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Long-Term Effects of Metal-Rich Sewage Sludge Application on Soil Populations of *Bradyrhizobium japonicum*

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The application of sewage sludge to land may increase the concentration of heavy metals in soil. Of considerable concern is the effect of heavy metals on soil microorganisms, especially those involved in the biocycling of elements important to soil productivity. *Bradyrhizobium japonicum* is a soil bacterium involved in symbiotic nitrogen fixation with *Glycine max*, the common soybean. To examine the effect of metal-rich sludge application on *B. japonicum*, the MICs for Pb, Cu, Al, Fe, Ni, Zn, Cd, and Hg were determined in minimal media by using laboratory reference strains representing 11 common serogroups of *B. japonicum*. Marked differences were found among the *B. japonicum* strains for sensitivity to Cu, Cd, Zn, and Ni. Strain USDA 123 was most sensitive to these metals, whereas strain USDA 122 was most resistant. In field studies, a silt loam soil amended 11 years ago with 0, 56, or 112 Mg of digested sludge per ha was examined for total numbers of *B. japonicum* by using the most probable number method. Nodule isolates from soybean nodules grown on this soil were serologically typed, and their metal sensitivity was determined. The number of soybean rhizobia in the sludge-amended soils was found to increase with increasing rates of sludge. Soybean rhizobia strains from 11 serogroups were identified in the soils; however, no differences in serogroup distribution or proportion of resistant strains were found between the soils. Thus, the application of heavy metal-containing sewage sludge did not have a long-term detrimental effect on soil rhizobial numbers, nor did it result in a shift in nodule serogroup distribution.

The effects of heavy metals on natural microbial communities have attracted increased attention. Heavy metals are being added to the environment from a variety of sources including municipal, industrial, and agricultural wastes as well as dredge spoils (1, 17, 30). Of considerable concern is the disposal of heavy metal-containing sewage sludge on agricultural land. This practice has raised concerns as to the effect of sludge on soil microorganisms, especially those involved in the biocycling of elements such as carbon, nitrogen, and sulfur.

One important component of the soil microbial community is the rhizobia, which are involved in nitrogen-fixing symbiotic relationships with legumes such as soybeans (*Glycine max* (L.) Merr.). An examination of the effects of heavy metals on rhizobium ecology in soils is important to assess the impact of increasing heavy metal concentrations in soil on symbiotic nitrogen fixation. The effect of heavy metals on rhizobia in soils is presently unclear. The current investigation was initiated as a result of previous research which suggested that soils containing high levels of heavy metals resulted in decreased nitrogen fixation by soybeans (J. R. Heckman, M.S. thesis, University of Maryland, 1985).

The addition of heavy metals can have several important effects on a microbial community. First, heavy metals may alter microbial biomass. Brookes and McGrath (6) found that soil microbial biomass, as measured by chloroform fumigation, was much less in sludge-amended soils than in comparable soils amended with manure, presumably due to the heavy metal content of the sewage sludge. In a separate study with various sludge-soil mixtures, populations of *Bradyrhizobium japonicum* were found to be reduced much faster at higher sludge/soil ratios (25). Reddy et al. (25) suggested that this may have been due to heavy metal toxicity. Other studies have shown that Pb and Cd reduce the nodule weight of soybeans grown in a sand-vermiculite mixture and that high concentrations of various heavy metals reduce the nodule numbers on red clover (*Trifolium pratense* (L.) Linnaeus) grown in soils (16, 22). In contrast, Rother et al. (27) reported that nitrogenase activity was not decreased significantly in white clover (*Trifolium repens* (L.)) grown in soils contaminated with Cd, Pb, and Zn; this study, however, did not examine the number of clover rhizobia in the soil, nor did it report the effect of increasing heavy metal concentrations on nodule number. Decreased numbers of nodules on the plant may not have affected nitrogenase activity due to compensation by the remaining nodules.

Second, the addition of heavy metals to a microbial community may bring about, through selective pressure, an increase in the proportion of heavy metal-resistant strains in the community. This change has been shown in a variety of habitats, since a positive correlation between concentration of heavy metals and proportion of heavy metal-resistant strains has been demonstrated in sediments (30), soil (11, 12, 23), and leaf litter (21). In contrast, Borges and Wollum (3, 4) examined the microsymbiont of soybeans, *B. japonicum*, for Cd sensitivity. These authors reported that, although there were significant differences in Cd sensitivity among strains of *B. japonicum*, the addition of Cd to soil did not affect the serogroup distribution in the nodules or the amount of nitrogen fixed by symbiotic soybean plants grown in the soil. Increasing soil Cd levels did, however, reduce nodule numbers.

The third effect that heavy metal additions may have on a microbial community is to change the diversity of the community. Studies have shown a decrease in the diversity of aquatic and sediment bacterial communities as a result of

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heavy metal pollution (15, 33). The same effect has not been clearly demonstrated in soil microbial communities (2).

The objectives of the current study were (i) to examine the natural level of heavy metal resistance in different strains of soybean rhizobia, (ii) to examine the long-term effects of metal-rich sewage sludge applications on the total number of soybean rhizobia in the soil, and (iii) to examine the long-term effects of metal-rich sewage sludge applications on the diversity and proportion of metal-resistant strains in a soybean rhizobia population.

MATERIALS AND METHODS

Source and maintenance of microorganisms. All *B. japonicum* and *Rhizobium fredii* strains were obtained from the U.S. Department of Agriculture (USDA), Beltsville, Md., *Rhizobium* Culture Collection. Stock cultures were maintained on yeast extract-mannitol (YEM) agar slants and stored at 4°C (31).

Heavy metal media. The metal sensitivity of all rhizobium strains was determined on solid agar media. The HM minimal medium of Cole and Elkan (8) was used. The medium was solidified with the addition of 1.5% agar (Difco Laboratories), and the pH was adjusted to 6.6 with 5 N NaOH before autoclaving. Filter-sterilized L-arabinose was added after autoclaving to a final concentration of 0.1%. The metals were added as $PbCl_2$, $CdCl_2 \cdot 2H_2O$, $HgCl_2$, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnCl_2$, $FeCl_3 \cdot 6H_2O$, and $AlCl_3 \cdot 6H_2O$. Stock solutions of 5 g of each metal per liter were used, except for iron, which was prepared fresh for each experiment. The metal solutions were autoclaved separately before use and then added to sterile HM medium. Both the arabinose and metal solutions were added to molten HM medium, which was cooled to approximately 55°C. The initial concentrations of heavy metals used for sensitivity testing were obtained from previous studies which examined the metal sensitivity of rhizobia as well as other soil bacteria (8, 10, 12, 26, 30).

Field plots. The soil examined in all experiments was a Sassafras sandy loam (Typic Hapludult, fine, loamy, siliceous, mesic) located at the University of Maryland Plant Research Farm, Fairland. The experimental design was a randomized complete block with three replications. The sludge plots were established in 1975 with a one-time application of 0, 56, or 112 Mg of anaerobically digested sewage sludge per ha (28). The sludge contained 4,400, 2,200, 140, 170, and 16 mg of Zn, Cu, Pb, Ni, and Cd, respectively, kg of $soil^{-1}$ on a dry weight basis. The soil pH of all plots ranged from 6.4 to 6.9. Clark soybeans were planted in May of both 1984 and 1985. Previous studies conducted on these plots involved the growing of a variety of crops, including soybeans and corn. The plots were not inoculated with rhizobia.

Serotyping. The short agglutination test of Vincent (31) was used for serotyping of field isolates. Dense (10^9 cells per ml) cultures of rhizobia in YEM broth were steamed at 100°C for 20 min to inactivate flagellar antigens. Rabbit antisera prepared against 17 different serotype strains of *B. japonicum* were used at a 1/100 final concentration. Each isolate was tested against all 17 antisera.

Determination of heavy metal resistance. All laboratory strains of rhizobia were grown in HM broth to the late-log phase for use as inoculants. Single loopfuls of inoculant were streaked onto plates containing metal-amended HM agar. Plates were amended with only one metal, and each inoculant was tested on different concentrations of all eight metals. All plates were replicated three times and incubated at 28°C. Plates were scored at 10 days for *B. japonicum* and at 5 days for *R. fredii*. Initially, the plates were also scored at 14 and 10 days, respectively, but no differences were found. The MIC was the lowest concentration of each heavy metal which completely inhibited visible growth of the microorganism.

Soil isolates of *B. japonicum* were obtained by harvesting 50 nodules from soybeans grown in the control plots in 1984. The nodules were surface sterilized as described by Vincent (31). The nodule isolates were grown up and serotyped by agglutination. Rhizobia from seven different serogroups were obtained, and the MICs for all eight metals were determined for one or two isolates from each serogroup.

Determination of rhizobia numbers. Composite soil samples from the top 10 cm were taken from each of the nine plots before planting. Two soil samples from each plot were analyzed for rhizobial numbers by the most-probable-number technique. Fivefold dilutions of the soil were used, and four samples from each dilution were added to pregerminated Clark soybeans grown in plastic growth pouches. Uninoculated control pouches were used to check for rhizobial contamination; no contamination was observed. The pouches were placed in a greenhouse and watered when needed with nitrogen-free nutrient solution (34). The number of rhizobia per gram of dry soil was determined by using the tables of Brockwell (5).

Determination of serogroup distribution and proportion of resistant strains. Five soybean root systems were randomly harvested from each plot in August of 1985, and nodule samples were removed from each root system. Fifty randomly selected, surface-sterilized nodules from each plot were used to isolate soybean rhizobia on YEM plates containing 150 mg of cyclohexamide per liter. Colonies were picked for transfer to YEM plates amended with 25 mg of congo red per liter and then transferred to fresh YEM slants for maintenance at 4°C. The serogroups of isolated soybean rhizobia were determined by agglutination, and the MICs for Zn, Ni, Cu, and Cd were determined for each strain.

RESULTS AND DISCUSSION

Determination of heavy metal resistance. The MICs that we determined for Pb (400 $\mu g\ g^{-1}$), Fe (70 $\mu g\ g^{-1}$), and Hg (3 to 5 $\mu g\ g^{-1}$) were virtually identical for all fast- and slow-growing rhizobium strains tested. The *B. japonicum* strains had higher MICs for Al and Cd and much higher MICs for Cu, Ni, and Zn than the *R. fredii* strains (Table 1). The *R. fredii* strains, as a group, did not vary in their MICs for all metals, except for Zn. In contrast, the *B. japonicum* strains varied in their resistance levels. Strains USDA 62, 110, and 122 were more tolerant of Cu, and strains USDA 62 and 122 were also more tolerant of Cd, as compared with other strains tested. Strain USDA 123 showed markedly less resistance to Cu, Zn, and Cd than did the other *B. japonicum* strains. The difference in diversity of MICs between the *R. fredii* strains and the *B. japonicum* strains may be attributed to the relatively few sites from which the *R. fredii* were originally isolated (18). Thus, the *R. fredii* strains examined probably represent a less varied germplasm collection than the *B. japonicum* strains in this study.

Soil isolates of *B. japonicum* from seven different serogroups, including 6, 31, 62, 76, 110, 122, and 125, were isolated from soybeans grown in the control soil. The soil isolates exhibited identical MICs for all eight metals in comparison with those of the reference laboratory strains within the same serogroup. Vincent (32) originally cautioned

against associating the placement of a strain within a serogroup with any other property; however, several such associations have since been demonstrated (20).

To further investigate the consistency of heavy metal resistance among serogroup strains, the MICs of additional laboratory strains were examined. Strains USDA 94, 110, 122, and 123, each of which is the type strains within its respective serogroup, had distinctive MIC patterns for Zn, Ni, Cu, and Cd (Table 1). Strains within these four serogroups, from the most diverse original sources available, were obtained from the USDA culture collection, and their MICs were determined for Ni, Zn, Cu, and Cd (Table 2). When the MICs of the strains are compared with those of the serogroup type strain (Table 1), it is evident that they have the same MIC patterns. Strains USDA 2, 5, 28, and 105, all in serogroup 123, are all relatively sensitive to the four metals, whereas strains USDA 133, 136, and 143, which are in serogroup 122, are all relatively tolerant. Interestingly, strain 129, which is placed in both serogroups 122 and 123, exhibited metal tolerance properties between those of the other strains in serogroups 122 and 123. Strains within serogroups 94 and 110 also have MICs for all four metals similar to those of their serogroup type strains. It appears, therefore, that there is some correlation between serogroup placement and heavy metal tolerance levels among strains of *B. japonicum*.

The division of *B. japonicum* into different groups based on heavy metal tolerance, especially to Cu and Cd, also supports the grouping reported in studies using divergence of gene structure as a measure of diversity (29). Both DNA homology studies and the current study examining heavy metal resistance support the placing of strains USDA 62, 110, and 122 as a distinct group.

It is not possible to predict the MIC of metals in soil for rhizobia from the MICs determined in the laboratory. Soil is a complex substrate for microbial growth and contains both clay and organic matter, which have been shown to specifically bind heavy metals (14). In addition, heavy metals often are bound by medium components; medium composition thus has been found to greatly influence the heavy metal sensitivity of microbes (7). Yeast extract, which is a normal component of rhizobium media, has been shown to extensively bind heavy metal ions (24). Minimal medium was used in the current study, therefore, to facilitate comparisons of MICs with those from other research.

TABLE 1. Heavy metal tolerance of rhizobial serogroup type strains

Strains	Metal MIC ($\mu g\ g^{-1}$)				
	Cu	Al	Ni	Zn	Cd
B. japonicum					
USDA 125	40	70	20	400	40
USDA 124	10	70	10	300	15
USDA 123	5	70	15	30	10
USDA 122	130	70	40	500	90
USDA 110	120	70	20	700	50
USDA 94	60	70	70	400	40
USDA 76	30	70	40	600	40
USDA 62	100	70	40	600	90
USDA 46	30	70	20	600	40
USDA 31	10	70	40	500	20
USDA 6	60	70	10	300	40
R. fredii USDA 191, 192, 193, 194, 201, 205, 206, 208, 214, 217, and 257	2	40	1	5–30	10

TABLE 2. Heavy metal tolerance of *B. japonicum* strains within specific serogroups

Strain	Original source	Serogroup	Metal MIC ($\mu g\ g^{-1}$)			
			Cu	Cd	Zn	Ni
2	Iowa	123	5	10	100	15
5	Indiana	123	5	10	100	15
28	Florida	123	5	10	100	15
105	Mississippi	123	5	10	100	20
129	Iowa	122/123	40	50	300	15
133	Louisiana	122	100	90	600	60
136	Maryland	122	100	90	600	60
143	India	122	100	90	600	60
16	North Carolina	110	90	80	500	30
20	Wisconsin	110	100	80	500	30
30	Iowa	110	100	80	500	30
335	China	110	90	80	500	30
97	North Carolina	94	60	40	400	85
98	North Carolina	94	60	40	400	85
99	North Carolina	94	60	40	400	85
119	South Carolina	94	50	40	400	85

All of the heavy metals examined in this study become more available and thus more toxic at low pH levels. In soils, free Al is rarely found in significant quantities at pHs above 5.5 and is seldom a problem in temperate soils. Aluminum and iron resistance were examined, however, because they are commonly added during sewage treatment as flocculating agents. A slightly acid medium for bacterial growth was used to examine heavy metal tolerance to ionic species of the heavy metals that would be present in well-managed agricultural soil. The U.S. Environmental Protection Agency requires a minimum soil pH of 6.5 for the application of sewage sludge to land used in growing food-chain crops (13).

Borges and Wollum (3, 4), who examined the effect of Cd on various strains of *B. japonicum*, reported values of metal tolerance that were similar to those found by our present study, despite the fact that there is a difference in medium composition. Their ranking of *B. japonicum* strains for Cd tolerance was similar to that reported in our study, with USDA 123 the least tolerant and USDA 110 the most tolerant. The order of toxicity of metals to *B. japonicum* reported in the current study is also similar to that in other reports (10, 23).

Determination of rhizobial numbers. The number of soybean rhizobia per gram of soil increased with increasing rates of sludge application. The mean number of rhizobia for the plots receiving 0, 56, and 112 Mg of sludge per ha was 14.1×10^4, 32.2×10^4, and 69.2×10^4 per g of dry soil, respectively. The mean number of rhizobia for the plots receiving 112 Mg of sludge per ha was significantly higher than the mean number of rhizobia for the control plots, but the mean number of rhizobia for the plots receiving 56 Mg of sludge per ha was not significantly different from either the control plots or the plots receiving 112 Mg ha^{-1}. The observed increase in soybean rhizobial numbers is in contrast with the study of Brookes and McGrath (6), who reported a decrease in microbial biomass as a result of sewage sludge applications. This discrepancy may be due to the fact that the sludge applied in the Brookes and McGrath study appeared to have been much higher in metal content than that applied in the present study. Another possible difference between the two studies is that Brookes and McGrath examined the total community of microbes, whereas we examined only one component of the community, *B. japonicum*.

TABLE 3. *B. japonicum* serogroup distribution of nodule occupants

Sludge treatment (mg ha^{-1})	% of nodule occupants in serogroup:										
	4	6	31	46	62	76	94	110	122	125	130
0	2	28	4	2	0	4	27	10	7	10	2
56	0	19	5	2	5	5	27	11	9	15	0
112	1	22	5	2	7	4	21	7	11	17	2

Another study examined the survival of *B. japonicum* in various sludge-soil mixtures; the numbers of rhizobia decreased more rapidly in high-sludge mixtures (25). The sludge used by Reddy et al. (25) was very similar in metal content to that used in the current study. The difference in results between their study and the current study is probably due to the fact that their experiment was short term (42 days) and done in a greenhouse with air-dried soil. The decline in rhizobial numbers may have been caused by either toxic organic compounds or salts present in recently applied sewage sludge. These components would normally be degraded or leached from the soil over time. The metals in a freshly applied sewage sludge may also be in a relatively available form. Over time metals may be bound to soil components and made less available.

The application of sewage sludge actually increased the number of soybean rhizobia in the current study. Thus, possible decreases in nitrogen fixation in the high-sludge plots would not be a result of decreased rhizobial numbers. The increase in rhizobial numbers may have been caused by the addition of specific nutrients and organic matter. In addition to stimulating rhizobial numbers directly, the nutrients and organic matter may have stimulated plant growth over the past 11 years, which may have resulted in increased soybean rhizobial numbers. The organic matter from sludge application may also have improved the physical properties of the soil such as its water-holding capacity, drainage, and aeration, all of which are favorable for rhizobial growth. There was an observable improvement in the tilth of the soil in sludge-amended plots, even 11 years after sludge application.

Determination of serogroup distribution and proportion of resistant strains. If the heavy metals added to the soil by sludge application acted as a selective pressure, there are two ways that the rhizobial population may have responded. First, since there are differences in metal tolerance between different serogroups of soybean rhizobia, the metals may have selected for the more tolerant serogroups and against the most sensitive serogroups. This did not occur (Table 3). Soybean rhizobia from 11 different serogroups were identified, including rhizobia from serogroups 62, 110, and 122. The sludge applied had considerable amounts of Cu, Zn, Cd, and Ni. These are all metals to which soybean rhizobia in serogroups 62, 110, and 122 are resistant in comparison to soybean rhizobia in other serogroups.

A second response that the rhizobial population may have exhibited was the appearance and proliferation of strains more resistant to heavy metals than the other strains in the same serogroup. This may occur through spontaneous mutation or through dissemination of plasmid-mediated heavy metal resistance among the strains. It is possible that heavy metal resistance plasmids even may have been present in strains of bacteria from the original sludge applied to the soil. Heavy metal resistance may be linked with antibiotic resistance; antibiotic-resistant bacteria are often found in sewage sludge bacterial communities (9). Selection for heavy metal-resistant strains was not observed in this study; the heavy metal resistance of all of the isolated *B. japonicum* strains was tested on laboratory medium (data not shown), and no differences were found between their MICs and those of laboratory strains of the same serogroup.

All of the field experiments examined rhizobia that were isolated from the soil through soybean nodules. Thus the results reflect the effects of heavy metals only on the rhizobia which nodulate soybeans. However, the results can be generalized to most *B. japonicum* strains found in this soil since the soybean host used is highly promiscuous. Clark soybeans were shown to be nodulated by each of 14 different rhizobial strains tested in one study (19). In the current experiment, using only 17 different antisera, we identified a wide variety of rhizobia in the nodules, representing 11 different serogroups.

Since the increase in rhizobial numbers was not a result of strain shifts or an increase in resistant bacteria, it is evident that this soil had a buffering capacity that was sufficient to reduce the toxic effects on the soybean rhizobia. The addition of sewage sludge to soil would be expected to increase the buffering capacity of soil by the concurrent addition of organic matter. Therefore, it is unlikely that the limited application of heavy metal-contaminated sewage sludge to well-buffered soils would have an adverse long-term effect on *B. japonicum*.

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