inclusions in readily weathering ilmenite (S. E. Church, oral communication). However, at the Keystone deposits, the correlations suggest that the rare earths must be present in a biologically available form. The considerable differences in solubility among the major rare-earth-containing minerals suggests that the observed effect must be heavily dependent on the soil mineralogy of these elements.

The consistently positive correlations between the Ca/La ratio and the percent penicillin-resistant Bacillus spp. (Table 3) is consistent with the above interpretation that the lanthanide elements are interfering with calcium transport, the idea being that high Ca/La ratios are favorable to penicillin production.

The correlations with beryllium are most puzzling. The beryllium content of these soils is low and the variation minor (0.25 to 0.49 ppm). On this basis alone, one might suppose that these correlations are fortuitous. But even lacking an organic rationale, it is most improbable that beryllium should correlate so well with penicillin resistance throughout the range of test concentrations. We tentatively conclude, therefore, that beryllium in some way frustrates penicillin production even more effectively than do the rare-earth elements, although we have no idea by what means this might occur.

The ecology of penicillin resistance. Selman Waksman (1947, 1951) was of the opinion that antibiotic antagonism and hence antibiotic production, despite its evident usefulness in nature, is not the decisive factor in determining the numerical hierarchy of microorganisms in most settings. Otherwise, he reasoned, the antibiotic-producing organisms would now dominate the microbial

world, and clearly they do not. Brian (1957), although he stated that "the capacity to produce antibiotics is a character conducing to fitness," nevertheless concluded in his review of experiments attempting to measure the production of antibiotics in soils that most antibiotics are produced in extremely minor amounts under natural conditions and even then, in such restricted microenvironments as to be virtually without effect in "the bulk soil as a whole." This conclusion appears to be the concensus of most of the investigators who have undertaken the difficult task of demonstrating the presence of antibiotics in soil. But U.S. patent laws may also be of some significance to the existance of this opinion because they discourage the patenting of natural substances. It may thus be partly a consequence of these laws that the view that antibiotics, by and large, are produced only with the aid of clever biochemists, has its adherents and has so often been put forward.

In a brief review of the literature on antibiotic production and antibiotic resistance in soils, we have found only a single study (Hill, 1972) on both antibiotic production and antibiotic resistance in soil and none on either antibiotic production or antibiotic resistance in metalliferous soils. The lack of such studies (if indeed there are none) is interesting in view of the well-known coincidence of metal- and antibiotic-resistance genes on bacterial plasmids. It seems rather elementary, in view of this genetic coincidence, to ask whether bacteria encounter unusual concentrations of metals and antibiotics in the same environmental settings. The genetic knowledge however came somewhat after the excitement about antibiotics had peaked.

A basic assumption of our investigation has been that the documented coincidence of metal- and penicillin-resistance genes on bacterial plasmids may be evidence that these metals and penicillin are commonly encountered by bacteria in the same natural settings and perhaps at a similar frequency. Other persuasive, strictly genetic reasons for the common occurrence of antibiotic and heavy-metal-resistance genes on plasmids can be forwarded, but we do not think they invalidate our assumption. Resistance genes specific for copper have not yet been identified in bacteria (A. O. Summers, personal communication).

Our conclusions about the ecology of penicillin resistance and soil metal are summarized in the flow diagram in Figure 3. The diagram is based on a conjectural interpretation of our data. What follows is evidence from the literature for this interpretation.

Penicillin production in nature is not limited to the organisms used for its commercial production, but is an ability common to dozens of different species of molds belonging to at least nine genera (Pollock, 1967). Penicillin-producing molds of the two genera which account for most of these organisms that had been identified by the end of World War II are listed in Table 5. In trying to gain insights into the possible coincidence of penicillin-production and metal resistance in these molds, we looked at the documented metal resistances of the known penicillin-producing organisms. The result of cross referencing penicillin production to metal resistance (simply using the papers in our files) is shown in Table 6. A systematic literature search would undoubtedly result in a larger list. The impression gained from this exercise is that the penicillia and aspergilli most noted for their

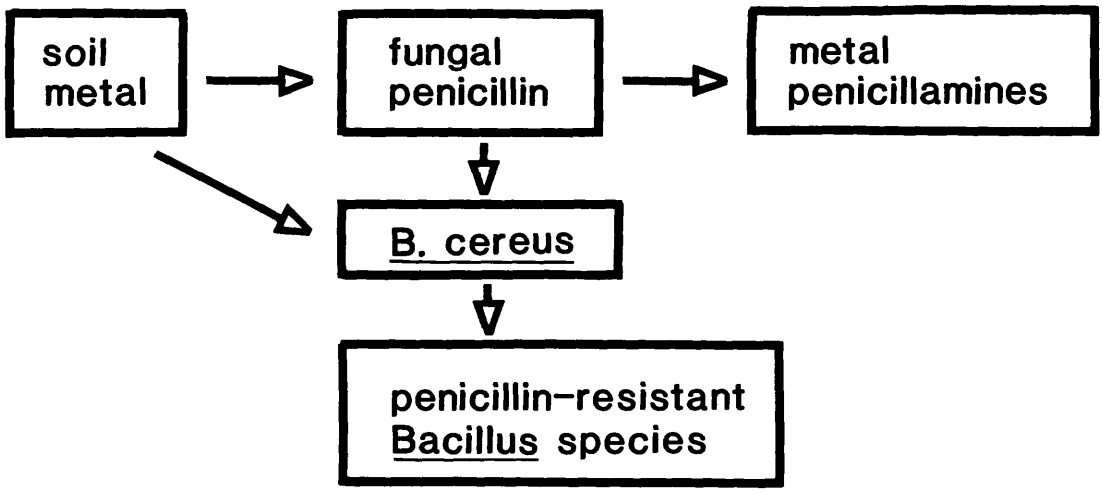


Figure 3. A flow diagram of ecological causality according to our interpretation. Our data furnish evidence for the soil metal-*B. cereus*-penicillin resistance relationship. The soil metal-fungal penicillin-penicillamines relationship is conjectural.

TABLE 5. Fungi that produce penicillin

Genus PENICILLIA
P. turbatum Westling
P. cinerascens Biourge
P. rubens Biourge
P. chloro-leucon Biourge
P. steckii Zaleski
P. chrysogenum Thom
P. chlorophaeum Biourge
P. griseo-roseum Dierckx
P. notatum Westling
P. citreo-roseum Dierckx
P. cyaneo-fulvum Biourge
P. brunneo-rubrum Dierckx
P. baculatum Westling
P. fluorescens Laxa
P. asperulum Bainier
P. meleagrinum Biourge
P. raciborskii Zaleski
P. roseo-citreum Biourge
P. griseo-fulvum Dierckx
P. avellaneum Thom et Turesson
P. crateriforme Gilman and Abbott
P. baarnense von Beyma
P. euglaucum von Beyma
Genus ASPERGILLI
A. giganteus Wehmer
A. nidulans Eidam
A. flavipes Thom and Church
A. niger van Tieghem
A. oryzae Cohn
A. parasiticus Speare
A. flavus Link

Original references in: Florey et al, 1949, Antibiotics, vol 1., p. 674 and following, Oxford

TABLE 6. Metal resistance of the penicillin-producing fungi

Aspergillus nidulans (Eidam)	Mn^{++} Zn^{++} Co^{++} Ni^{++} Fe^{+++} Ba^{++} Sn^{++}	Elorza, V., 1969, Microbiologia Espanola 22: 131-138.
Aspergillus niger (Thom)	Cu^{++}	Starkey, R. L., 1973, J. gen. microbiol. 78: 217-225
	Hg^{++}	Ashworth, L. J., Jr., and Amin, J. V., 1964, Phytopathology 54: 1459-1463.
	Pb^{++}	Zlochevskaya, I. V., 1968, Microbiology 37: 709-714.
	$AsO_3^{\equiv}$	Terui, G., and others, 1960, Tech. Rept. Osaka Univ. 10: 279-290.
	Au^{+++}	Mineyev, G. G., and others, 1975, cited in: Mineyev, G. G., 1976, Geochem. International 13(2): 164-168.
Penicillium chrysogenum (Thom)	Cu^{++}	Jarvis, F. G. and Johnson, M. J., 1950, J. Bact. 59: 51-60.
	$AsO_3^{\equiv}$	Challenger, F., 1944, Chem. Rev. 36: 315-361.
	$SeO_3^{\equiv}$	
	$TeO_3^{\equiv}$	
Penicillium notatum (Westling)	Cu^{++} Hg^{++}	Partridge, A. D., and Rich, A. E., 1962, Phytopathology 52: 1000-1004.
	$AsO_3^{\equiv}$	Challenger, F., 1944, ibid.
	$SeO_3^{\equiv}$	
	$TeO_3^{\equiv}$	
Aspergillus oryzae (Ahlburg) Cohn	Cu^{++}	Starkey, R. L., 1973, ibid.
	$AsO_3^{\equiv}$	Terui, G., 1960, ibid.
	Au^{+++}	Mineyev, G. G., 1976, ibid.
Aspergillus flavus (Link)	Cu^{++}	Starkey, R. L., 1973, ibid.
Penicillium Thomii (Maire)	Cu^{++}	Kendrick, W. B., 1962, Can. J. Microbiol. 8: 639-647.

resistance to several toxic metals share in common the ability to produce penicillin. We note that copper resistance is something of a common denominator in this group. Why might penicillin-production confer resistance to heavy metals, particularly copper? We will now attempt to answer this question.

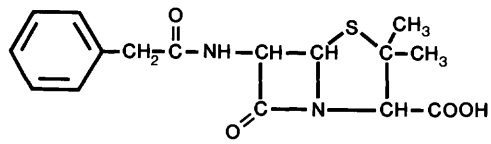
Penicillin-metal interactions. While trying to prepare salts of penicillin, early investigators noticed that the substance was inactivated by a large number of metal cations (Abraham and Chain, 1942). The metals with the most powerful inactivating effect on penicillin were copper, lead, zinc, and cadmium; but other heavy metals such as nickel, mercury, and uranium also exerted the inactivating effect. They did not understand the mechanism of this inactivation. Abraham and Chain (1942) noted that no antibiotic activity could be recovered by decomposing the inactivated penicillin with acid and extracting with ether. Although they did not deduce from this that the (still unresolved) molecule was broken, they guessed that complex formation was taking place between the metals and the penicillin. Within the year Abraham and others (1943) had purified the primary penicillin hydrolysate and named it penicillamine. They did not, apparently, associate this compound with their previous observations of the chemical reactivity of penicillin toward metals.

The presently understood route of penicillin hydrolysis, omitting an unstable intermediate in the conversion of penicilloic acid to penicillamine, penamaldate, is shown in Figure 4 (Davis and others, 1980).

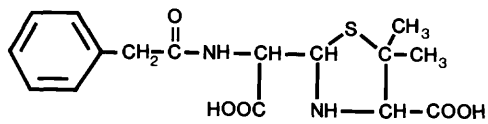
In 1955, it occurred to an English physician, J. M. Walshe (Walshe, 1981) that the penicillamine molecule might have a suitable structure to act as a copper-binding agent for use in the treatment of Wilson's disease, a toxic buildup of copper that causes destructive lesions in the brain and liver. He obtained a few grams of the scarce compound from Merck, and administered 0.5 g to himself. The next morning he gave a similar dose to a patient suffering from Wilson's disease. These early experiments showed that administration of 1 g of penicillamine by mouth led to a 10- to 20-fold increase in the urinary excretion rate of copper. By the late 1950's, penicillamine had revolutionized the prognosis of this hitherto invariably fatal disease. Since then, penicillamine, which also forms strong complexes in vitro with a wide range of heavy metals, has been used experimentally in treating poisoning due to lead, mercury, cadmium, and gold (Gergely and Sovago, 1979; Lyle, 1981). The various metal complexes which form with penicillamine, as listed by these authors, are shown in Figure 5. It now appears to us that the formation of these strong penicillamine chelates adequately explains the well-documented destruction of the penicillin molecule by these metals. We thus believe that penicillin may have at least two roles in nature, that of an antibiotic and that of a heavy metal detoxifying agent.

SUMMARY

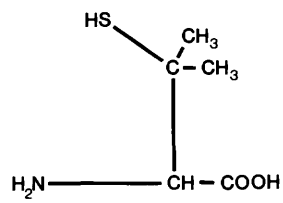
In response to a conclusion by Timoney and others (1978) that metals can create a selective pressure for antibiotic resistance, we tested the penicillin resistance of a normally penicillin-sensitive group of bacteria (Bacillus spp.) in the naturally metalliferous soils associated with two ore deposits. In both cases, we found significant correlations between soil metal content and penicillin resistance. Further investigation of the Poorman Creek soils revealed that the numbers of one organism, B. cereus, the most



PENICILLIN

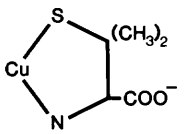


PENICILLOIC ACID



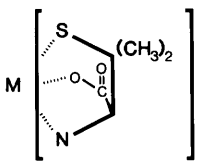
PENICILLAMINE

Figure 4. Steps in the hydrolysis of penicillin. A more elaborate account is in Davis and others (1980).



COPPER PENICILLAMINE COMPLEX

M = Cu^{++} Sn^{++}
Pb^{++} Ni^{++}
Hg^{++} Ag^{+}
Ga^{+++} Au^{+++}
In^{+++}
Zn^{++}
Cd^{++}
Sb^{+++}
Cr^{+++}
Mo^{+++}
Fe^{++} $^{+++}$
Co^{++}
Pd^{++}



PENICILLAMINE COMPLEXES

Gergely and Sovago, 1979
Lyle, 1981

Figure 5. The copper penicillamine complex and a general form of other known heavy metal penicillamine complexes.

constitutively penicillin resistant of the group, explains the correlations between soil metal content and penicillin resistance. We conclude from this and from other circumstantial evidence having to do with the metal resistance of the penicillin-producing molds, the chemistry of the penicillin molecule, and the extraordinary metal-binding properties of its most characteristic hydrolysate, penicillamine, that penicillin production may confer a selective advantage on penicillin-producing molds in metalliferous soils, particularly those containing copper. We think that these findings may bear on the evolution of the common plasmid loci of metal- and antibiotic-resistance genes. We also conclude that B. cereus is an authentic heavy-metal indicator microbe whose presence in soil, water, and stream-sediment samples should be investigated further for its potential as an indicator of mineralization. The ease with which this organism can be enumerated on egg-yolk agar commends it for investigation as a rapid geochemical assay.

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