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# Ecological Interactions Between Metals and Microbes

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**RESULTS TO DATE:** 1. Analyses of chromium resistant microbes. Culturable xylene-degrading and chromate-resistant microbes were obtained from chronically cocontaminated soil using a microcosm enrichment technique, and shown to correlate to dominant soil populations using culture independent techniques. The soil microbial community proved able to mount a respiratory response to addition of xylene in the presence of chromate. The majority of isolates belonged to the ubiquitous but poorly studied high %G+C Gram positive genus Arthrobacter, and exhibited considerable genotypic and phenotypic variability. Phenotypic assays uncovered a wide variation in the levels of chromate resistance, even between very closely related strains. Primers designed against conserved motifs in the known chrA chromate efflux gene failed to detect similar sequences among the chromate resistant Arthrobacter isolates obtained through enrichment. These results suggest Arthrobacter species may represent dominant populations in chromate-contaminated soils, and may contribute to continued degradation of xenobiotic compounds; furthermore, the isolates obtained may possess novel chromate resistance genes. A predicted plasmid-borne chromate resistance gene cluster from an unrelated Arthrobacter strain was isolated, sequenced, and characterized. This cluster was found to possess putative homologues of well known chr chromate resistance genes, but in a highly unusual arrangement. The sequencing and annotation of the predicted chromate resistance loci revealed that chrA is 1359 bp long, with a 65% G+C content; chrB is 930 bp long, with a 69% G+C content. The translated product of the chrA gene from pCHR15 shared greatest similarity with predicted chromate efflux proteins from Mesorhizobium loti (50.2%), Magnetospirillum magnetotacticum (49.6%), and Sinorhizobium meliloti (49.7%). Much less similarity existed between ChrA and previously described chromate transporters from R. metallidurans (31.1%) and P. aeruginosa (31.1%). The two closest matches with other Gram-positive organisms were for predicted chromate transporters in Bacillus halodurans (28.7%) and B. cereus (28.6%). The translated product chrB shared greatest similarity with a predicted chromate resistance signal protein from R. solanacearum (34.9%), an uncharacterized conserved protein from Burkholderia fungorum (35.0%), R. metallidurans ChrB2 (36.6%), R. metallidurans ChrB1 (32.6%), a putative chromate resistance protein from captured environmental plasmid pB4 (37.2%), and a predicted chromate resistance signal protein from Chromobacterium violaceum (31.6%). The chrB and chrA genes were found to be separated by over 7 kbp of plasmid DNA, containing ORFs that did not resemble genes from previously characterized chr clusters. Furthermore, the chr genes appeared to have a Gram negative ancestry. The chr genes conferred chromate resistance to a previously sensitive Escherichia coli recipient, though at severely reduced levels compared to the original Arthrobacter host. The resistance assay indicated chrB has positive effects on hosts that are independent of chromate, while chrA had inhibitory effects at lower concentrations, and positive effects at higher concentrations. The low degree of chromate resistance conferred to sensitive hosts transformed with chrA and chrB sequences suggest other genes may encode additional functions involved in the high resistance phenotype.

2. Analyses of Pb resistant Arthrobacter. Previously we had isolated a lead resistant Arthrobacter sp. from the sampling site at Seymour IN, denominated SI.1. From these strain we generated a mutant lead sensitive strain denominated SI.1.1. It was of particular interest to determine the death rate of SI.1.1 upon exposure to to 75micromolar Pb. Growth experiments were performed, in which both strains, SI.1 (wild type/lead resistant) and SI.1.1 were inoculated on mXBM + or - Pb and were grown to mid-logarithmic phase, cells were washed, followed by a dilution on: mXBM (-) glucose + 75 micromolar Pb and mXBM (-) glucose + water (control). Samples were taken and diluted on MES buffer + 0.5 mM Na citrate. Dilutions were spread on LB plates following incubation at 30 C. CFU's were calculated. As a result SI.1.1 (lead sensitive strain) was

not killed upon exposure to Pb, on the contrary these cells started to grow exponentially 26 hrs after exposure to Pb. We were not able to determine the CFU's because the dilution plate was too numerous to count. The wild type and the controls grew up to 108 cells. Since the lead sensitive strain, appears to grow on Pb, our next objective was to determine if Pb genes are still present on sensitive strain SI.1.1. After SI.1.1, started to grow on the presence of Pb, bacteria were isolated of mXBM + Pb plates. Whole cell PCR was performed with primers designed from SI.1.1 Pb resistant gene. DNA of all the isolates tested had positively amplified by PCR; these results indicated that lead gene was now present in the mutants after exposure to 75 micromolar Pb, even though a PCR product was not found in the sensitive strain before selection on Pb. Lead resistant gene (plasmid) of Arthrobacter sp. SI.1 (pSI.1) had been sequenced. Our research had been focused in determining the mechanism of lead resistance of SI.1. Complementation experiments in a lead sensitive E. coli (RW3110), using the lead resistant plasmid were performed using PBLUESCRIPT II SK, as vector. A 17.7 kb fragment was transformed in to RW 3110 and Pb resistance was obtained, this strain was denominated pKJ60. This fragment contains: (ORF 69) arsR/merR type transcriptional regulator, (ORF 3) P-type ATPase, similar to cad A, (ORF 63) lipoprotein signal peptidase, (ORF 44) ccdA like protein, (ORF 54) cad D like protein and other fractions of the pSI.1 plasmid. The five ORF listed are our focus in determining the mechanisms of Pb resistance, therefore 2 more mutants were created: pKJ61 which included ORF 69, 3 and 63 and pKJ62 which included all 5 ORFs. Both pKJ61 and pK62 were able to grow on Pb up to 200 micromolar. We proceeded to eliminate ORF's from the mutant strain pKJ61 by restriction digest. Each ORF was eliminated independently, and it was determined that ORF 3 and ORF69 (the regulatory gene and the P-type ATPase) are required for Pb resistance. The direction of the research now, is to determine the MIC's of all the mutant strains upon exposure to Pb and other metals like Zn, Cd, Ni and Co. Our current hypothesis is that the mechanism for Pb resistance is similar to the one observed in Zn and Cd resistance. Also it is of special interest to test other metals because they have similar physical-chemical properties to Pb and therefore it is likely that the same mechanism of resistance is involved.

3. Responses of anaerobic microbial communities to chromium stress. Anaerobic microcosms were created with soil, an (i) organic energy source - glucose or protein, (ii) a terminal electron acceptor (Fe (III) or NO3-) and (iii) no, low, medium or high levels of Cr(VI) - concentrations that produced reductions of acute microbial activity of 0, 33, 50, or 75%, respectively. Some microcosms were subjected to a second addition of Cr(VI). The addition of glucose stimulated respiration in the microcosms without an apparent lag regardless of the presence of Cr. In addition, Cr(VI) was not detected in any microcosm 48 hours after set-up. These data suggest the presence of a Cr-resistant community, capable of utilizing glucose, in these soils. Chromium had an inhibitory effect on the rate of CO2 production with higher doses of Cr(VI) producing a larger rate decrease. Respiking only had an effect on microcosms which received high doses of Cr(VI), by reducing both the rate of CO2 production and the maximum CO2. These results suggest that detoxification of Cr(VI) is needed before the added carbon source can be used. The addition of protein to microcosms without Cr(VI) stimulated respiration without an apparent lag. In microcosms which received protein and Cr(VI), the CO2 concentration increased in two phases - an initial phase of slow CO2 accumulation and a second phase of faster accumulation. The length of the initial slow phase increased with increasing concentration of added Cr(VI). Chromium (VI) was detected in all Cr amended gelatin microcosms on day 2 at levels of about 10% of the theoretically added. When the different treatments were sampled next - at the start of the fast CO2 accumulation phase - Cr(VI) was not detected. The addition of a second Cr(VI) dose had an immediate inhibitory effect on respiration in all three Cr treatments, which was most pronounced in microcosms with high levels of Cr(VI). PCR-DGGE was used to study the changes in the microcosm soil microbial communities. We have only analyzed the data from Fe(III) amended microcosm. In glucose amended microcosms, the number of intense bands in microcosms increased (a) with time across all treatments and (b) in treatments in which Cr(VI) was added. Communities in microcosms which received Cr differed from those in which no Cr was added, however, there was no effect of the amount of Cr added. In protein microcosms, the number of intense bands across all treatments significantly increased at the beginning and remained constant for the rest of the experiment, however there were no significant effects of Cr addition.

There were four major bands that were present in most of the microcosms that received protein. We are currently sequencing dominant bands from PCR-DGGE profiles. We isolated a total of 101 and 70 CrR strains, respectively, from glucose and protein microcosms amended with Fe(III) and medium Cr levels. Nearly all of the isolated strains were also able to grow at 0.5 mM Cr(VI) and a substantial number were able to grow to levels of up-to 10 mM Cr(VI) in the media. We are currently working on sequencing those isolates which match dominant bands on the PCR-DGGE profiles. Real-time PCR was used to follow the population changes of members of the Geobacteraceae family in some Fe(III) amended microcosms. In microcosms which received carbon source but no Cr(VI), copy numbers of the 16S rDNA gene for Geobacteraceae increased when CO2 was produced at the maximum rate and then decreased to the same levels which were present at the beginning of the experiment. In contrast, in microcosms amended with high levels of Cr, the copy number of the 16S rDNA gene for Geobacteraceae remained constant throughout the entire experiment, suggesting that the iron-reducing bacteria in these soils were adversely affected by Cr(VI) addition.

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