

The background of the slide is a composite image. The top half shows an oil rig at sea during a sunset, with the sky in shades of orange and red. The bottom half shows a satellite-style map of South America, with Brazil highlighted in green. The text is overlaid on the map.

Screening for Novel Biodegraders in Metagenomic Libraries of Petroleum-Associated Environments

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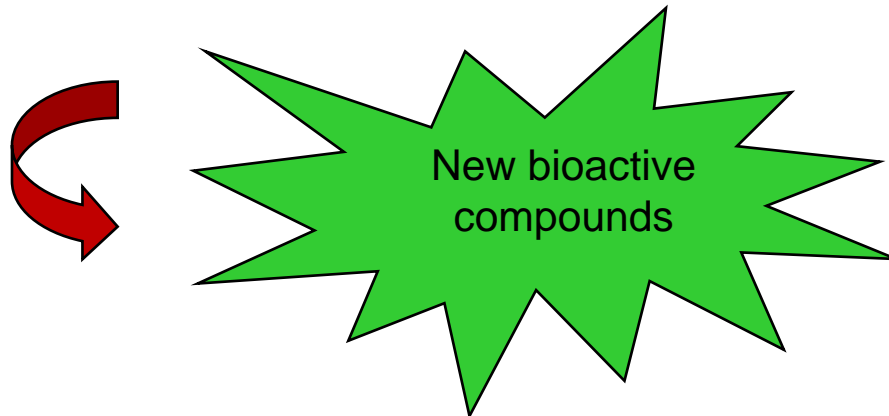


Bioprospection: technology *versus* nature

- Soil microorganisms
 - ✓ Source for the production of natural compounds with biological activity
 - ✓ actinobacteria, *Bacillus* spp., *Pseudomonas* spp., etc.
- Majority of biotechnological products available
 - ✓ Isolation and screening of microorganisms and plants
 - ✓ Applications : medical, industrial and agricultural areas
- **High rate of “rediscovery” of new products**
 - ✓ Screening of culturable microorganisms
 - ✓ Continuous sampling of the same environments

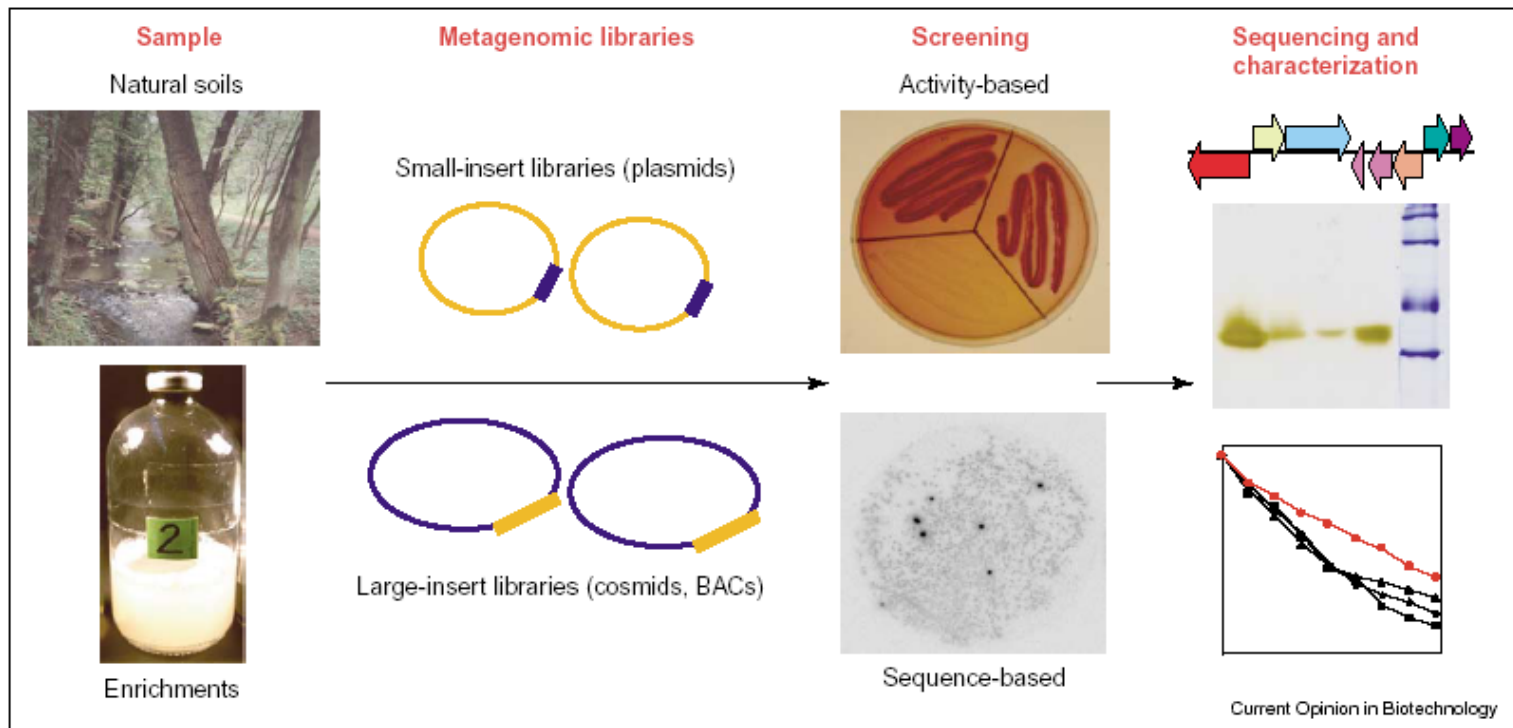
Trends in bioprospection

- **New approaches in the search of microbial products**
 - ✓ **New environments**
 - Endophytic microorganisms, marine, polluted and extreme environments
 - ✓ **New strategies**
 - New methods for selective isolation
 - Cultivation-independent methods: ***“metagenomics”***



Why Metagenomics?

- Allow the access to the metabolic diversity of uncultured microorganisms (the unseen majority)



Strategy to access and exploit the soil metagenome through the construction and screening of DNA libraries derived from soil samples or soil enrichments.

Daniel, R., 2004, *Current Opinion in Biotechnology* 15:199–204.

img/m  **INTEGRATED MICROBIAL GENOMES with MICROBIOME SAMPLES**

- [IMG/M Home](#)
- [Find Genomes](#)
- [Find Genes](#)
- [Find Functions](#)
- [Compare Genomes](#)
- [Analysis Carts](#)
- [MyIMG/M](#)
- [Using IMG/M](#)

Genome Browser *Microbiome Tree Loaded.

- 01 ▾ ● ● *Microbiome (119) ←
- 02 ▾ ● ● Engineered (15)
- 02 ▾ ● ● Environmental (65) ←
- 03 ▾ ● ● Air (2)
- 03 ▾ ● ● Aquatic (56)
- 04 ▾ ● ● Biofilm (1)
- 05 ▾ ● ● Mine drainage biofilm (1)
- 04 ▾ ● ● Groundwater (2)
- 05 ▾ ● ● Freshwater (2)
- 06 ▾ ● ● Contaminated (1)
- 08 [Uranium Contaminated Groundwater FW106](#) [D] (JGI)
- 04 ▾ ● ● Surface water (53)
- 05 ▾ ● ● Freshwater (6)
- 06 ▾ ● ● Lake sediment (6)
- 05 ▾ ● ● Marine (9)
- 05 ▾ ● ● Saline and alkaline lakes and closed seas (10)
- 06 ▾ ● ● Salt crystallizer ponds (10)
- 05 ▾ ● ● Thermal spring (28)
- 03 ▾ ● ● Fossil (3)
- 03 ▾ ● ● Soil (4)
- 02 ▾ ● ● Host-associated (36)
- 02 ▾ ● ● Modeled communities (3)

Metagenome sequencing of extreme environments



40/119!!

Functional Metagenomics

Chemistry & Biology, Vol. 12, 895–904, August, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.06.020

Microbial E Urania Dee

Appl Microbiol Biotechnol (2007) 74:688–698
DOI 10.1007/s00253-006-0691-0

ENVIRONMENTAL BIOTECHNOLOGY

APPLIED AND ENVIRONM
0099-2240/05/\$08.00+0
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Novel bacterial sulfur oxygenase reductases from bioreactors treating gold-bearing concentrates

New TI
Si

Z.-W. Chen · Y.-Y. Liu · J.-F. Wu · Q. She · C.-Y. Jiang ·
S.-J. Liu

Cloned from a Metagenomic Library

Jin-Kyu Rhee, Dae-Gyun Ahn, Yeon-Gu Kim, and Jong-Won Oh*

The ISME Journal (2009) 3, 243–251
© 2009 International Society for Microbial Ecology All rights reserved 1751-7362/09 \$32.00
www.nature.com/ismej



ORIGINAL ARTICLE

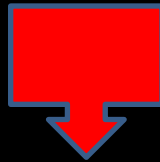
Functional metagenomics reveals diverse β -lactamases in a remote Alaskan soil

Heather K Allen^{1,2}, Luke A Moe¹, Jitsupang Rodbumrer^{1,3}, Andra Gaarder¹
and Jo Handelsman¹



An aerial photograph of a large offshore oil rig in the middle of a deep blue ocean. The rig is a complex of steel structures, including a tall derrick, various platforms, and cranes. The water is a vibrant blue with some whitecaps.

Novel enzymes ????



Petroleum industry



Screening of phenol degrading activity in microbial metagenome from petroleum refinery wastewater



Cynthia Canêdo da Silva & Valéria Maia de Oliveira



or

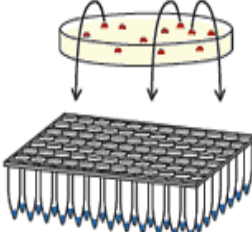


Cloning-Ready CopyControl™
pCC1FOS™ Vector



Ligate

Package
(lambda phage)



Transformation in *E. coli* EPI 300

**Membrane Bioreactor +
high phenolic load
(68.5 mg phenol/ 30 days)**

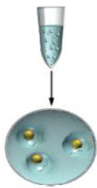
**Phenol batch
enrichment (1000 mg
phenol/30 days)**

Isolate genomic
DNA ~40 kb

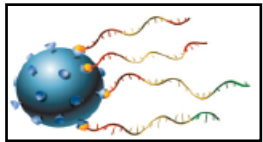
End-repair
~40 kb DNA



Pyrosequencing



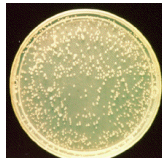
Emulsion PCR



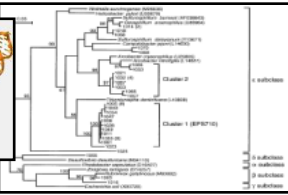
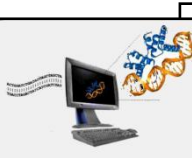
FLX Titanium library



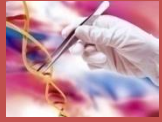
**Fosmid extraction
Large Construct Kit
(Qiagen)**



Screening (phenol
degradation)

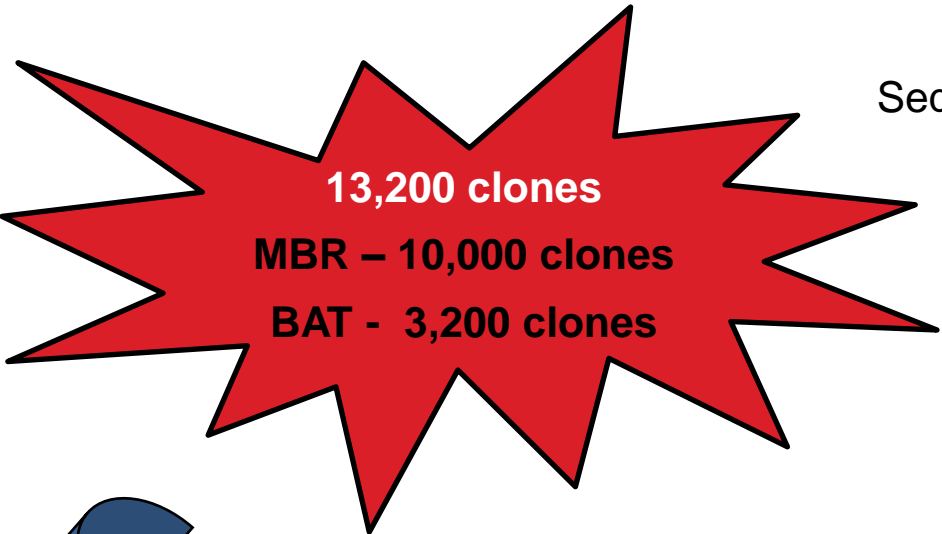


**Bioinformatics (phylogenetic
and functional diversity)**

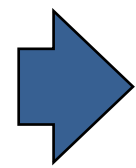


Screening by PCR

phenol hydroxylase large subunit gene (LmPHs)



Sequence-based screening

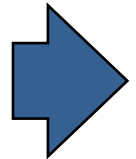
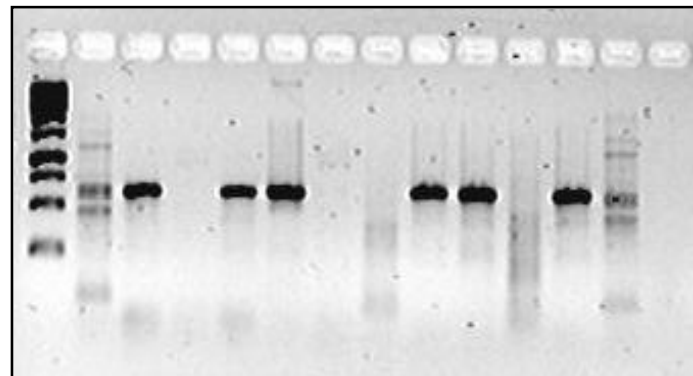


1st PCR: pools with 10 clones

2nd PCR: individual clones of positive pools (identification of positive clones in each pool)



Results:



1- MBR library - 10 positive clones
2- BAT library - 11 positive clones

✓ Positive control: *Acidovorax* sp. isolated from the enrichment after acclimation

Functional Screening: colorimetric assay

13,200 clones

MBR – 10,000 clones

BAT - 3,200 clones

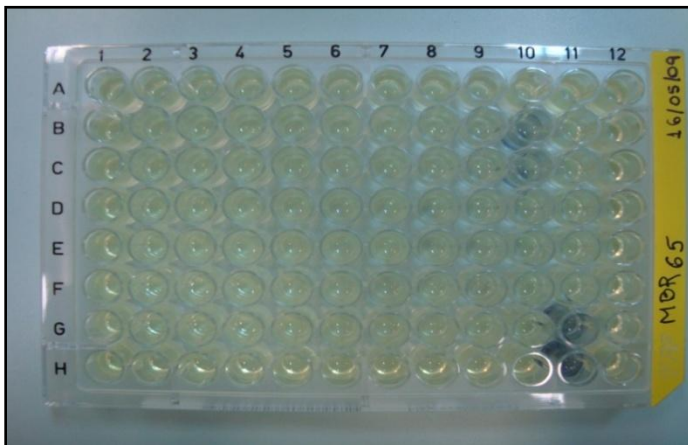
Activity-based
screening

Mineral medium + 0.02%
phenol (microplate assay)

48 h / 37°C

Thiazolyl blue [3-(4,5-dimethyl-2-
thiazolyl)-2,5-diphenyl-2H-tetrazolium
bromide] (1h / 37°C):

detection of clones able to grow on phenol
as sole carbon source (violet color)



1- MBR libray - 211 positive clones

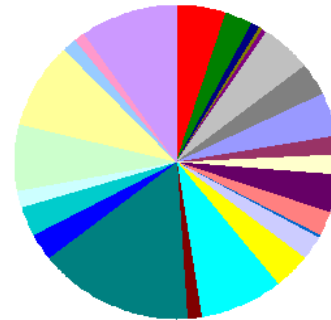
2- BAT library - 202 positive clones

✓ chromatographic assays will be performed (quantitation and identification of subproducts)

New genes related to phenol degradation in metagenomic libraries derived from petroleum refinery sludge

Cynthia C. Silva, Tim Sawbridge, Helen Hayden, Maira P. Souza, Ana P.R. Torres, Vânia M.J. Santiago & Valéria M. Oliveira (*in preparation*)

MG-RAST – Metabolic profile



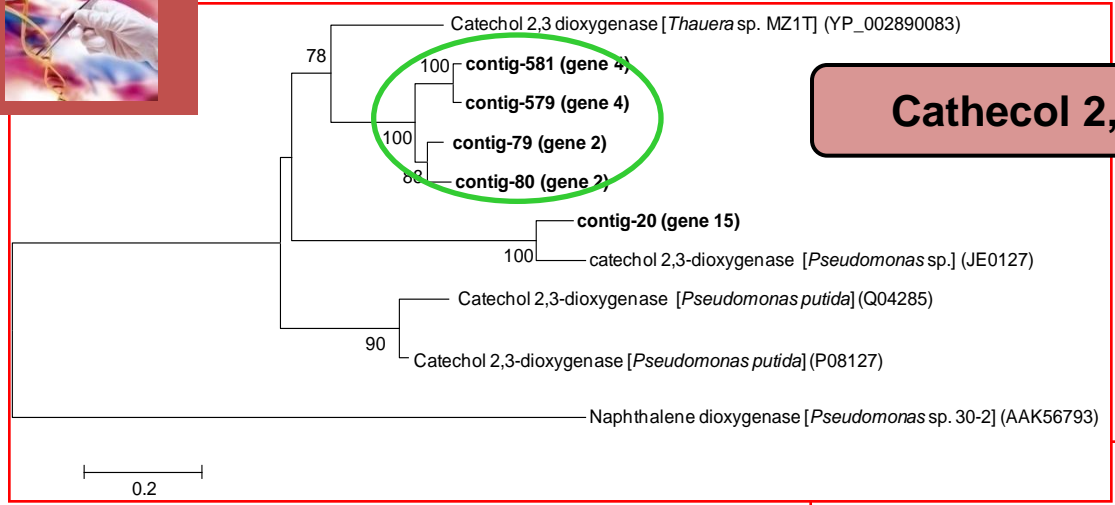
Cofactors, Vitamins, Prosthetic Groups, Pigments	5.09% (79)
Cell Wall and Capsule	2.84% (44)
Potassium metabolism	0.90% (14)
Photosynthesis	0.06% (1)
Miscellaneous	0.64% (10)
Membrane Transport	5.09% (79)
RNA Metabolism	3.22% (50)
Protein Metabolism	4.51% (70)
Nucleosides and Nucleotides	2.06% (32)
Cell Division and Cell Cycle	2.06% (32)
Motility and Chemotaxis	3.80% (59)
Regulation and Cell signaling	2.51% (39)
Secondary Metabolism	0.32% (5)
DNA Metabolism	2.51% (39)
Prophage	0.13% (2)
Unclassified	3.61% (56)
Virulence	8.25% (128)
Macromolecular Synthesis	0.06% (1)
Nitrogen Metabolism	1.23% (19)
Clustering-based subsystems	15.60% (242)
Respiration	2.90% (45)
Stress Response	2.90% (45)
Sulfur Metabolism	1.68% (26)
Metabolism of Aromatic Compounds	6.96% (108)
Amino Acids and Derivatives	8.96% (139)
Fatty Acids and Lipids	1.48% (23)
Phosphorus Metabolism	1.10% (17)
Carbohydrates	9.48% (147)

Positive clones for phenol degradation

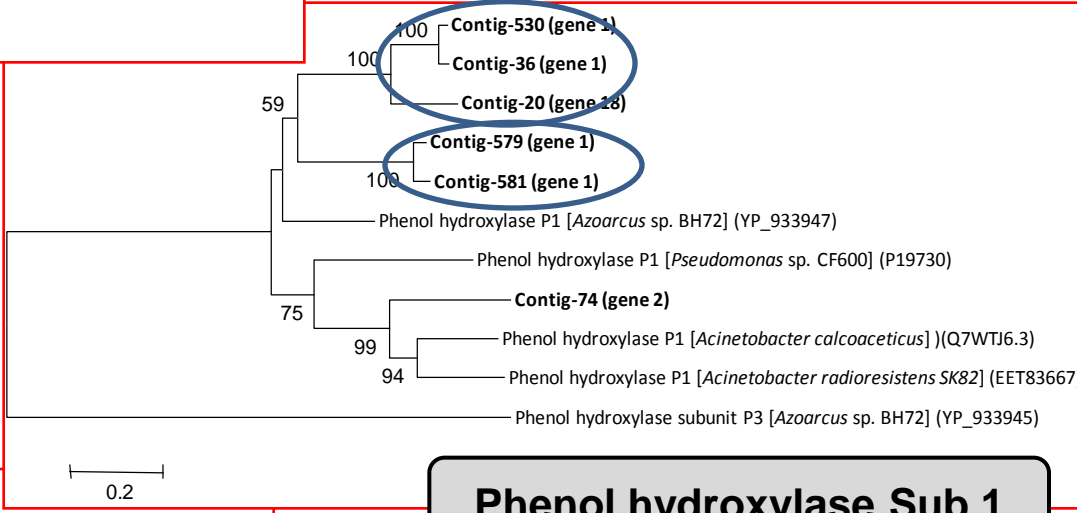


Fosmid DNA pool

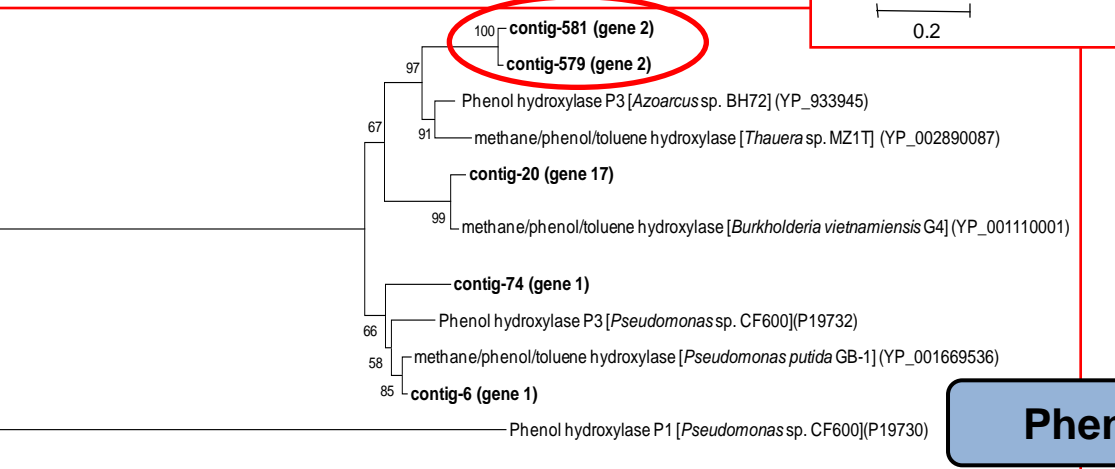
454 RUN DATA
129,635 reads
341 contigs (>1000 bp)



Cathecol 2,3-dioxygenase



Phenol hydroxylase Sub 1



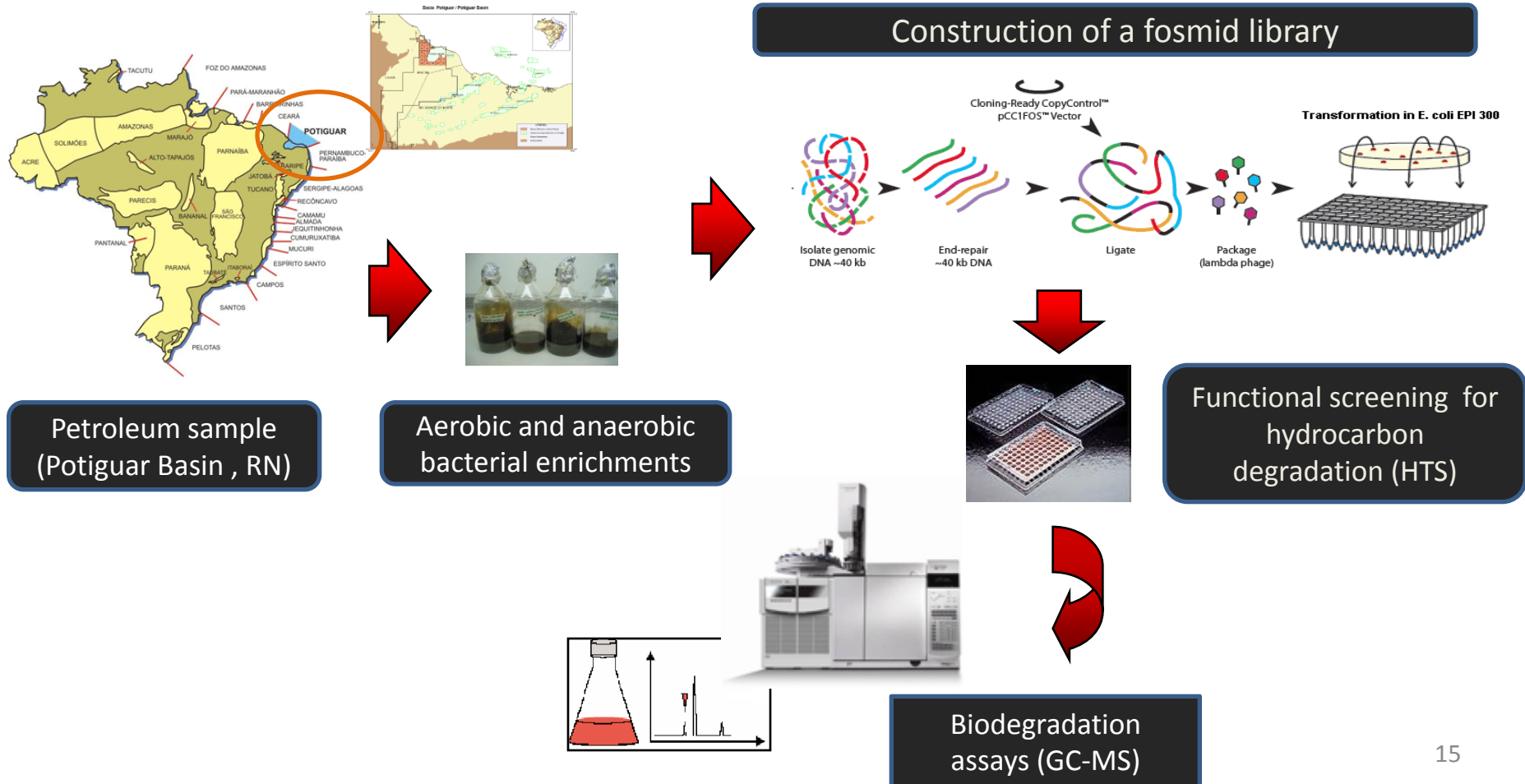
Phenol hydroxylase Sub 3

Future work

- ✓ Chromatographic analyses to select the best performing clones for phenol degradation;
- ✓ Evaluation of the ability of metagenomic clones to degrade other phenol derivatives;
- ✓ Genetic characterization of positive fosmids able to phenol degradation (new genes or pathways?);

Metagenomic studies of microbial community in petroleum samples and investigation of its biocatalytic potential

Suzan Pantaroto de Vasconcellos & Valéria Maia de Oliveira





Contents lists available at ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem

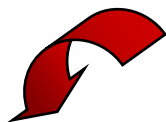
Screening for hydrocarbon biodegraders in a metagenomic clone library derived from Brazilian petroleum reservoirs

Suzan Pantaroto de Vasconcellos^a, Célio Fernando Figueiredo Angolini^b, Isabel Natalia Sierra García^a, Bruna Martins Dellagnezze^a, Cynthia Canedo da Silva^a, Anita Jocelyne Marsaioli^b, Eugenio Vaz dos Santos Neto^c, Valéria Maia de Oliveira^{a,*}

^aMicrobial Resource Division, Research Center for Chemistry, Biology and Agriculture (CPQBA), Campinas University – UNICAMP, CP 6171, CEP 13081-970 Campinas, SP, Brazil

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^cPETROBRAS R&D Center, Cidade Universitária, Quadra 7, Rio de Janeiro, RJ 21949-900, Brazil



- ✓ Metagenomic library (aerobic + anaerobic enrichment of biodegraded oil + hexadecane as carbon source): 31,000 clones

✓ Assay for hexadecane degradation:

- microplates (*colorimetric assay*): 72 positive hits among 5,000 clones evaluated
- chromatographic analyses: 1 positive pool (9 clones) in 8 pools evaluated
- **Pool CO8** – 5 clones were able to degrade hexadecane (>70%)

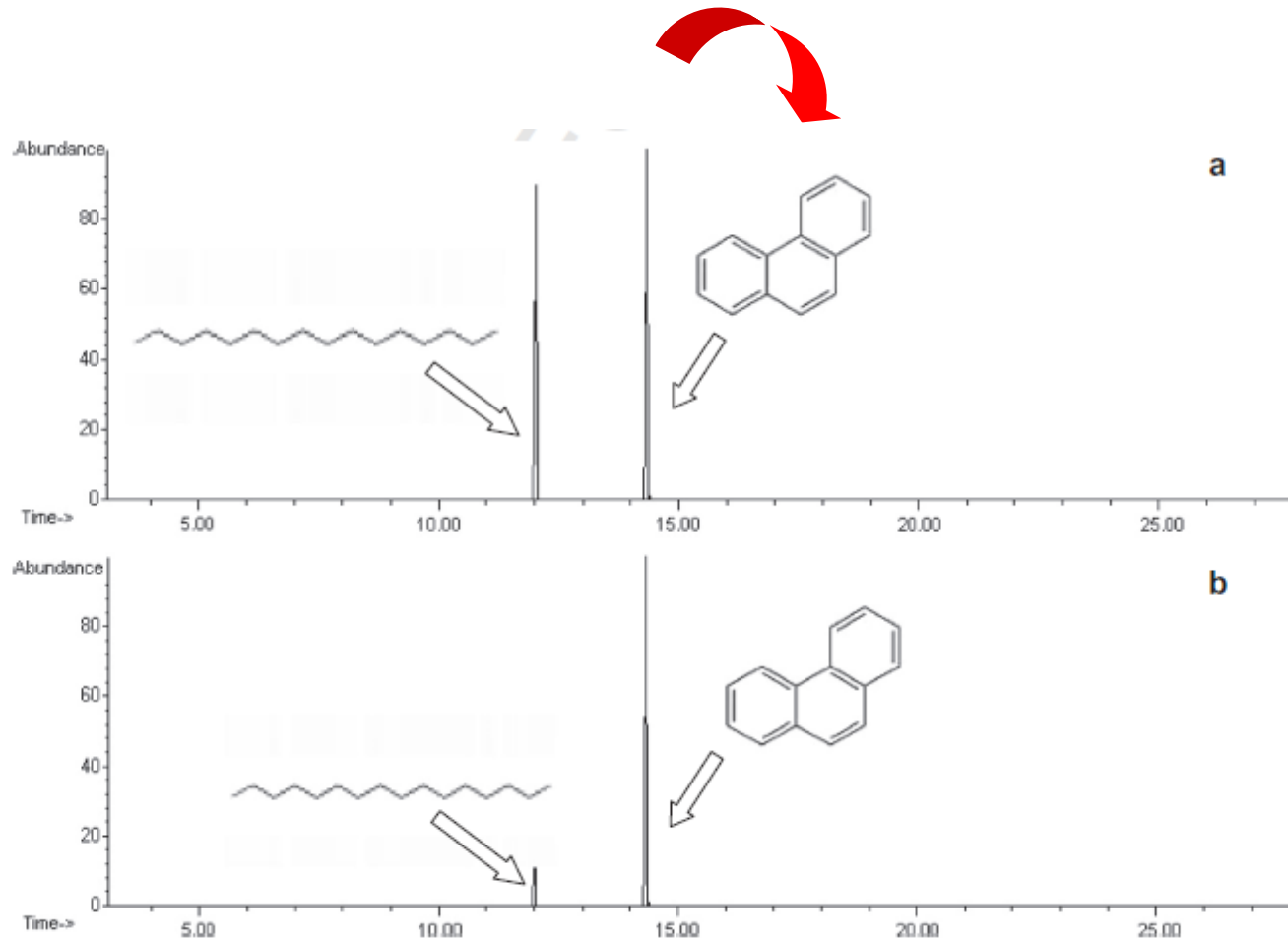


Fig. 4. GC-MS total ion chromatogram showing hexadecane biodegradation by the clone 2B. (a) Time 0; (b) after 28 days of incubation (98% biodegradation level).

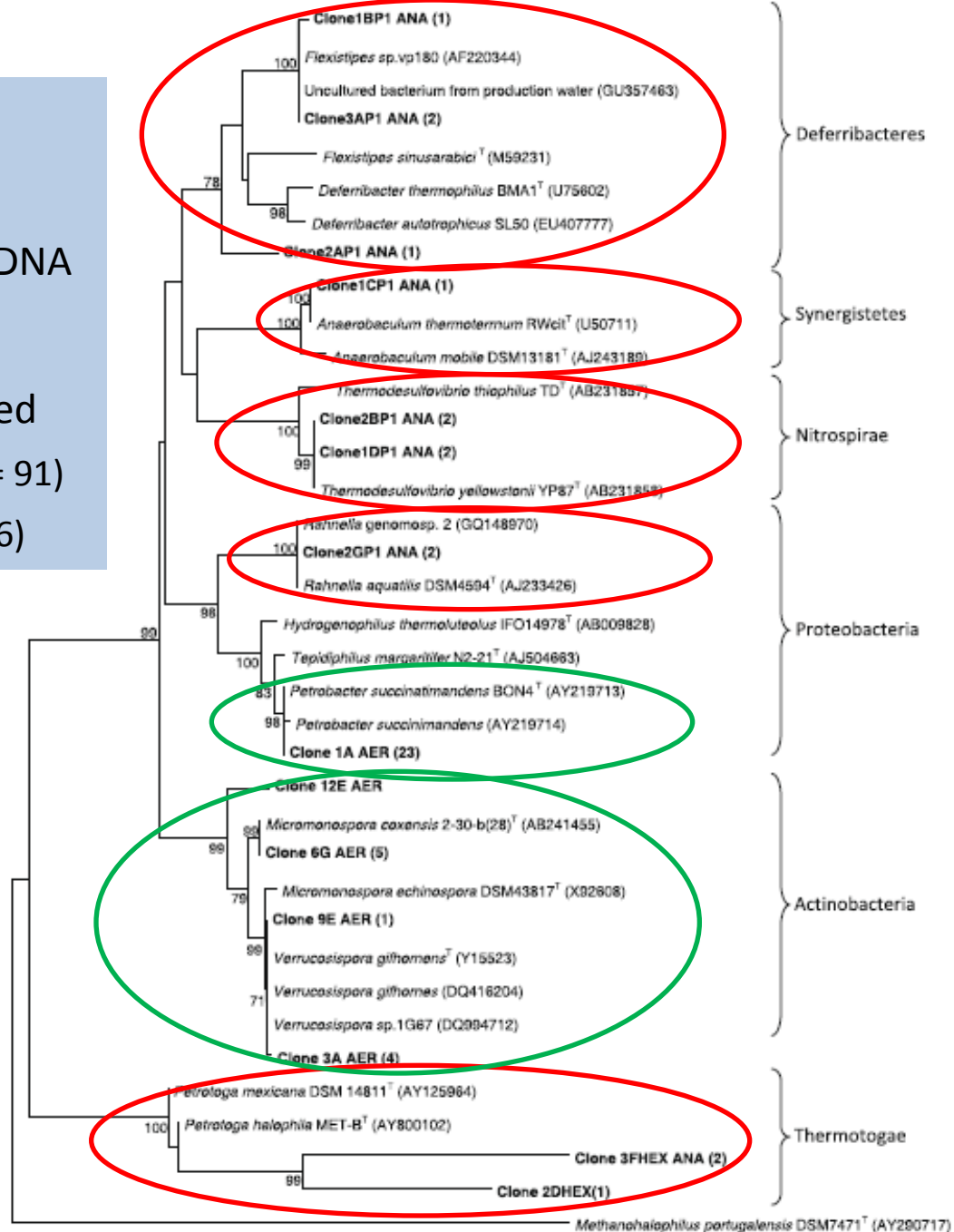
✓ Aerobic and anaerobic bacterial enrichments

✓ Diversity analysis (16S rDNA libraries)

✓ 6 bacterial phyla detected

— Anaerobic enrichment ($N = 91$)

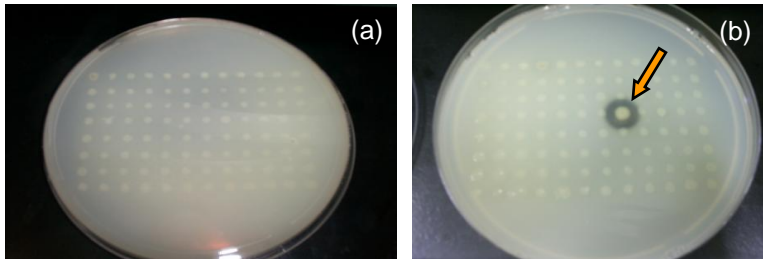
— Aerobic enrichment ($N = 96$)



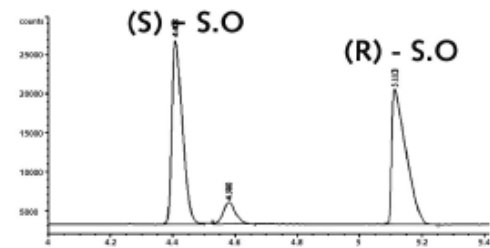
Functional Screening: lipases and proteases

- ✓ **Lipases**: screening of 5,000 clones in plate assays with tributyrin
- ✓ **Proteases**: screening of 7,800 clones in plate assays with skin milk

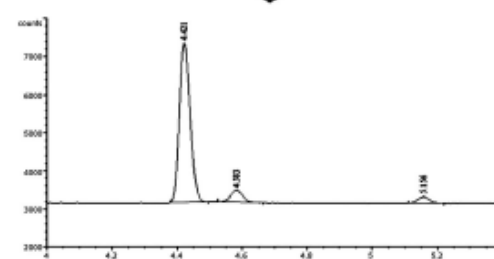
Results:



- ✓ 20 positive hits for protease
- ✓ 28 positive hits for lipase



Incubation



3 clones = higher than 85%
tributyrin conversion

Structural characterization of genes encoding hydrocarbon degradation activity in microbial metagenome derived from petroleum reservoirs



Isabel Natalia Sierra Garcia & Valéria Maia de Oliveira

Positive fosmid clones
(HC degradation)

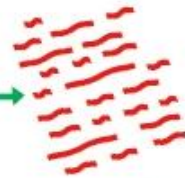


Extract DNA



Random shotgun library construction

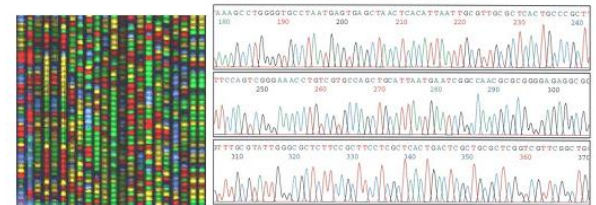
Sonicate



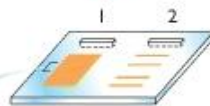
DNA fragments of various sizes



Sequencing and
Data analysis



Agarose gel electrophoresis



Purify DNA from
the gel

LANE 1: Sonicated *H. influenzae* DNA
LANE 2: DNA markers

DNA fragments – 1.6–2.0 kb



Prepare a clone library



Assembly and gene
characterization



Biodegradation evaluation of metagenomic clones

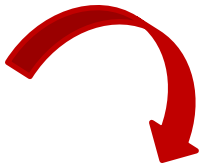
HTS assays
(Vasconcellos et al., 2010)



1 pool: **CO8**
58% hexadecane degradation



9 clones: 5 clones **> 70%**
hexadecane degradation

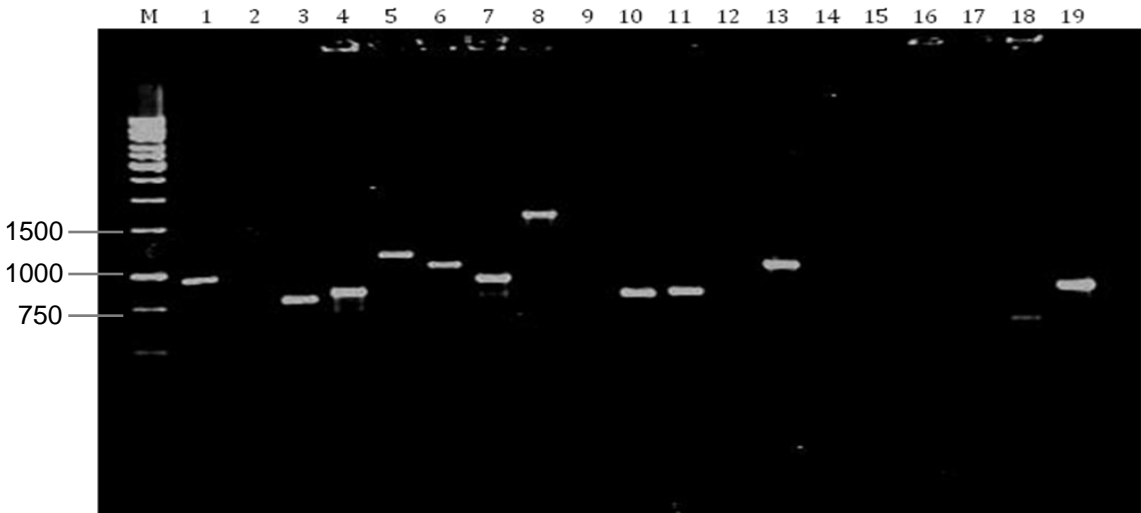
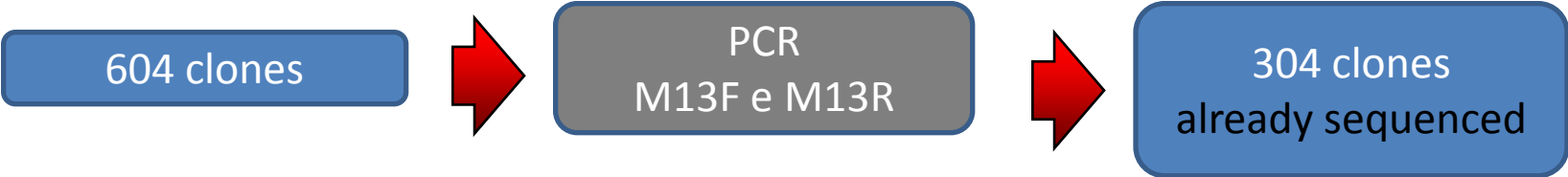


Clones (CO8)	Hexadecane degradation
10A	86%
1A	91%
6G	92%
3B	70%
2B	98%

Clones (CO8)	Phenanthrene degradation
10A	49%
1A	5%
6G	15%
3B	21%
2B	44%



Sequencing of shotgun library (clone 1A)

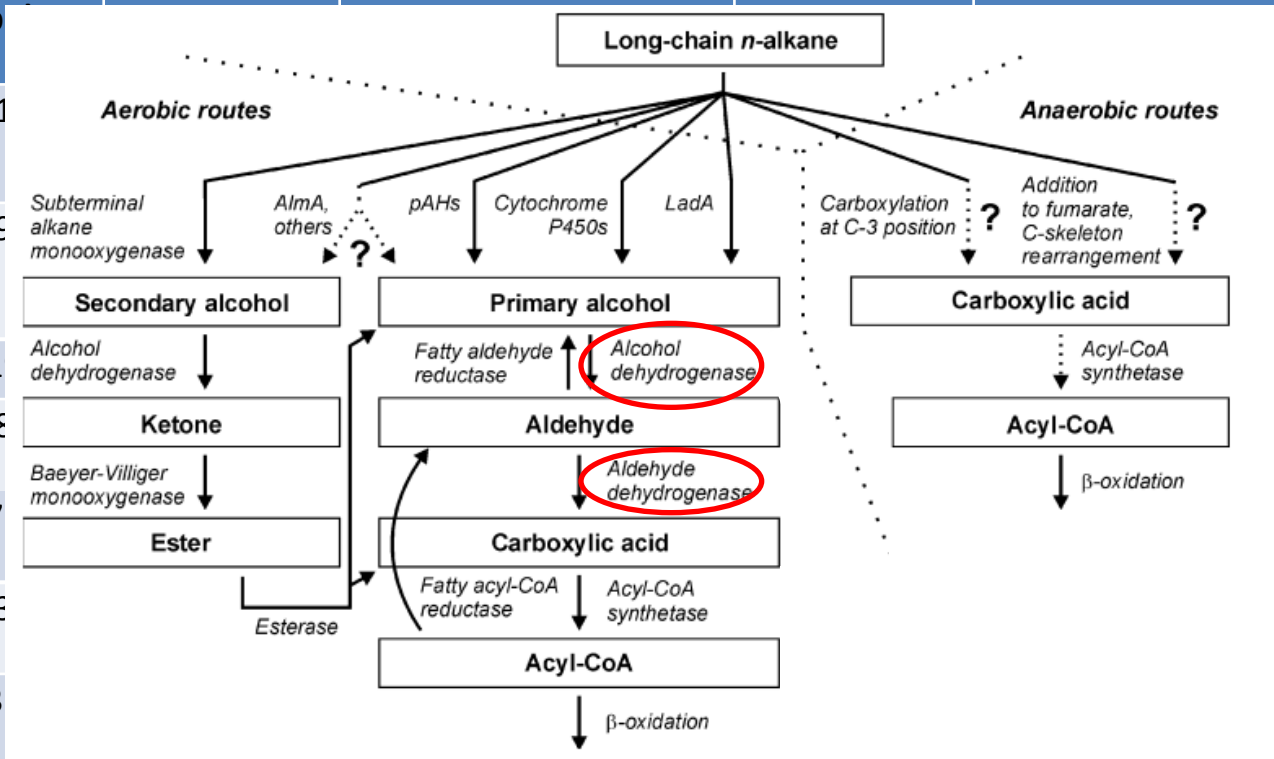


Amplicons of shotgun clones derived from fosmid 1A with *primers* M13F e M13R.

General statistics	
#Clusters	100
#Contigs	24
#singlets	76
Total length	47088
Mean contig size	1774
N50: 700	22 contigs

Sequencing of shotgun library (clone 1A)

Contig	Contig size (bp)	Number of	Top BLASTX hit	Identity (%)	Organism type	Target Accession
Contig 39	61					NP_770638.1
Contig 35	99					YP_002355505.1
Contig 11	11					YP_935028.1
Contig 44	88					YP_158609.1
Contig 25	17					YP_934701.1
Contig 46	43					YP_934701.1
Contig 3	13					YP_315294.1
					25259	



Future work and Comments

- ☞ Sequencing of additional clones derived from fosmid 1A is being conducted in order to achieve the complete coverage of the insert (~40 kb) responsible for hexadecane degradation (92%)
- ☞ Shotgun cloning and complete sequencing of fosmid 2B, able to degrade hexadecane (98%) and phenanthrene (44%), are also being carried out
- ☞ Genetic sequences responsible for oil degradation processes in Brazilian reservoirs are being for the first time identified by means of the metagenome approach
- ☞ Results reinforce the huge potential of metagenomics for bioprospecting hydrocarbon degradation genes from extreme environments

Microbial Ecology and Metagenomics Group:

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- ✓ Leandro da Costa Lima Verde, D
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- ✓ Patricia Ferreira Lopes, M
- ✓ Paula Brandão Miqueletto, M
- ✓ Tiago Rodrigues e Silva, M
- ✓ Daniela Ferreira Domingos, D
- ✓ Julia Ronzella Ottoni, D



Colaborators:

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- ✓ Profa. Dra. Anete Pereira de Souza (IB/UNICAMP)
- ✓ Profa. Dra. Anita J. Marsaioli (IQ/UNICAMP)
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Support:





Florianópolis