Stable-Isotope Probing of PAH-Degrading Bacteria in a Bioreactor Treating PAH-Contaminated Soil

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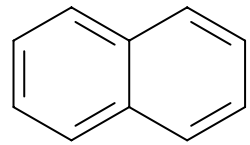
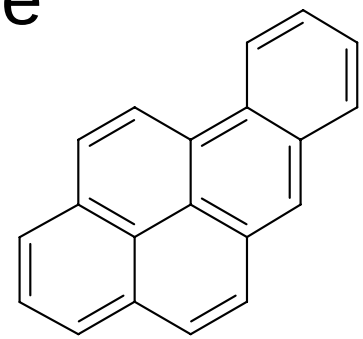
Outline

- Overview of PAH biodegradation
  - challenges for remediation in the field
  - complexity of PAH-contaminated systems
- Limitations of “conventional” tools in microbial ecology
- Overview of stable-isotope probing (SIP)
- Application of SIP to bioreactor treating PAH-contaminated soil

PAHs of concern

- EPA priority pollutant PAHs
  - 16 compounds ranging in size from two rings to six rings (“total” PAH, or tPAH)
- Seven are considered to be “probable human carcinogens” (cPAH)
  - five are 5- or 6-ring compounds (high-molecular-weight)
- Cleanup requirements can be based on tPAH, cPAH or both





Biodegradation of PAHs

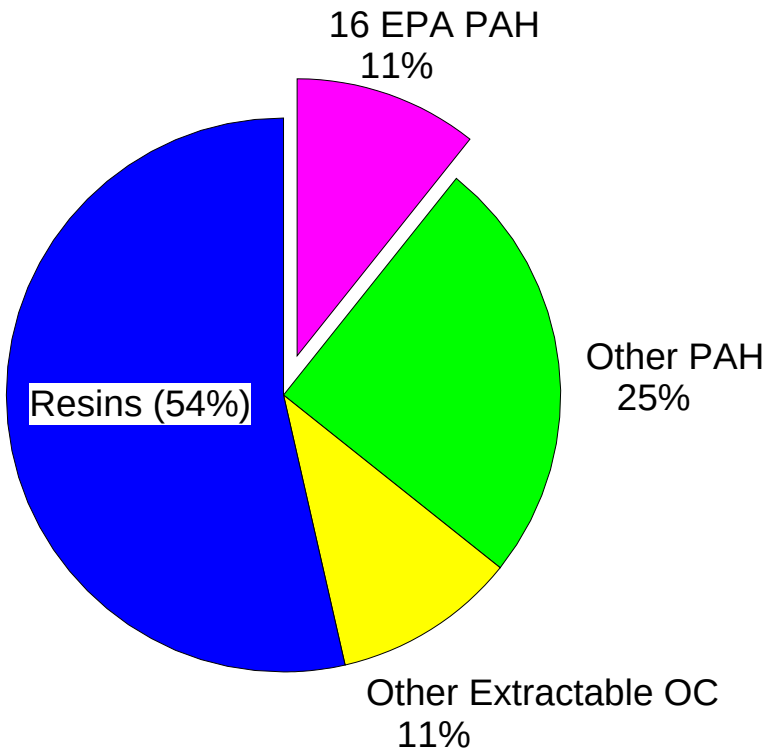
- Removal of PAHs usually is incomplete after biological treatment, even with aggressive bioremediation
- High-molecular-weight (HMW) compounds are less extensively removed
  - less removal of cPAH than LMW PAH
- Bioremediation may not be technically feasible or cost-competitive
- Currently no predictive tools for HMW PAH degradation in the field

PAH-contaminated systems are complex

- Complex mixtures of contaminants
  - 16 regulated PAHs but many more unregulated
- Complex microbial communities
- Complex microbial responses to contaminants
  - growth, mineralization without growth, or incomplete metabolism
  - competition for growth substrates by different organisms
  - competitive metabolism of multiple substrates by a given organism

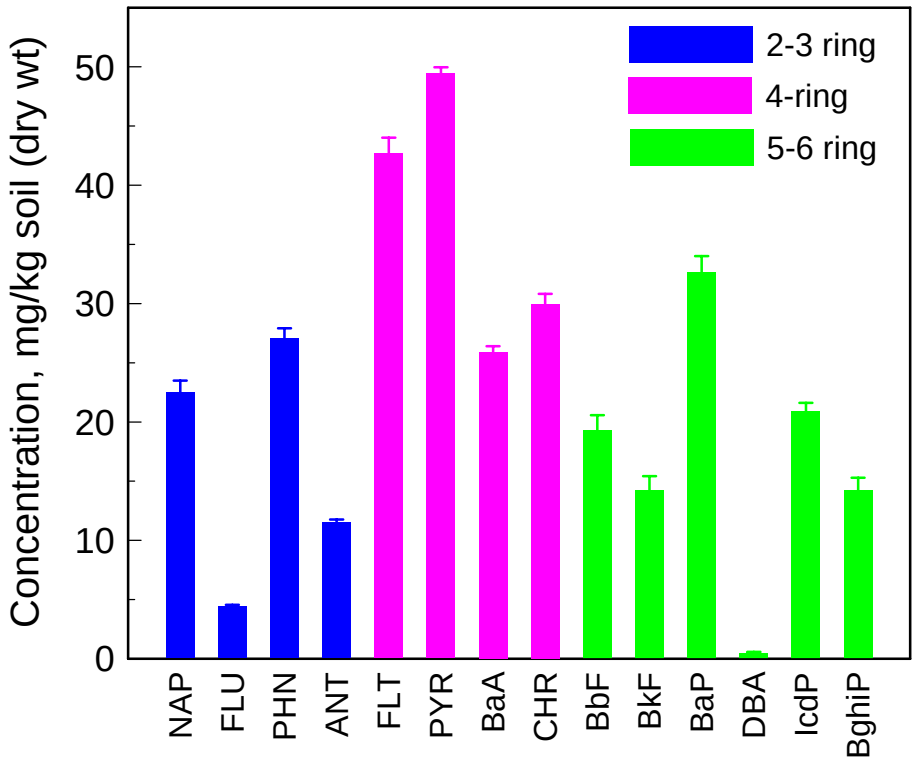
Distribution of contaminants in soils from MGP sites

Extractable organic carbon (~ 30% of total organic carbon)

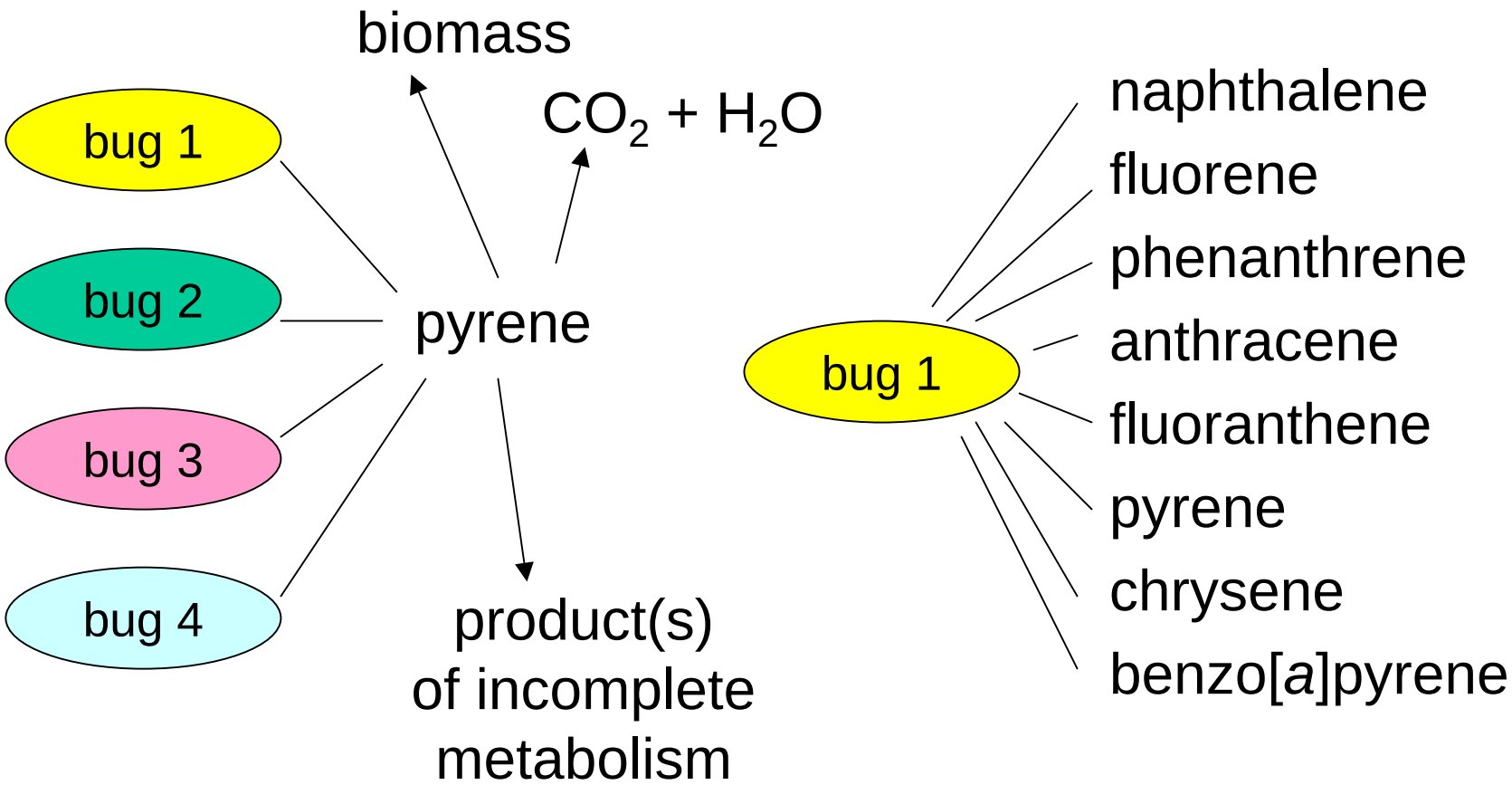


Haeseler et al. (1999) *Environ. Sci. Technol.*, 33: 825-830

EPA-regulated PAHs in soil from MGP site in Charlotte



Complex communities and responses



No organism known to grow on a 5- or 6-ring PAH
(must be growing on something else!)

Factors potentially limiting biodegradation

- Compounds are not biodegradable
- Recalcitrant compounds are not bioavailable
- Required organisms are not present in the system (or present at very low numbers)
- Inherently slow degradation kinetics
- Degradation of HMW compounds declines when LMW compounds are depleted
- Competitive metabolism of LMW and HMW compounds within a given organism
- Accumulation of inhibitory products

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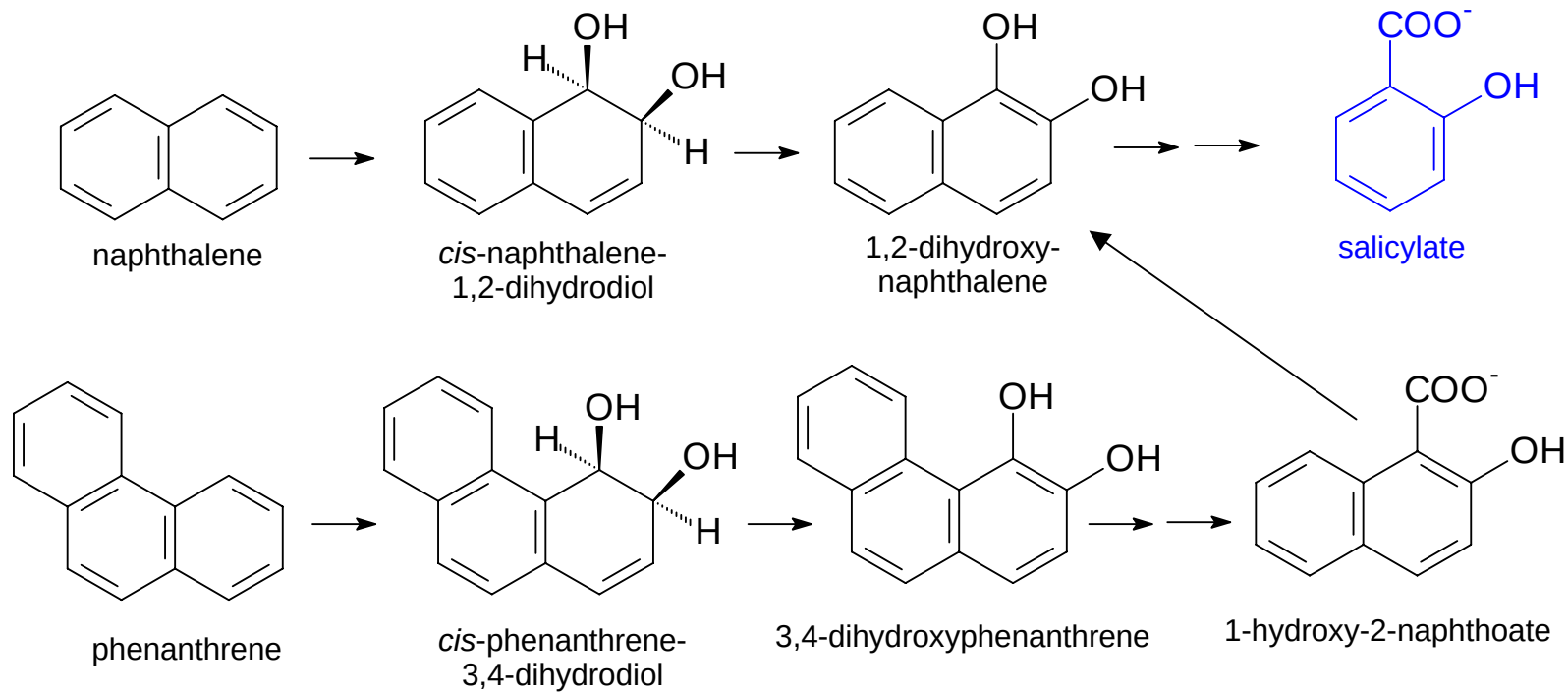
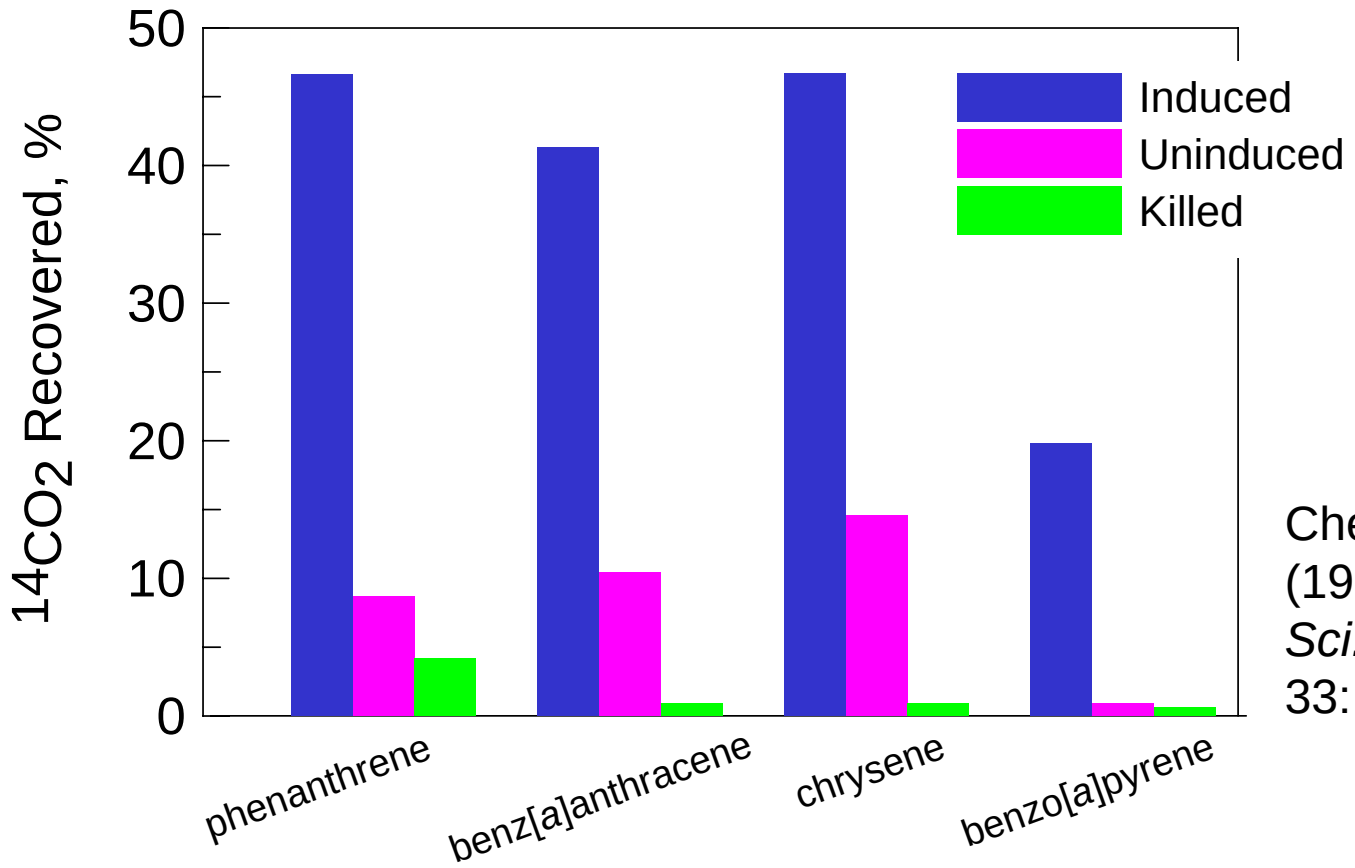
PAH degraders use common enzymes or pathways for multiple compounds



level of enzyme specificity for different PAHs and intermediates is poorly understood

Salicylate induces the mineralization of multiple PAHs by *P. saccharophila* P15



Chen and Aitken (1999) *Environ. Sci. Technol.*, 33: 435-439

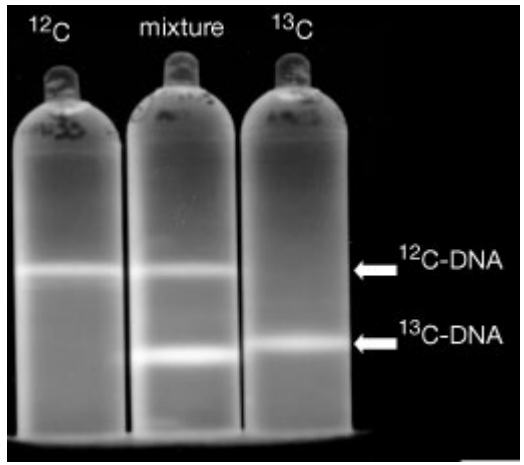
How do we identify other organisms that mineralize BaP?

Conventional tools in microbial ecology

- Cultivation
  - most organisms not culturable
- Clone libraries
  - identifies who's there, not what they're doing
- "Fingerprint" methods
  - terminal restriction-fragment length polymorphism (TRFLP)
  - denaturing-gradient gel electrophoresis (DGGE)
  - can see shifts in community but difficult to assign functions
- Gene probing/fluorescent in situ hybridization (FISH)
  - need to know what you're looking for; overlooks diversity and unknowns

Stable-isotope probing

- Incubate community with ^{13}C-labeled carbon source (preferably uniformly labeled)
- Extract DNA or RNA
- Separate labeled and unlabeled nucleic acids by density-gradient ultracentrifugation



Radajewski et al., *Nature*, **403:**646-649

- Perform "conventional" molecular analyses
- *SIP answers "who's doing what?"; relevance of conventional techniques improved*

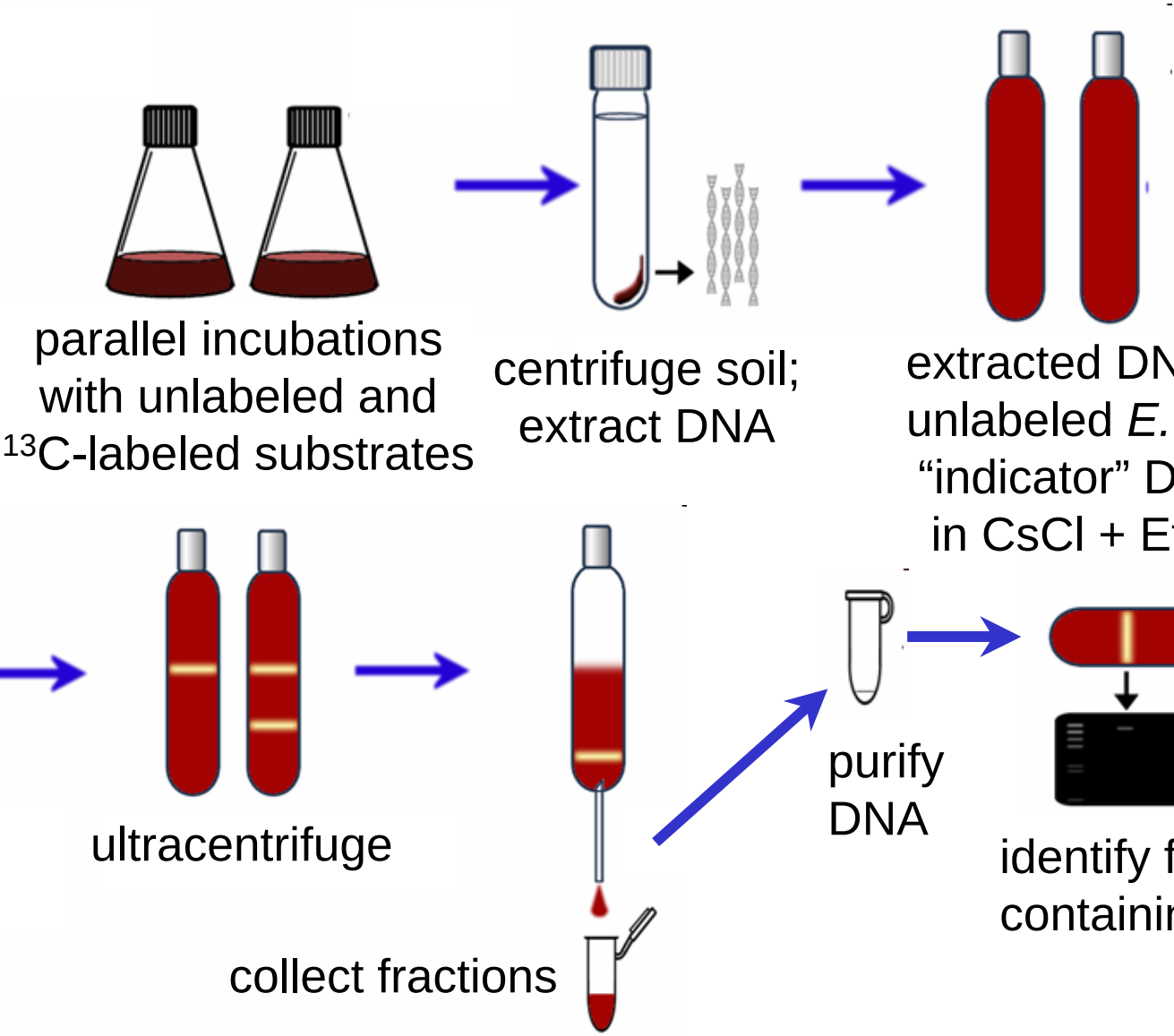
Experimental system

- Lab-scale, slurry-phase bioreactor
  - soil from MGP site in Charlotte
  - 20% solids
  - continuously mixed and aerated
- Semi-continuous (draw-and-fill) operation
  - every two weeks, replace 20% of contents with untreated soil slurry
  - solids residence time ~ 70 days

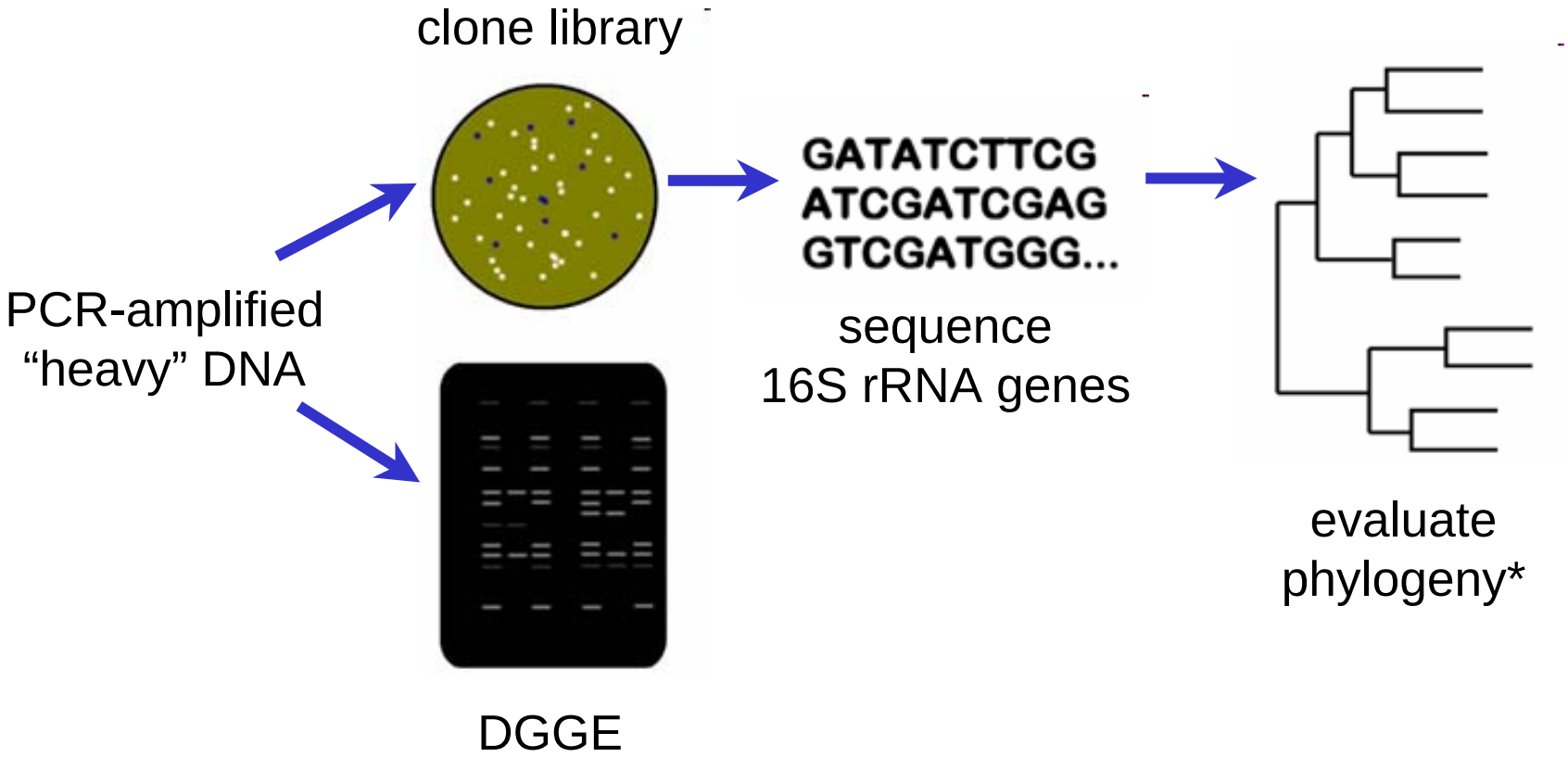
SIP experimental design

- Questions:
  - do the same organisms degrade naphthalene and phenanthrene?
  - does salicylate select for organisms that degrade either compound?
- Synthesize ^{13}C-labeled salicylate, naphthalene, and phenanthrene
- Incubate with slurry (~1:5 dilution in reactor buffer)
  - salicylate or naphthalene for 2 days
  - naphthalene or phenanthrene for 7 days

SIP protocol

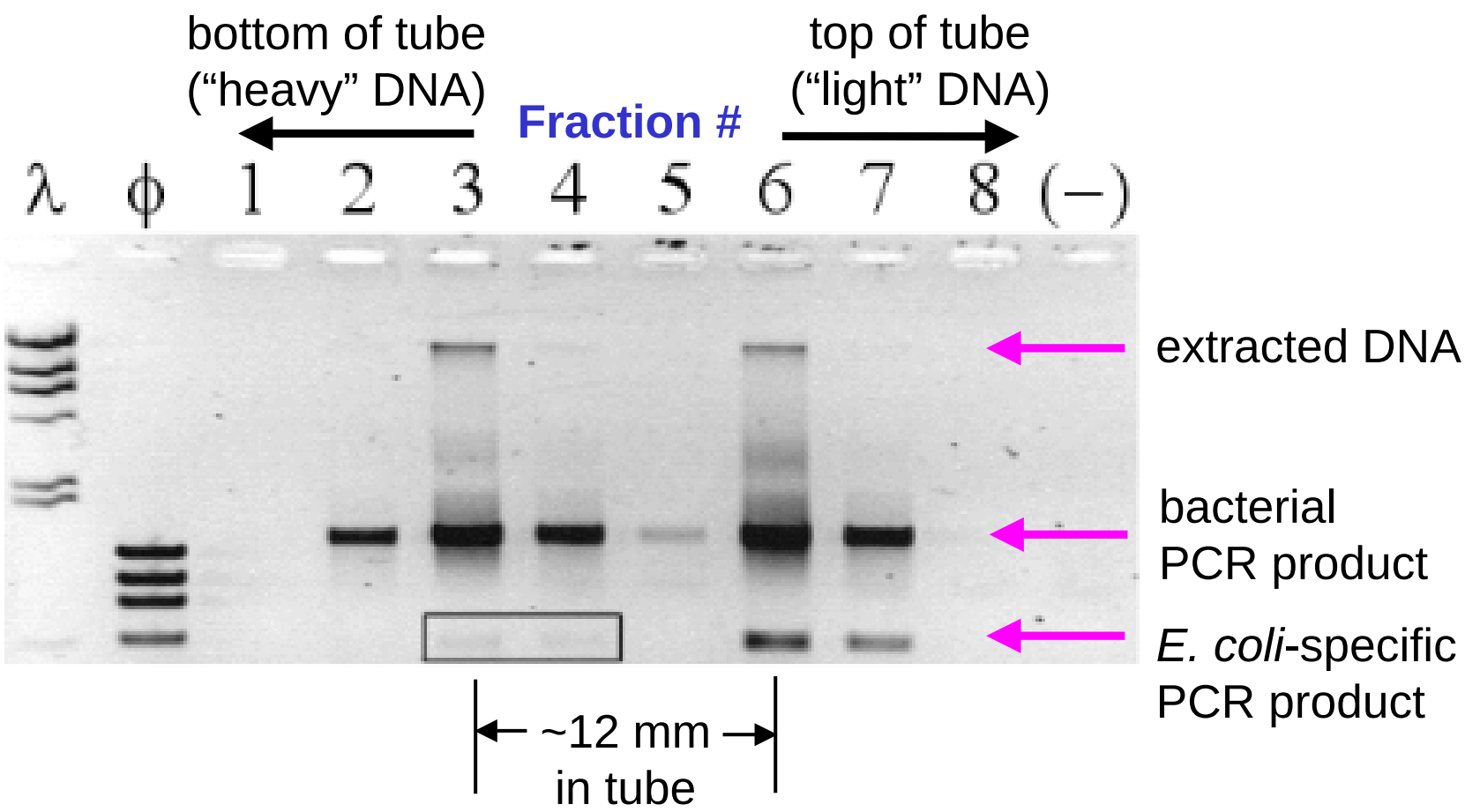


SIP protocol (continued)

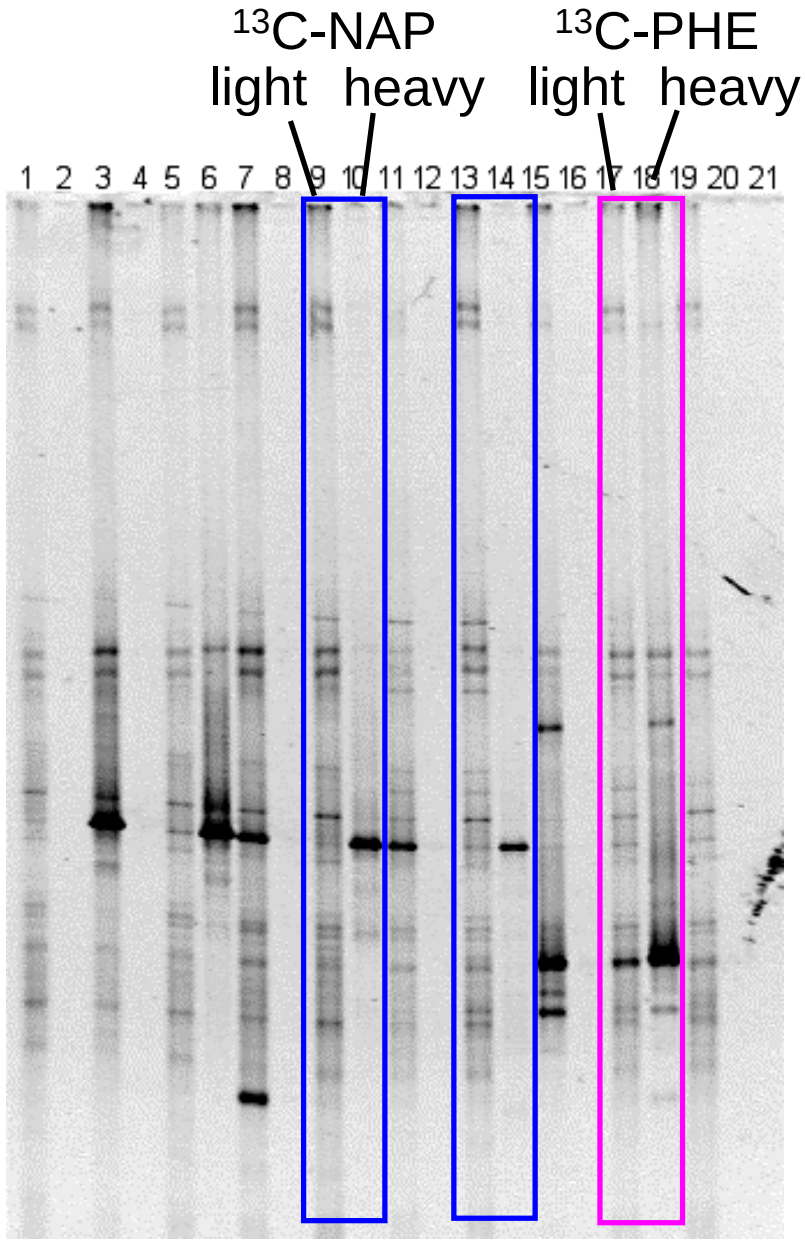


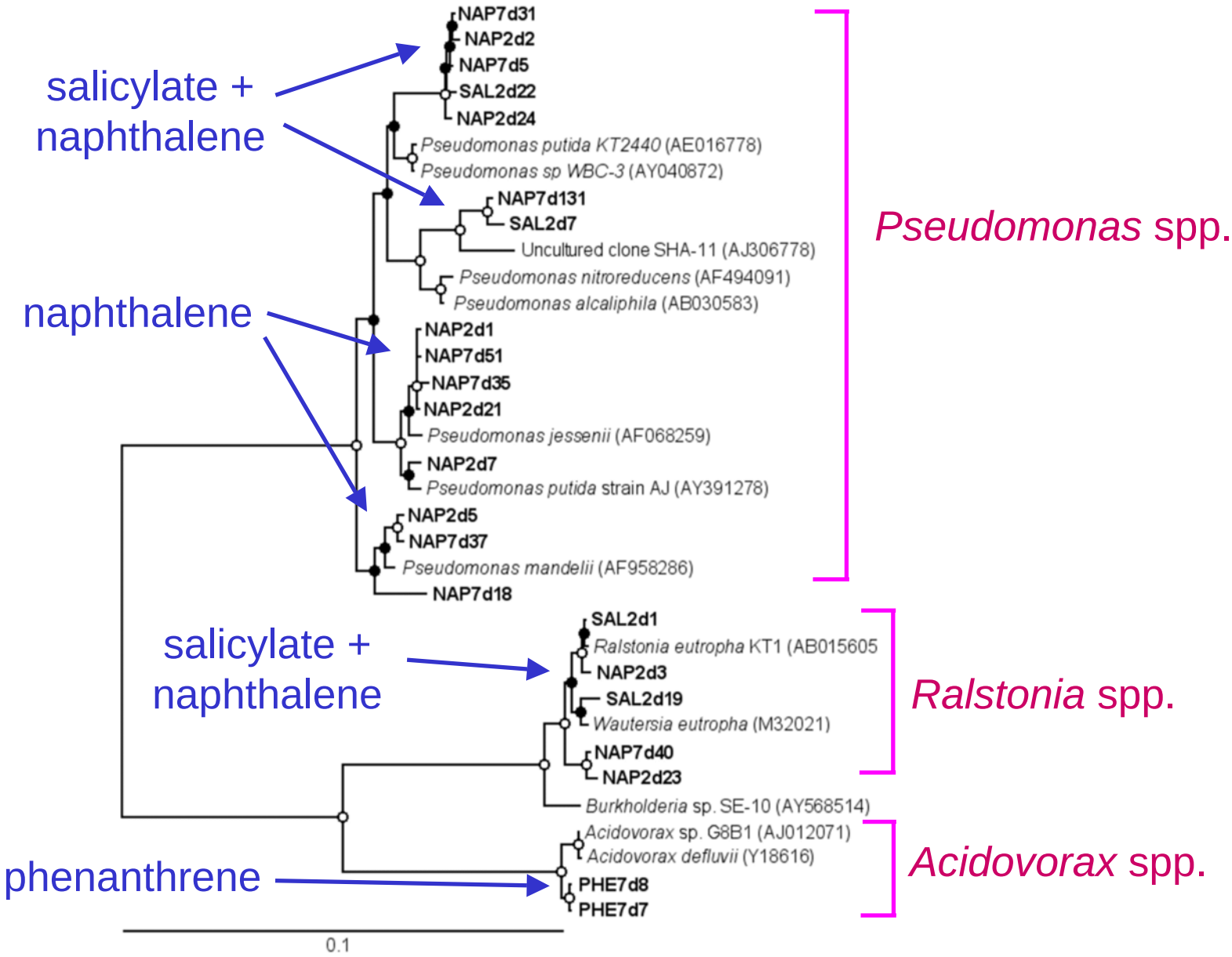
*no singleton sequences considered; sequences recovered at same or lower frequency than *E. coli* sequence were also not considered

Recovery of DNA in fractions after separation (incubation with phenanthrene)



DGGE analysis




salicylate +
naphthalene
naphthalene
salicylate +
naphthalene
phenanthrene
NAP7d31
NAP2d2
NAP7d5
SAL2d22
NAP2d24
Pseudomonas putida KT2440 (AE016778)
Pseudomonas sp WBC-3 (AY040872)
NAP7d131
SAL2d7
Uncultured clone SHA-11 (AJ306778)
Pseudomonas nitroreducens (AF494091)
Pseudomonas alcaliphila (AB030583)
NAP2d1
NAP7d51
NAP7d35
NAP2d21
Pseudomonas jessenii (AF068259)
NAP2d7
Pseudomonas putida strain AJ (AY391278)
NAP2d5
NAP7d37
Pseudomonas mandelii (AF958286)
NAP7d18
SAL2d1
Ralstonia eutropha KT1 (AB015605
NAP2d3
SAL2d19
Wautersia eutropha (M32021)
NAP7d40
NAP2d23
Burkholderia sp. SE-10 (AY568514)
Acidovorax sp. G8B1 (AJ012071)
Acidovorax defluvii (Y18616)
PHE7d8
PHE7d7
0.1
Pseudomonas spp.
Ralstonia spp.
Acidovorax spp.

Summary of results

- Naphthalene-degrading bacteria in bioreactor cluster with *Pseudomonas* spp. (γ-Proteobacteria) and *Ralstonia* spp. (β-Proteobacteria)
- Phenanthrene-degrading bacteria are similar to *Acidovorax* spp. (β-Proteobacteria)
- No overlap between naphthalene and phenanthrene degraders (no sequence appearing more than once)
- Salicylate degraders were very similar to naphthalene degraders but not phenanthrene degraders
- Some naphthalene degraders were not closely associated with salicylate degraders

Results (continued)

- Little effect of incubation time (2 or 7 days)
  - "cross-feeding" not a concern
  - less DNA from longer incubation
- *E. coli* indicator:
  - sensitive *E. coli*-specific PCR program recovered *E. coli* DNA in all fractions
  - two clones (of 51) in phenanthrene library had *E. coli* sequences
  - no *E. coli* sequences in naphthalene or salicylate clone libraries
- No archaeal or fungal DNA recovered

Preliminary interpretations

- “Division of labor” in PAH degradation in field-contaminated systems?
- Limited diversity of bacteria enriched with salicylate: implications for stimulation of HMW PAH degradation?
  - naphthalene-degrading bacteria typically metabolize narrower range of PAHs

Beyond SIP

- Develop probes to quantify specific degraders in bioreactor
  - FISH
  - quantitative PCR
- Isolate pure cultures known to be significant degraders for physiological studies (which ones degrade BaP?)
- Interrogate “heavy” DNA for other genes (e.g., genes for PAH metabolism)
- Metagenomic analysis of “heavy” DNA

Acknowledgments

Molecular Work

David Singleton

Sabrina Powell

^{13}C Syntheses

Ramiah Sangaiah

Avram Gold

Louise Ball

Funding

NIEHS (5 P42 ES05948)

NSF (BES-0221836)