Effect of Toxic Metals on Indigenous Soil β-Subgroup Proteobacterium Ammonia Oxidizer Community Structure and Protection against Toxicity by Inoculated Metal-Resistant Bacteria

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Received 8 April 1998/Accepted 17 July 1998

Toxic metal wastes from defense-related activities, industry, and municipal sources have routinely entered the environment through disposal in landfill sites or by accidental release in accidents such as that which occurred at Chernobyl. These practices have resulted in surface contamination problems, transport to groundwater, and/or bioaccumulation of radionuclides and toxic metals (see, e.g., references 8, 9, 21, and 31). Metals such as Cs, Sr, Cd, and, to a lesser extent, Co are prevalent in soils near industrial centers (see, e.g., references 7 and 21) at concentrations up to 50 μg of Cs/g, 350 μg of Cd/g, and 500 μg of Sr/g (31). As cocontaminants, toxic metals are often inhibitory to other bioremediative processes, e.g., hydrocarbon degradation (34).

Due to biotic and abiotic chemical dynamics, microbial metal toxicity is reduced by 1 to 2 orders of magnitude in soils relative to solution, depending on factors such as soil type, aeration conditions, metal speciation, carbon sources, pH, and Eh (2, 12). Microbial communities are of primary importance in bioremediation of metal-contaminated soils and represent a substantial proportion of the in situ biomass and metabolic diversity. The structure and diversity of soil microbial communities have been shown to change in soil in the presence of toxic metals (2, 13, 14, 28). Microorganisms can alter metal chemistry and mobility through reduction, accumulation, mobilization, and immobilization (1, 5, 20, 39). Since metal ion species are generally more readily soluble in acidic environments, acidojenic microbial metabolic activities may contribute to the introduction of metals into groundwater from contaminated soils.

In soils, the ammonia oxidizers that are members of the beta subgroup of the Proteobacteria (β-subgroup ammonia oxidizers) form an important part of the bacterial community, being chiefly responsible for the first step in the oxidation of immobile ammonia to highly mobile nitrate via nitrite (29). This process involves a concomitant release of protons, which can lead to significant soil acidification where the ammonia input is high (4). The coherent phylogenetic and physiological characteristics of the β-subgroup ammonia oxidizers has provided the opportunity to view them as an indicator species for environmental change (16, 18, 33, 37). By using 16S rDNA as a marker, specific changes in β-subgroup ammonia oxidizer populations have been observed to occur with changing pH (37), with addition of swine manure (16) to soil, through effects of salmon farm waste in marine sediments (37), and with proximity to the ocean and aging in coastal sand dunes (18). It is now practical to use a second molecular marker in environmental studies, amoA, the gene encoding the α-subunit of ammonia monooxygenase. Rotthauwe et al. (33) have demonstrated the use of a highly specific set of PCR primers to

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amplify a fragment of \textit{amoA} from a variety of pure cultures of β-subgroup ammonia oxidizers and environmental samples. In this study, we use \textit{amoA} as a functional molecular marker to assess whether any changes in the population structure of β-subgroup ammonia oxidizers could be induced by an 8-week exposure of an indigenous soil community to high levels of a mixture of toxic metal ions. This study had two aims. First, we wished to determine whether all indigenous species of β-subgroup ammonia oxidizers were equally susceptible to damage by toxic metals through observation of changes in the structure of different clones of libraries. Observation of unequal susceptibility may provide the basis for using β-subgroup ammonia oxidizers as an indicator group for the end point of metal removal or immobilization, defined as return of the impacted communities to control values. Our initial approach was to assess the natural diversity of \textit{amoA} genes by PCR amplification, cloning, and sequence analysis of selected clones (33). The second aim of this study was to assess whether inoculation of soil with a group of metal-resistant bacteria could reduce the bioavailability of toxic metal ions to the indigenous microflora. A second set of soil microcosms was created as above, but these were also inoculated with several bacteria which have been considered metal resistant in the literature. Here, the aim was to assess whether any changes to the β-subgroup ammonia oxidizer population would also be observed in the presence of these added bacteria or whether the activities of these bacteria might protect the indigenous β-subgroup ammonia oxidizer community from the toxic effects of the metals.

\section*{MATERIALS AND METHODS}

\subsection*{Soil microcosms.} Microcosms consisted of 150-ml polypropylene beakers (VWR Scientific) containing 75 g (dry weight) of sieved (2-mm) agricultural loam top soil (depth, 0 to 100 mm) from the University of Tennessee Agricultural Experiment Station in Alcoa (Sequatchie series). The soil was slightly acidic (pH 5.5) and contained 0.06% (wt/wt) organic carbon and 0.03% (wt/wt) total nitrogen. Indigenous \(\text{NH}_4\text{Cl} \cdot \text{NH}_2\text{O} \cdot \text{H}_2\text{O} \text{(dry weight)} \) and clay, and silt, and clay were measured at 35.3%, 32.9%, and 13.6% respectively. After metal or inoculation additions (final water content, 17% [wt/wt]), the microcosms were thoroughly mixed and the soils were compacted to 1.2 g/cm\(^3\) and loosely covered with foil for aerobic incubation. Metal extractions and soil pH.

\subsection*{Metal extraction was performed by shaking soil for 2 h in distilled water at 1:10 (soil dry weight/volume) solution. Filtrates were collected after centrifugation (1,500 \(\times\) g for 5 min). The soil pH was determined by using a pH combination electrode. (Plant and Soil Science Department, University of Tennessee, Knoxville, Tenn.), were measured by monitoring inductively coupled argon plasma atomic emission spectrometry (30). Samples were stored at 4°C for 1 to 4 weeks. Sr, Co, Mn, Fe, and Cd were measured after monitoring inductively coupled argon plasma atomic emission spectrometry (30). 

\subsection*{Statistical analysis.} Chi-square and Student \(t\) tests were performed with an Excel spreadsheet (Microsoft Office 97, Microsoft Corp.).

\subsection*{RESULTS AND DISCUSSION}

Bioavailability of metals in soil microcosms. No significant differences in the solubility of any of the metals added were detected between the inoculated and noninoculated soils. The percent availability of each metal at each time point consistently followed the order Sr > Co > Cs > Cd. The water-soluble fraction of each averaged 324.8 ± 14.7, 284.7 ± 17.1, 560.6 ± 198.1, and 213.6 ± 50.8 ppm, respectively (means ± standard deviations of 30 readings taken over 8 weeks).

\subsection*{Effect of metal addition on \(\text{NH}_4\) and \(\text{NH}_3\) levels in soil.} The ammonia and ammonium level in the test soil at time zero was...
25.0 ppm (Fig. 1). Ammonia and ammonium levels in the microcosms which were not treated by the addition of metals fell by 80% of this value over the first 4 weeks and then were stable until the end of the sampling period. The ammonia and ammonium levels in the metal-treated microcosms fell by only approximately 30% of the initial level over the first 4 weeks and then accumulated between weeks 4 and 8.

**FIG. 1. Ammonia and ammonium levels in soil microcosms.** Ammonia and ammonium levels were determined as described previously (36). Week 0 values are given for the combined analysis of all microcosms \((n = 12)\). All others are given for triplicate microcosms. \(\text{NH}_3\) and \(\text{NH}_4^+\) levels fell rapidly in uncontaminated soils and stabilized by week 4. \(\text{NH}_3\) and \(\text{NH}_4^+\) levels fell more slowly in metal-contaminated microcosms and then accumulated between weeks 4 and 8.

**Recovery of amoA gene fragments from soil.** Single PCR products of the predicted size (ca. 490 bp) were recovered from each soil sample taken from week 0 and week 4 microcosms by primary amplification with the primers \(\text{amoA-1F}^*\) and \(\text{amoA-2R}\). Amplification products were generated from all week 8 microcosms which were not treated by the addition of metals and stabilized by week 4. 

**amoA** gene fragments from soil. Cloned **amoA** gene fragments were amplified from the cloning vector by using primers directed at the T7 and M13 reverse RNA polymerase binding sites, producing a fragment with approximately 70 bp of vector sequence on each end. The vector sequence contained no \(\text{MspI}\) recognition sites. Products were digested with a twofold excess of \(\text{MspI}\) for 1 h, analyzed by electrophoresis on a 2% agarose gel with TAE buffer, and visualized by ethidium bromide fluorescence. Lanes: 1, molecular size marker (100-bp ladder; Boehringer); 2, 9, and 19 pattern 1 amoA; 10, 17, and 18, pattern 2 amoA; 3 to 8, 11 to 16, and 20, pattern 3 amoA clones.

**Diversity of recovered sequences.** Neighboring-joining analysis revealed four clusters of **amoA** sequences and 10 single sequences that did not fall within any of the four clusters. Independent analysis of the 3’ 205 bases and the 5’ 244 bases demonstrated poor stability in placement of 9 of the 10 single sequences, which were therefore tentatively regarded as chimeric artifacts of the PCR amplification process (37). Bootstrap analysis (100 replicates) also provided values of less than 50 for placement of these sequences. Bootstrap values of 100% values were recovered for support of each cluster and the grouping of sequence NAB8_C11 with clone SP-1 (32). Sequence clusters B and C also grouped together with 100% bootstrap support. The only systematic difference between the translated protein sequences of the clusters is the conservative exchange at position 195 of an isoleucine in clusters A, B, and C (typical of published Nitrosomonas-related AmoA primary sequences) with a valine in cluster D sequences (typical of published Nitrosospira-related AmoA primary sequences). Comparison with the available data from cultured species (compiled in reference 33) suggested that all the sequences recovered in this study were derived from members of the genus Nitrosospira (Fig. 3). Despite selection of all variants of \(\text{MspI}\) restriction digestion patterns from the first 213 randomly selected clones analyzed, no sequences related to the genus Nitrosomonas were recovered. This may reflect the apparently small number of Nitrosomonas cells compared to Nitrosospira cells as previously recorded in agricultural soil (16, 37). Equally, it may suggest that the Nitrosomonas species inferred to exist in soil carry **amoA** sequences that are not compatible with the primer set chosen. Rotthauwe et al. (33) predicted that this may be the case for some untested lineages of the genus Nitrosomonas.

Although it would be naive to attempt to determine the number of ammonia oxidizer species in these samples from the sequences of a single gene, some points of interest can be made. Multiple copies of **amoA** are carried by a number of \(\beta\)-subgroup proteobacterium ammonia oxidizers. Nitrosospira multiformis, Nitrosospira sp. strain AV, and Nitrosospira sp. strain 39-19 (24, 25) carry three nearly identical copies of **amoA** (Fig. 3). This near identity is reflected in the short
branch lengths separating the gene copies. The much longer branch lengths separating the four clusters and the one isolated representative of amoA sequences presented here suggest that they have been derived from at least five distinct strains of β-subgroup ammonia oxidizers.

Comparison of the population structures of clone libraries. MspI digestion pattern analysis of amoA clones from these microcosms was selected as an analytical tool due to its rapidity and the observation that some phylogenetic information was maintained, inasmuch as two of the four supported clusters could be identified. amoA sequence clusters B (pattern 1) and C (pattern 2) could be distinguished from each other and from clusters A and D (pattern 3) with 100% accuracy as judged from the initial survey of 20 recovered sequences within these groups (Fig. 3). Clusters A and D could not be differentiated by this method; therefore, any changes in the abundance of these
groups relative to each other were not detected. Clone libraries were generated from each soil microcosm and between 36 and 47 clones analyzed for each soil sample (Fig. 4). At time zero, clone libraries were dominated by sequences related to clusters A and D (80%), and the remainder were split equally between clusters B and C. After the 8-week microcosm incubation, the structures of all recovered libraries had changed significantly as judged by chi-square analysis. The greatest positive change was a relative increase in the proportion of cluster B (pattern 1) sequences.

Effect of toxic metals. The effect of metal addition after 8 weeks was seen in a comparison of libraries recovered from microcosms treated or not treated with toxic metals. These populations were significantly different, with cluster C (pattern 2) sequences being present in higher relative abundance after metal treatment, suggesting that the source organisms carried a selective advantage over other detectable β-subgroup ammonia oxidizers in the toxic-metal-treated soils.

Effect of the metal-resistant inoculum on metal solubility and changes to the β-subgroup ammonia oxidizer population. Extraction of metals from soils in water did not indicate that the presence of the inoculated metal-resistant bacteria had any significant effect on the solubility of any of the added metals (data not shown). The presence of the inoculum did not appear to influence the β-subgroup ammonia oxidizer population structure of noncontaminated microcosms, as seen in the comparison of libraries from 8-week-old untreated soil and untreated soil plus inoculum. Therefore, the inoculated bacteria did not affect the ammonia oxidizer population structure in the absence of toxic metals. A highly significant difference in population structure was, however, seen between amoA libraries recovered from metal-treated soils with and without the presence of the metal-resistant bacteria (P < 0.003). Further, there was no significant difference in the β-subgroup ammonia oxidizer population structure of 8-week-old untreated microcosms and that of metal-contaminated soils treated with the inoculum. These results demonstrated that the source organisms of amoA cluster C sequences gained no selective advantage over other indigenous β-subgroup ammonia oxidizers in the presence of toxic metals when the inoculum was present. We interpret this finding as evidence that the addition of the inoculum had lowered the bioavailability of the toxic metals to the β-subgroup ammonia oxidizer community sufficiently to protect it from specific metal-induced population change. The fact that no differences in metal availability were detected following water extraction may have been due to high standard errors on replicate samples, a weakness in the salt-free water extraction method (30).

Abundance of target amoA sequences in soil microcosms. To compare changes in the total number of ammonia oxidizers per gram of toxic-metal-contaminated soil, competitive PCR for amoA sequences was used. This demonstrated that the sequences targeted by these primers dropped from approximately 2.3 × 10^5 copies per g of soil to 7 × 10^3 to 8 × 10^4 copies per g of soil over the first 4 weeks of the experiment in the presence of metals, irrespective of the presence of the inoculum. The ability to retrieve amplification products from week 8 soil samples was too inconsistent to gain an accurate estimate of target numbers, presumably since their numbers had dropped close to detection limits. These values were similar to those for the non-metal-treated soils (data not shown). Thus, the decrease in numbers of amoA target molecules was attributed to incubation under laboratory conditions. The presence of the inoculum did not protect the β-subgroup ammonia oxidizer population from this effect (Fig. 5).

Conclusions. Due to the incomplete nature of the available amoA data set with respect to cultured organisms of the 16S phylogeny proposed previously (17), it is impossible to state what proportion of the soil β-subgroup ammonia oxidizer community was targeted by the PCR primers used. This is particularly notable in that phylogenetic interpretation of all available 16S rDNA sequence data from cultures, enrichments, and environmental clones suggests that the amoA sequences available from cultured organisms represent members of only 16S rDNA clusters 3 and 6/7 (32, 37). However, the amoA DNA sequence data presented here strongly suggests that the target organisms in the soil tested are quite distinct from any cultured organisms for which amoA sequence data is available. In this system, the source organisms of cluster C amoA sequences carried a demonstrable selective advantage over the other target organisms within the β-subgroup ammonia oxidizers following exposure to toxic metals. The rapid decline in amoA targets and the change in community structure associated with incubation of the soil under laboratory conditions preclude any strong conclusions on the effect of toxic metals on this group in the field. Nonetheless, sufficient evidence has been gathered to provide a working hypothesis which can now be tested at contaminated sites. Should the β-subgroup ammonia oxidizer community structure, measured as described, show elevated levels of cluster C amoA over neighboring control sites, a rapid and sensitive method will be available for the determination of a defensible end point to toxic-metal bioremediation. Reinstatement of the ratios of amoA sequence types to local control values may become a valuable measure of metal bioavailability.

The finding that the changes induced in the indigenous β-subgroup ammonia oxidizer population by toxic metals were abolished by the addition of exogenous bacterial species also supports the use of metal-resistant bacteria in reducing the bioavailability of metal species at sensitive sites. Determination of which member(s) of the five-species inoculum was responsible for this effect is under investigation.

ACKNOWLEDGMENTS

This work was supported by Department of Energy, Office of Energy Research, grant DE-FC02-96ER62278White as part of the Assessment Component of the Natural and Accelerated Bioremediation Research Program (NABIR), administered by John Houghton to D.C.W.
We thank Werner Liesack for helpful discussion and Julia Brüggemann for thoughtful comments on the manuscript.

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