New cultivation-based approaches for mining the metabolic potential of microorganisms

Karsten Zengler, Diversa Corporation

The Introduction of Solid, Defined Media and Pure-Culture Methods Marked a True Revolution*

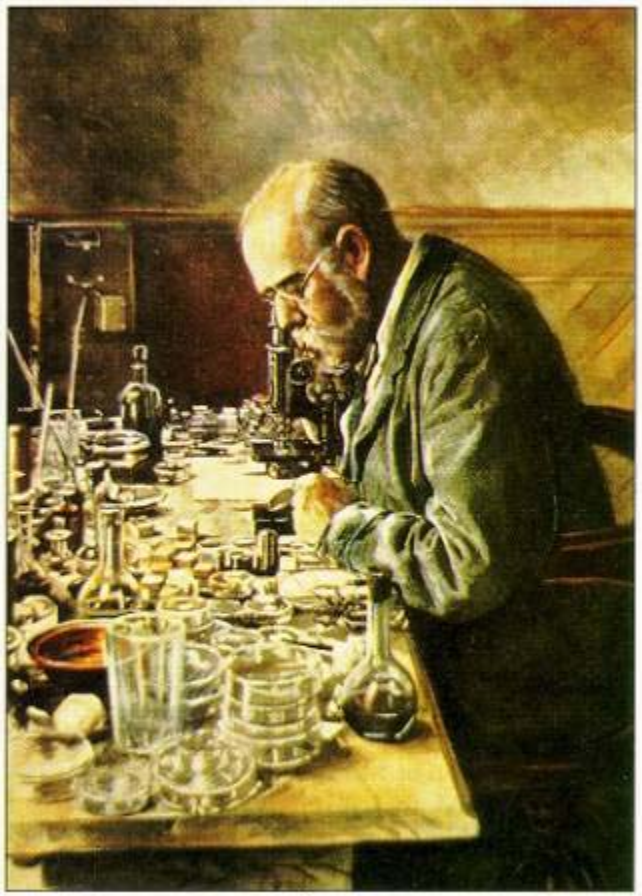

Figure 1.11 Robert Koch, another giant in microbiology, in his laboratory.

* from: Biology of the Procaryotes; Lengeler, Drews, Schlegel

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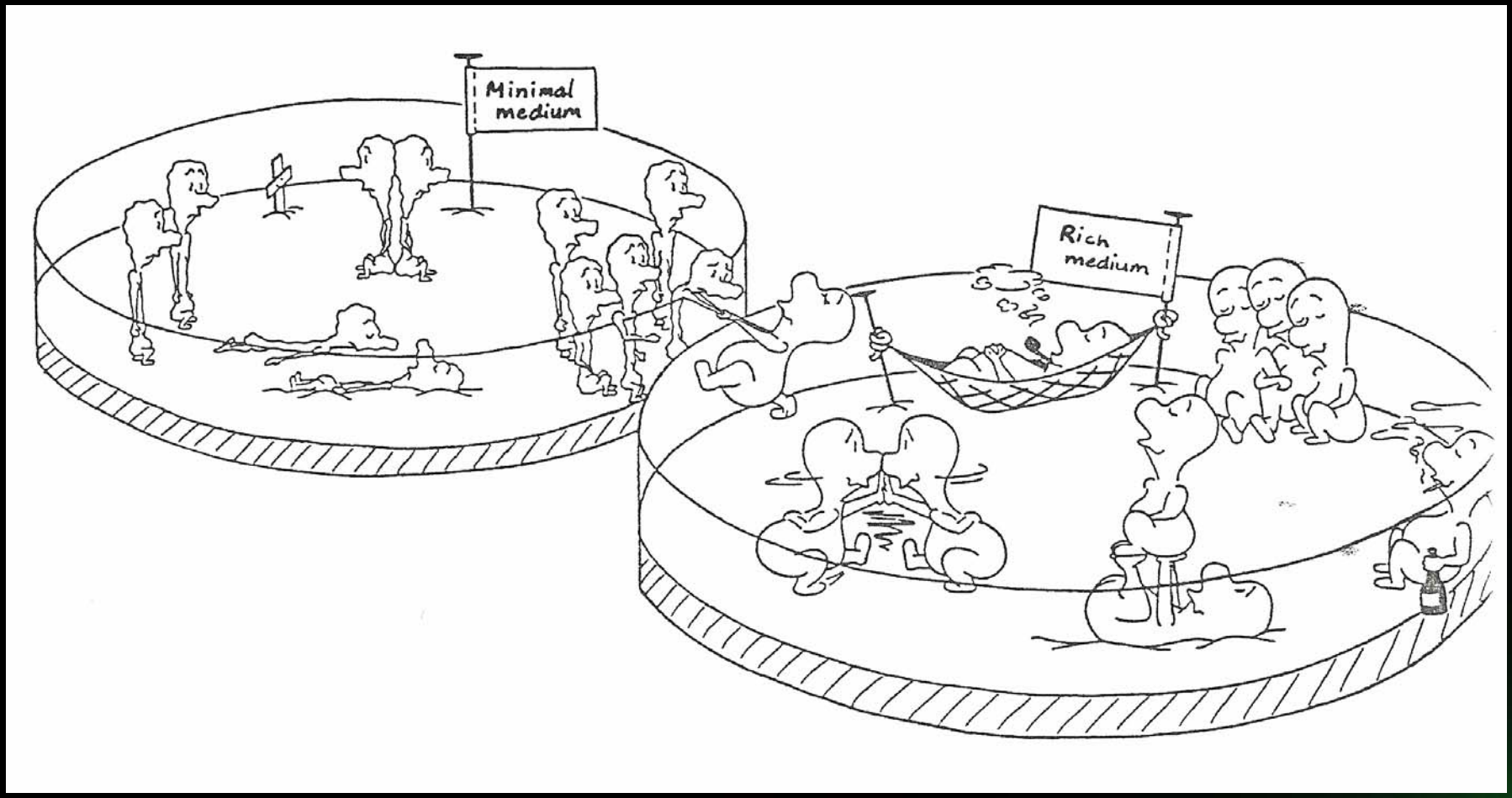
The Theory…



* from: What's so funny about microbiology? / Joachim Czichos

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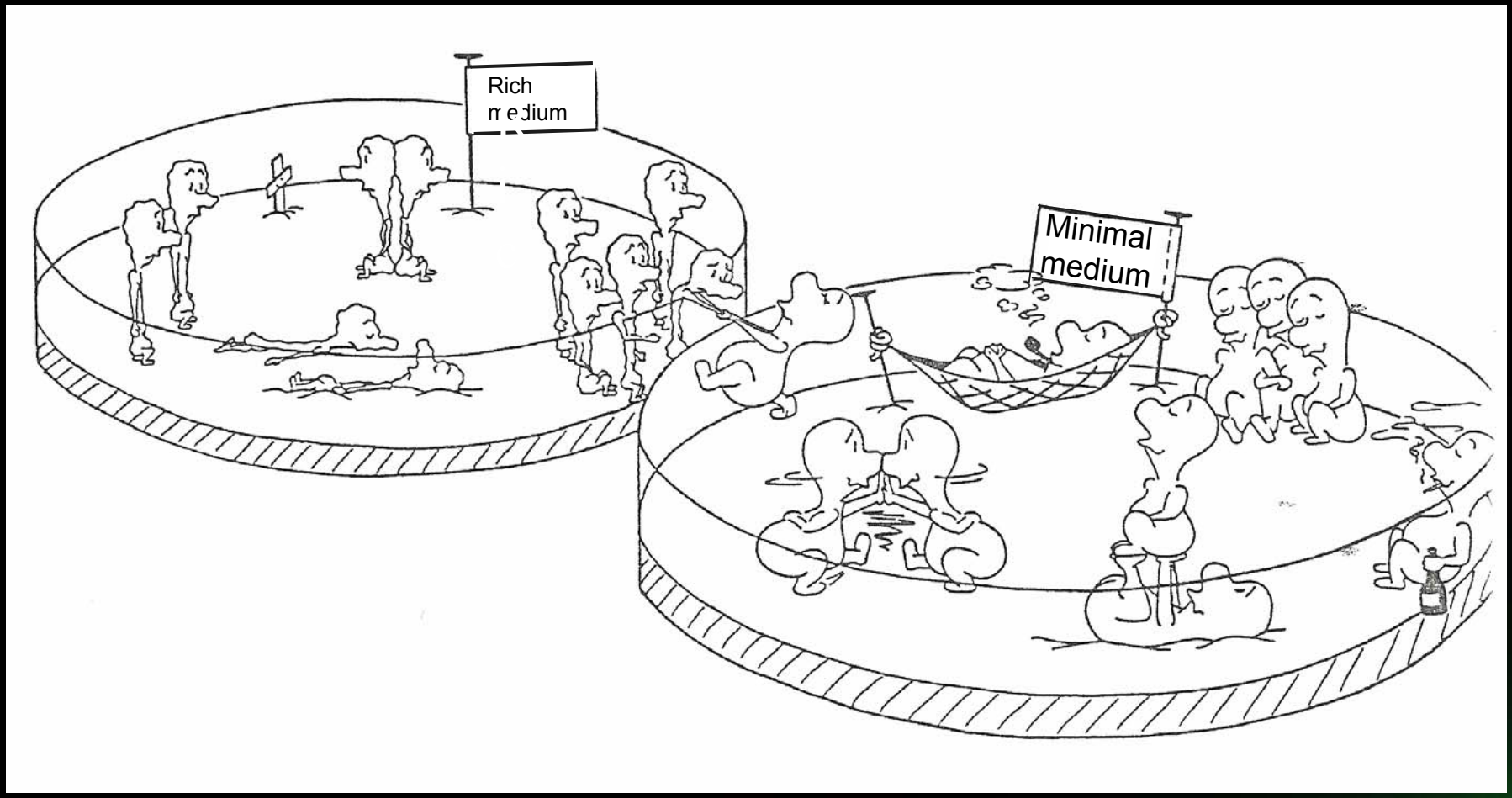
The Reality…



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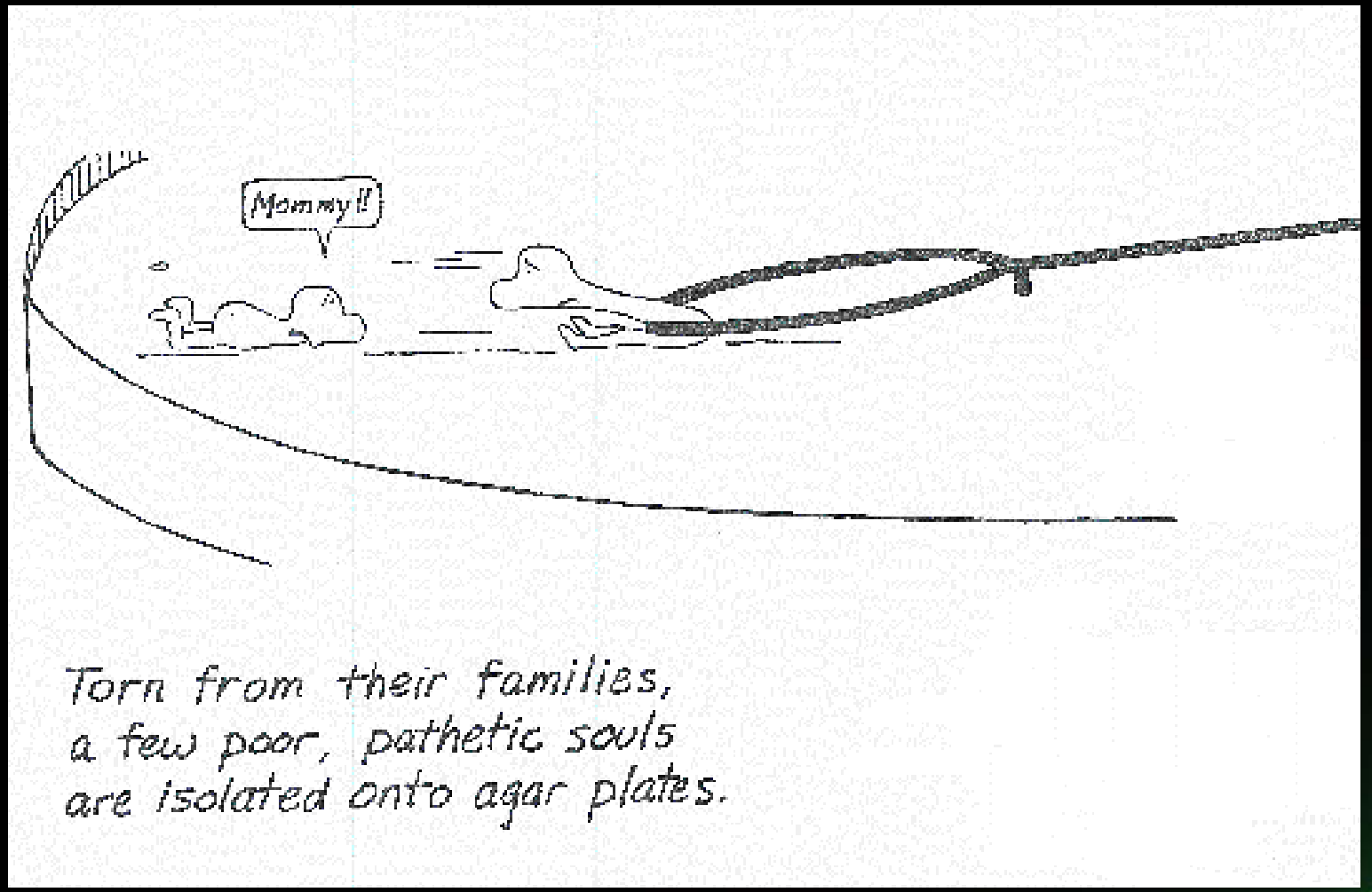
Laboratory conditions (media) are far off from the environment

Concentration of nutrients

Organics	Seawater	Media for Heterotrophs
Peptone	–	0.5 – 10 g/L
Dextrose	–	0.5 – 2 g/L
Yeast extract	–	0.3 – 5 g/L
Meat extract	–	0.5 – 10 g/L
Pyruvate	~ng/L	0.3 – 1 g/L
Succinate	~ng/L	0.5 – 1 g/L
Glucose	~ng/L	0.5 – 20 g/L
Amino acids	ng/L – μg/L	0.5 – 10 g/L

x 10^{10}

Some bacteria don’t like to live alone



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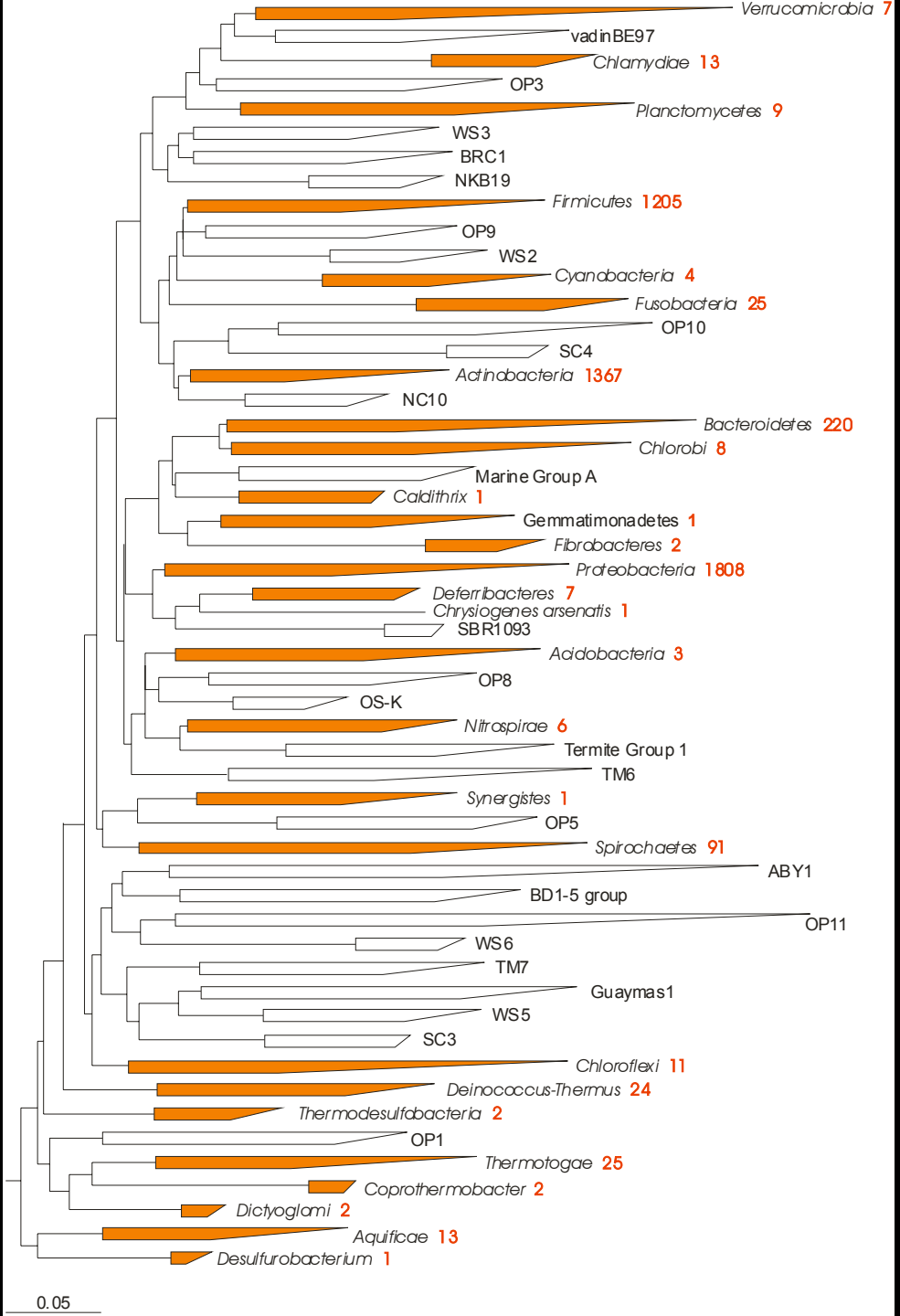

The bacterial world as of today…

- Phyla with cultivated representatives: 27
- Phyla without cultivated representatives: 26

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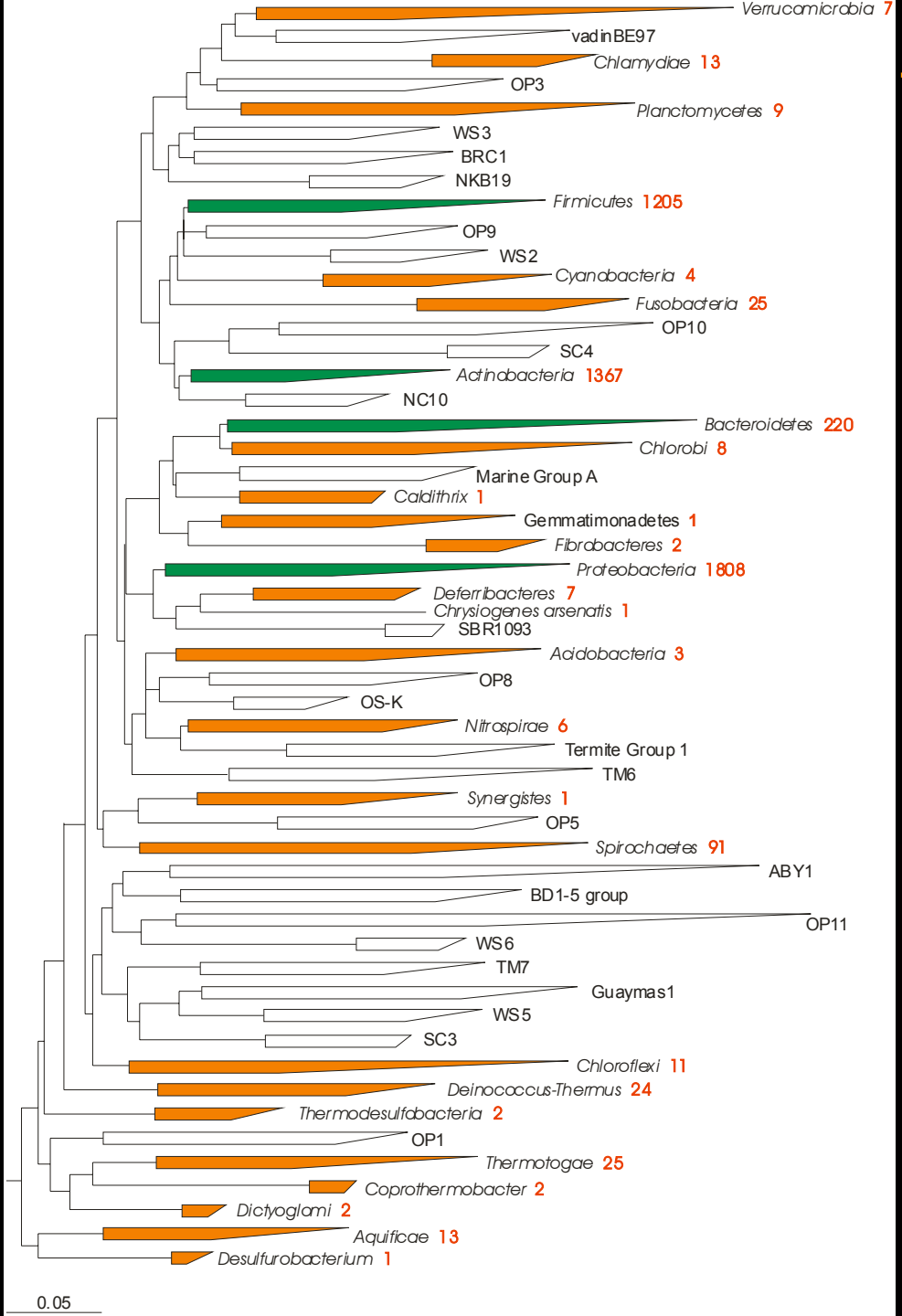

The bacterial world as of today…

Phyla with cultivated representatives: 27

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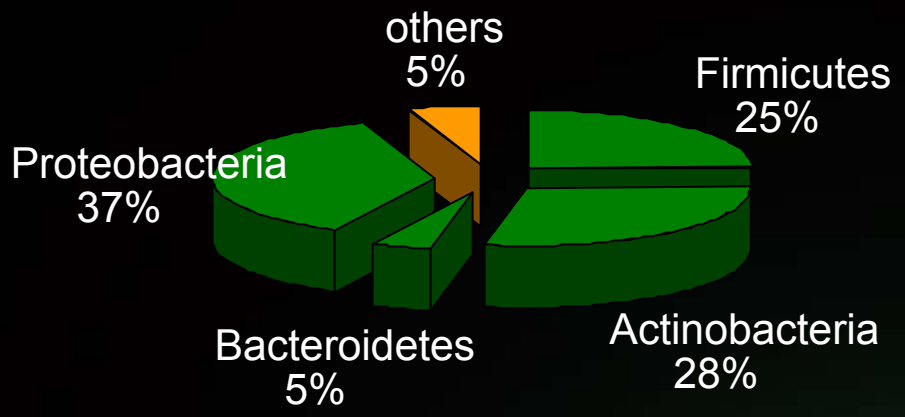

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Development of a Novel Cultivation Approach

How can we grow more than the microbial weeds?

Do we have to move away from the traditional understanding of a pure culture?

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Development of a Novel Cultivation Approach

- novel
- easy to automate
- universal
- detection of small amount of cells – 100 cells
- high-throughput
- low organic media
- cultivation together and apart

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FACS Technology

- Premier Diversa screening platform
- 3-Dimensional, single cell format
- Screening rates of 50,000 cells/second
- Multiple Screening systems
  - expression
  - hybridization
  - bioassays
- Encapsulation program

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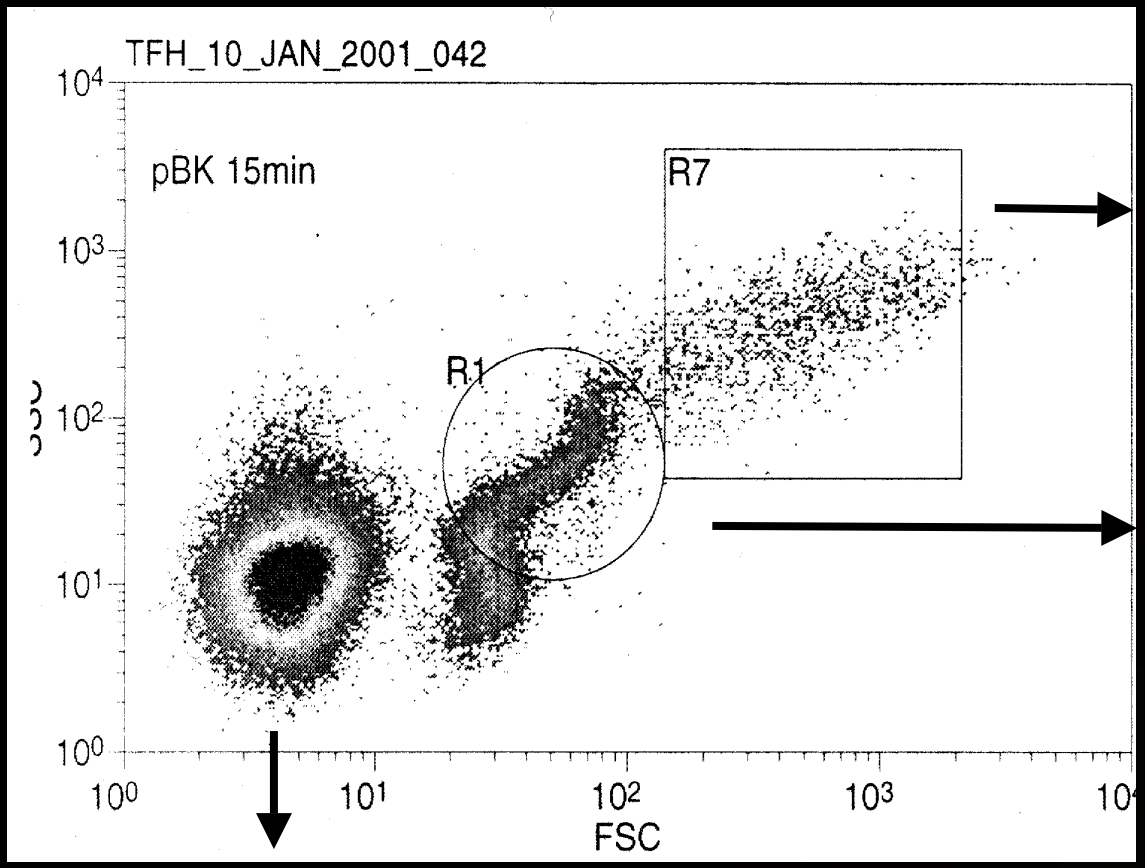
Sorting of Single and Multiple Occupied Microcapsules



Free Bacteria

Multiple occupied Microcapsules

Single occupied Microcapsules

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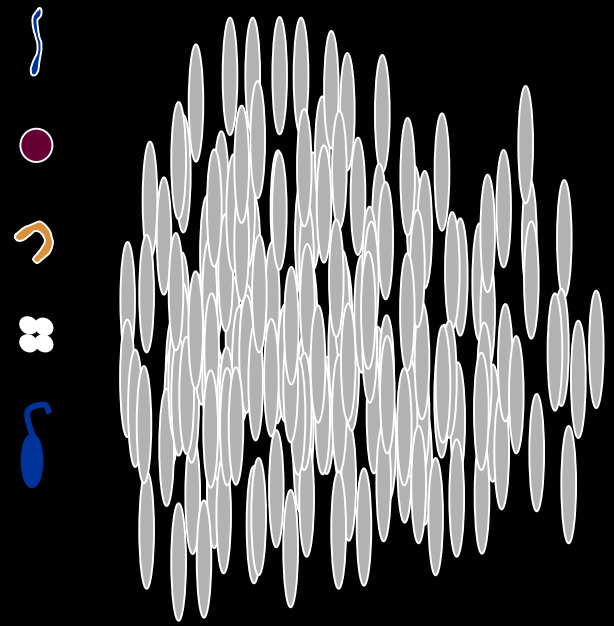
Traditional:

1. Grow



2. Isolate

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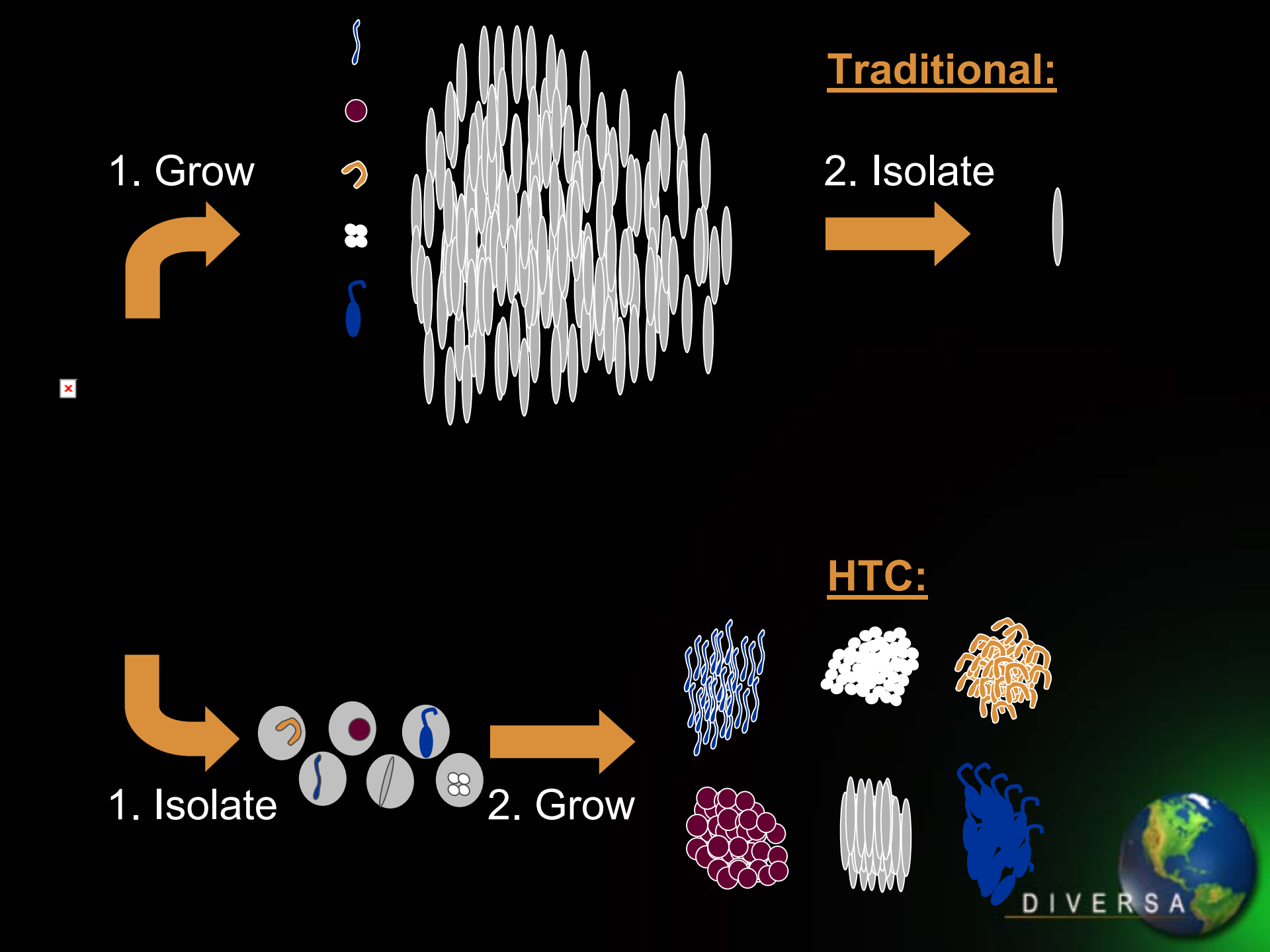
Traditional:
1. Grow
2. Isolate
HTC:
1. Isolate
2. Grow
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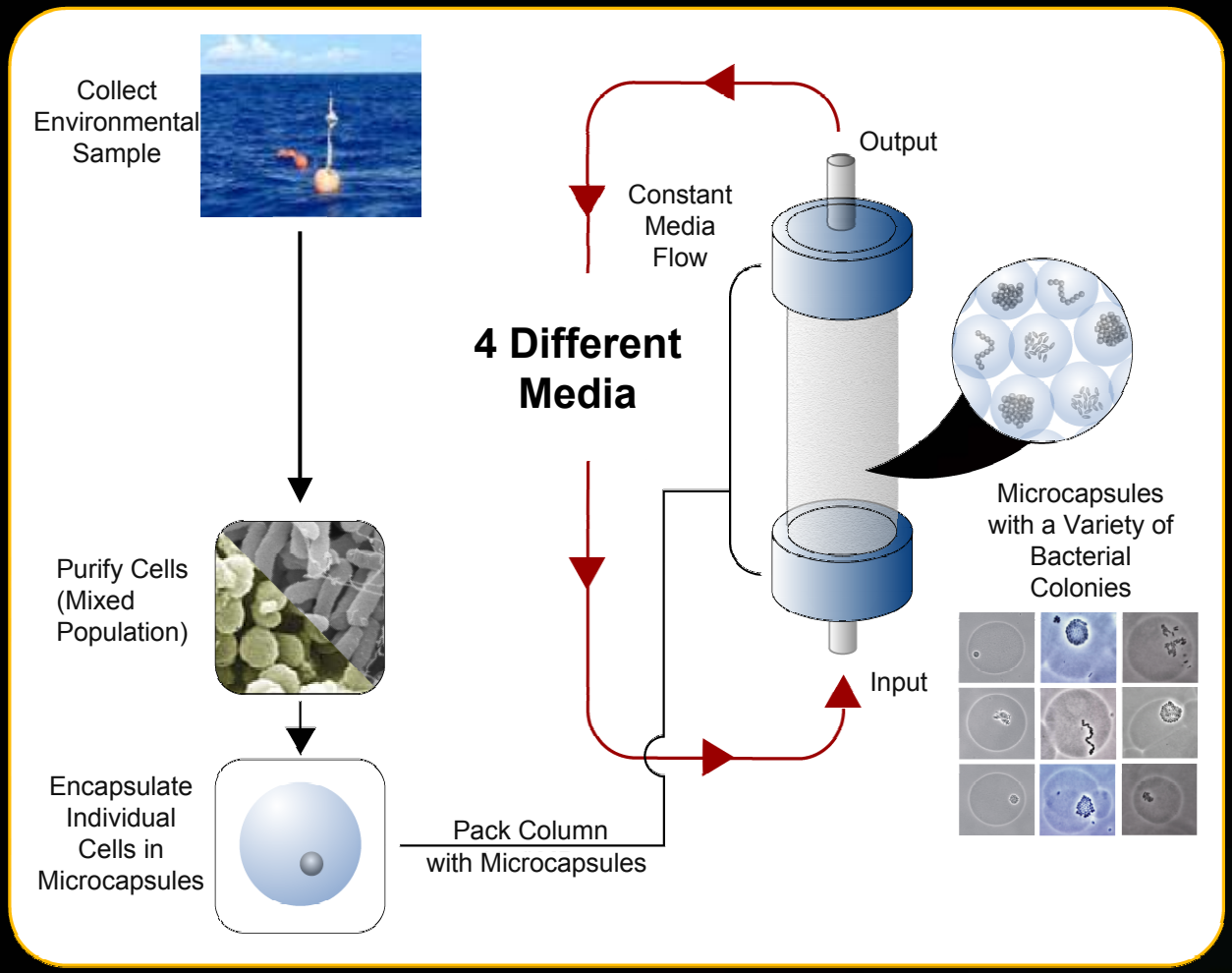
Cultivation of Sargasso Sea Microbes



4 different types of media:

- Marine rich media 1/100
- Sea water + amino acids
- Sea water + inorganic nutrients
- Sea water

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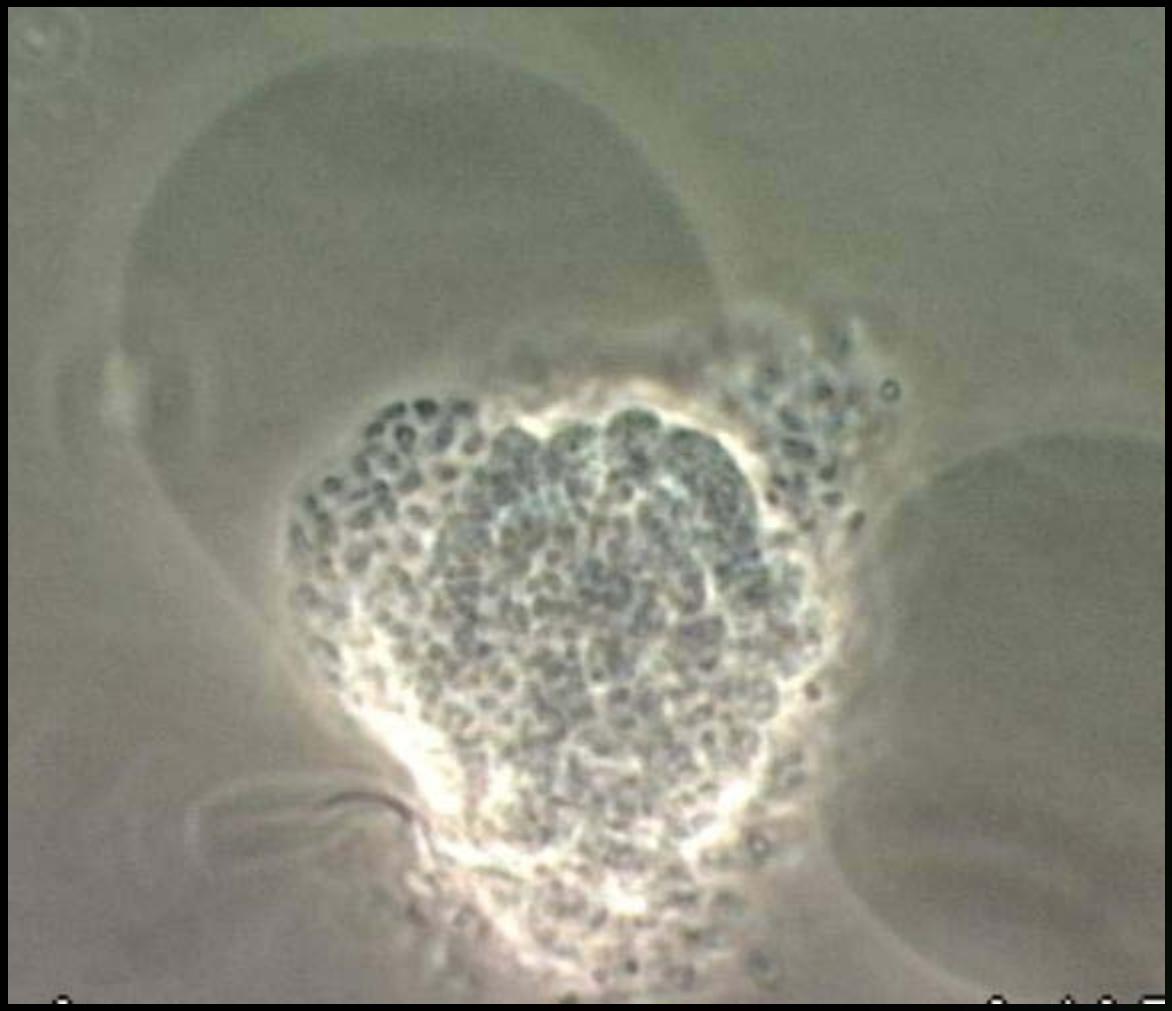
Microcolonie in Marine Rich Medium after 5 Weeks



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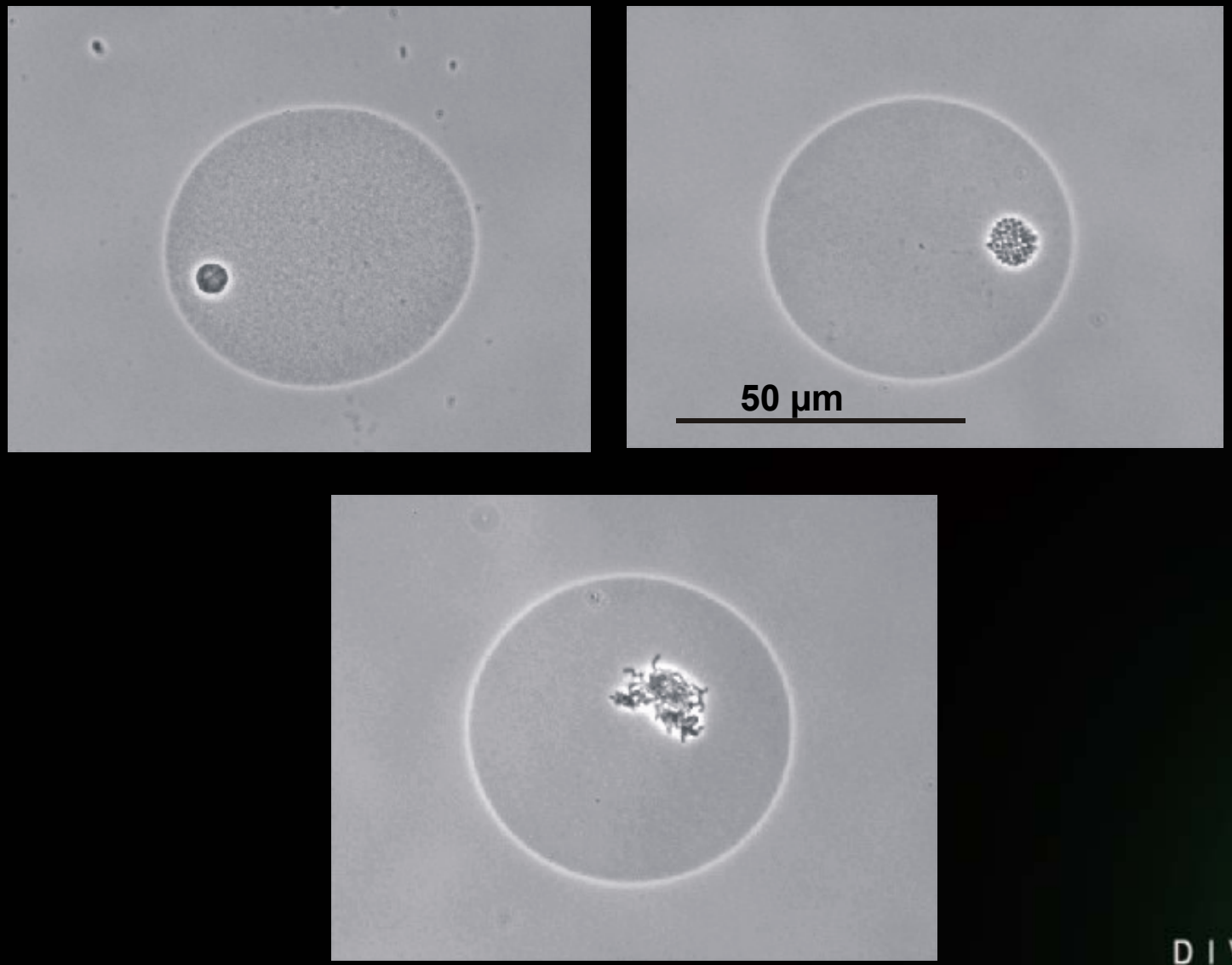
Microcolonies in Pure Seawater after 5 Weeks



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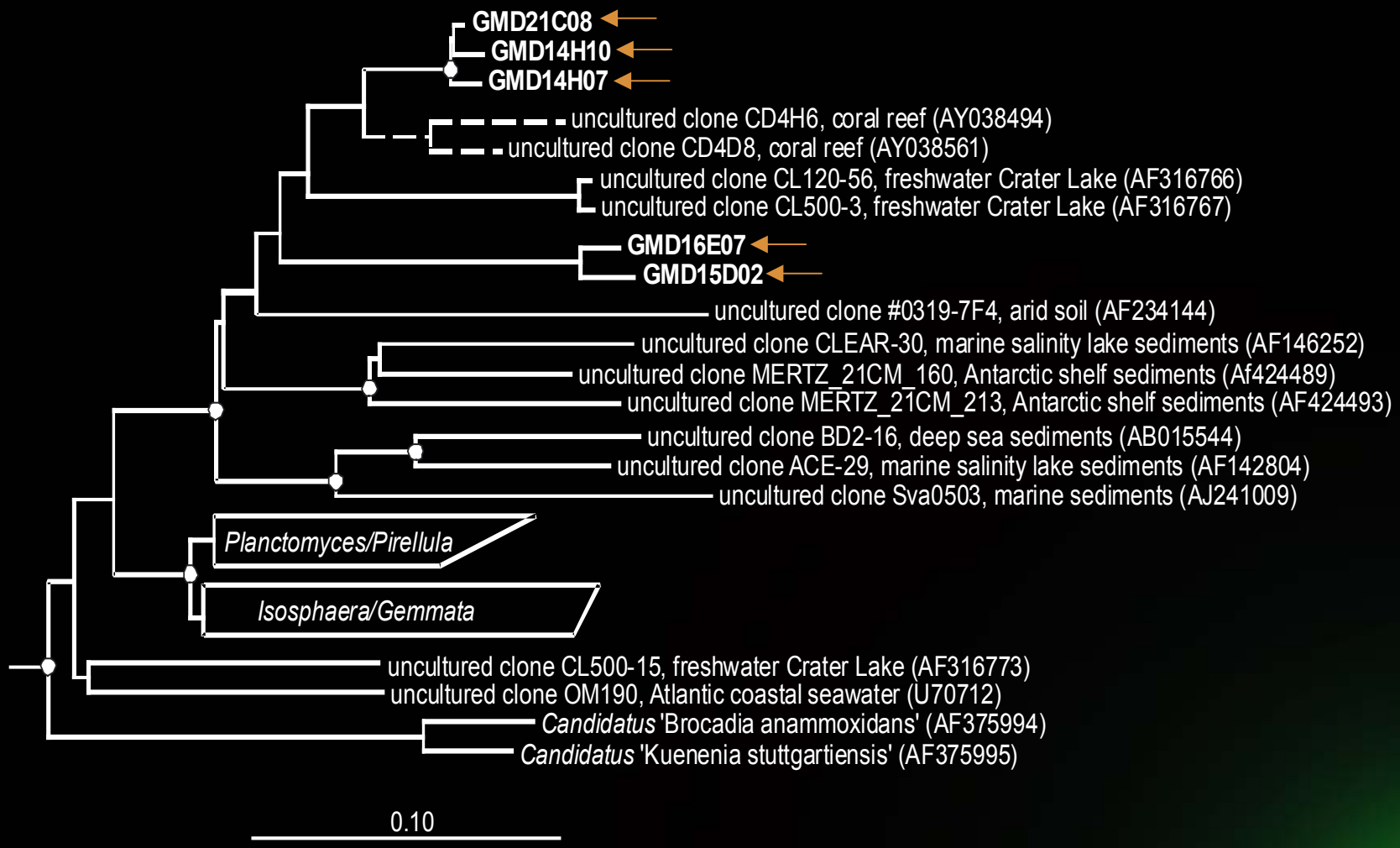
High Throughput Cultivation - Sargasso Sea



Planctomyces and Relatives

Verrucomicrobia and *Chlamydiae* sequences were used as outgroups

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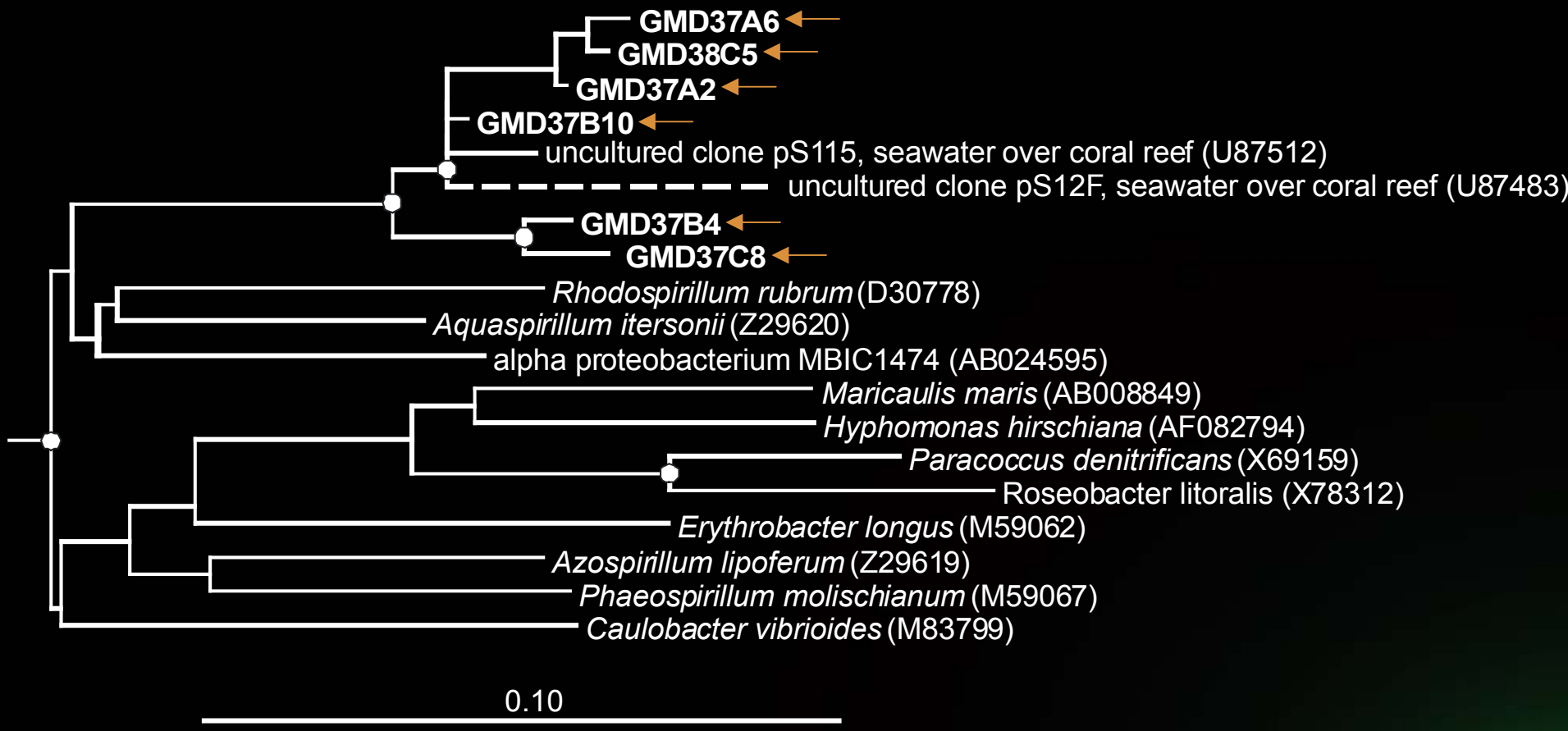
High Throughput Cultivation - Sargasso Sea



Alpha *Proteobacteria*

Gamma and Beta *Proteobacteria* sequences were used as outgroups

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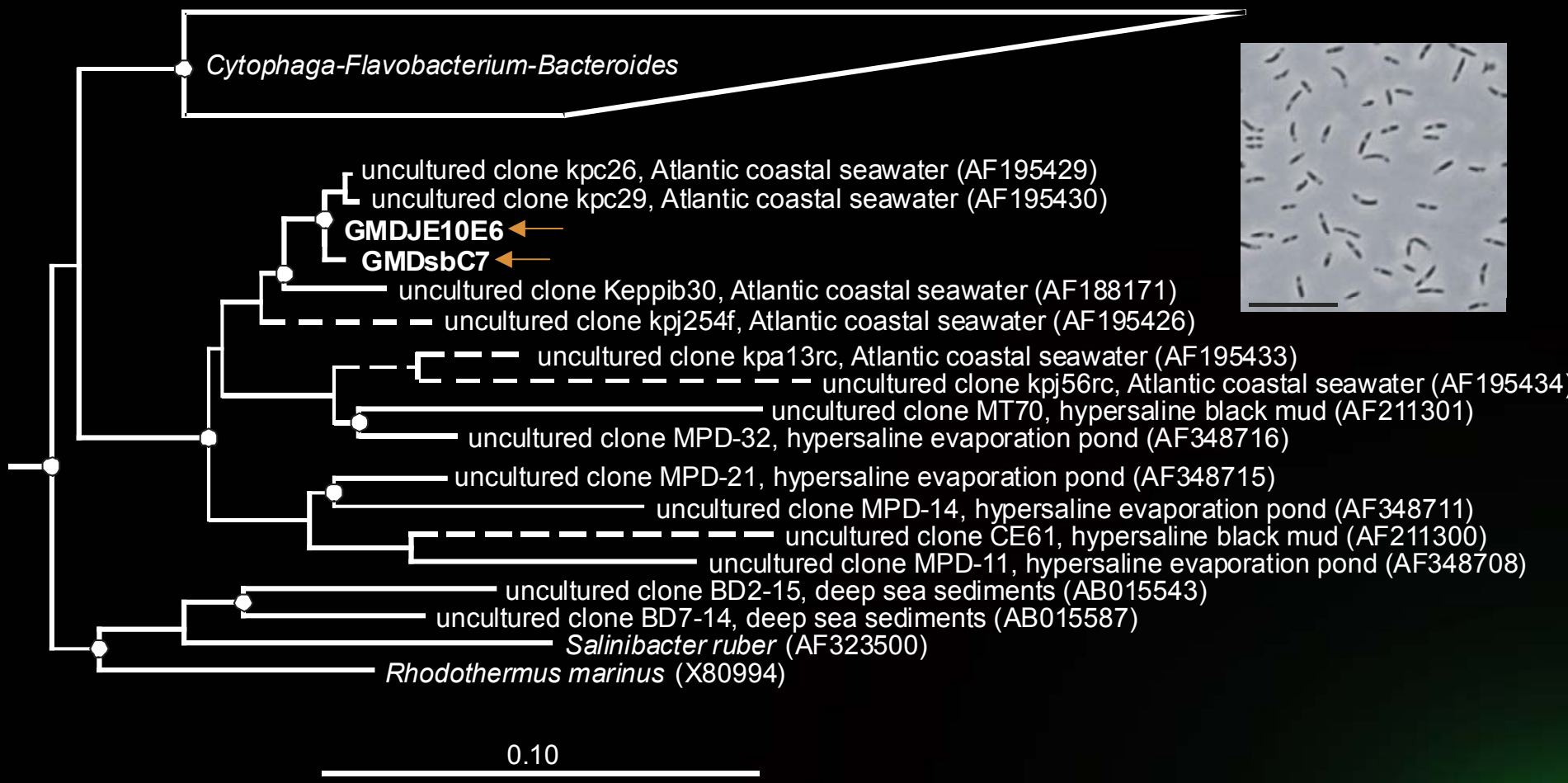
High Throughput Cultivation - Sargasso Sea



Cytophaga-Flavobacterium-Bacteroides and Relatives

Chlorobiaceae sequences were used as outgroups

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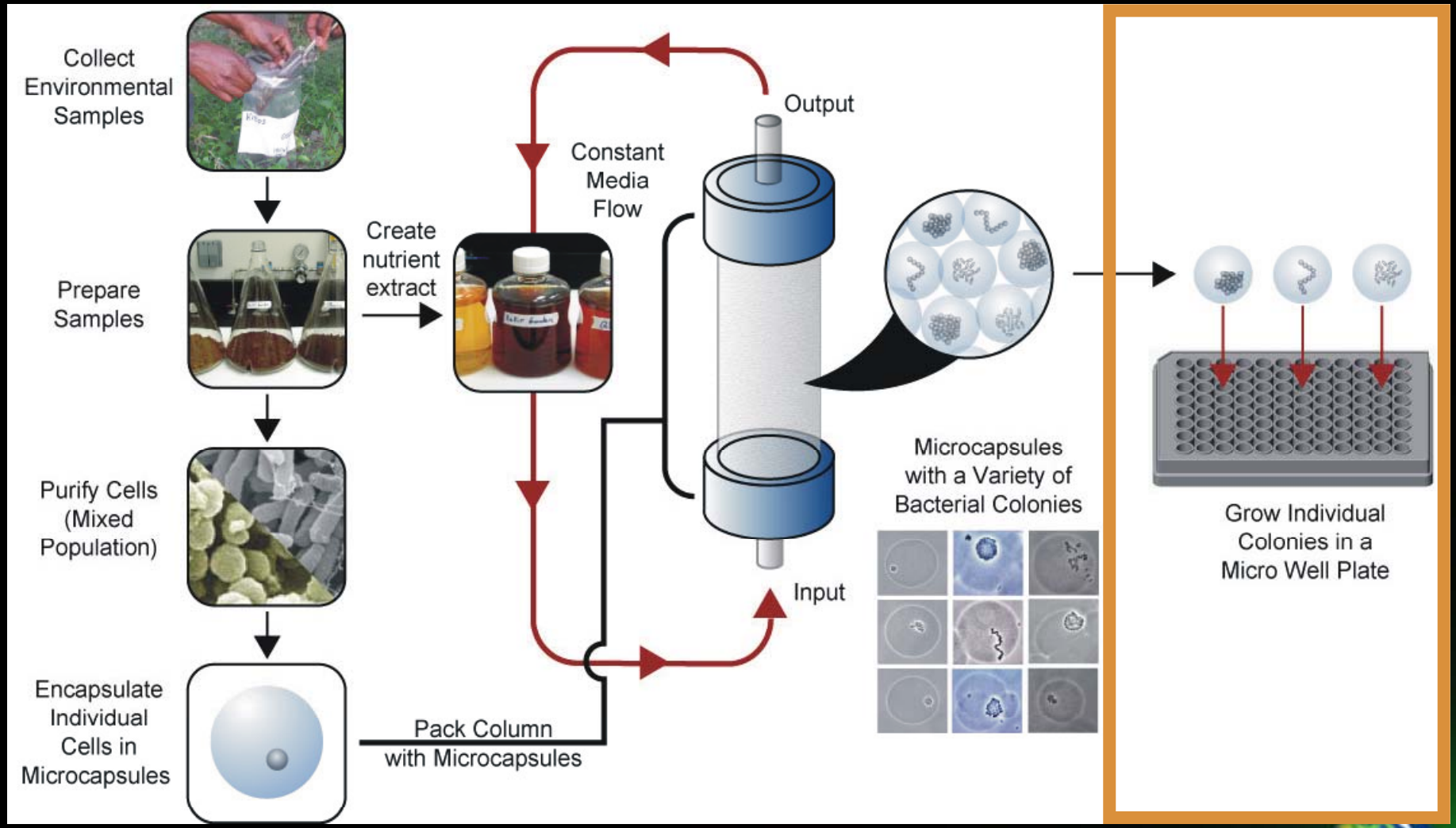
High Throughput Cultivation



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Targeted Biotopes

- Marine samples (sediment and water samples from surface and deep sea)
- alkaline Lake sediment (Kenya)
- Invertebrates (e.g., sponges)
- **Soils (e.g., Ghana)**

67% of all cultures (960) investigated reached OD_{600} >0.1

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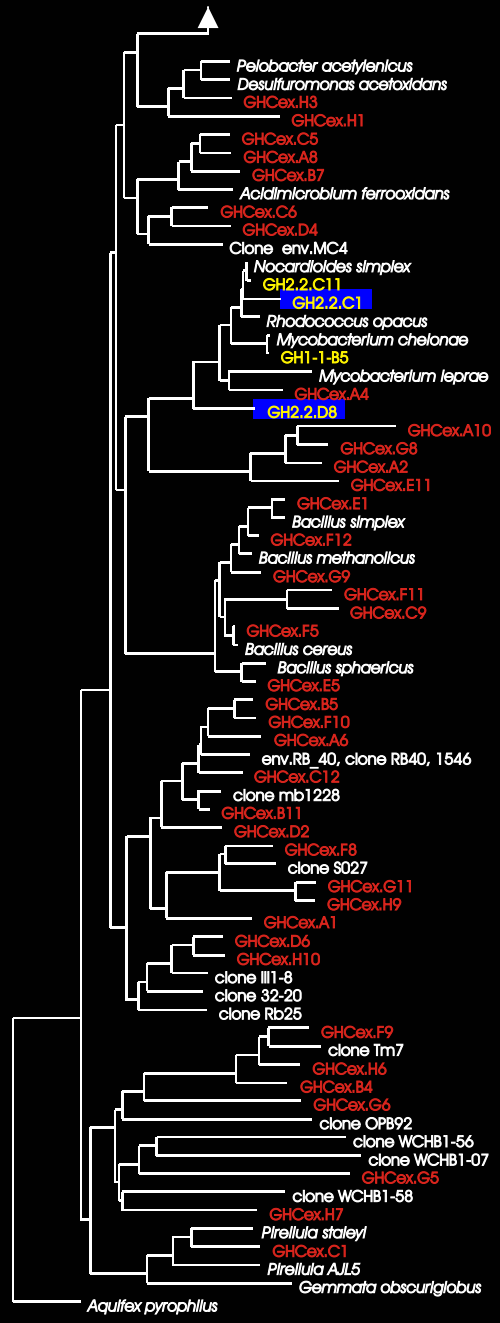
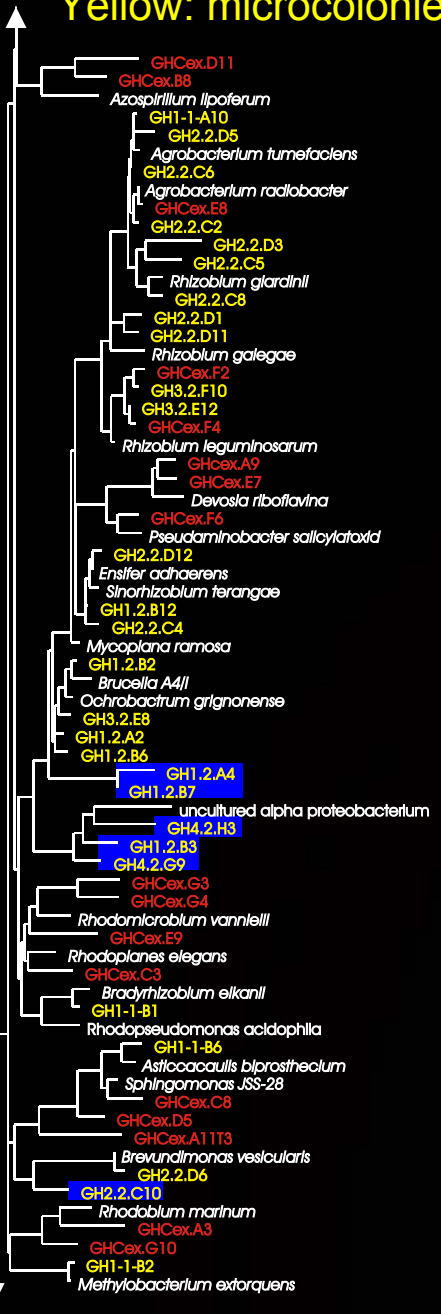
Ghana

8 new groups

Red: environmental clone library

Yellow: microcolonies





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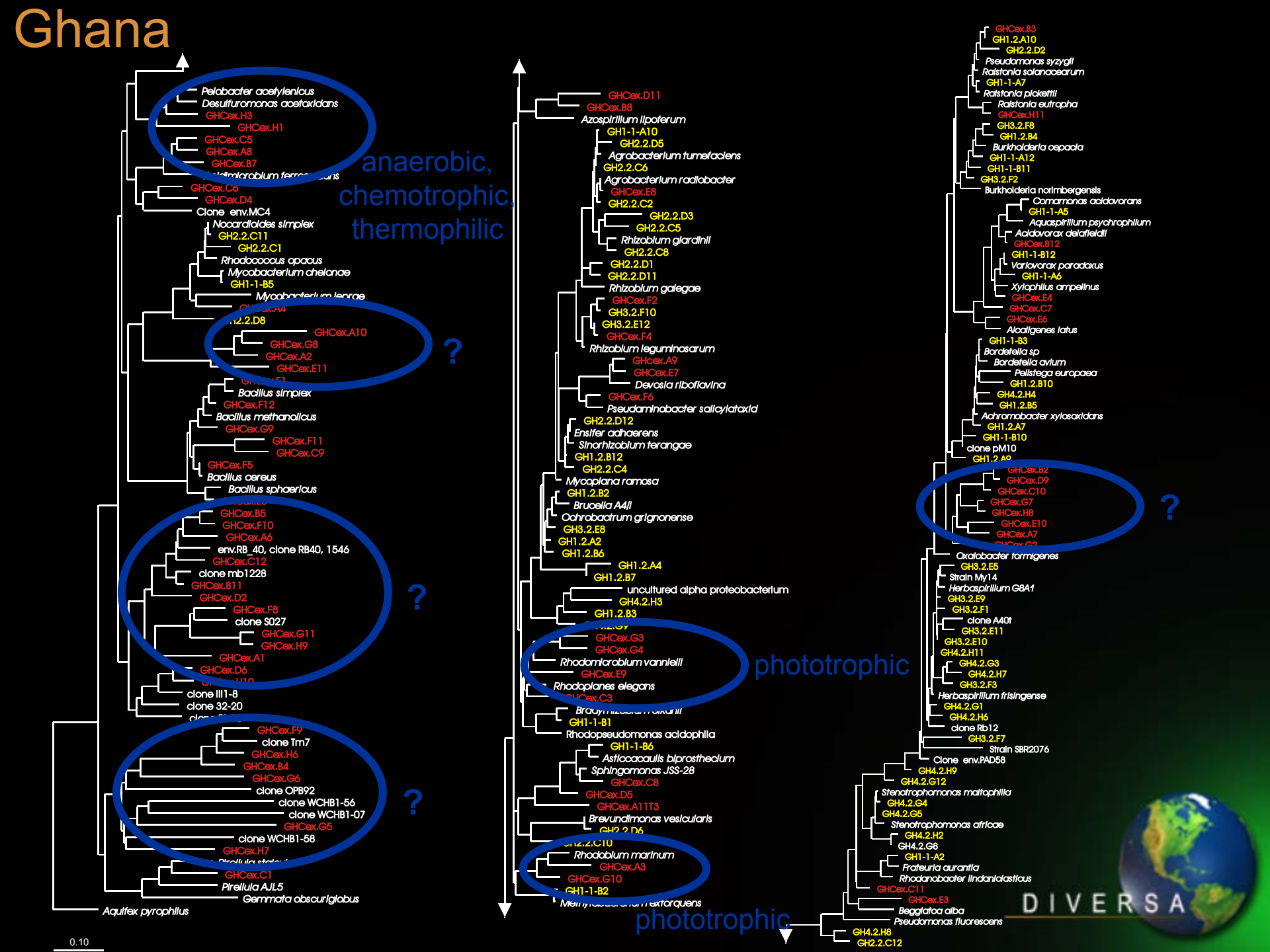
Ghana


Pelobacter acetylenicus
Desulfuromonas acetoxidans
GHCex.H3
GHCex.H1
GHCex.C5
GHCex.A8
GHCex.B7
GHCex.Co
GHCex.D4
Clone env.MC4
Nocardioides simplex
GH2.2.C11
GH2.2.C1
Rhodococcus opacus
Mycobacterium chelonae
GH1-1-B5
GHCex.A10
GHCex.G8
GHCex.A2
GHCex.E11
Bacillus simplex
GHCex.F12
Bacillus methanolicus
GHCex.G9
GHCex.F11
GHCex.C9
GHCex.F5
Bacillus cereus
Bacillus sphaericus
GHCex.B5
GHCex.F10
GHCex.A6
env.RB_40, clone RB40, 1546
GHCex.C12
clone mb1228
GHCex.B11
GHCex.D2
GHCex.F8
clone S027
GHCex.G11
GHCex.H9
GHCex.A1
GHCex.D6
clone III1-8
clone 32-20
GHCex.F9
clone Tm7
GHCex.H6
GHCex.B4
GHCex.G6
clone OPB92
clone WCHB1-56
clone WCHB1-07
GHCex.G5
clone WCHB1-58
GHCex.H7
GHCex.C1
Pirellula AJL5
Gemmata obscuriglobus
Aquifex pyrophilus
0.10
anaerobic, chemotrophic, thermophilic
?
?
?
GHCex.D11
GHCex.B8
Azospirillum lipoferum
GH1-1-A10
GH2.2.D5
Agrobacterium tumefaciens
GH2.2.C6
Agrobacterium radiobacter
GHCex.E8
GH2.2.C2
GH2.2.D3
GH2.2.C5
Rhizobium giardinii
GH2.2.C8
GH2.2.D1
GH2.2.D11
Rhizobium galegae
GHCex.F2
GH3.2.F10
GH3.2.E12
GHCex.F4
Rhizobium leguminosarum
GHcex.A9
GHCex.E7
Devosia riboflavina
GHCex.F6
Pseudaminobacter salicylatoxid
GH2.2.D12
Ensifer adhaerens
Sinorhizobium terangae
GH1.2.B12
GH2.2.C4
Mycoplana ramosa
GH1.2.B2
Brucella A4/I
Ochrobactrum grignonense
GH3.2.E8
GH1.2.A2
GH1.2.B6
GH1.2.A4
GH1.2.B7
uncultured alpha proteobacterium
GH4.2.H3
GH1.2.B3
GHCex.G3
GHCex.G4
Rhodomicrobium vannielii
GHCex.E9
Rhodoplanes elegans
GH1-1-B1
Rhodopseudomonas acidophila
GH1-1-B6
Asticcacaulis biprosthecium
Sphingomonas JSS-28
GHCex.C8
GHCex.D5
GHCex.A11T3
Brevundimonas vesicularis
Rhodobium marinum
GHCex.A3
GHCex.G10
GH1-1-B2
phototrophic
phototrophic
GHCex.B3
GH1.2.A10
GH2.2.D2
Pseudomonas syzygii
Ralstonia solanacearum
GH1-1-A7
Ralstonia pickettii
Ralstonia eutropha
GHCex.H11
GH3.2.F8
GH1.2.B4
Burkholderia cepacia
GH1-1-A12
GH1-1-B11
GH3.2.F2
Burkholderia nordbergensis
Comamonas acidovorans
GH1-1-A5
Aquaspirillum psychrophilum
Acidovorax delafieldi
GHCex.B12
GH1-1-B12
Variovorax paradoxus
GH1-1-A6
Xylophilus ampelinus
GHCex.E4
GHCex.C7
GHCex.E6
Alcaligenes latus
GH1-1-B3
Bordetella sp
Bordetella avium
Pelistega europaea
GH1.2.B10
GH4.2.H4
GH1.2.B5
Achromobacter xylosoxidans
GH1.2.A7
GH1-1-B10
clone pM10
GHCex.B2
GHCex.D9
GHCex.C10
GHCex.G7
GHCex.H8
GHCex.E10
GHCex.A7
?
Oxalobacter formigenes
GH3.2.E5
Strain My14
Herbaspirillum G8A1
GH3.2.E9
GH3.2.F1
clone A40t
GH3.2.E11
GH3.2.E10
GH4.2.H11
GH4.2.G3
GH4.2.H7
GH3.2.F3
Herbaspirillum frisingense
GH4.2.G1
GH4.2.H6
clone Rb12
GH3.2.F7
Strain SBR2076
Clone env.PAD58
GH4.2.H9
GH4.2.G12
Stenotrophomonas maltophilia
GH4.2.G4
GH4.2.G5
Stenotrophomonas africae
GH4.2.H2
GH4.2.G8
GH1-1-A2
Frateuria aurantia
Rhodanobacter lindaniclasticus
GHCex.C11
GHCex.E3
Begglatoa alba
Pseudomonas fluorescens
GH4.2.H8
GH2.2.C12
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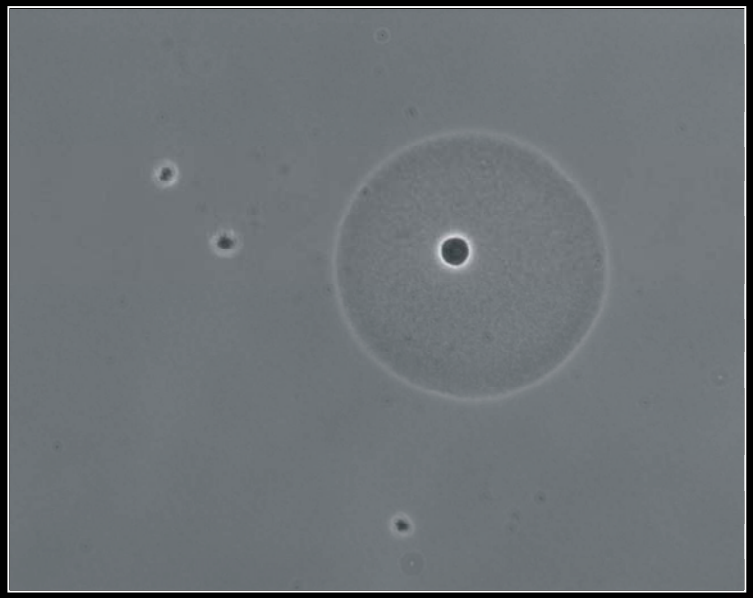
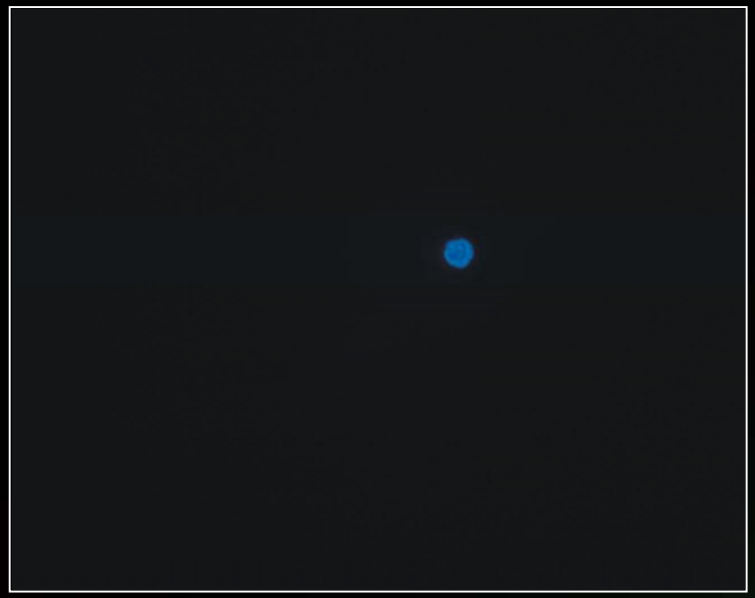
Encapsulation and Growth of Anaerobes

Methanococcus thermolithotrophicus



Phase contrast



Autofluorescence (F_{420})

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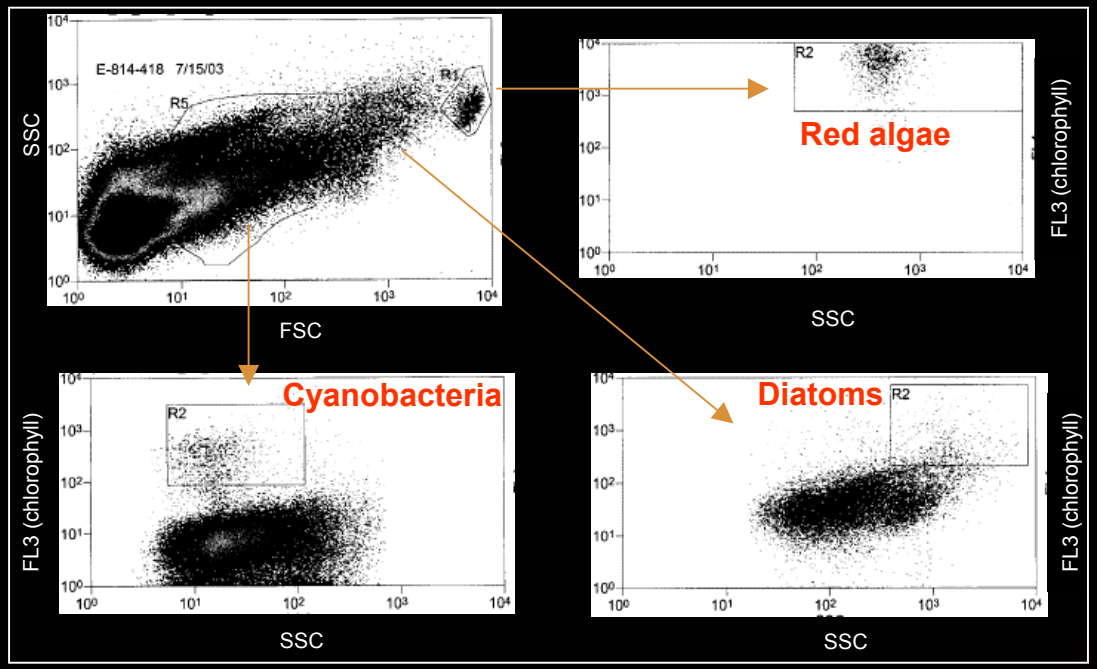
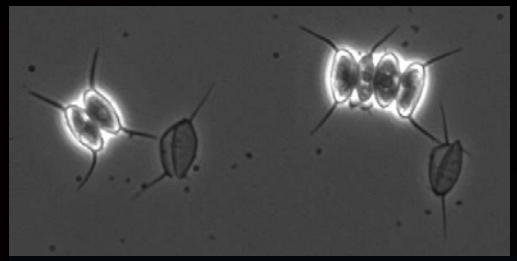
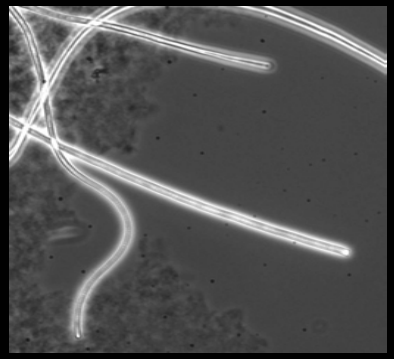
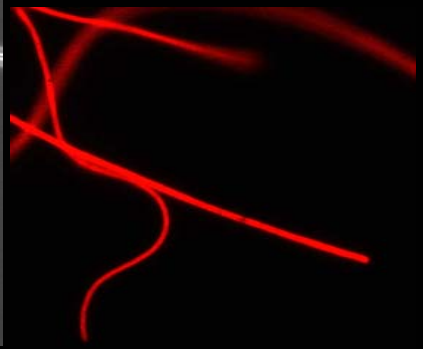
The Phototrophs:









Cyanobacteria: 91% Planktothrix

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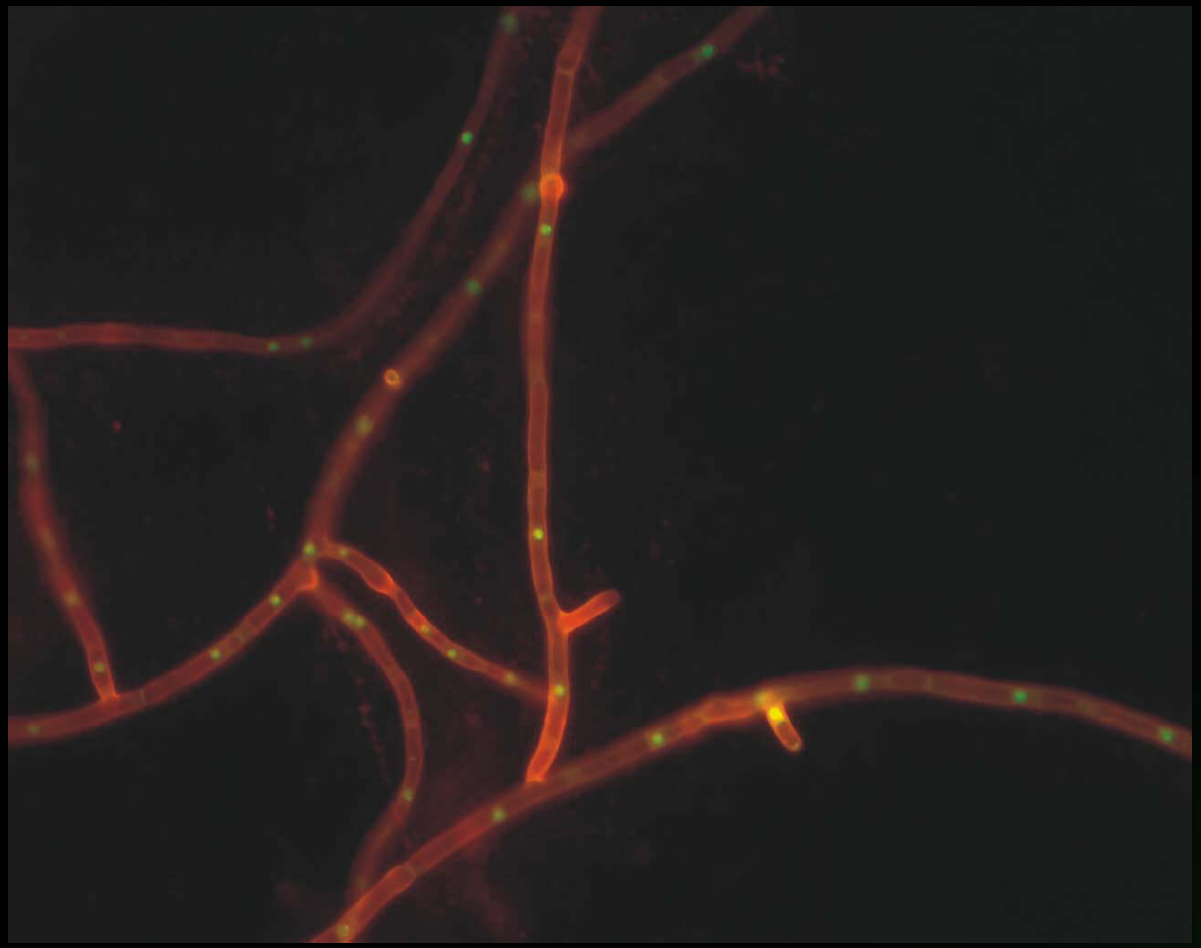
The Fungi:



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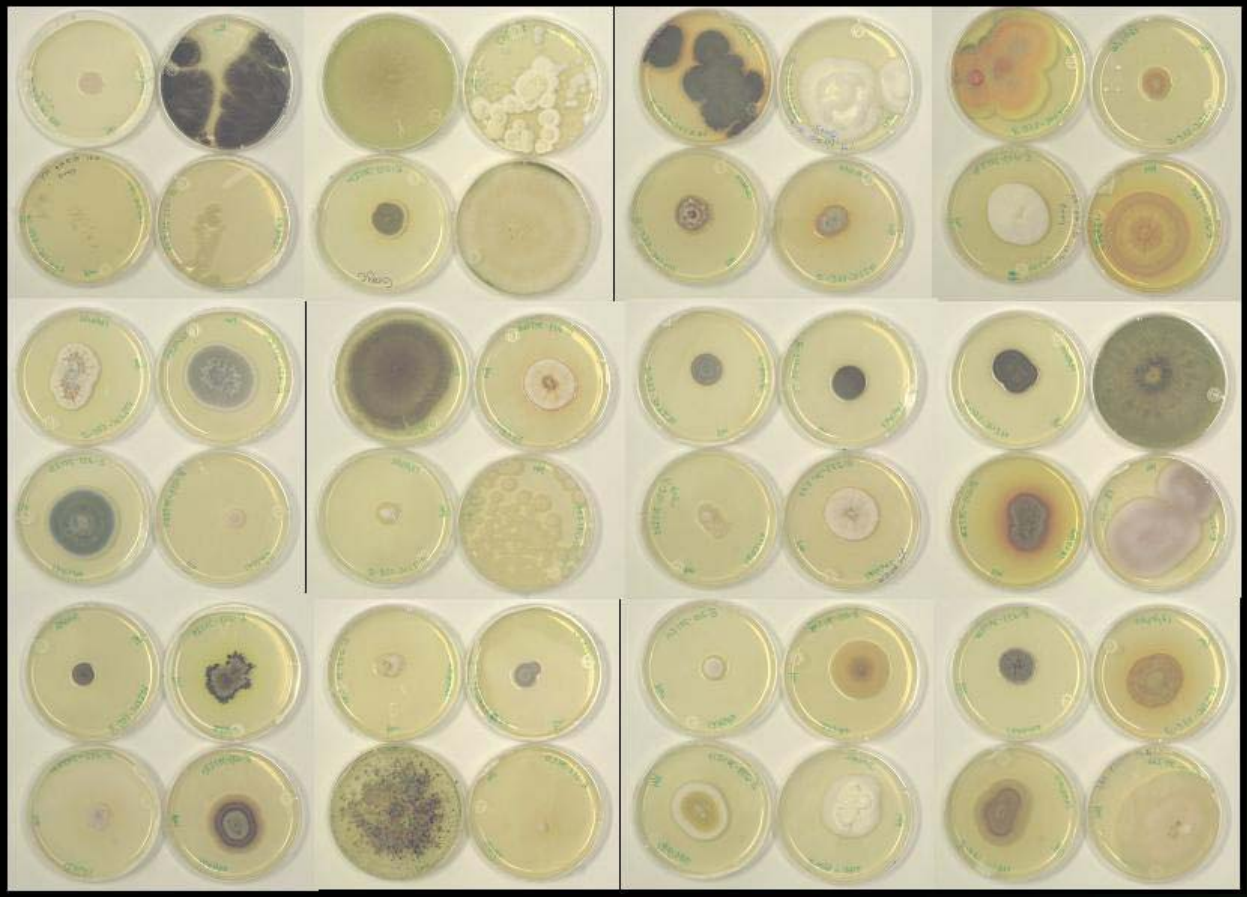
High Throughput Cultivation for Filamentous Fungi



Many morphological different fungi from a single environmental sample (average of 40 fungi/sample)

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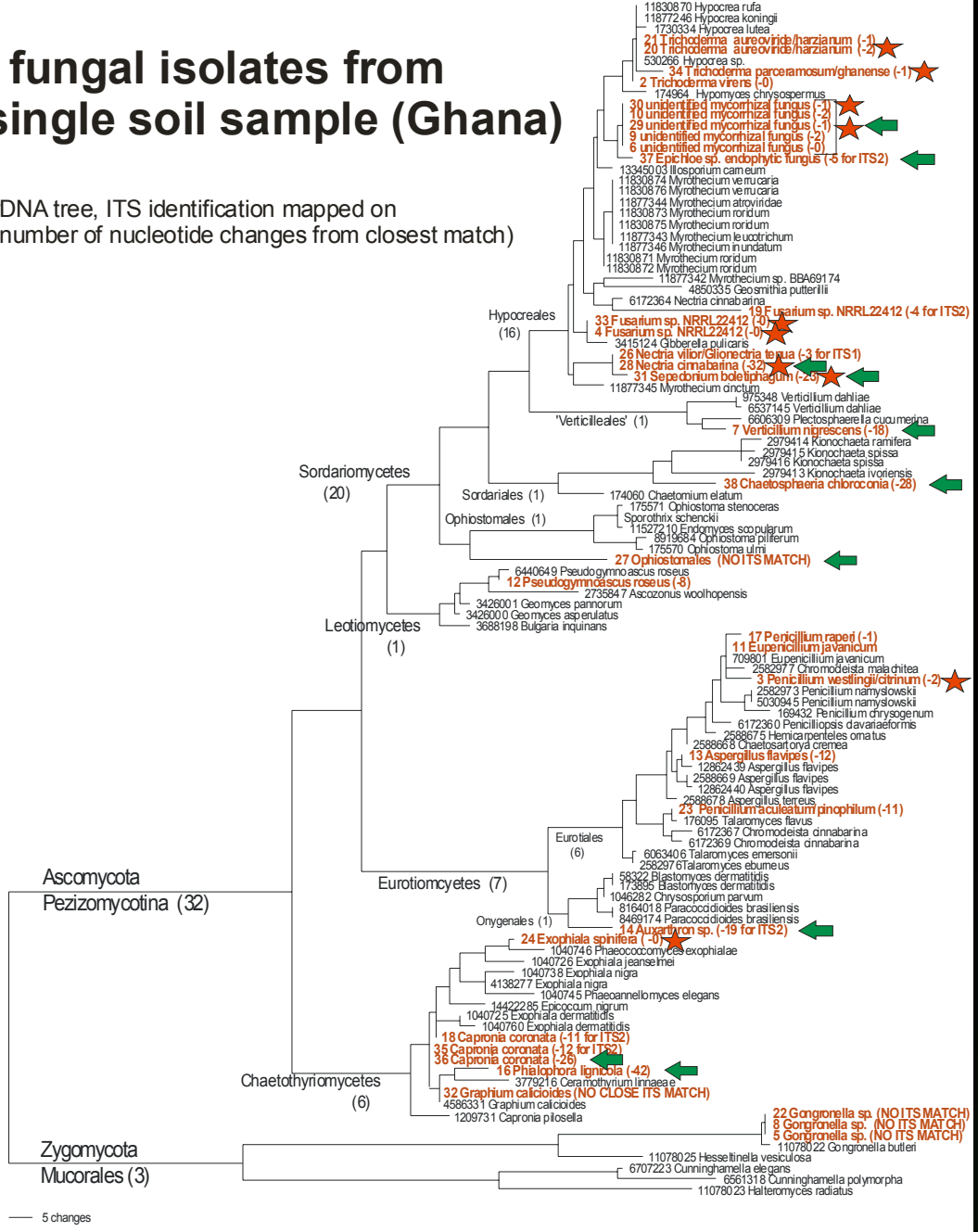
35 fungal isolates from a single soil sample (Ghana)

18S rDNA tree, ITS identification mapped on
(with number of nucleotide changes from closest match)



← = novel culture
★ = hit in Pharma screen

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High Throughput Cultivation
Novel Tool to Access Natures Biodiversity

- easy to multiplex
- cultivating novel microorganisms
- hundreds of different bacteria and fungi per sample

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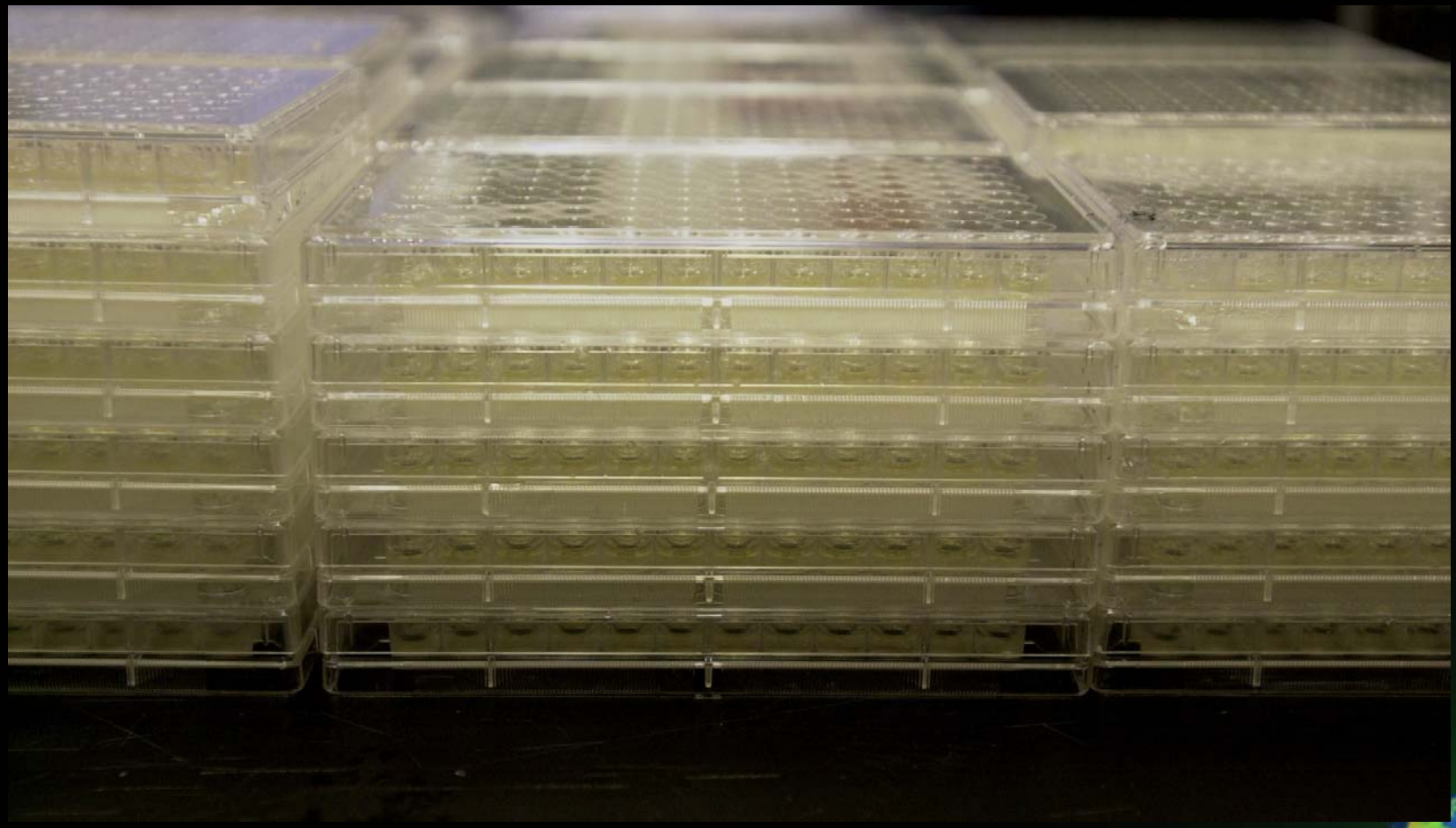
High Throughput Cultivation



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How do we determine different cultures and reduce redundancy ?

Options:

16S rRNA gene sequencing: time consuming, expensive

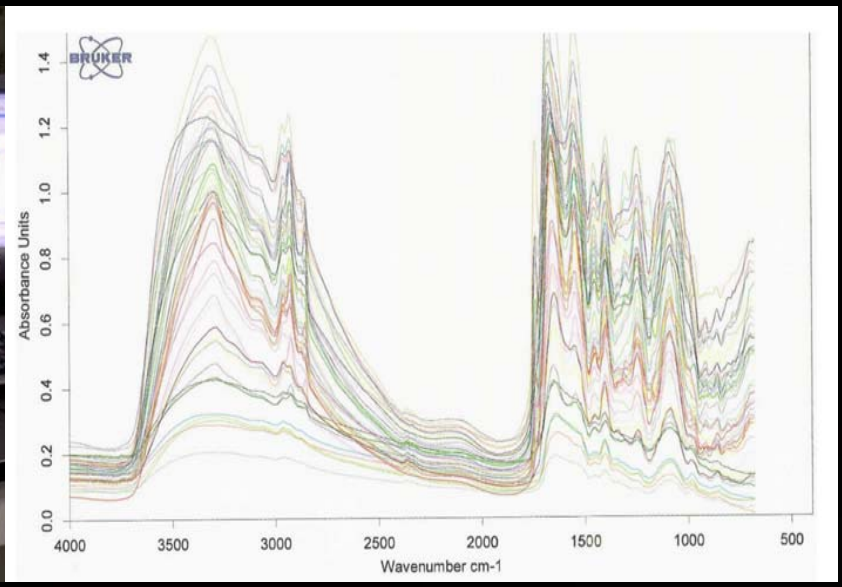
Mass Spectroscopy: no database, sample preparation

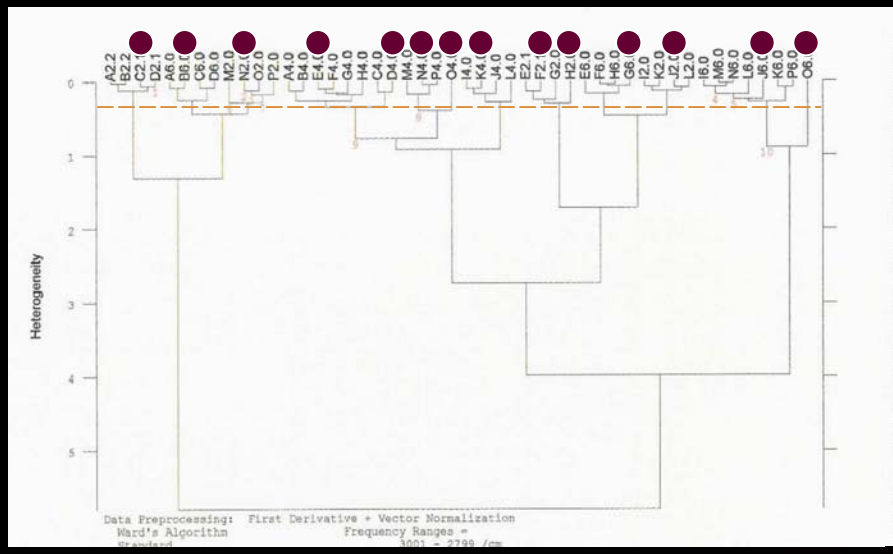
FT-IR (Fourier transform infrared spectroscopy):

no database

→ fast (30 min/384 samples), inexpensive

Bacterial Deconvolution by FT-IR*





Well	Plate
	control strains.PIK
A2	control strains
D2	control strains
C6	control strains
D6	control strains
A6	control strains
M2	control strains
A4	control strains
G4	control strains
I2	control strains
K2	control strains
I4	control strains
L4	control strains
C4	control strains
F4	control strains
E2	control strains
H2	control strains
G2	control strains
E6	control strains
L2	control strains
I6	control strains
P6	control strains
J6	control strains
N6	control strains
M4	control strains
P4	control strains
O4	control strains
O6	control strains

* FT-IR: Fourier-transform Infrared Spectroscopy

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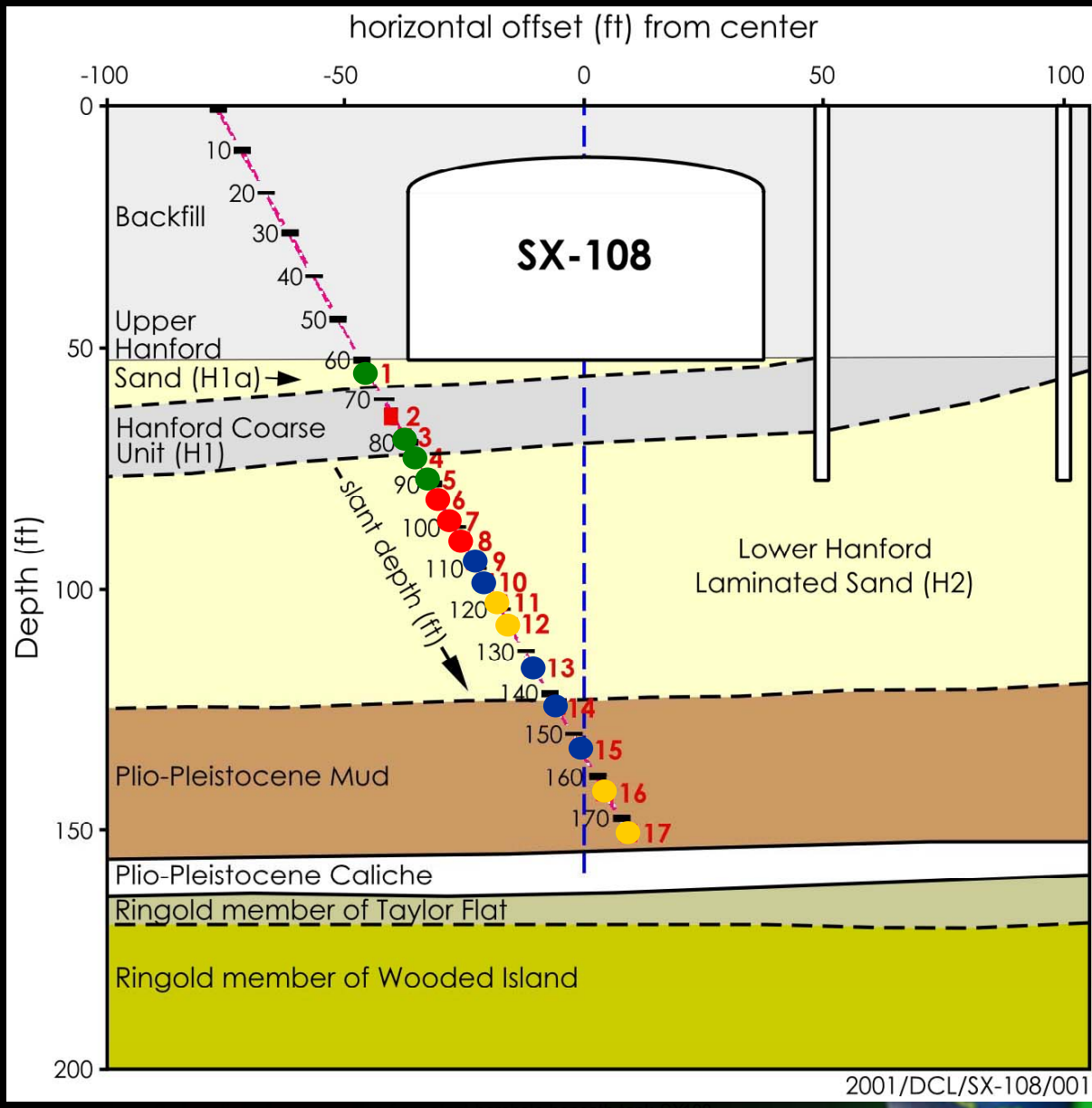
Hanford Site

SX-108 Zone 1 ●

SX-108 Zone 2 ●

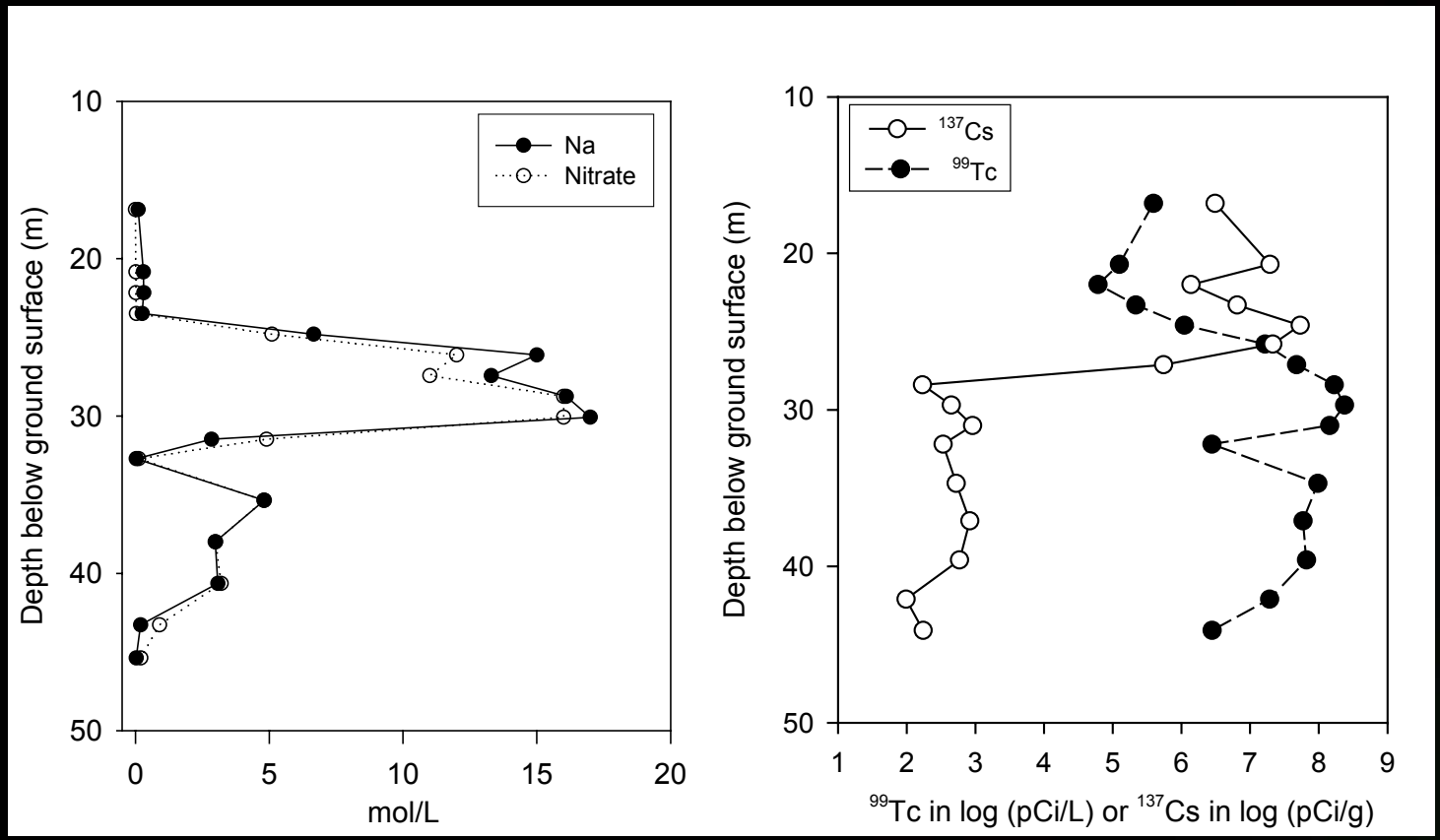
SX-108 Zone 3 ●

SX-108 Zone 4 ●



Jim Fredrickson, Fred Brockman/PPNL

Pooled sample	Total cells
SX-108 zone 1	2.9×10^4
SX-108 zone 2	2.8×10^4
SX-108 zone 3	9.0×10^5
SX-108 zone 4	9.3×10^5



Jim Fredrickson, Fred Brockman/PPNL

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SX-108 Zone 1

Aquatic bacterium RUB_NH4_10	96%	ALPHA
aquatic bacterium RUB_NH4_10	99%	ALPHA
Arthrobacter chlorophenolicus	99%	HIGH GC G+
Arthrobacter chlorophenolicus	97%	HIGH GC G+
Bradyrhizobium elkanii	100%	ALPHA
Ralstonia solanacearum	96%	BETA
Ralstonia solanacearum	98%	BETA
Burkholderia cepacia	99%	BETA
Methylobacterium radiotolerans	99%	ALPHA
Methylobacterium fujisawaense	99%	ALPHA
Pseudomonas fluorescens	99%	GAMMA
Ralstonia pickettii	100%	BETA
uncultured eubacterium WD208	98%	ALPHA
unidentified Actinomycete BD4-12	100%	HIGH GC G+

SX-108 Zone 3

Arthrobacter chlorophenolicus	99%	HIGH GC G+
Blastobacter denitrificans	99%	ALPHA
Ralstonia solanacearum	98%	BETA
Gordonia polyisoprenivorans	100%	HIGH GC G+
Pseudomonas fluorescens	100%	GAMMA
Ralstonia pickettii	100%	BETA
Staphylococcus epidermidis	97%	LOW GC G+

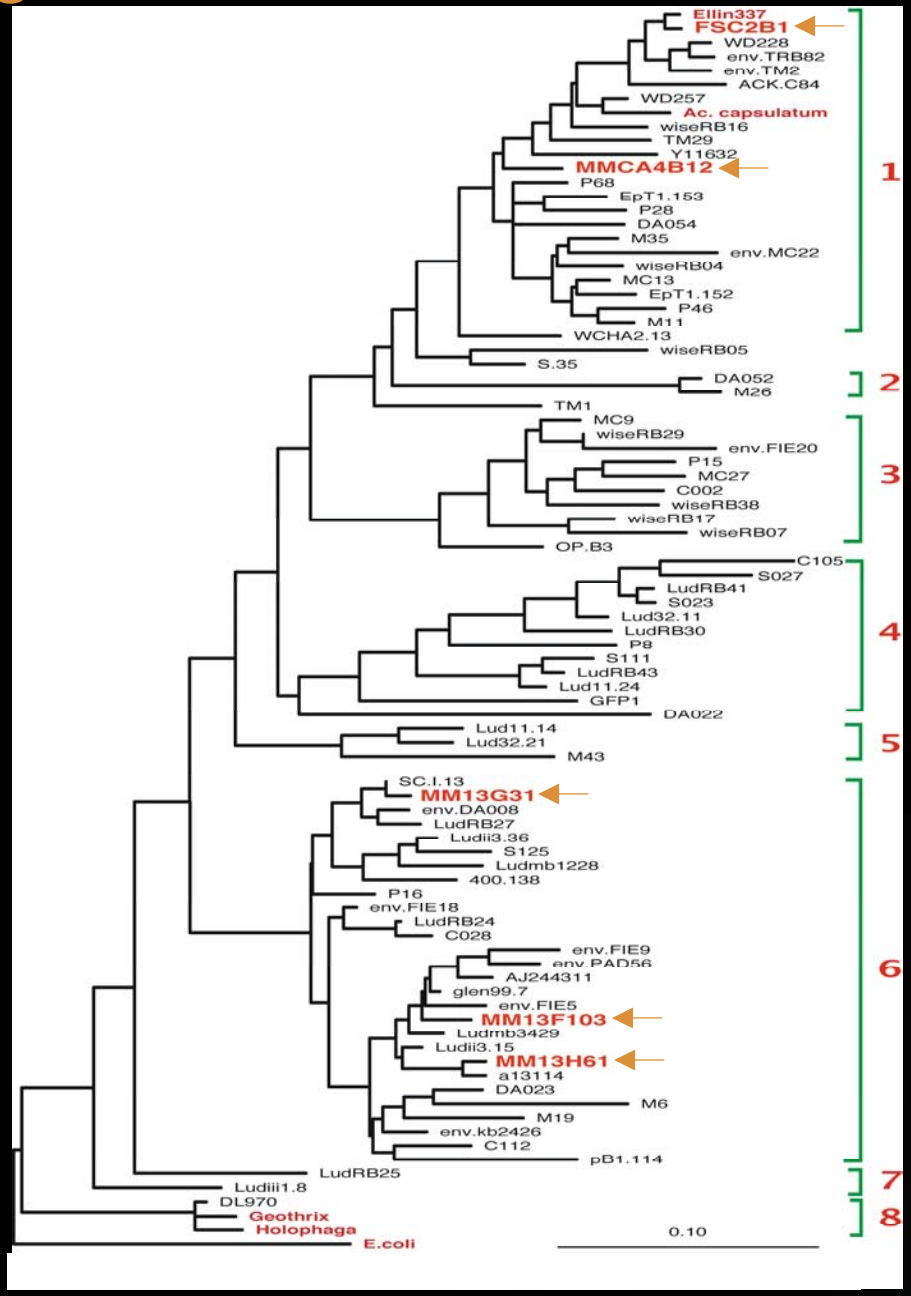
SX-108 Zone 2

Aquatic bacterium RUB_NH4_10	98%	ALPHA
Bradyrhizobium elkanii	100%	ALPHA
Burkholderia cepacia	100%	BETA
Gordonia polyisoprenivorans	100%	HIGH GC G+
Mesorhizobium amorphae	98%	ALPHA
Methylobacterium sp.	99%	ALPHA
Pseudomonas fluorescens	99%	GAMMA
Pseudomonas sp. BUVI	100%	GAMMA
Ralstonia pickettii	100%	BETA
Rhizobium sp. RM1-2001	100%	ALPHA

SX-108 Zone 4

Arthrobacter chlorophenolicus	99%	HIGH GC G+
Arthrobacter globiformis	99%	HIGH GC G+
Arthrobacter ramosus	98%	HIGH GC G+
Bacillus megaterium	100%	HIGH GC G+
Gordonia polyisoprenivorans	100%	HIGH GC G+
Mesorhizobium amorphae	98%	ALPHA
Methylobacterium sp.	99%	ALPHA
Ralstonia sp. APF11	100%	BETA
Rhizobium sp. RM1-2001	100%	ALPHA
Streptococcus mitis	99%	LOW GC G+

Targeted Isolation: *Acidobacteria*



Fred Brockman (PNNL), Cheryl Kuske and Sue Barns (LANL)

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Limited Biomass…

Total environmental sequencing (metagenomics):

+ Tremendous amount of information
- For complex communities no individual genome coverage

Single genome sequencing:

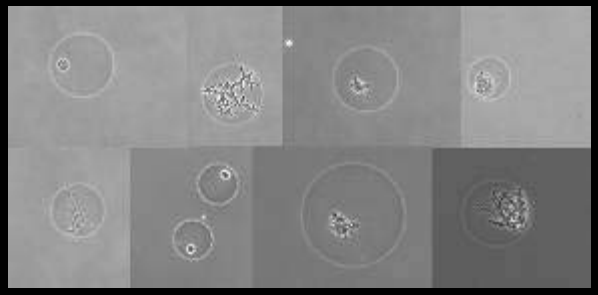
+ Link between diversity and function
- Access to individual species

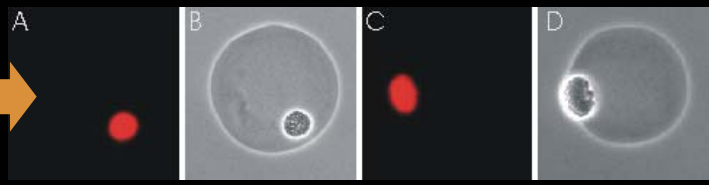
Genomes to Life

- Diversa
- Los Alamos National Laboratory (LANL)
- Pacific Northwestern National Laboratory (PNNL)

"*Access genomes and function of so-far uncultivated microorganism*"

GTL scheme

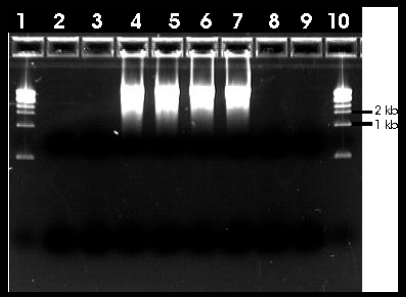




Sample

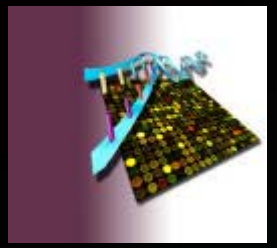
High-throughput cultivation

FISH



Rolling circle amplification

whole Genome Sequencing



Microarray

Gene expression experiments

Proteomics

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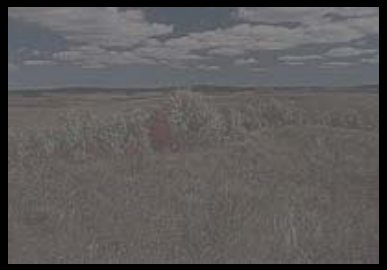
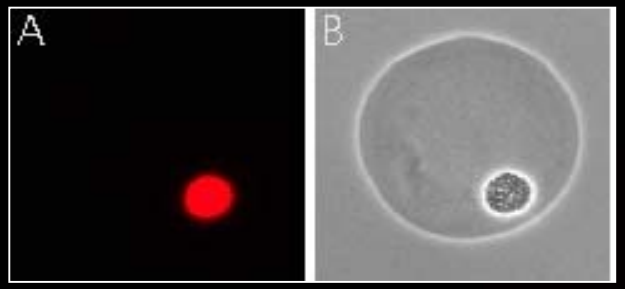
GTL scheme



In Situ
CULTIVATION



GENOMICS

METABOLOMICS

PROTEOMICS

DIVERSA

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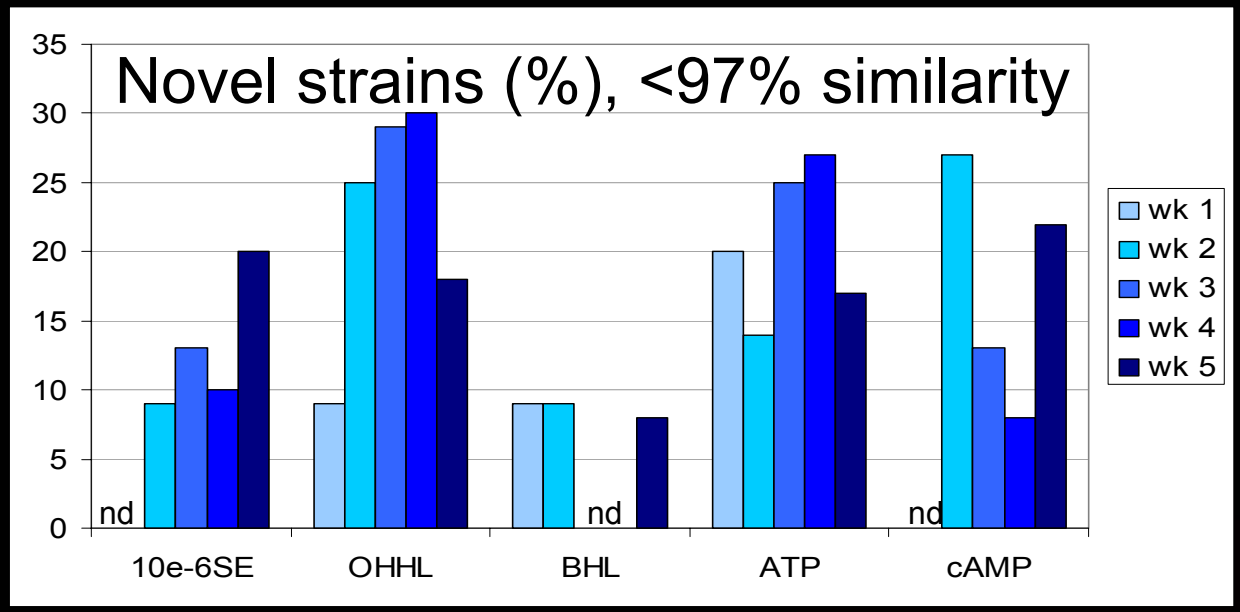
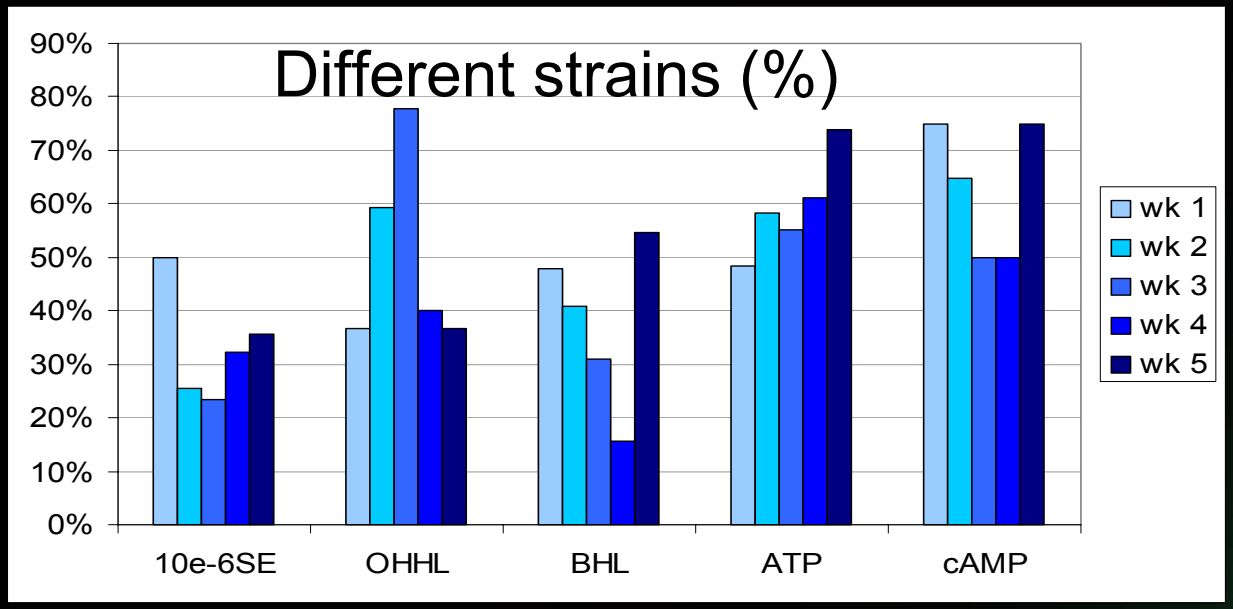
Fred Brockman

LANL:
Cheryl Kuske
Susan Barns

Diversity:

Costa Rican soil





OHHL: *N*- oxohexanoyl-DL-homoserine lactone

BHL: *N*-butyryl homoserine lactone

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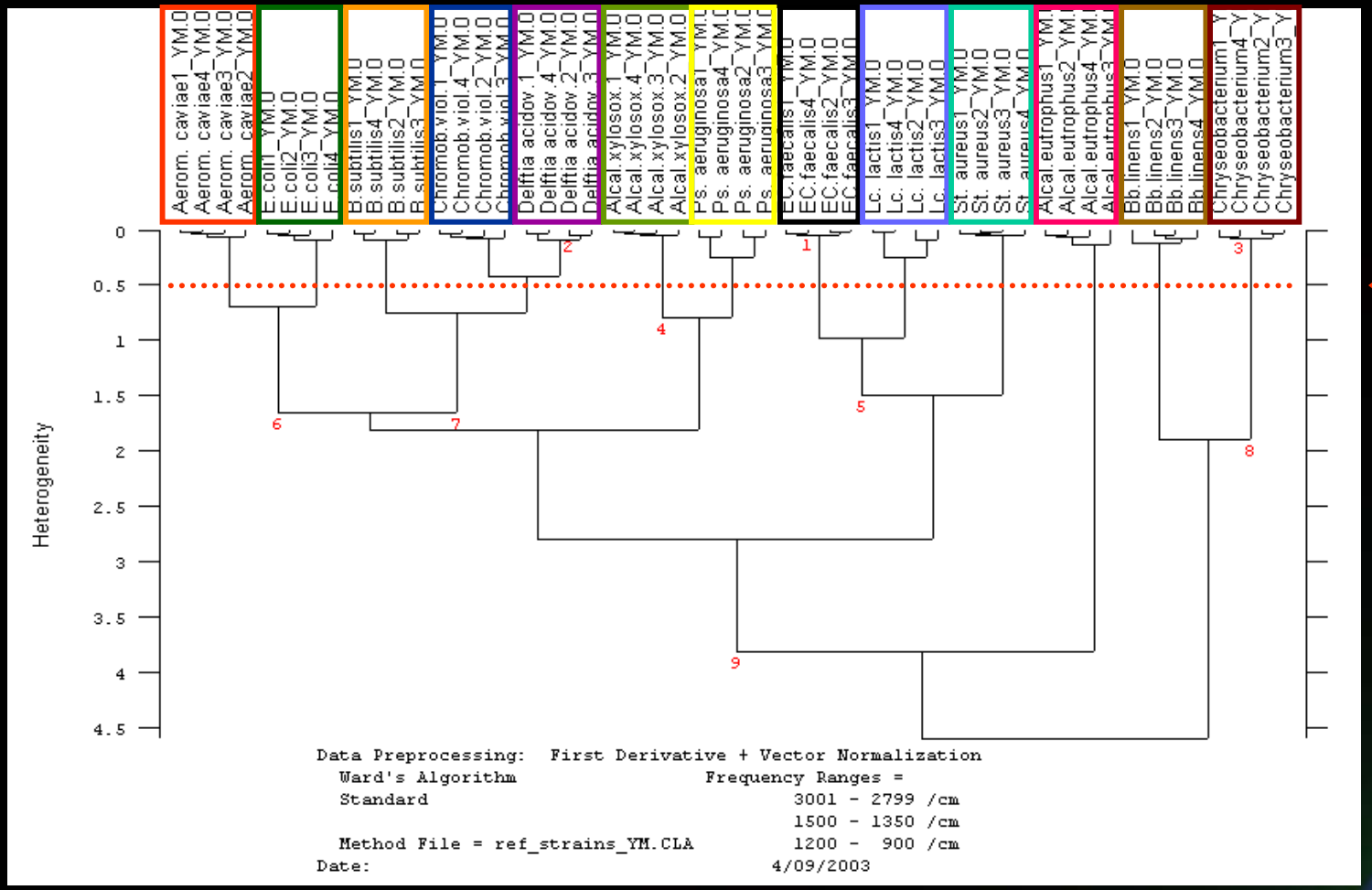
FT-IR reference strains (13):



Variable cut-off

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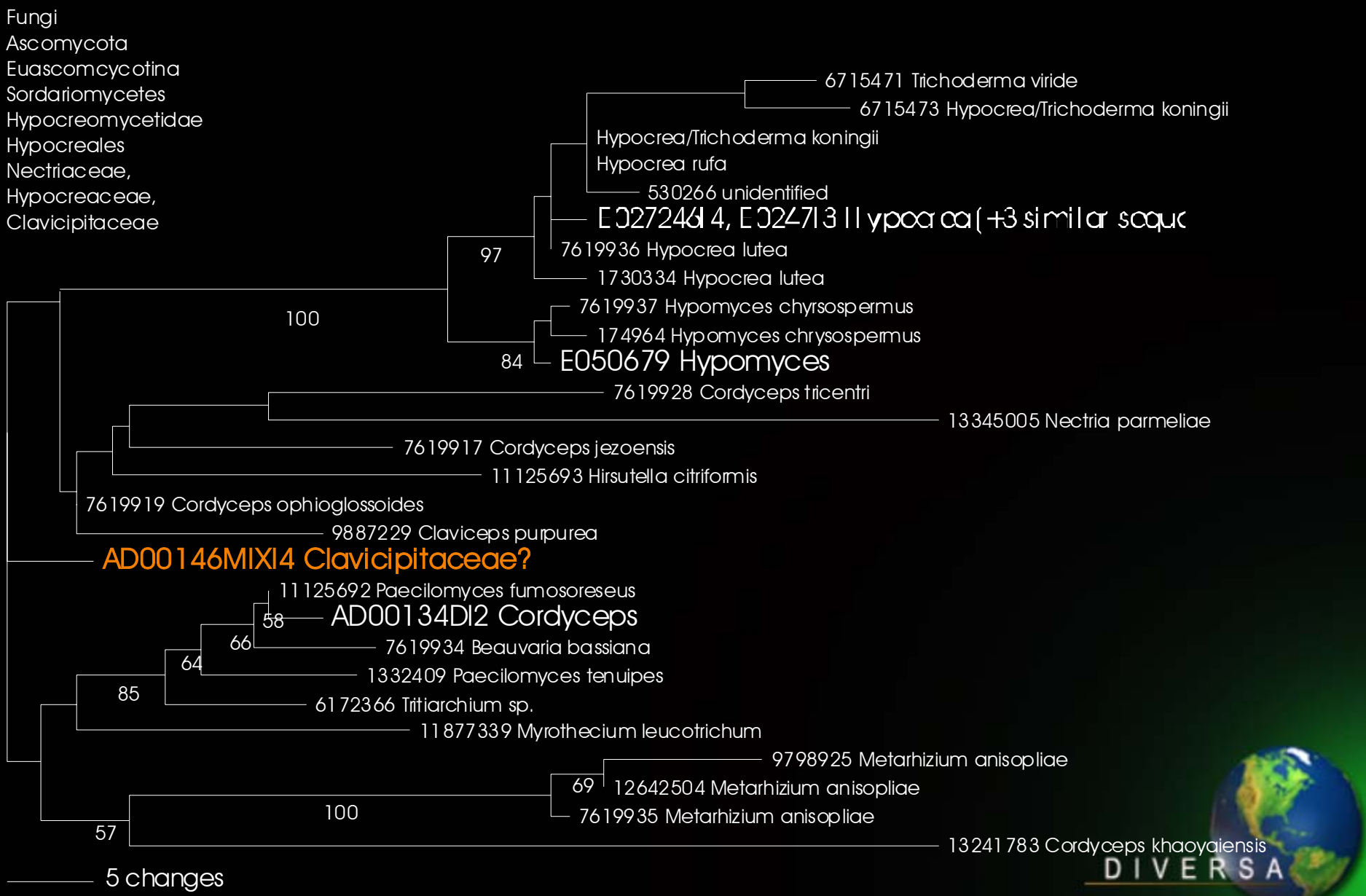
Phylogeny of NCE-producing fungus



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