Functional Metagenomics to Capture the Soil Resistome

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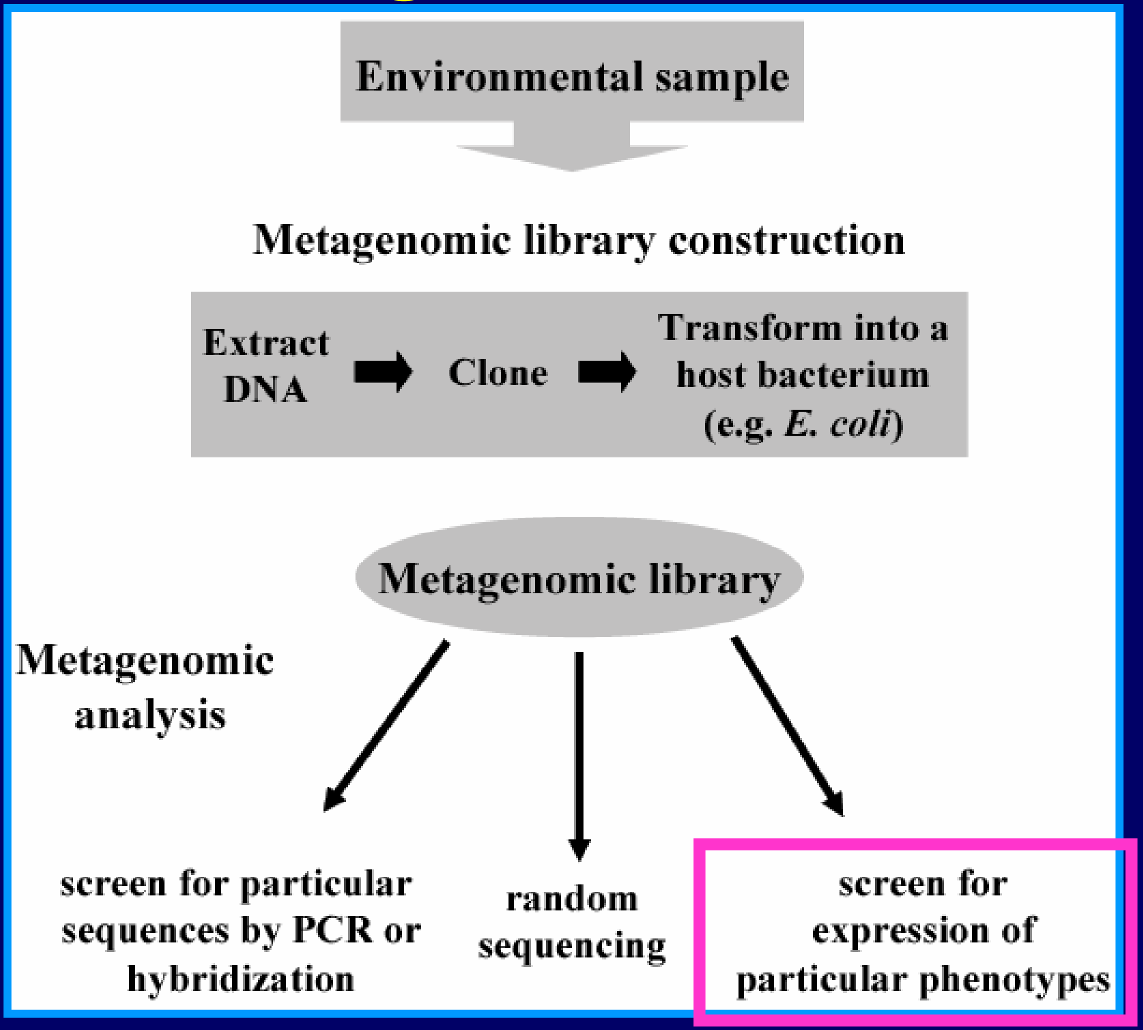
One Origin of Metagenomics: Communication in Microbial Communities

- Ecological roles of antibiotics in communities
- Antibiotics as signal molecules
- Proteins that interact with antibiotics and signals
- Discovery of antibiotics for human and agricultural use

Functional Metagenomics to Capture the Soil Resistome

- Functional anchor strategy
- Choice of function
- Choice of habitat and sites
- Antibiotic resistance determinants
- Conclusions and future directions

Where do functional anchors fit into metagenomics?



Sequence clones: choose randomly or based on a common feature

Doing Metagenomics with Functional Anchors

Unite expressed function with genomic analysis of uncultured microorganisms

1. Identify clones that express a shared function (the “functional anchor”)

2. Characterize genes responsible for function

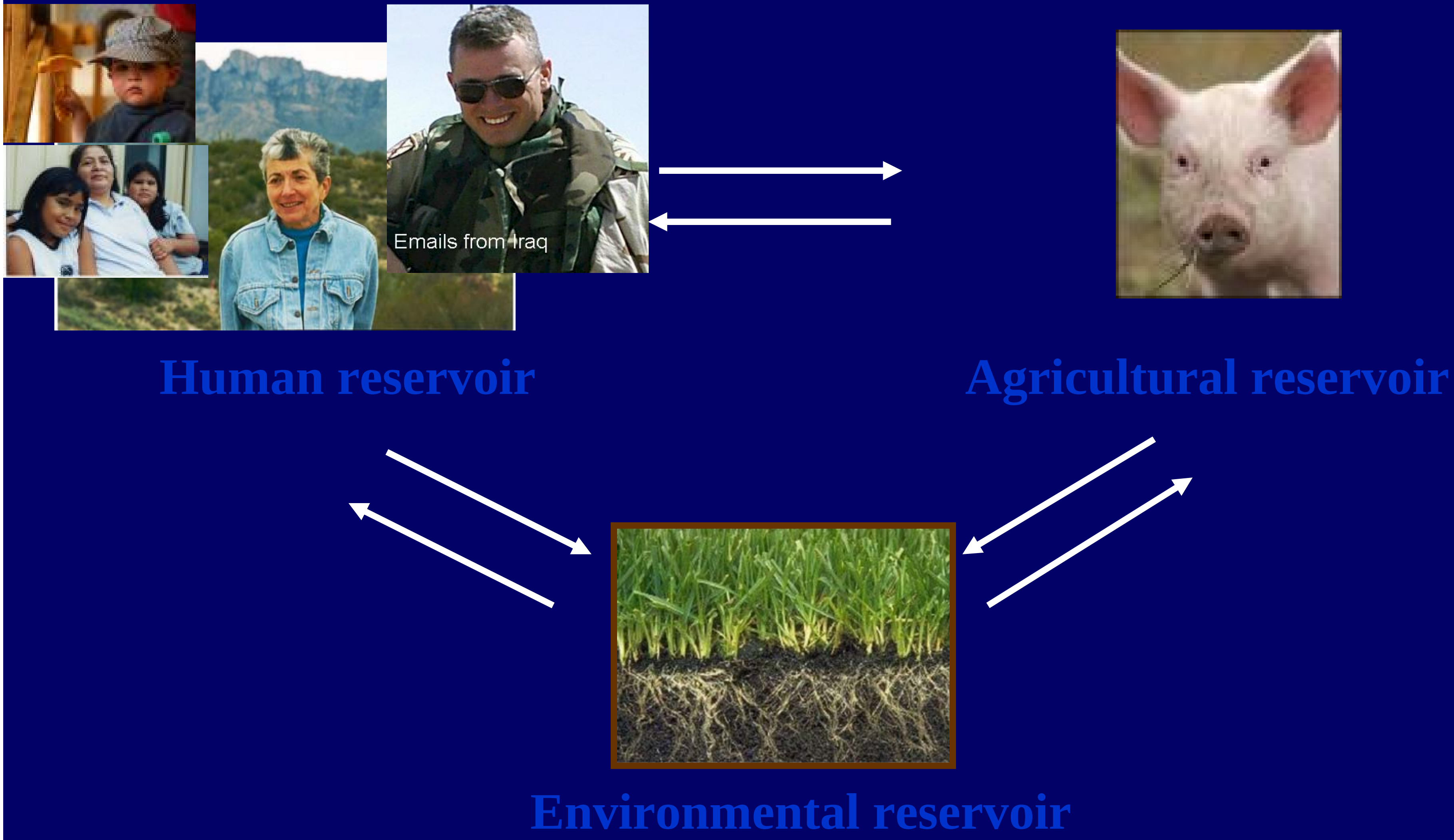
3. Sequence active clones and assess phylogenetic, genomic, and physiological context for functional gene

Functional anchor–based metagenomics identifies genes whose functions could not have been predicted based on sequence.

Antibiotic Resistance As Functional Anchor

- Powerful selections
- Important medical and environmental issue
- Little known about soil resistome
- Little known about uncultured sources of antibiotic resistance

Reservoirs of antibiotic resistance

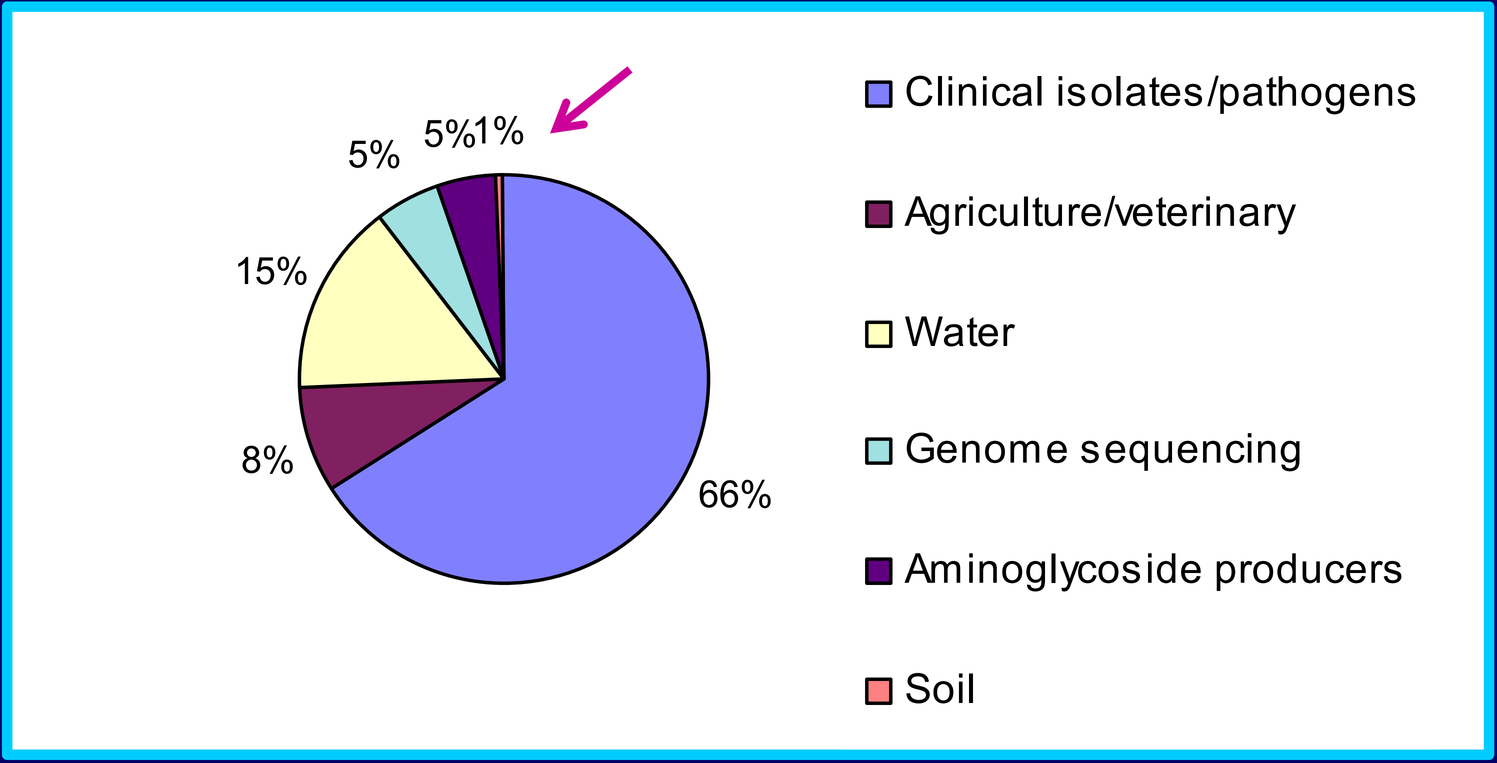


Human reservoir

Agricultural reservoir

Environmental reservoir

Information about antibiotic resistance genes in soil is scarce



150 aminoglycoside resistance genes from GenBank were assigned to one of six categories based on their source

Habitat for Metagenomic Analysis

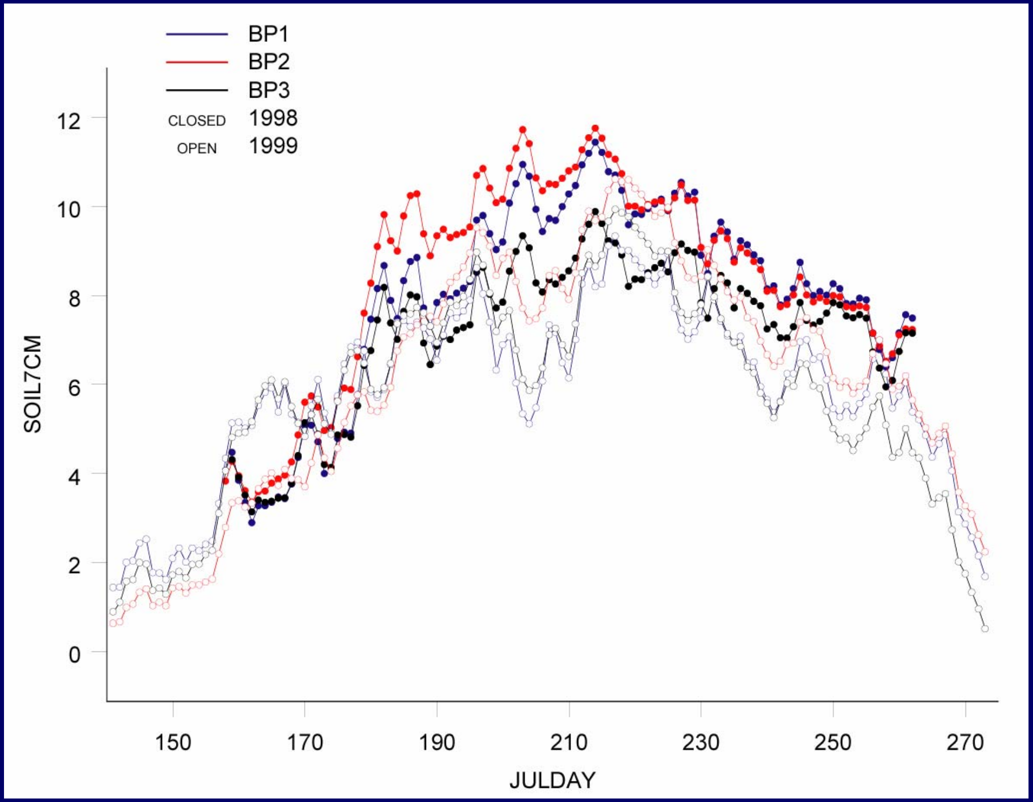
Soil -- ~5,000 species based on 16S rRNA and statistical modeling

Genetic and chemical diversity much greater than that

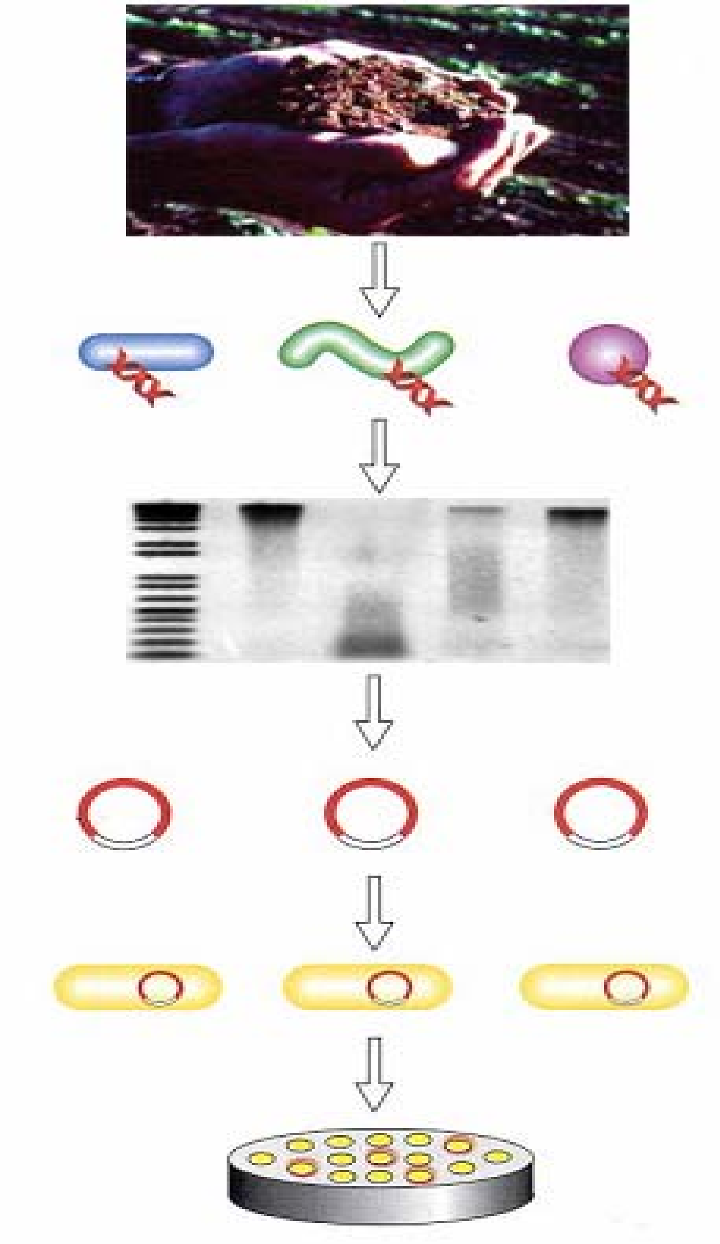
West Madison Agricultural Research Station

Alaskan Microbial Observatory

Soil Description



Metagenomic library construction



Collect soil

↓

Extract DNA

↓

Digest

↓

Ligate into vector

↓

Transform *E. coli*

↓

Screen transformants

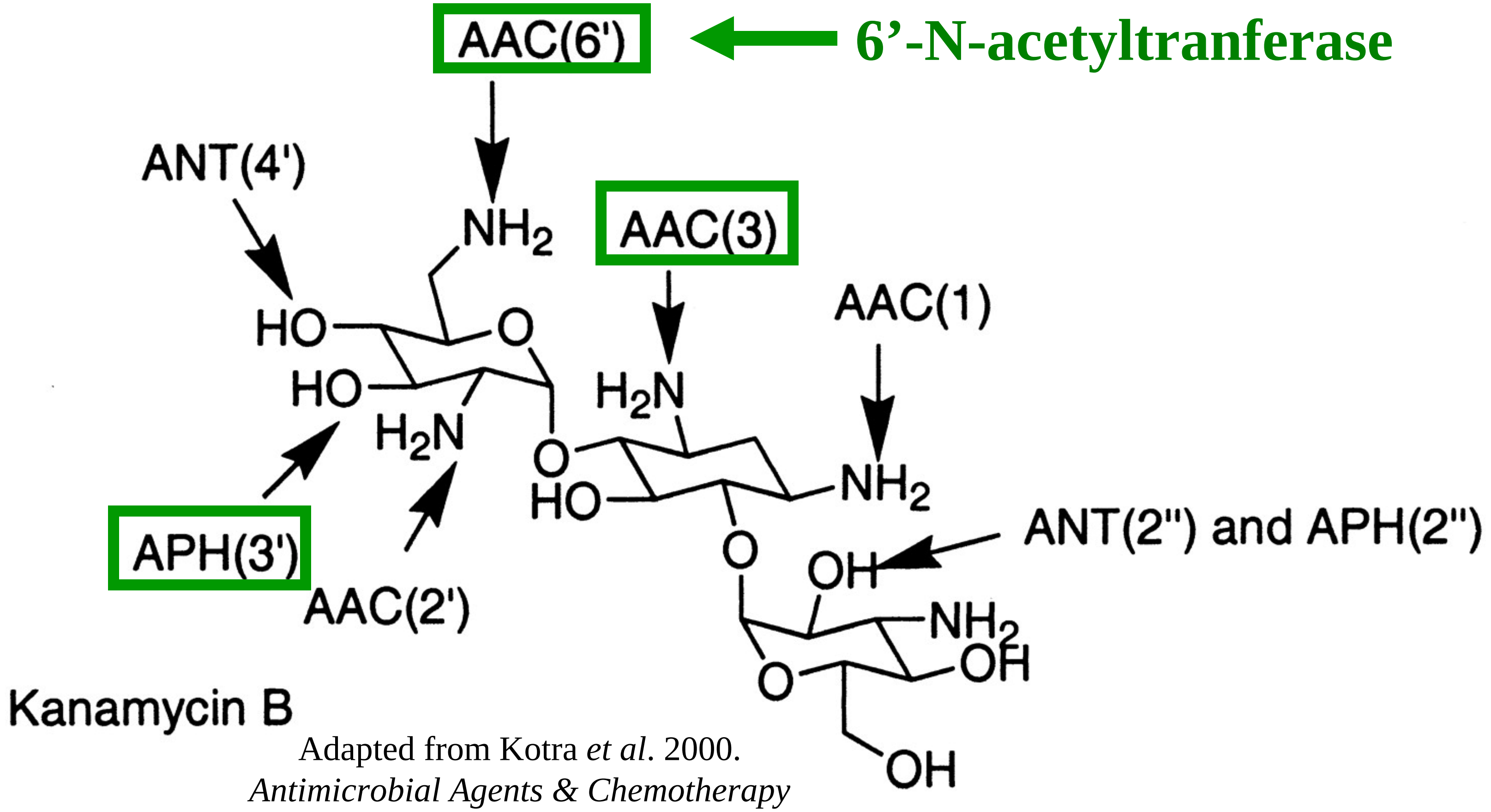
Soil Libraries

- Wisconsin Soil Metagenomic Libraries
  - 28,000 clones; inserts average 43 kb
  - ~ 1 Gb DNA
- Wisconsin Soil Metagenomic Libraries
  - 650,000 clones; inserts average 2 kb
  - ~ 1.3 Gb DNA
- Alaskan Soil Metagenomic Libraries –
  - 500,000 clones inserts 2 to 200 kb
  - ~ 10 Gb DNA

Functional Anchor Approach to the Soil Resistome

- Identify clones that express antibiotic resistance
- Fully sequence all of them
- Identify gene(s) responsible for activity
- Identify phylogenetically informative genes and conduct a genomic analysis
- Conduct a designed experiment: replicate, associate gene frequency with bacterial or human activity

Aminoglycoside modification sites



6'-N-acetyltranferase

Kanamycin B

Adapted from Kotra *et al*. 2000.
Antimicrobial Agents & Chemotherapy

Deduced amino acid sequences suggest three different aminoglycoside resistance types

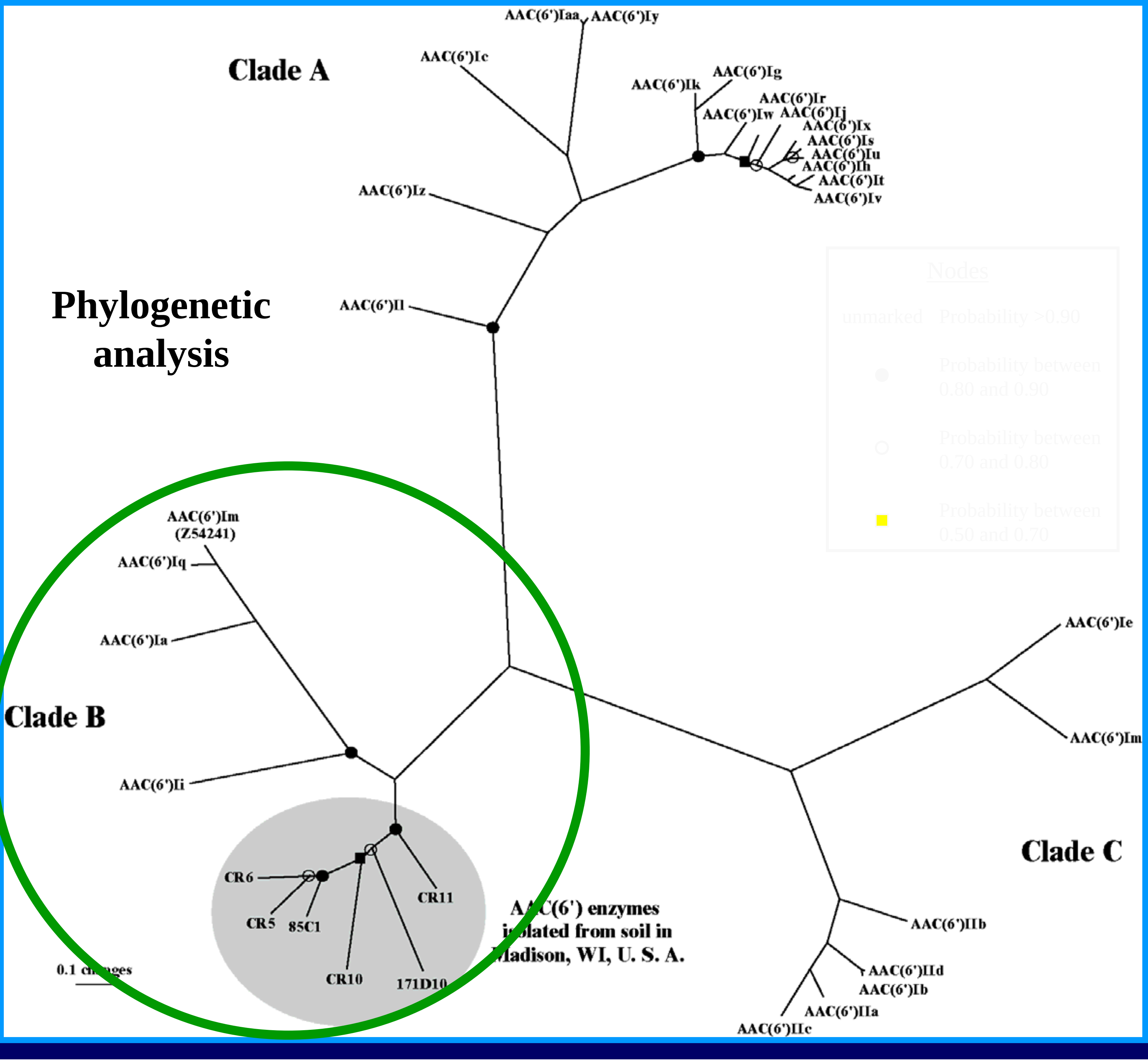
Selections for Antibiotic Resistance

Kanamycin Tetracycline Nalidixic Acid

10 aminoglycosides

13 unique clones from Wisconsin soil
One clone from Alaskan soil
Gene from Alaskan soil is a near match to the Wisconsin genes

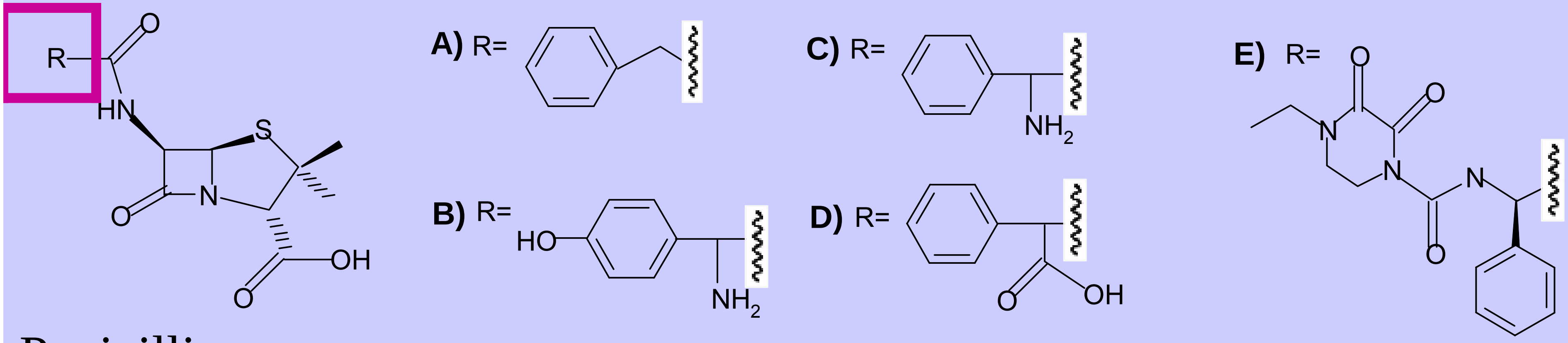
Phylogenetic analysis



Aminoglycoside Resistance Genes

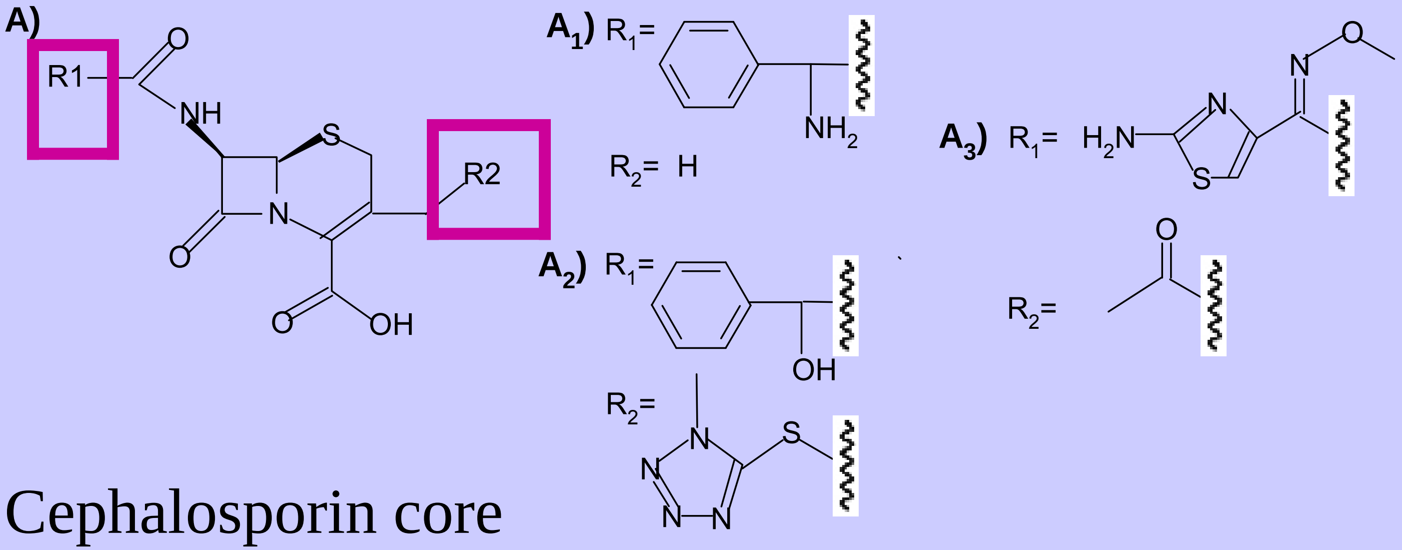
- All new sequences, some diverge deeply from known genes
- Discovered two from screening libraries in *Agrobacterium*, not expressed in *E. coli*
- One of these has no significant sequence similarity to any gene in GENBANK
- Alaskan gene is close to Wisconsin cluster

β-Lactam antibiotics



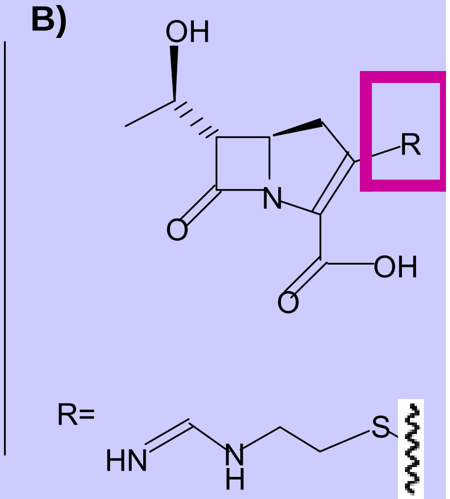
Penicillin core

A. Penicillin G. **B-E**, semi-synthetic β-lactam antibiotics.
B. Amoxicillin. **C**. Ampicillin. **D**. Carbenicillin. **E**. Piperacillin.



Cephalosporin core

A1. Cephalexin. **A**2. Cefamandole. **A**3. Cefotaxime.



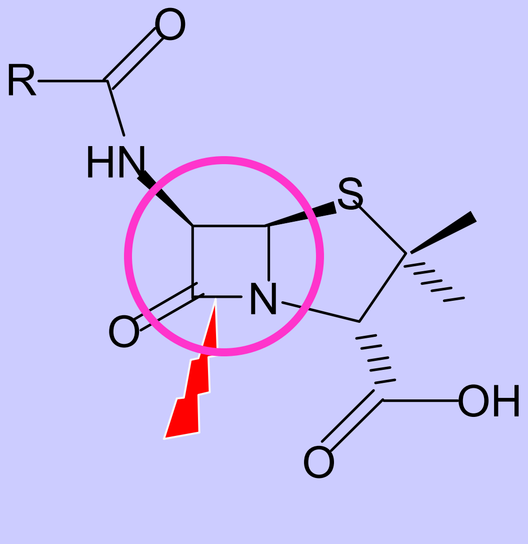
Carbapenem core

B. R = imipenem.

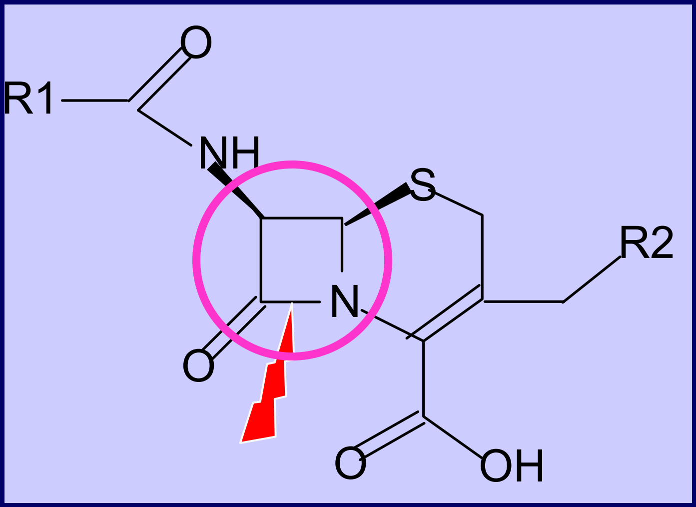
Enzymatic Inactivation of β-lactams

Classes of β-lactamases

- A, C, and D = serine β-lactamases
- B = metallo-β-lactamases

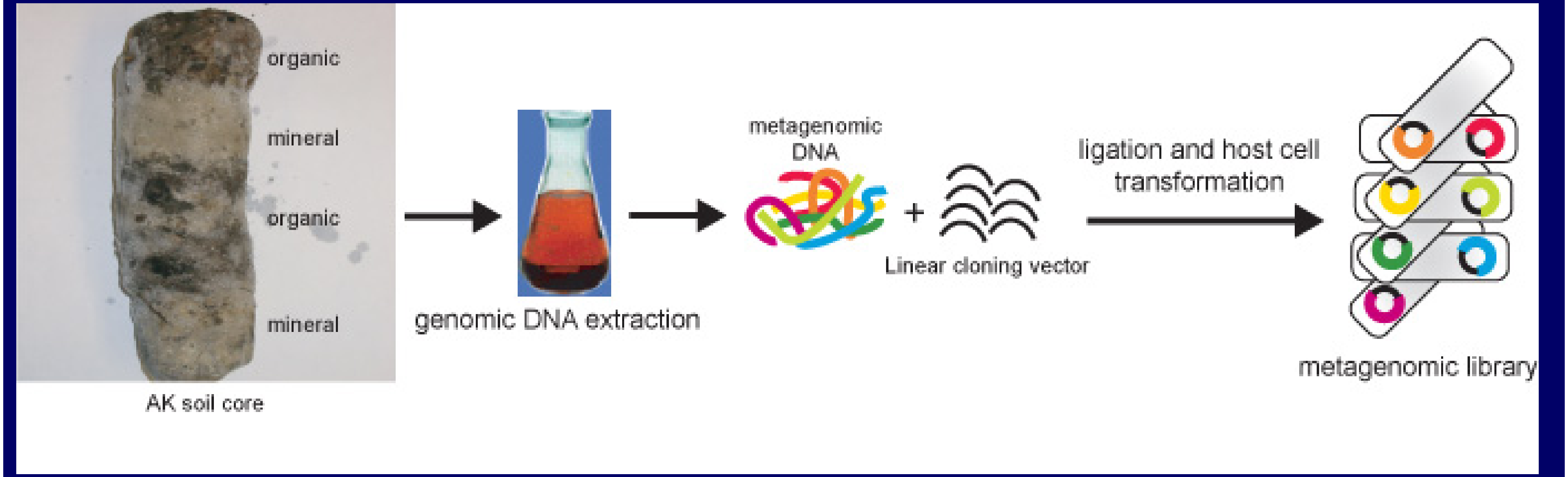


Penicillin core



Cephalosporin core

Metagenomic Library Construction -- Alaska



•Small-insert libraries: 2- to10-kb inserts

•Large-insert libraries: BAC (20 to190-kb inserts)
Fosmid (40-kb inserts)

- >739,061 clones
- >13.6 Gb = >2,472 *E. coli* genome equivalents

β-lactam Resistance in Small-insert Libraries

300,000 clones/2 Gb DNA

900,000 (3x coverage) selected
on 8 antibiotics

Up to 250 colonies/antibiotic

Resolved duplicates

4 clones fully sequenced

β-lactam Resistance in Fosmid Libraries

Screened 13 Gb DNA
8 clones partially characterized
Resistant to either penicillins or cephalosporins

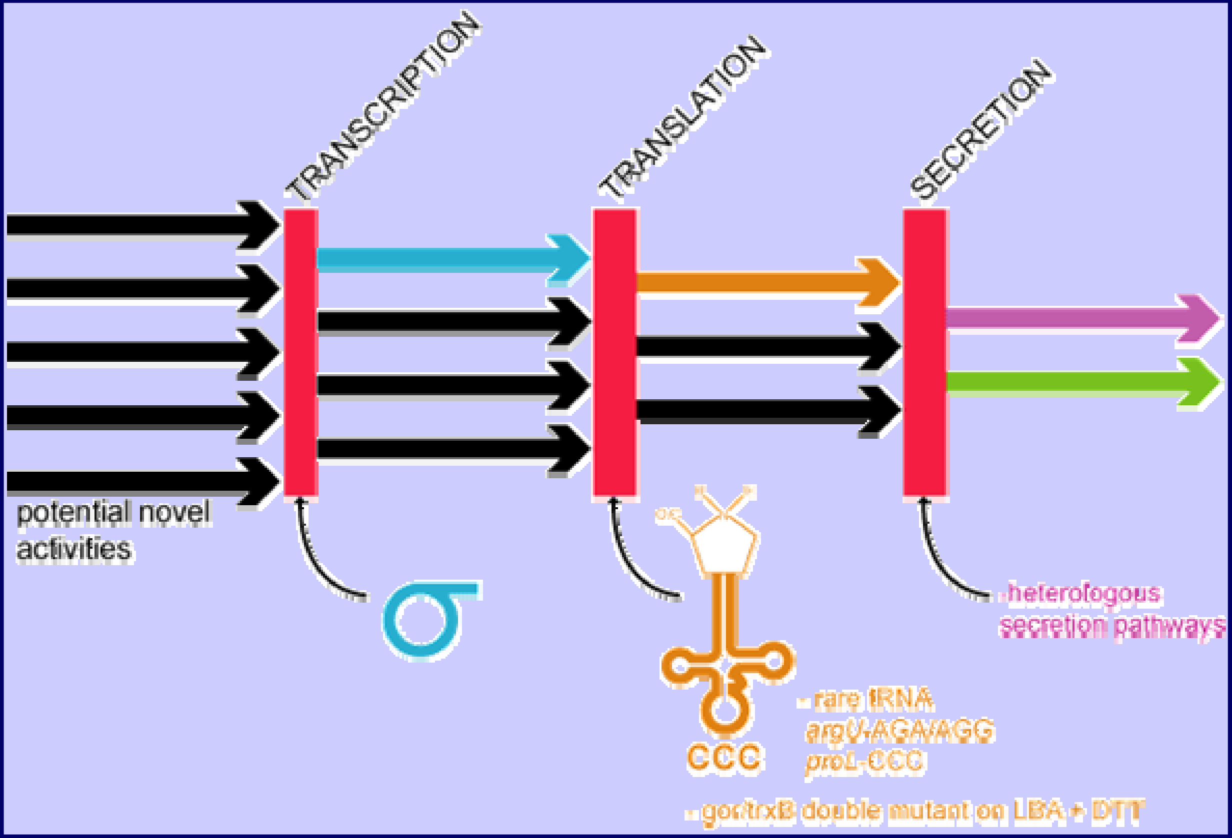
2 clones -- 35% amino acid identity/54% similarity to probable class A β -lactamase from *Gloeobacter violaceus*

3 *clones* -- No ORFs with recognizable β-lactamase (sequence incomplete)

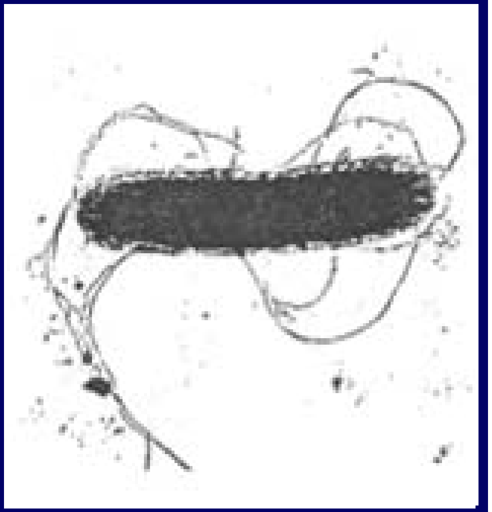
2 clones -- Similarity to class B carbapenemase from *Elizabethkingia meningoseptica*

1 clone -- Similarity to class C β-lactamase from *Mycobacterium smegmatis*

Overcoming Roadblocks in Heterologous Expression



Acidobacteria



- Acidobacteria are ubiquitous but poorly characterized
- few cultured isolates
- represent between 15% and 35% of soil microorganisms (16S analysis) and 16% of community in Alaskan soil

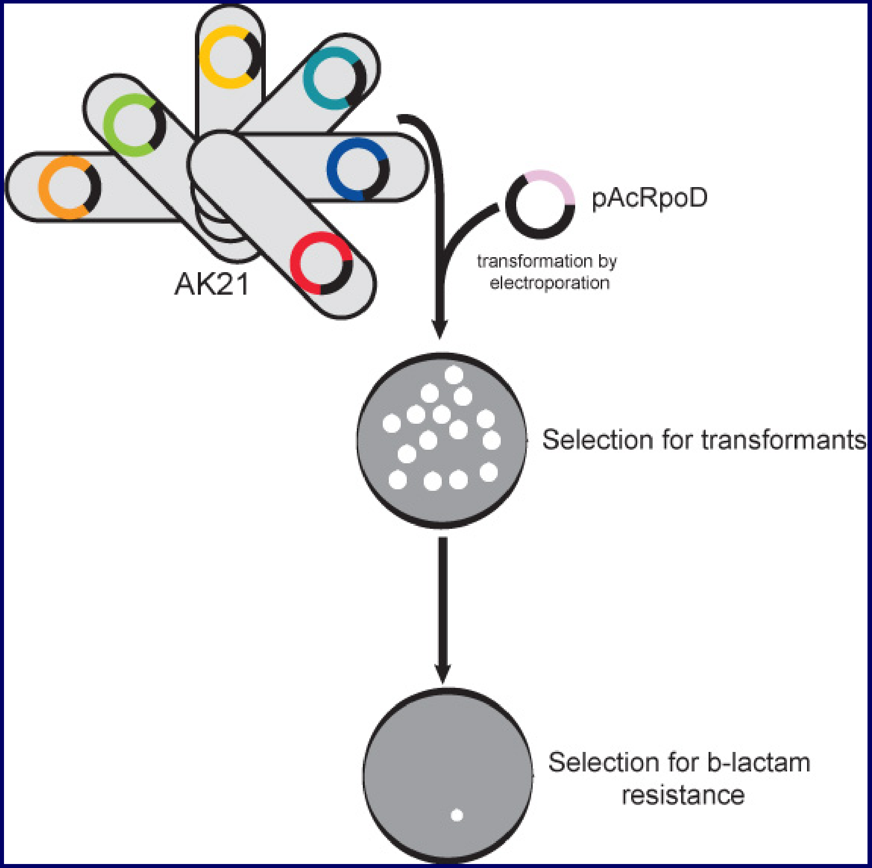
Hypothesis

Expression of Acidobacteria genes is limited by the sigma factors available in E. coli

Strategy

Acidobacterium capsulatum *major sigma factor RpoD (AcRpoD) in* E. coli

Screening large-insert library with the *A. capsulatum* major sigma factor, RpoD present in clones



Retrofit AK21 library with *Ac*RpoD

↓

Select for β-lactam resistance
- screen 415,000 clones/β-lactam
  (~1.5X coverage)
- 10,000 clones/plate

↓

Isolate *Ac*RpoD-dependent resistant clones

Screening β-lactam-resistant (R) clones for *Ac*RpoD-dependent phenotype

Electrocure β-lactamR clones of pAcRpoD (Tet^{R})

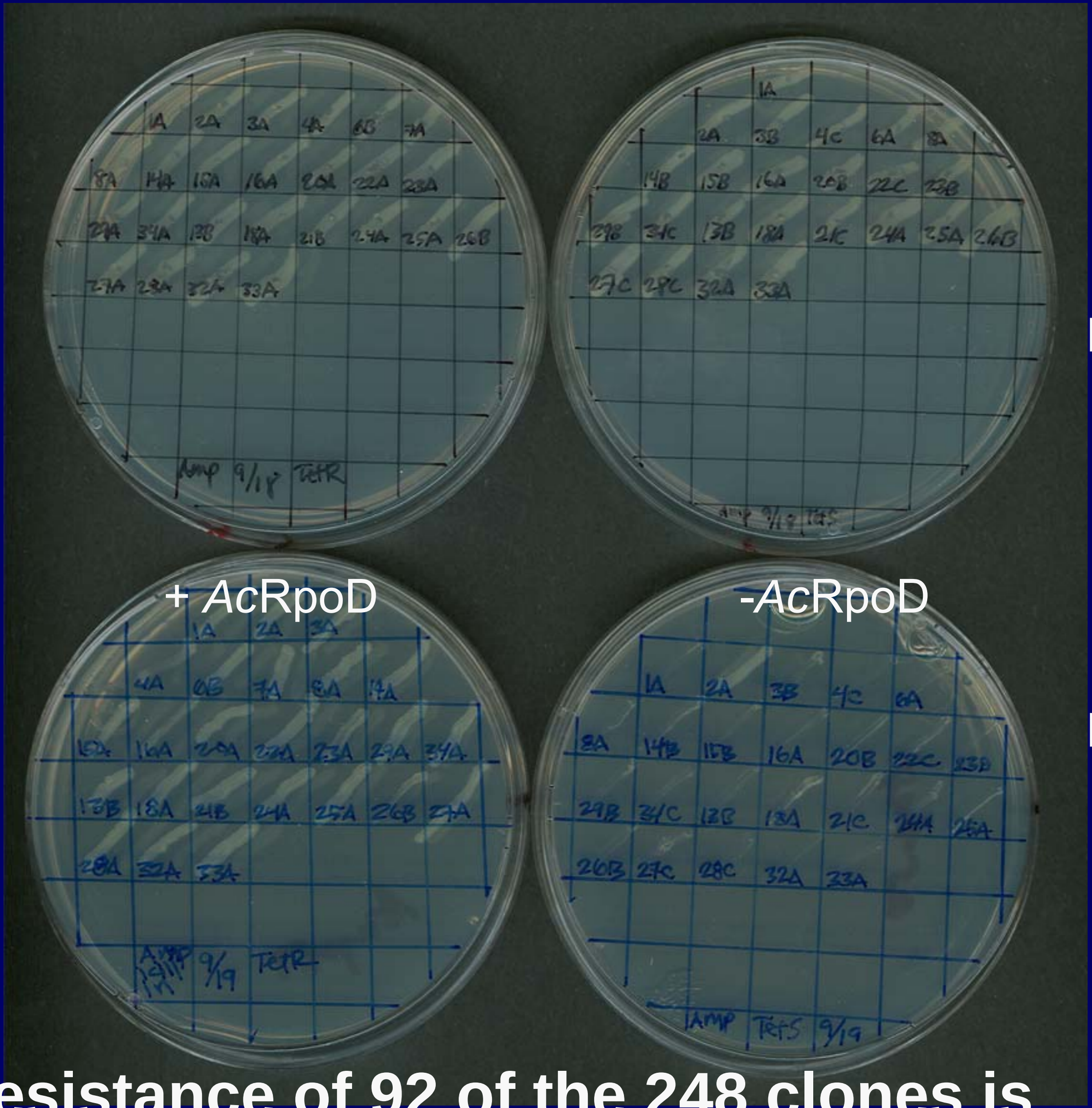
↓

Confirm Tet^{S} phenotype
Confirm loss of Ac *rpoD* by
PCR using AcRpoD-specific primers

↓

Test β-lactam sensitivity

β-lactam resistance of clones +/- *Ac*RpoD



+ *Ac*RpoD

-*Ac*RpoD

LBA+Cm

LBA + Amp

The resistance of 92 of the 248 clones is AcRpoD dependent

Future Directions

- Full-length sequencing and assembly of fosmid clones
- Characterization of novel proteins – enzymology and crystallography
- Study impact on soilborne antibiotic resistance genes of
  - streptomycin use in apple production
  - β-lactam use in dairy operations

Antibiotic Resistance Conclusions

- A functional anchor approach to metagenomics reveals new members of known gene families and new types of resistance genes

- Heterologous gene expression can be enhanced by using a different host species or adding a sigma factor from a member of another bacterial phylum

The Dirty Dozen

(the soil group)

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Brad Kasavana
Brad Borlee

Howard Hughes Medical Institute
National Institutes of Health
National Science Foundation
University of Wisconsin-Madison

Antibiotic Resistance in Wisconsin vs. Alaska

Wisconsin	**Alaska**
Agricultural	Pristine
Disturbed	undisturbed
Single freeze	Multiple freeze-thaw cycles
Maximum temp 16-20^{o} C	Maximum temp 12^{o} C
Stable environment	periodic river floods