ABSTRACT

Distinct microbial communities had been found in contaminated soils that varied in their concentrations of Pb, Cr and aromatic compounds. It is difficult to distinguish between their effects as their presence is highly correlated. Microcosms were constructed in which either Pb^{+2} or CrO_4^{-2} was added at levels that produced acute modest or severe acute effects (50 or 90% reduction). We previously reported on changes in microbial activity and broad patterns of Bacterial community composition. These results showed that addition of an organic energy source selected for a relatively small number of phylotypes and the addition of Pb or Cr(VI) modulated the community response. We sequenced dominant phylotypes from microcosms amended with xylene and Cr(VI) and from those with the simple addition of glucose only. In both cases, the dominant selected phylotypes were diverse. We found a number of distinct *Arthrobacter* strains, as well as several *Pseudomonas* spp. In addition, the high GC-content bands belonged to members of the genera *Nocardioides* and *Rhodococcus*. The focus of amended microcosm work has now shifted to anaerobic processes. The reduction of Cr(VI) to Cr(III) as a detoxification mechanism is of greater interest, as is the specific role of particular physiological groups of anaerobes in mediating Cr(VI) detoxification.

The correlation between microbial activity, community structure, and metal level has been analyzed on 150 mg of soil collected at spatial scales <1, 5, 15 and 50 cm. There was no correlation between metal content and activity level. Soils <1 cm apart could differ in activity 10-fold and extractable Pb and Cr 7-fold. Therefore, we turned to geostatistical analysis. There was spatial periodicity which is likely to reflect the heterogeneous distribution of active microbes and metal contaminants. Variograms indicated that the range of spatial dependence was up to 20 cm. To visualize the spatial relationships between the primary variate (activity) and its covariates (lead and chromium content), block kriging was used. The kriging maps suggest that areas exist where increased metal concentrations have zones of decreased metabolic microbial activity.

Cr(VI) resistant bacteria have been isolated from two contaminated sites. Most isolates are *Arthrobacter*, *Rhodococcus*, or *Pseudomonas* spp. A *chrA* gene has been cloned from *Arthrobacter* strain CR15 isolated from Cannelton, MI. PCR-primers have been produced against conserved motifs analyzed from 8 *chrA* sequences. Of the 96 Cr-resistant isolates from Cannelton, 85% gave a positive reaction to these primers. In contrast, none of the 38 isolates from Seymour, IN were positive. Therefore, at least for the culturable community, a particular resistance determinant appears to be widespread at a geographical site but rare (absent) at another site. The phylogenetic relatedness of the *Arthrobacter* strains is being evaluated via the distribution of repetitive elements as well as genome-wide restriction fragment analysis. Work to date on the latter has also suggested that *Arthrobacter* genomes are small (<2.5 Mbp). Gene capture experiments demonstrated that chromate-sensitive Gram-negative bacterial strains could obtain resistance from Cr-contaminated soil. However, frequency of transfer is low (10-6-10-8). Genetic diversity of the acquired chromate resistance mechanism is being assessed.

Role of *Arthrobacter* in Chromate Contaminated Soils

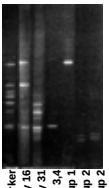
Objectives

INDOT soils spiked with Cr^{6+} and xylenes in aerobic microcosms become enriched in *Arthrobacter* populations, detected using molecular approaches (DGGE) and selective plating. Bacteria were isolated as either Cr^R or as xylene degraders (Xy^+) and those within the genus *Arthrobacter* were described using various molecular techniques.

Methods

- Isolates were obtained from microcosms dosed with 18 mg kg^{-1} Cr(VI) over a one month period. Soil dilutions were plated on media selective for xylene degradation, Cr resistance, or both. Xy^+ and Cr^R isolates were readily obtained; no growth occurred on Cr plates if xylene was the sole carbon source.
- Isolates were purified and grouped based on 16S rDNA enzyme (*Rsa*I) digest patterns, and subgrouped based on Rep PCR patterns (BOX primers) and 16S sequence data.
- Total genome restriction patterns were obtained for selected *Arthrobacter* strains (*Xba*I. digest, fragments separated by PFGE 50-500 kb).

Isolate 16S digest profiles

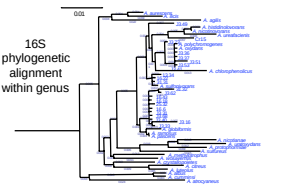


DGGE profiles for 16S group isolates:

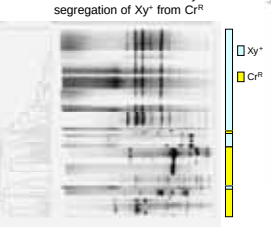
16S rRNA BLAST analysis

Xy^+ isolates,
1: *Pseudomonas* (15 strains)
2: *Rhodococcus* (23 strains)
3: *Arthrobacter* (66 strains)
Cr^R isolates:
4: Arthrobacter (38 strains)

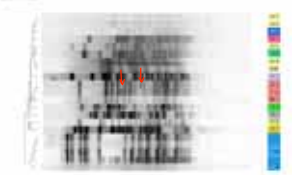
16S phylogenetic alignment within genus



Arthrobacter BOX PCR analysis: segregation of Xy^+ from Cr^R

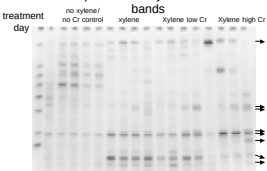


Arthrobacter *Xba*I. restriction mapping: Grouping is similar to BOX-groups (indicated by colors). Differences in pattern were observed within Box groups (see →).



Genome size estimation: 2.3 – 2.5 Mb

Microcosm DGGE profiles and sequence identity of dominant bands



1) *Pseudomonas* spp. 2) *Brevibacillus* 3) *Arthrobacter* 4) *Nocardiodes* 5) *Rhodococcus*

Isolation of Cr^R Genes in *Arthrobacter* Species

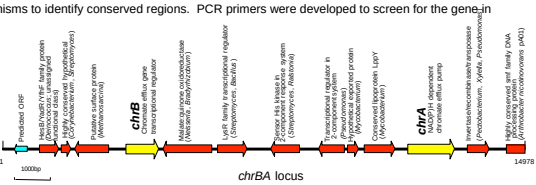
Objectives

Determine the role of mobile elements that confer chromate resistance. Resistant microbes continue to be physiologically active at the site, catabolizing organic compounds and generating reductive metabolites for Cr^{6+} detoxification. These microbes act as reservoirs for transferable metal resistance.

Methods

- *Arthrobacter* Cr15 was isolated from Cannelton Industries tannery site in Sault Ste. Marie, MI. Cr15 tolerates 20 mM Cr^{6+}. Resistance was traced to a 50 kb plasmid, pCR15, which was enzymatically digested into fragments that were shotgun cloned into an *E. coli* cDNA library for sequencing.
- A potential Cr resistance gene was aligned with ones from other organisms to identify conserved regions. PCR primers were developed to screen for the gene in other *Arthrobacter* isolates.

- Sequence data suggests pCR15 is conjugative, with competence factors enabling environmental DNA uptake; loci might be further mobilized via transposition
- Putative chromate response genes were identified in a domain rich with sensory/response elements and genes for cell surface proteins

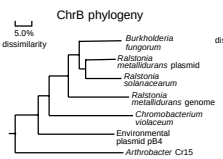


chrBA locus

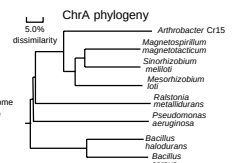
Designing PCR Primers for Cr^R in *Arthrobacter*

- *chrA*/*chrB* genes are described for *Pseudomonas aeruginosa* (Alvarez *et al.*, 1999, J Bacteriol 181: 7398-7400) and *Ralstonia metallidurans* (Juhnke *et* al., 2002, Arch Microbiol 179: 15-25). ChrA: NAD(P)H-dependent active chromate efflux mechanism. ChrB: transcriptional regulator for ChrA
- Potential *chrA* genes are identified in numerous bacterial genomes and plasmids; *chrB* genes are uncommon

ChrB phylogeny



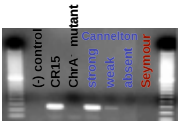
ChrA phylogeny



ChrA conserved amino acid motifs

Cr15	AAIVTFGGAYAVLAYV	SVLVT.WVTFVPSFLF
		GGLLTTWVTFAPCFLW
		GALLTTWVTFVPCFLW
Mag	MAMVTFGGAYAVLSYV	GALLTLWVTFTPCFFW
Sin	MAMVTFGGAYAVLSYV	GACVAAAATFLPCYLF
Mes	MAVVTFGGAYAVLAYV	GAVAMTVGVFLPAFAF
Ral	GAFV.FGSGLAIVPFL	GGLIATVAIFLPAFLL
Pse	GLL.TFGGAYTAIPFV	GAILATIAIFLPAFLL
B ha	GALV.FGGGHVVLPLL	
B ce	FGG motif GGHVVLPLL	FVP motif

forward primer — reverse primer



PCR results:
- 96 Cr^R *Arthrobacter* isolates from three independently established Cannelton culture collections:
  Positive: 67 Weakly positive: 13 Negative: 16
  (ChrA*, some binding site degeneracy)
- 38 Seymour isolates: all negative

Capture of Cr Resistance Genes from Heavy Metal Contaminated Soil

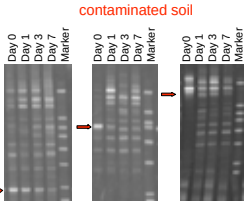
Objectives

Determine the ability of various bacterial strains to become Cr^R from heavy metal contaminated soil. Asses the effect of chromate concentration on gene capture efficiency.

Methods

Soil samples from Seymour site (contaminated with chromium and lead) were inoculated with chromate sensitive (MIC < 1 mM), rifampicin resistant strains: *Pseudomonas* sp., *Arthrobacter* sp., and *Escherichia coli.* Inoculum size was 10^7-10^{10} cells/g soil. Samples were screened for Cr^R, Rif^R transformants on Day 0, Day 1, Day 3, Day 7. Survival of the inoculum was monitored by DGGE and culturing. Controls were uninoculated and sterile soil samples.

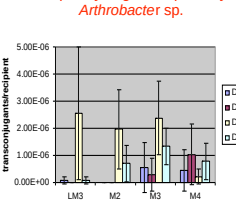
Survival of inoculum strains in the heavy metal contaminated soil



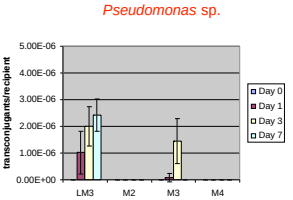
Arthrobacter sp. *E. coli* *Pseudomonas* sp.

Intensity of inoculum bands decreased with time. *E. coli* band was not visible by Day 3. All inoculant strains were reisolated on Day 7; their counts were 0-2 orders of magnitude lower than the original.

Frequency of gene capture by *Arthrobacter* sp.



Frequency of gene capture by *Pseudomonas* sp.



Preliminary results on frequency of gene capture show a low frequency of 10^{-6}-10^{-8} transconjugants/recipient for all recipient strains. Variability within and between soil samples was high. Reisolation of the original inoculum was confirmed by Box-PCR. Isolates are currently being screened for the presence of acquired plasmids. Low number of Cr^R mutants were obtained from the sterile soils as well, indicating that conjugation is not exclusively responsible for resistance. Spontaneous Cr^R mutants in the absence of soil were not observed.