Genomics-GTL Addressing DOE Environmental Science Needs in:

- *In situ* characterization of contaminated environments
- Understanding contaminant fate and transport
- Development of strategies for *in situ* control or remediation of contaminated sites
Tools From Genomes-GTL Applicable to DOE Environmental Restoration Needs

• *In situ* characterization of contaminated environments
  Molecular (mRNA) analysis of the *in situ* metabolic state of the microbial community via whole-genome analysis to reveal:
  - environmental stresses
  - nutrient requirements

• Understanding contaminant fate and transport
  Molecular (mRNA) analysis of *in situ* rates of metal reduction from levels of expression of key respiratory gene

• Development of strategies for *in situ* control or remediation of contaminated sites
  Prediction of fate of contaminants under natural attenuation or engineered bioremediation options by coupling *in silico* microbial models with the appropriate hydrological and geochemical models
Principle Investigators
Derek Lovley, UMASS, ecology, physiology, and biochemistry of Geobacteraceae
Maddalena Coppi, UMASS, genetics of Geobacteraceae
Stacy Ciufo, UMASS, bioinformatics, environmental genomics
Barbara Methe, TIGR, bioinformatics, DNA microarray gene expression
Pablo Pomposiello, UMASS, analysis of gene expression in response to stress
Steve Sandler, UMASS, microbial genetics
Cinthia Nunez, UMASS, microbial genetics, regulation of gene expression
Daniel Bond, UMASS, anaerobic microbial physiology, electron transfer to electrodes
Susan Childers, UMASS, microbial physiology, metabolic responses in Geobacter
Carol Giometti, Argonne National Laboratory, proteomics
Julia Krushkal, University of Tennessee, bioinformatics
Christophe Shilling and Bernard Palsson, Genomatica, in silico modeling
The primary goal of this research is to develop conceptual and computational models that can describe the functioning of complex microbial communities involved in microbial processes of interest to the Department of Energy.

**Microbial Communities to be Investigated**
1. Microbial community associated with the *in situ* bioremediation of uranium-contaminated groundwater.
2. Microbial community that is capable of harvesting energy from waste organic matter in the form of electricity.

**DOE Needs Addressed**
1. Remediation of metals and radionuclides at DOE sites
2. Development of cleaner forms of energy
3. Biomass conversion to energy
Analysis of Microbial Communities Will Focus Exclusively on the *Geobacter* Component in the First Three Years

**Rationale:**

1. *Geobacters* account for ca. 50-90% of the total microbial community in the environments of interest.

2. *Geobacters* are the primary organisms carrying out the processes of interest in these environments.

3. Environmental genomics studies enhanced by parallel pure culture studies.

4. A meaningful evaluation of the other highly diverse components of the microbial communities in the environments of interest is not currently feasible.
Microbial Bioremediation of Uranium

Uranium Contamination Removal Documented:
Groundwaters from DOE Hanford Site

Surface water from DOI site

Washings from DOD contaminated soil

**In situ Uranium Bioremediation Strategy**

*Geobacter* species comprise as much as 85% of the microbial community in the subsurface during the most active phase of *in situ* uranium bioremediation.

Geobacter Can Use Electrodes as an Electron Acceptor
Harvesting Power From Aquatic Sediments and Other Sources of Waste Organics

Cathode Reaction:
\[ 2O_2 + 8H^+ + 8e^- \rightarrow 4H_2O \]

Anode Reaction:
\[ C_2H_4O_2 + 2H_2O \rightarrow 2 CO_2 + 8H^+ + 8e^- \]

Geobacter species Comprise ca. 50% of the Microbial Community On the Anode

Fermentation

Pure Culture Geobatteries
Traditional Microbial Fuel Cell

- Organic Electron Donor
- Incomplete Oxidation Products
- Oxidized Electron Shuttle
- Reduced Electron Shuttle

GeoBattery

- Organic Electron Donor
- Carbon Dioxide
- Direct Electron Transfer to Electrode

Anode

- H₂O
- O₂

Cathode

- H₂O
- O₂
<table>
<thead>
<tr>
<th>Comparison of Geo Batteries with Previous Microbial Fuel Cells</th>
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<tr>
<td><strong>Previous Microbial Fuel Cells</strong></td>
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<tr>
<td>Oxidation of Organic Fuel</td>
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<td>Requirement for Toxic Electron Shuttles to Function</td>
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<tr>
<td>Recovery of Electrons As Electricity</td>
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<td>Long-Term Stability</td>
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<td>Ability to Function in “Open” Environments</td>
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Potential Applications of GeoBatteries

- Powering Monitoring Devices in Remote Locations
- Powering Electronic Devices from Renewable Energy Sources
- “Gastrobots”-robots fueled from food or organic waste
- Decentralized domestic power source
- Novel sensing devices
- Conversion of waste organic matter to electricity instead of methane
- Conversion of renewable biomass to electricity instead of ethanol
- Bioremediation of contaminated environments
- Powering automobiles
Application of Environmental Genomics and Systems Biology to Uranium Bioremediation and Harvesting Electricity from Waste Organic Matter

- Environmental Genomic DNA of “As-Yet-Uncultured” Geobacters
- Novel Culturing Strategies to Isolate Environmentally Relevant Geobacters
- Previously Cultured Geobacters
- Genome Sequencing
- Geobacter Genetic Potential
- Analysis of Gene Expression in Relevant Environments with Environmental Genome Arrays and Proteomics
- Functional Genomics Elucidation of Regulatory Systems Analysis of Gene Expression Physiological, Biochemical Studies
- In Silico Model of Cell Function
- In Silico and Conceptual Models for Optimizing Uranium Bioremediation and Electrical Energy Harvesting
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Discoveries from GTL with Direct and Immediate Application to NABIR

• Demonstration that *Geobacter* species can grow with oxygen as the terminal electron acceptor

• Elucidation of novel mechanism for *Geobacter* species to find and access Fe(III) oxides

• Elucidation of genes encoding for key respiratory proteins

• Elucidation of systems regulating expression key respiratory genes

• Elucidation of systems regulating:
  - growth under slow, environmentally relevant conditions
  - response to environmental stress
  - response to nutrient limitation

• Elucidation of novel central metabolism genes
Discoveries from GTL with Direct and Immediate Application to NABIR (continued)

• Development of an *in silico* model that can:

  -- Predict the response of *Geobacter* to different environmental conditions including strategies for manipulating the environment to promote bioremediation

  -- Aid in elucidating the likely outcome of genetically engineering novel metabolic capabilities in *Geobacter*

• Discovery of significant similarities in genomes of as-yet-uncultured *Geobacter* species and pure cultures of *Geobacter* species
Discoveries from GTL with Direct and Immediate Application to NABIR

• Demonstration that *Geobacter* species can grow with oxygen as the terminal electron acceptor

Provides explanation for the reservoir of *Geobacter* species in aerobic aquifers that can so rapidly respond to introduction of acetate and immediately start removing uranium from contaminated groundwater.
Genome-based Model for the Reduction of Oxygen and the Detoxification of Reactive Oxygen Species by *G. sulfurreducens*

**Outer membrane**

$\text{O}_2 \cdot^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O}$

**Inner membrane**

$\text{O}_2 \cdot^- + \text{H}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$

$\text{O}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{NAD} + \text{H}^+ + \text{H}_2\text{O}_2$

$\text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O}$

$\text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O}$

$\text{O}_2 + \text{H}_2\text{O}_2$

$\text{NADH oxidase(s)}$

$\text{ruberythrin(s)}$

$\text{thioredoxin peroxidase(s)}$

$\text{catalase}$

$\text{O}_2 + \text{H}_2\text{O}$
Genome-based Model for the Reduction of Oxygen and the Detoxification of Reactive Oxygen Species by *G. sulfurreducens*

**Outer membrane**

\[ \text{superoxide reductase} \quad \text{cyto } c \text{ peroxidase} \]

\[ \text{O}_2^\cdot - + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} \]

**Inner membrane**

- **Cyto bc\textsuperscript{1} oxidase**
- **Cyto c oxidase**
- **NiFe Hydrogenase(s)**

\[ \text{H}_2 + \text{O}_2 + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O} \]

- **ruberythrin(s)**
- **thioredoxin peroxidase(s)**
- **catalase**

\[ \text{O}_2 + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \]

\[ \text{O}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{NAD} + \text{H}^+ + \text{H}_2\text{O}_2 \]

\[ \text{H}_2\text{O}_2 + 2\text{H}^+ \rightarrow 2\text{H}_2\text{O} \]

\[ \text{O}_2 + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O} \]
Growth of *G. sulfurreducens* can grow with oxygen as the sole terminal electron acceptor.

Growth of *Geobacter* on oxygen provides an explanation for how *Geobacter* survives in low numbers in aerobic subsurface environments and then rapidly responds to the development of anaerobic conditions when uranium bioremediation is initiated.

Knocking out the cytochrome oxidase genes inhibits growth of *G. sulfurreducens* on oxygen

- Cytochrome oxidase is comprised of four genes, ORFs 374, 376, 378, and 380.
- Mutant is a deletion of 374, 376 and replaced with antibiotic resistance cassette.
- Mutant does not grow with O₂ but still can consume O₂.
- Implications:
  - Terminal oxidase is responsible for growth with low % O₂.
  - Inactivation of terminal oxidase does not affect the activity of oxidative stress enzymes.
Discoveries from GTL with Direct and Immediate Application to NABIR

• Elucidation of novel mechanism for *Geobacter* species to find and access Fe(III) oxides

Solves the mystery of how *Geobacter* species, which were thought to be non-motile, can efficiently access Fe(III) oxides via chemotaxis and thus compete for Fe(III) oxides even though *Geobacter* species require direct contact with Fe(III) oxides in order to reduce them.
Geobacter Specifically Expresses Flagella when only Fe(III) Oxide is Available as an Electron Acceptor.

Geobacter follows Fe(II) Gradient to Locate Fe(III) Oxides.

Geobacter May use Flagella to Make Initial Contact with Fe(III).

Geobacter uses Pili to “Twitch” Along Sediment Surface and Contact Fe(III).

Discoveries from GTL with Direct and Immediate Application to NABIR

- Elucidation of genes encoding for key respiratory proteins

Provides molecular targets for estimating rates of metal reduction in the subsurface.
Electron Transfer to Extracellular Electron Acceptors Such as Metals and Electrodes is Fundamentally Different than the Reduction of Commonly Considered Soluble Electron Acceptors

O₂, NO₃, SO₄

Fe(III) Fe(II)

Reduction at inner membrane or in the cytoplasm

???
Geobacter sulfurreducens has a unusually high percentage of genes devoted to electron transport, many of which encode for c-type cytochromes.
Model for Electron Transfer to Fe(III) in *Geobacter*

- **NADH**
- **NAD**
- **DH**
- **omcB**
- **OmpA**
- **OmcD**
- **MacA**
- **PppA,C,D,E**
- **Fe(III)**
- **Fe(II)**
- **MQ_{ox}**
- **MQ_{red}**
- **OmpB**
- **OmcE**
OmcB but not OmcC is Required for Fe(III) Reduction

Direct Correlation Between levels of \textit{omcB} mRNA and Rates of Fe(III) Reduction in Acetate-Limited Chemostats of \textit{Geobacter sulfurreducens}
Discoveries from GTL with Direct and Immediate Application to NABIR

- Elucidation of systems regulating expression key respiratory genes

This makes it possible to predict under which environmental conditions respiratory genes necessary for metals bioremediation will be expressed.
Environmental stimuli lead to signals that activate regulatory cascades to control transcription. These cascades include sigma factors, two-component systems, and other global regulators. Sigma factors like RpoS and RpoE, histidine kinases, response regulators, RelA, and Fur are involved. The ultimate response involves electron transfer, regulating gene expression.
Stationary phase may more closely represent physiological state in subsurface environments or on electrodes
Knocking out \textit{rpoS} affects the expression of at least 100 other genes
\textbf{Genes regulated include cytochrome genes required for Fe(III) reduction}
First definition of role of \textit{rpoS} in \textit{δ}-proteobacteria
Defining the RpoE regulon in *G. sulfurreducens*

An *rpoE* mutation affects the expression of at least 200 other genes

- **RpoE REGULON:**
  1. **Cytochrome genes** (7) and cytochrome biogenesis genes involved in Fe(III) reduction
  2. **Oxidative stress** regulon different from RpoS
  3. **Biofilm metabolism and development**
  4. **Biofilm electron transfer via H₂**
Rel A Plays an Important Role in Regulating Growth and Metabolism in *Geobacter sulfurreducens* under Environmentally Relevant Conditions

**Phenotype of relA Mutant**
- Increase growth under nutrient limitation
- Decrease growth in presence of oxygen
- Upregulation of genes involved in:
  - protein biosynthesis
  - cell division
  - transport
- Downregulation of genes for:
  - stress response
  - signal transduction
  - insoluble Fe(III) reduction

**Slower Growth** (protein synthesis, nutrient transport)
**Increased resistance to Oxidative Stress Resistance**
**Increased production of cytochromes for Fe(III) Reduction**
Microarray Results Comparing Wild Type to the fur Mutant

Regulators
Up regulation of 9 regulatory genes was found, including dtx, another iron regulated repressor.

Metabolism
Up regulation of 15 genes involved in metabolism including HydB, the hydrogenase responsible for hydrogen dependant growth.

Metal Uptake
Up regulation of 16 possible metal uptake genes was found, including FeoB, a ferrous iron cytoplasmic membrane transporter.

Cytochromes
Up regulation of 7 cytochrome genes was found, including OmcB and OmcD.

Unknown Proteins
Up regulation of 32 genes with an unknown function.
Knocking out a Histidine Kinase Sensor Inhibits Cytochrome Production

1-D and 2-D SDS-PAGE stained for c-type cytochromes (heme)
Fe(III)-Specific Regulation of Fumarate Respiration

Discoveries from GTL with Direct and Immediate Application to NABIR

• Elucidation of systems regulating:
  growth under slow, environmentally relevant conditions
  response to environmental stress
  response to nutrient limitation

Provides information necessary to interpret the *in situ* metabolic state of *Geobacter* species in the subsurface.
Discoveries from GTL with Direct and Immediate Application to NABIR

• Elucidation of novel central metabolism genes

For example, the novel, eucaryotic-like citrate synthase provides a unique molecular marker for tracking *Geobacter* species and their activity in the subsurface.
Discoveries from GTL with Direct and Immediate Application to NABIR
(continued)

• Development of an *in silico* model that can:

  -- Predict the response of *Geobacter* to different environmental conditions including strategies for manipulating the environment to promote bioremediation

  -- Aid in elucidating the likely outcome of genetically engineering novel metabolic capabilities in *Geobacter*
Contributions of Iterative *In Silico* Model Building to Understanding of the Environmental Responses of *Geobacter*
Total Number of Genes: 3532
Included Genes: 583 (17 %)
Percentage of the annotated genome: (29%)
Total Number of Reactions: 520
Gene/(Non-gene) associated: 466 (54)
Number of Proteins: 431
Number of Metabolites: 537
Given a limited number of constraints, growth rate, yield, and flux through metabolic network can be quantitatively predicted.
Phylogenetically Distinct Fe(III) Reducers Have Different Mechanisms for Fe(III) Reduction

*Geobacter* has to directly contact Fe(III) oxide in order to reduce it


*Geothrix* releases electron shuttles and chelators which alleviate the need for direct microbe-Fe(III) oxide contact

Analysis of Metabolic Cost to Release a Quinone-Based Electron Shuttle

Simulations carried out for varying

- Quinone secretion rates
- Different Sized Molecules

Significant growth rate reduction due to both ATP requirements and carbon requirements for shuttle synthesis

Provides likely explanation for the predominance *Geobacter* over *Geothrix* in subsurface environments
Discoveries from GTL with Direct and Immediate Application to NABIR

(continued)

• Discovery of significant similarities in genomes of as-yet-uncultured *Geobacter* species and pure cultures of *Geobacter* species

Suggests that models based on intensively studied pure cultures may have applicability to predicting the activity of as-yet-uncultured *Geobacter* species that predominate in uranium-contaminated subsurface environments.
*Geobacter uranibioremediacens*: Isolate from Rifle, Colorado Field Site

16S rDNA sequence identical with predominant *Geobacter* sequence in groundwater during uranium bioremediation

Direct Isolation on Solidified Medium with Aquifer Clay Fraction Serving as Fe(III) Source

Phase Contrast Micrograph of Cells Amongst Sediment Clay Fraction In Ground-Water Amended Medium
BAC clone from as-yet-uncultured *Geobacter* from subsurface sediments contains *omcB*, a gene for an outer-membrane cytochrome required for Fe(III) reduction in *G. sulfurreducens* in the same gene organization as seen in *G. sulfurreducens*.
Map of BAC 109

- ompB
- ompB
- lipoprotein
- efflux protein
- tetR
- sensory box histidine kinase
- dehydropantoate reductase
- hlyD
- acrB
- drug resistance
- drug resistance
- moeA
- fibronectin
- membrane protein
- efflux protein
- efflux protein
- LuxR
- response regulator
- tetR
General Secretion Pathway

BAC 83

% Similarity

G. sulfurreducens

% Similarity
Flagella

**BAC 84**

- flgB: 61.9%
- flgC: 65%
- fliE: 63%
- fliF: 71.9%
- fliG: 67%
- fliH: 66.2%
- fliJ: 66%

**G. sulfurreducens**

- flgB: 52.1%
- flgC: 66.3%
- fliE: 63.3%
- fliF: 63.2%

% Similarity: 66.3% 66.2% 63.2%
Pilin

BAC 96

% Similarity
62.6%  60.5%  56.5%  62.2%

G. sulfurreducens

% Similarity
62.6%  60.5%  56.5%  62.2%
Cytochrome Oxidase

BAC 82

% Similarity

64.5% 60% 62.5% 66.7%

G. sulfurreducens

subunit 1
subunit 3
subunit 4
subunit 2
Hydrogenase A

BAC 81
Maturation protein 56%
Large subunit 66.2%
Small subunit 58.2%
Competence F 64%

G. sulfurreducens
Maturation protein
Large subunit
Small subunit
Competence F
Hydrogenase B

**BAC 88**

- Small subunit: 63.8%
- FeS subunit: 68.5%
- Membrane protein: 61%
- Large subunit: 63.7%

**G. sulfurreducens**

- Small subunit
- FeS subunit
- Membrane protein
- Large subunit
Formate Dehydrogenase

BAC 147

G. sulfurreducens

fdhA  fdhB  fdhC  fdhD

60%  61.4%  63.3%  50%
Nitrogen Fixation Genes

BAC 74

% Similarity

G. sulfurreducens

nifK  nifD  nifH
62%  63%  61.9%
Environmental Genomic DNA of “As-Yet-Uncultured” Geobacters

Novel Culturing Strategies to Isolate Environmentally Relevant Geobacters

Previously Cultured Geobacters

Functional Genomics: Elucidation of Regulatory Systems Analysis of Gene Expression Physiological, Biochemical Studies

In Silico Model of Cell Function

In Silico and Conceptual Models for Optimizing Uranium Bioremediation and Electrical Energy Harvesting
$nifD$ and $recA$ expression in Acetate-amended and Control Subsurface Sediments before and after adding 100 $\mu$M $NH_4Cl$
**Geobacter** Genes Upregulated During Growth on Electrodes

- heat shock protein, Hsp20 family
- dnaJ domain protein
- heat shock protein, Hsp20 family
- heat shock protein, Hsp20 family
- heat shock protein, Hsp20 family
- heat shock protein, Hsp20 family
- **cytochrome c family protein**
- hypothetical protein
- NOL1/NOP2/sun family protein
- conserved domain protein
- C4-dicarboxylate transporter, anaerobic
- **cytochrome c family protein, putative**
- **cytochrome c family protein**
- ClpB protein
- NHL repeat domain protein
- metal ion efflux outer memb. prot. family, put.
- hypothetical protein
- hypothetical protein
- hypothetical protein
- ABC transporter, permease protein
- transcriptional regulator, MerR family
- hypothetical protein
Geobacter Genes Upregulated During Growth on Electrodes

- heat shock protein, Hsp20 family
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- hypothetical protein
- hypothetical protein
- hypothetical protein
- ABC transporter, permease protein
- transcriptional regulator, MerR family
- hypothetical protein
Effect of Deletion Mutation in *omcD* on Current Production

Wild type

OmcD Mutant-Complemented

OmcD Mutant
Support of NABIR by GTL in the Future

• Use of molecular techniques to assess *in situ* rates of metal reduction.

• Whole-genome analysis of *in situ* gene expression to determine the *in situ* metabolic state of microorganisms during uranium bioremediation which will help direct implementation of bioremediation strategies.

• Coupling *in silico* microbial models with geochemical and hydrological models to accurately predict the rate and extent of bioremediation in diverse environments under various bioremediation strategies.