Natural Gene Transfer to Develop Resistance to Metal Toxicity in Microbial Communities at the Oak Ridge FRC

Project Number EE-595-EEDA

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FRC conditions present challenges to in-situ bioremediation strategies

**Problematic conditions**

1. High nitrate (~1000 ppm in Area 2)
2. Acidity
3. Heavy metals (Ni, Al...)

**Result**

1. Too high redox for stimulating sulfate and Fe reducers
2. Affects metal bioavailability, and thus, toxicity
3. Inhibit nitrate reducers

Will introduction of nickel resistance into indigenous microbial community have a positive effect on nitrate reducers and stimulate iron and sulfate reducers?
Project overview

**Goal:** Immobilize uranium in contaminated sediments via microbial reduction and precipitation

**Problem:** Active uranium reducers are inhibited by co-contaminants in complex waste streams (e.g., heavy metals)

**Major project objectives**

Demonstrate application of natural gene transfer to improve community function under increased levels of toxic metal stress *(van der Lelie, BNL Biology Dept.)*

Demonstrate ability to enhance uranium immobilization in ORNL sediments by indigenous microorganisms that have adopted the toxic metal resistance marker *(Fitts, BNL Environmental Sci. Dept.)*
Project design schematic – presentation outline

- FRC soils
- Total community
- Nickel stress
  1. Community structure
  2. Improved nitrate red.
- Horizontal gene transfer (in vivo)
  – soil columns
- Model organisms
  Isolated from FRC fluidized bed reactor
  Strain construction (in vitro)
- Ni resistance
  mechanisms
  1. S and Fe reduction
  2. Uranium reduction and precipitation

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Construction of model organisms

- Ralstonia
- Cupriavidus

Metal resistant gene

- Plasmid pMOL222 (IncQ): broad host replication and mobilization
- Mini Tn5 single hopper transposons: provide stability due to loss of transposase gene

Minimum required for Ni resistance: nreB
nre gene provides Ni resistance to broad range of strains

1. Broad expression range for nre encoded Ni resistance in both proteo and gram positive bacteria
   - proven concept (Dong et al., 1998; Taghavi et al., 2001)

2. Kanamycin selection marker
### Ni resistance introduced into nitrate reducers

<table>
<thead>
<tr>
<th>Species</th>
<th>pMOL222 transconjugants</th>
<th>Tn5::ncc-nre transconjugants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic MIC (mM)</td>
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</tr>
<tr>
<td><strong>Enterobacter M-53</strong></td>
<td>3 - 6</td>
<td>3</td>
</tr>
<tr>
<td><strong>Enterobacter DM-S</strong></td>
<td>3-6</td>
<td>3</td>
</tr>
<tr>
<td><strong>Klebsiella DM-C3</strong></td>
<td>1-2</td>
<td>2 - ≥3</td>
</tr>
<tr>
<td><strong>Pseudomonas DM-Y2</strong></td>
<td>1 - 2</td>
<td>&lt;1 - 1</td>
</tr>
<tr>
<td><strong>Iodobacter DM-K3</strong></td>
<td>&lt; 1 - 2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Chromobacterium DM-N3</strong></td>
<td>&lt; 1 – 2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Janthinobacterium M-A11</strong></td>
<td>&lt; 1 – 3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Stenotrophomonas M-A15</strong></td>
<td>Not available</td>
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</tr>
<tr>
<td><strong>Shewanella MR-1</strong></td>
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### Nickel-resistant wild type

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<tr>
<td><strong>Pseudomonas DM-H2</strong></td>
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- **Organisms isolated from fluidized bed reactor treating FRC groundwater**
- **MIC values independent of plasmid vs. genomic insertion**
Successful gene transfer is confirmed on the genome level

**BOX PCR**
1: receptor P. DM-Y2
2: transconjugant
3: Donor E. coli CM2034 (in case of ncc-nre E. coli CM 2520)

**nreB PCR**
4: receptor P. DM-Y2
5: transconjugant
6: Donor E. coli CM2034 (in case of ncc-nre E. coli CM 2520)

* 1 kb ladder

Both plasmid and genomic insertion confirmed for all strains

*Pseudomonas* DM-Y2 pMOL222

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Plasmid transfer produces equally stable *nre* gene after 100 generations

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Both plasmid and genomic insertion exhibit equal stability in culture
Ni resistance mechanisms

Scanning Transmission X-ray Microscope imaging at the carbon K-edge

Washed cells exposed to Ni for 2 hrs

Rinsed cells are dried on microscope window

Carbonates not observed

O K-edge will be sensitive to NiO formation

Pseudomonas DMY2::ncc-nre exposed to 2 mM NiCl₂

Cluster image

Optical Density at 290eV

2.5μm

0.8μm

Carbonate in organic matrix

Carbonate in organic matrix

C=O carbonate

C=C carbonate

kx

280 285 290 295 300 305

Energy (eV)

Hz

1.2

1.0

0.8

0.6

0.4

0.2

0.0

Energy (eV)
Column experiments

Area 2 sediments (high nitrate low uranium)

Homogenization under atmospheric conditions

Inoculated columns with extracted indigenous community

Anaerobic mineral growth media w/ C:N:P of 100:10:1 (ethanol carbon source)

Operate under anaerobic conditions
Pseudomonas DMY2 tested in column studies

2,6 - FRC community
3,7 - FRC community + Pseudomonas wild type
4,8 - FRC community + Pseudomonas pMol222
5,9 - FRC community + Pseudomonas::ncc-nre

Kill 1, 2, 3, 4, 5, 6, 7, 8, 9
Geochemical interrogation: S, Fe & U at time zero

U distribution

U-Fe correlation

S speciation and redox state

U oxidation state at M5 edge

Area 2 FRC soil

Typical soil

Organic matter

Reduced organic S species

Inorganic sulfate

NSLS beamlines X27A & X15B
Geochemistry of columns after 65 days

Ni distribution in Column 2

Column effluent indicators

• Initial mobilization of Uranium
• Nickel breakthrough observed but significant adsorption occurs

Soil indicators by x-ray absorption spectroscopy

• Small increase in sulfide relative to kill (oxidation during transfer may be problem)
• Fe(III) oxides still dominate
• No reduction of Uranium observed
Column experiment after 78 days – nitrate analyses

• After 78 days significant nitrate reduction in the viable columns

• Negative effect of nickel on nitrate reduction
Column experiment after 78 days – sulfate analyses

- No significant reduction in sulfate is observed
Community analysis – Most Probable Number Counts

MPN Counts for nitrate, sulfate and iron reducers

- MPN for nitrate, sulfate and iron reducers
  - Increase of nitrate reducing & iron reducing organisms
  - Decrease of sulfate reducing organisms
  - Nickel has a negative effect on sulfate and iron reducing organisms
Community analysis - ongoing

- Relative number counts of nickel and kanamycin resistant bacteria
  - Enrichment of nickel resistant organisms
  - More nickel resistant organisms under pressure of nickel

Start

- 44 days with nickel
  - 1 mM Ni: 36%
  - 2 mM Ni: 34%
  - 3 mM Ni: 28%

- 44 days without nickel
  - 1 mM Ni: 77%
  - 2 mM Ni: 5%
  - 3 mM Ni: 3%

Km 43%
other 57%
Community analysis - ongoing

1. Constructed library with DNA extracted from community in the homogenized soil
   - TOPO TA cloning: 100 clones

2. Presently being sequenced; community composition will be determined at least on genus level
Findings and Future directions

1. Determine community composition of columns bioaugmented with engineered Pseudomonas DMY2

2. Quantitative PCR with nre targets to look for evidence of horizontal gene transfer in FRC column communities

3. Ni bioavailability measured with Ni biosensor, based on lux fusion (light production) with cnr (nickel resistance operon) in strain CH34

4. Explore Cupriavidus metallidurans strain CH34 which has natural resistance to a variety of metals

5. Extend findings to other systems, e.g. Hg (II) and Cr(VI) resistance
Acknowledgements

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Garry Crosson

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James Ablett (BNL NSLS) – Microprobe beamline X27A

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