Day 3

- Examine gels from PCR
- Learn about more molecular methods in microbial ecology

Genes We Targeted

1: dsrAB 1800bp

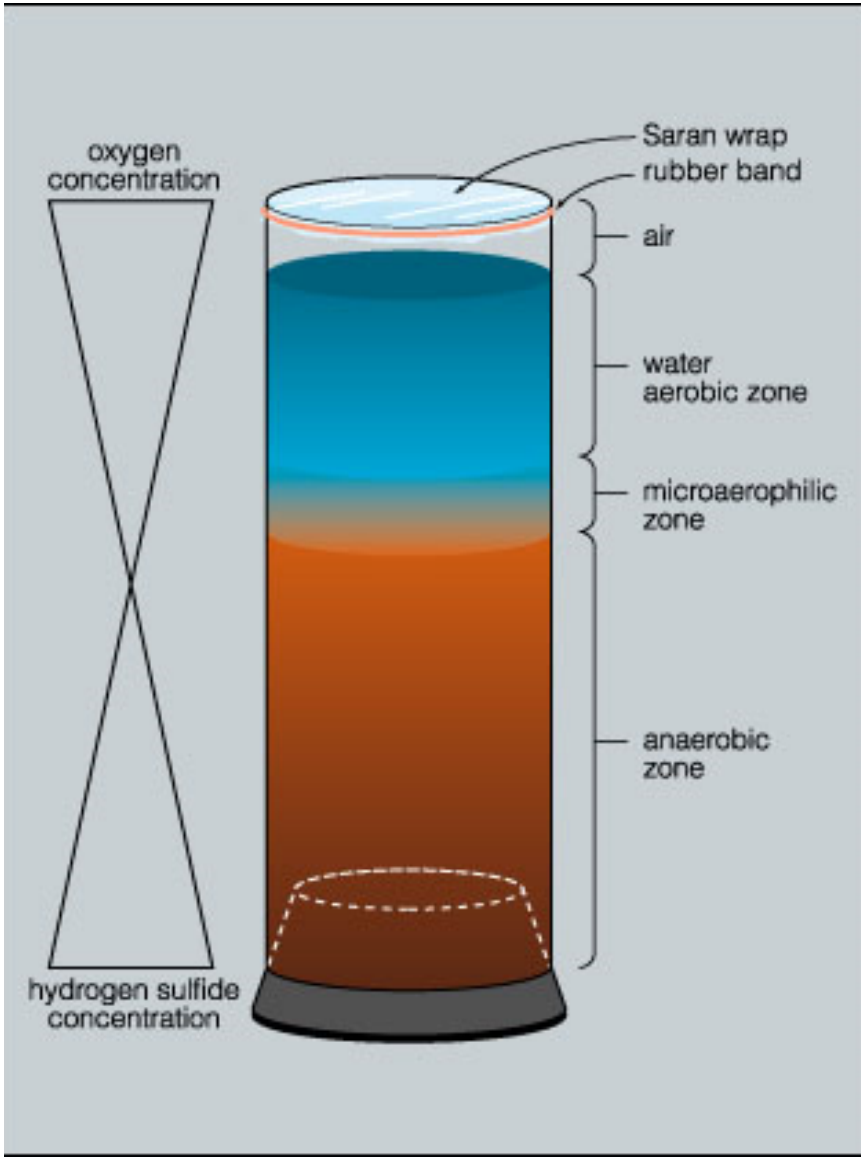
2: mcrA 750bp

3: Bacteria 1450bp

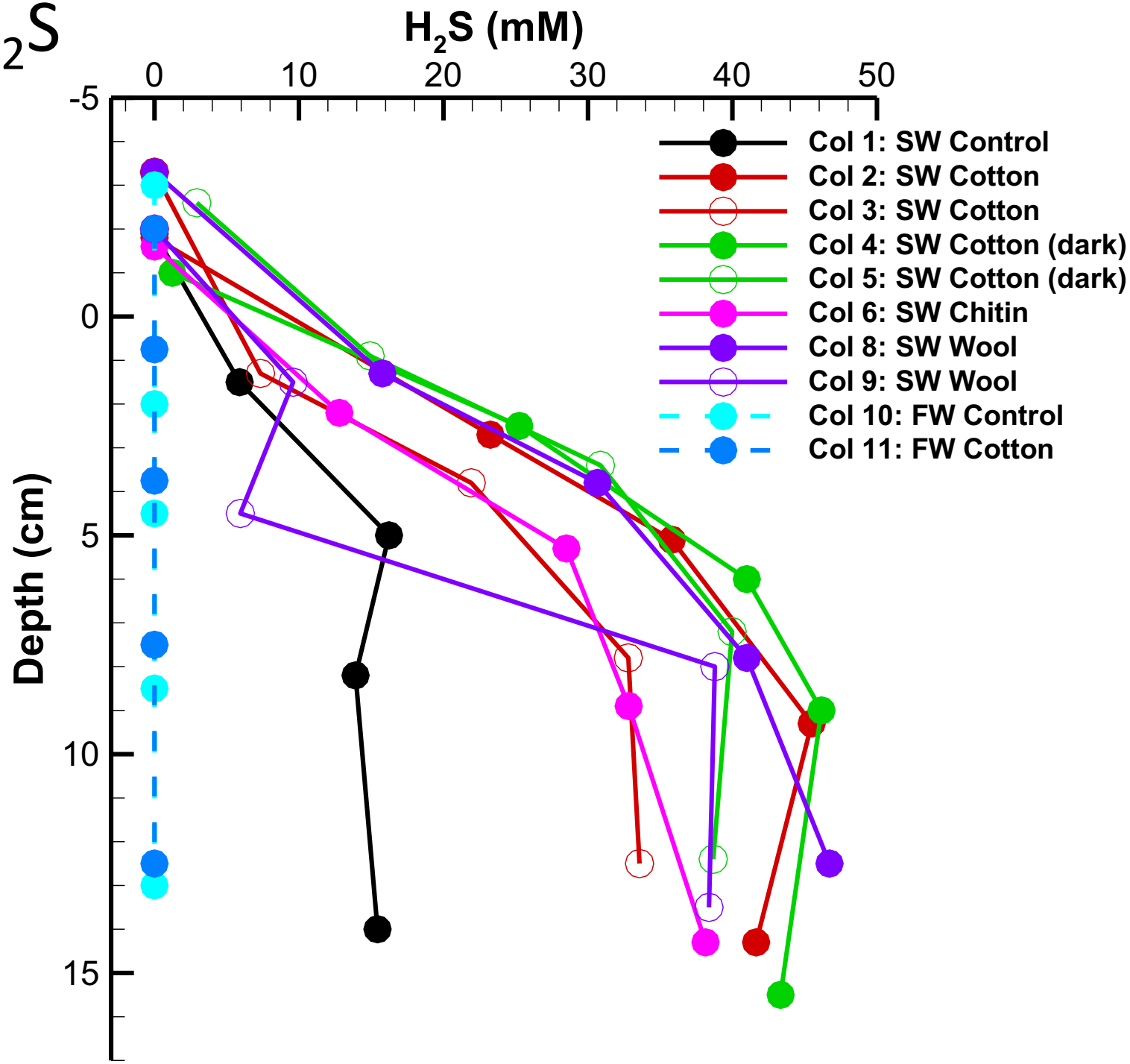
4: Archaea 950bp

5: Archaea + 950bp

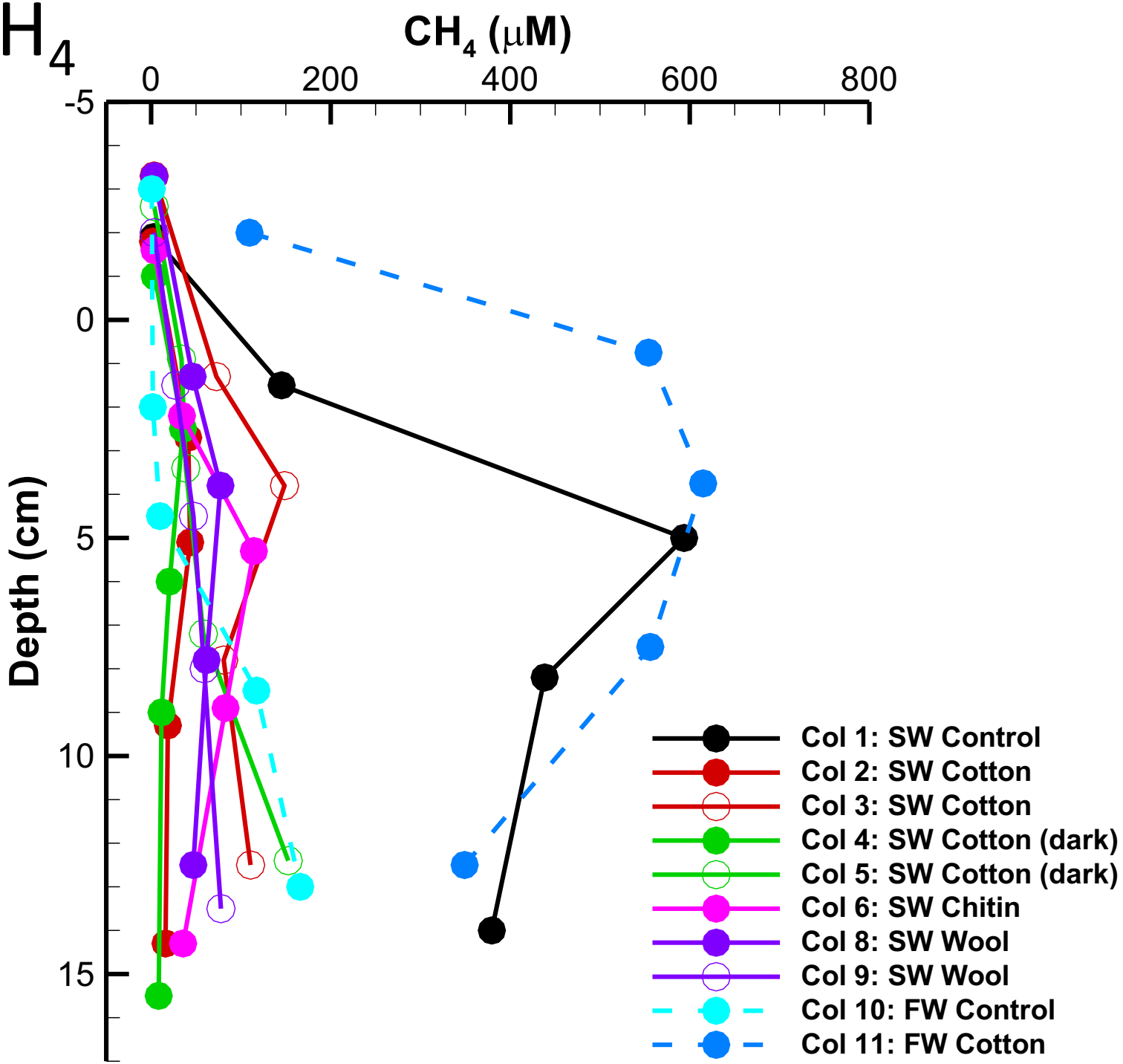
6: Negative control

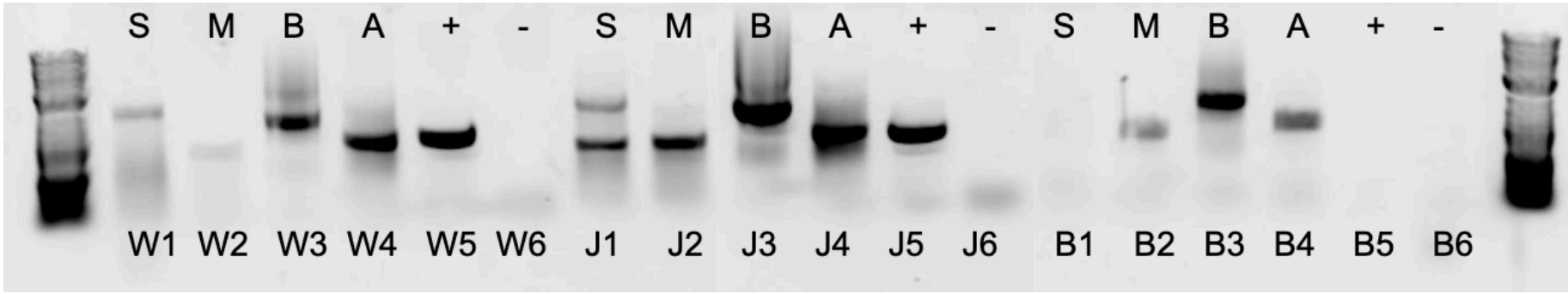


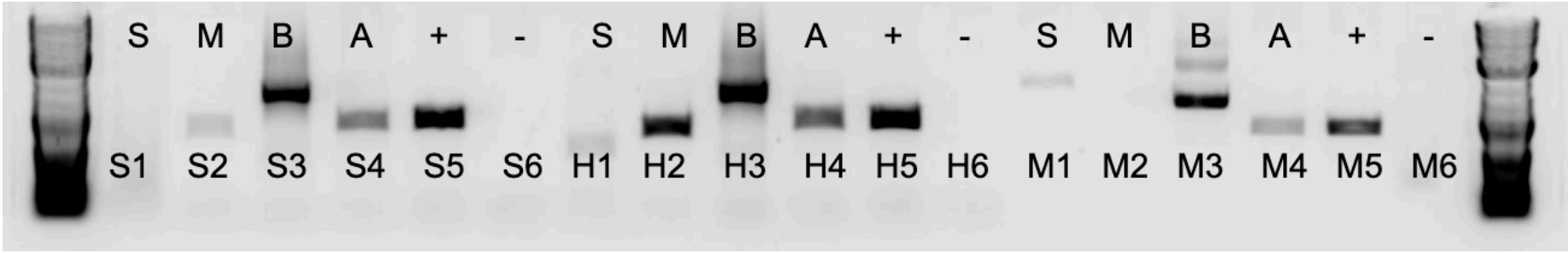
2018 H_2S

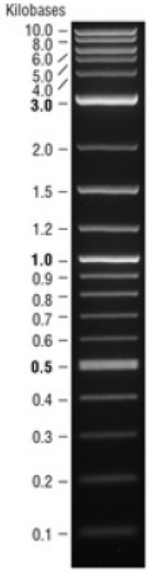

H2S (mM)
0
10
20
30
40
50
-5
0
5
10
15
Depth (cm)
Col 1: SW Control
Col 2: SW Cotton
Col 3: SW Cotton
Col 4: SW Cotton (dark)
Col 5: SW Cotton (dark)
Col 6: SW Chitin
Col 8: SW Wool
Col 9: SW Wool
Col 10: FW Control
Col 11: FW Cotton

2018 CH_4


CH4 (μM)
0
200
400
600
800
-5
0
5
10
15
Depth (cm)
Col 1: SW Control
Col 2: SW Cotton
Col 3: SW Cotton
Col 4: SW Cotton (dark)
Col 5: SW Cotton (dark)
Col 6: SW Chitin
Col 8: SW Wool
Col 9: SW Wool
Col 10: FW Control
Col 11: FW Cotton







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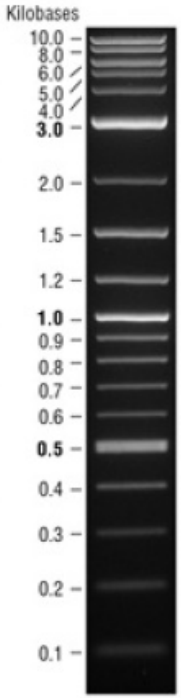
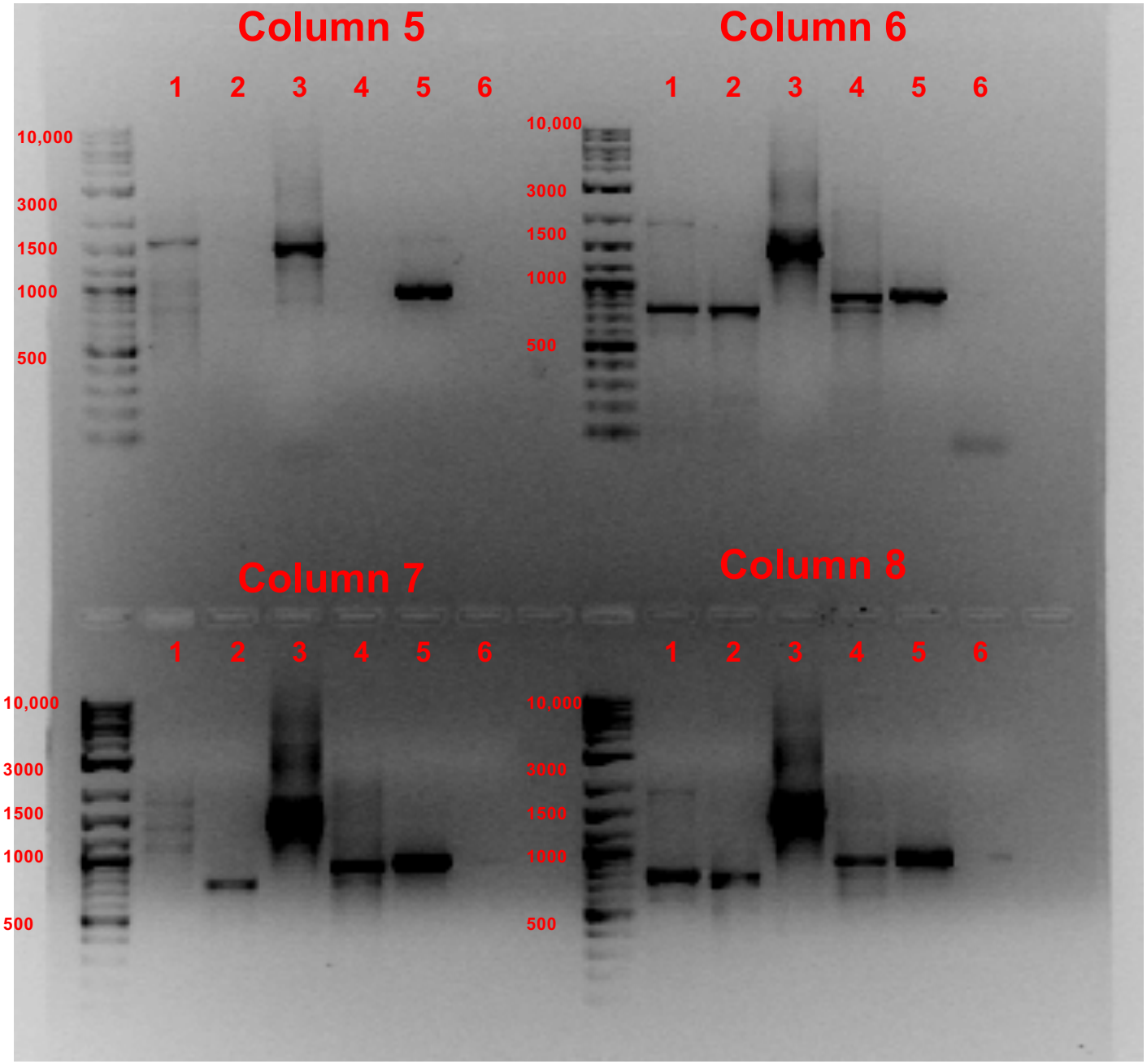




Some Problems with PCR

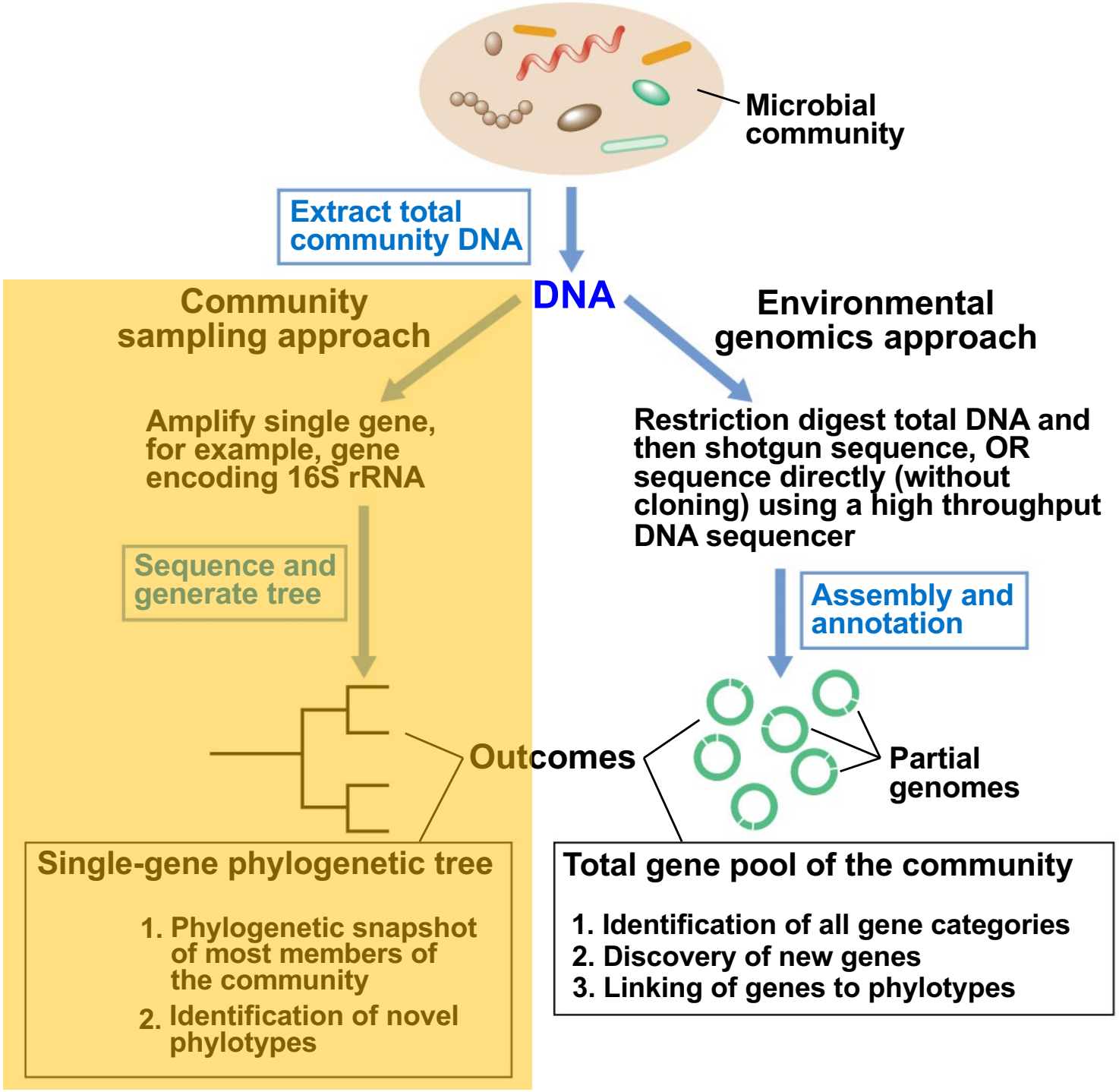
- Inhibitors in template DNA
- Amplification bias
- Gene copy number
- Limited by primer design
- Differential denaturation efficiency
- Chimeric PCR products may form
- Contamination w/ non-target DNA
- Potentially low sensitivity and resolution
- General screw-ups

(Some) Problems with Molecular Methods

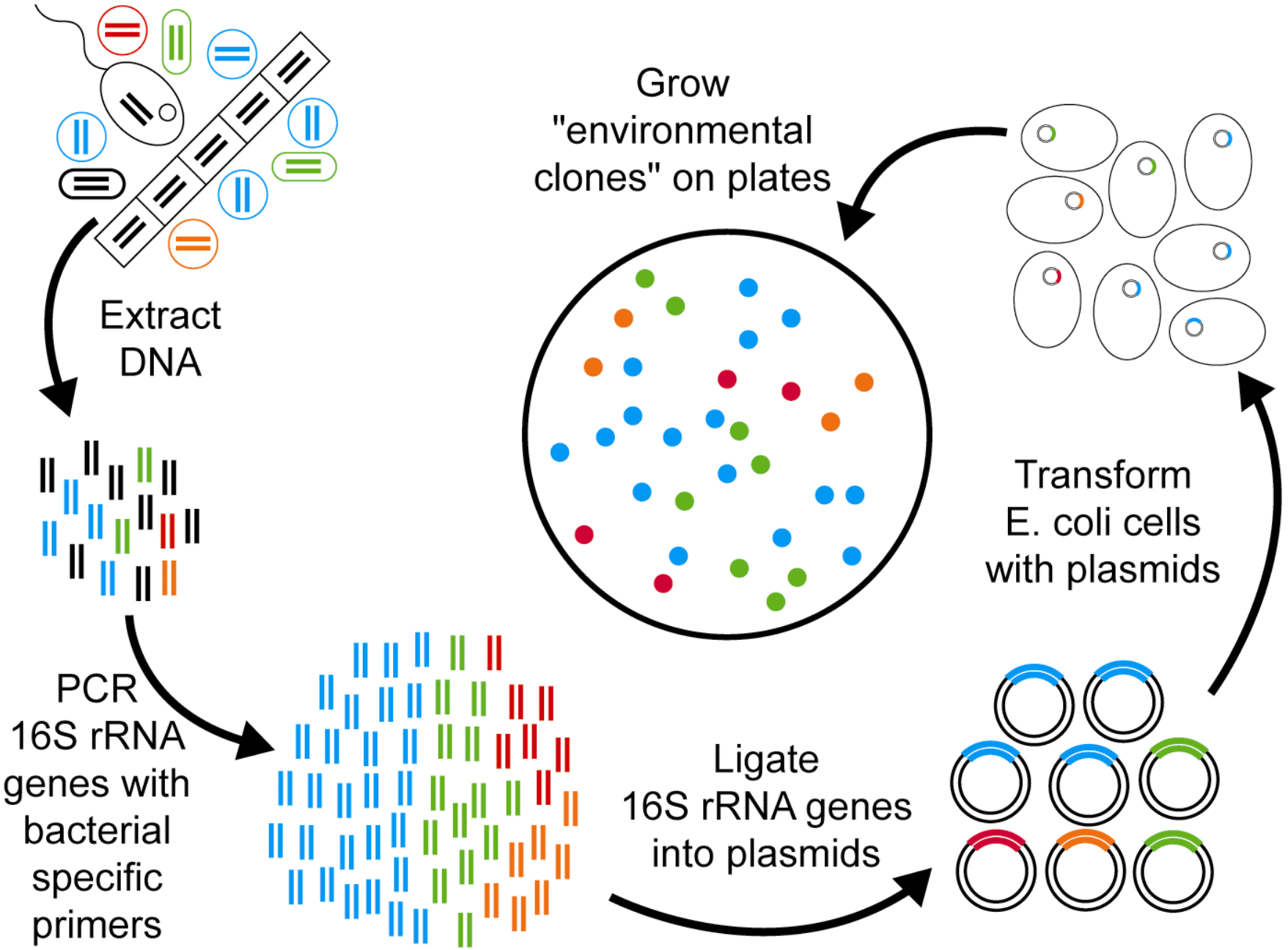
D/RNA extraction	Incomplete sampling
	Resistance to cell lysis
Storage	Enzymatic degradation
PCR	Inhibitors in template DNA
	Amplification bias
	Gene copy number
	Fidelity of PCR
	Differential denaturation efficiency
	Chimeric PCR products
Anytime	Contamination w/ non-target DNA

So you have a positive PCR product: Now what?

- Clone and sequence
- Get “community fingerprint” via T-RFLP, DGGE, etc.
- Design probes for imaging to provide spatial information
- Quantify
- Go straight into sequencing (next generation sequencing)



Traditional Gene Cloning

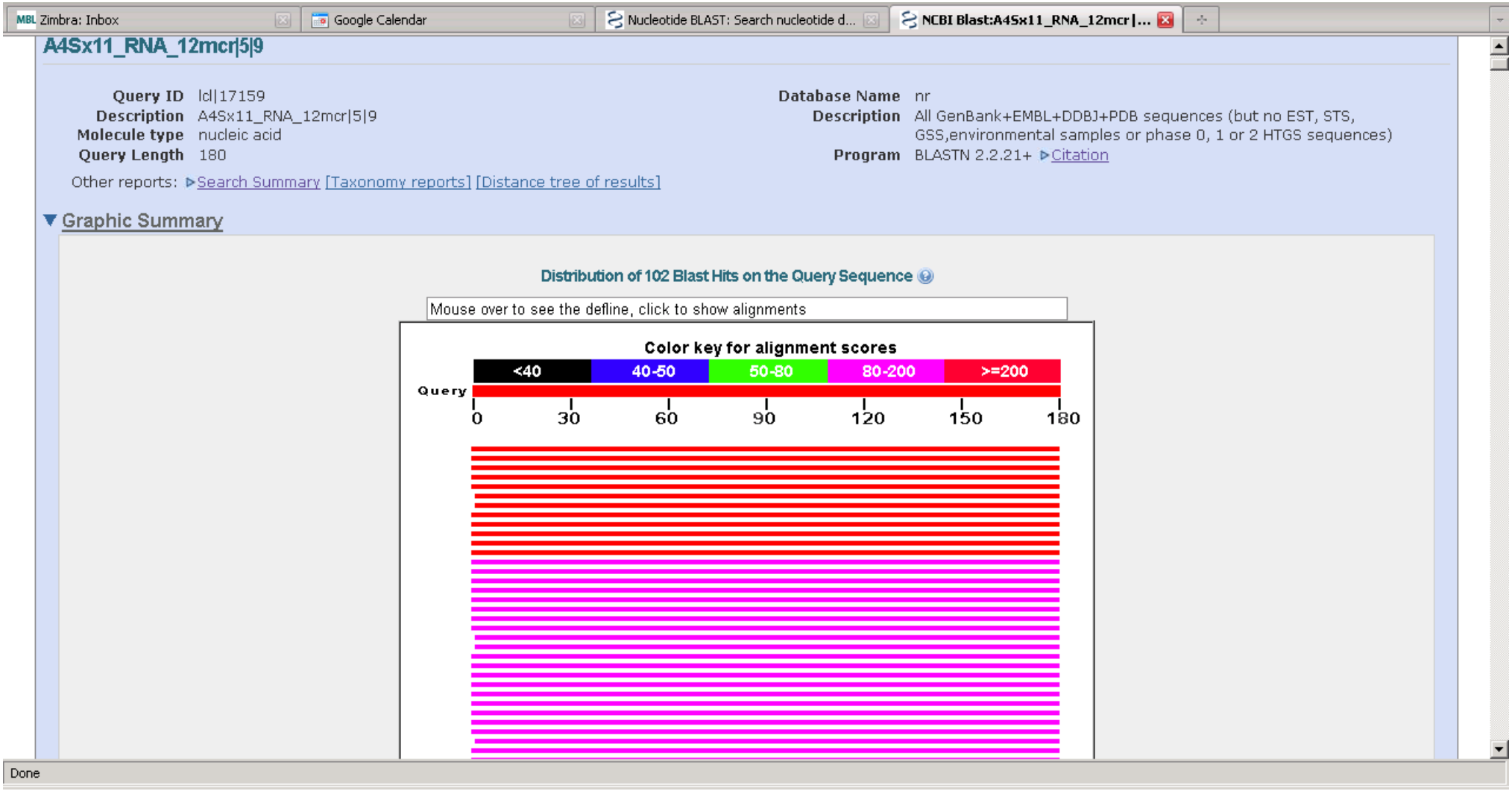


Schematic courtesy of B. Crump

What do you DO with sequences?

- Perform a similarity search
- Align the sequences
- Build a tree and classify
- Reconstruct genomes
- Categorize functions
- Compare organisms/samples
- Design probes and quantify
- Examine expression patterns
- Etc. Etc. Etc.

BLAST
Basic Local Alignment Search Tool



http://blast.ncbi.nlm.nih.gov/Blast.cgi

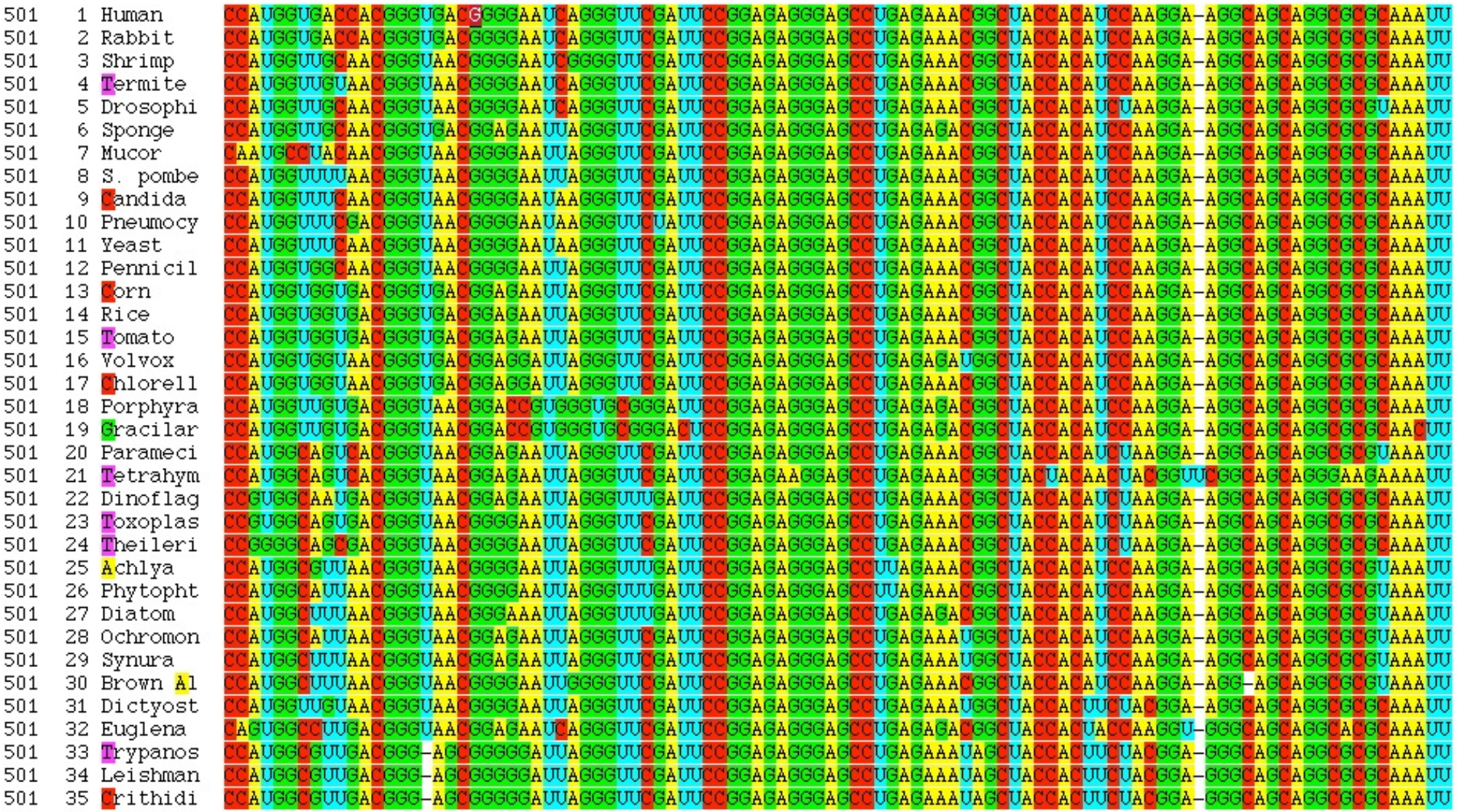
Making Sense of Sequences: Molecular Phylogeny

1. Align sequences so that "homologous" residues are juxtaposed.

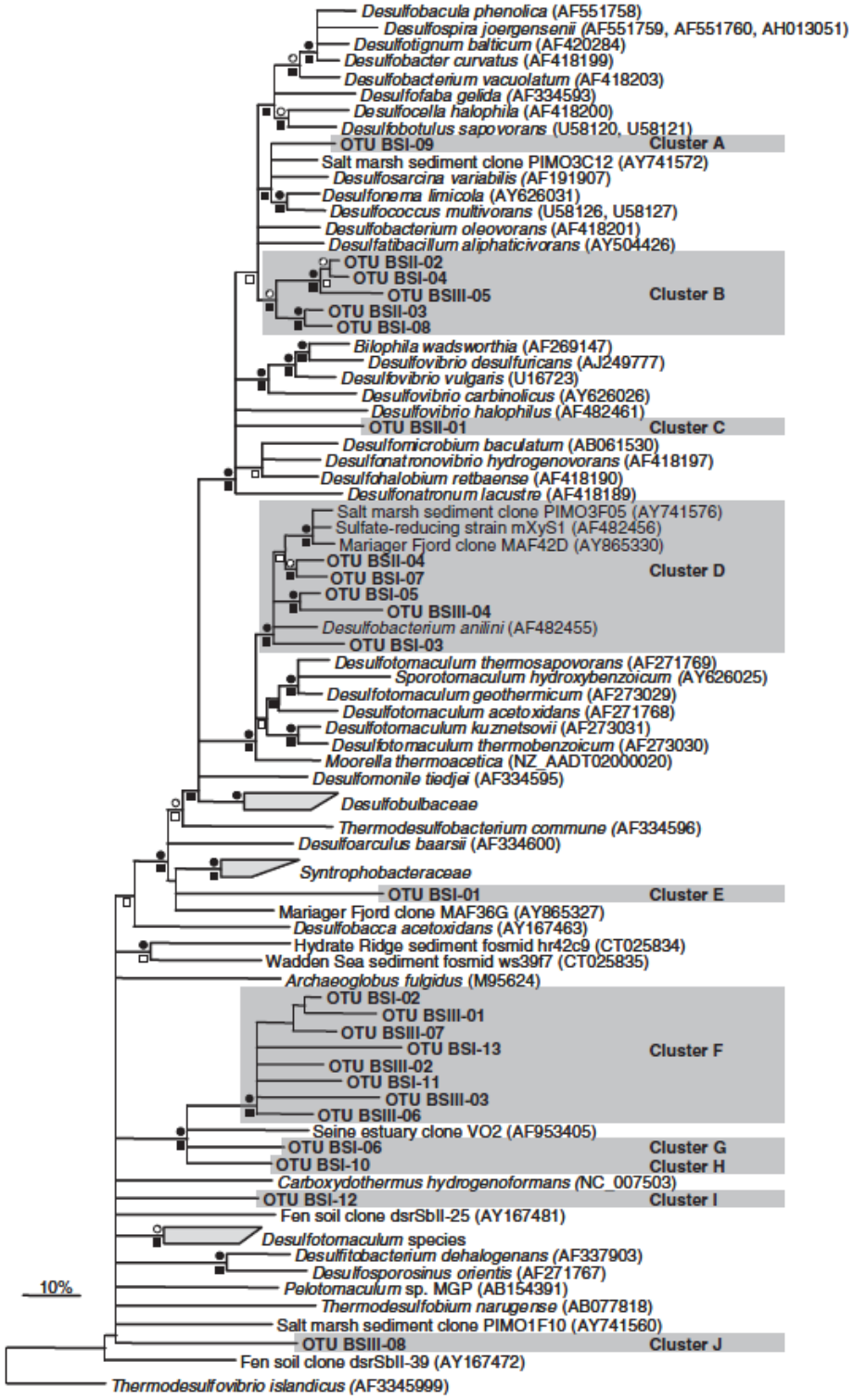
2. Count the number of differences between pairs of sequences; this is some measure of "evolutionary distance" that separates the organisms.

3. Calculate the "tree," the relatedness map, that most accurately represents all the pairwise differences.

Cursor positioned at nucleotide 521 of sequence number 1 (Human)

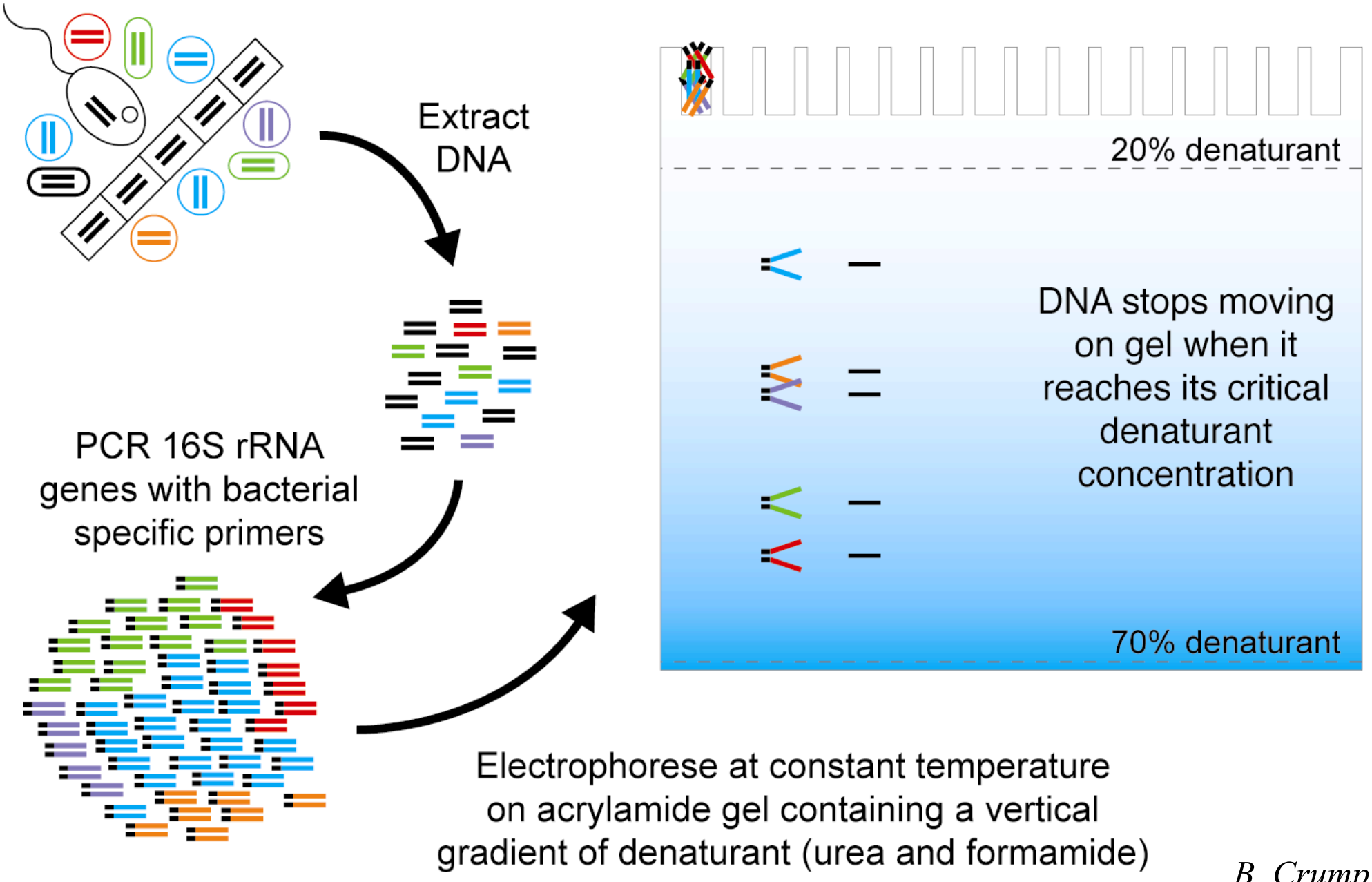



ursor positioned at nucleotide 1100 of sequence number 35 ( )
1050 1 Human
1050 2 Rabbit
1050 3 Shrimp
1050 4 Drosophi
1050 5 Sponge
1050 6 Mucor
1050 7 S. pombe
1050 8 Candida
1050 9 Pneumocy
1050 10 Yeast
1050 11 Pennicil
1050 12 Corn
1050 13 Rice
1050 14 Tomato
1050 15 Volvox
1050 16 Chlorell
1050 17 Porphyra
1050 18 Gracilar
1050 19 Parameci
1050 20 Tetrahym
1050 21 Dinoflag
1050 22 Toxoplas
1050 23 Theileri
1050 24 Achlya
1050 25 Phytopht
1050 26 Diatom
1050 27 Ochromon
1050 28 Synura
1050 29 Brown Al
1050 30 Dictyost
1050 31 Euglena
1050 32 Trypanos
1050 33 Leishman
1050 34 Crithidi



- Found similar novel *dsr* sequences in the sulfate-rich and methane-rich zones
- Different (and already known) *dsr* sequences in SMTZ

Denaturing Gradient Gel Electrophoresis (DGGE)



B. Crump

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2004, p. 1494–1505 Vol. 70, No. 3
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.3.1494–1505.2004

Microbial Biogeography along an Estuarine Salinity Gradient: Combined Influences of Bacterial Growth and Residence Time

Byron C. Crump,[1]* Charles S. Hopkinson,[2] Mitchell L. Sogin,[3] and John E. Hobbie[2]

Horn Point Laboratory, University of Maryland Center for Environmental Science, Cambridge, Maryland,[1] and The Ecosystems Center[2] and The Josephine Bay Paul Center for Comparative Molecular Biology and Evolution,[3] Marine Biological Laboratory, Woods Hole, Massachusetts

Received 1 August 2003/Accepted 16 December 2003

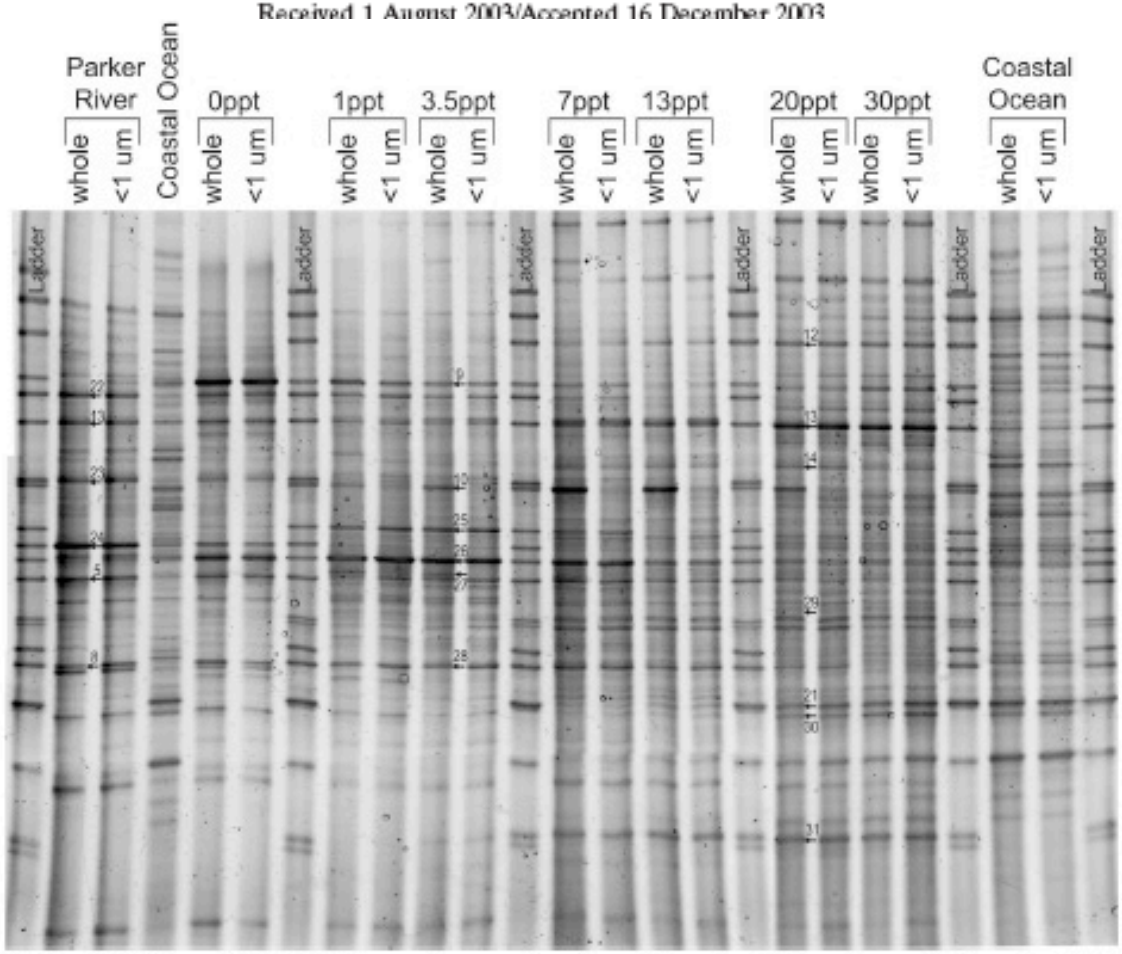
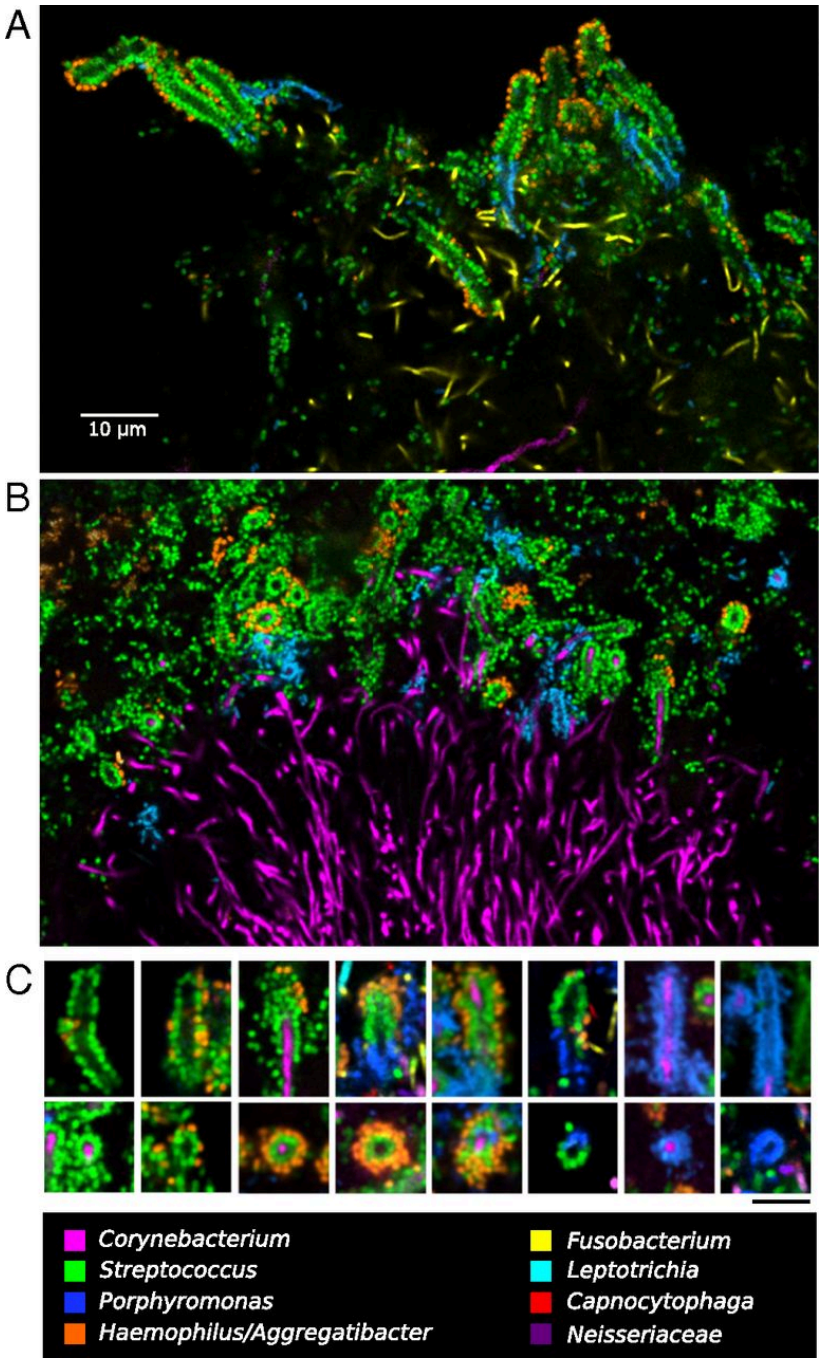
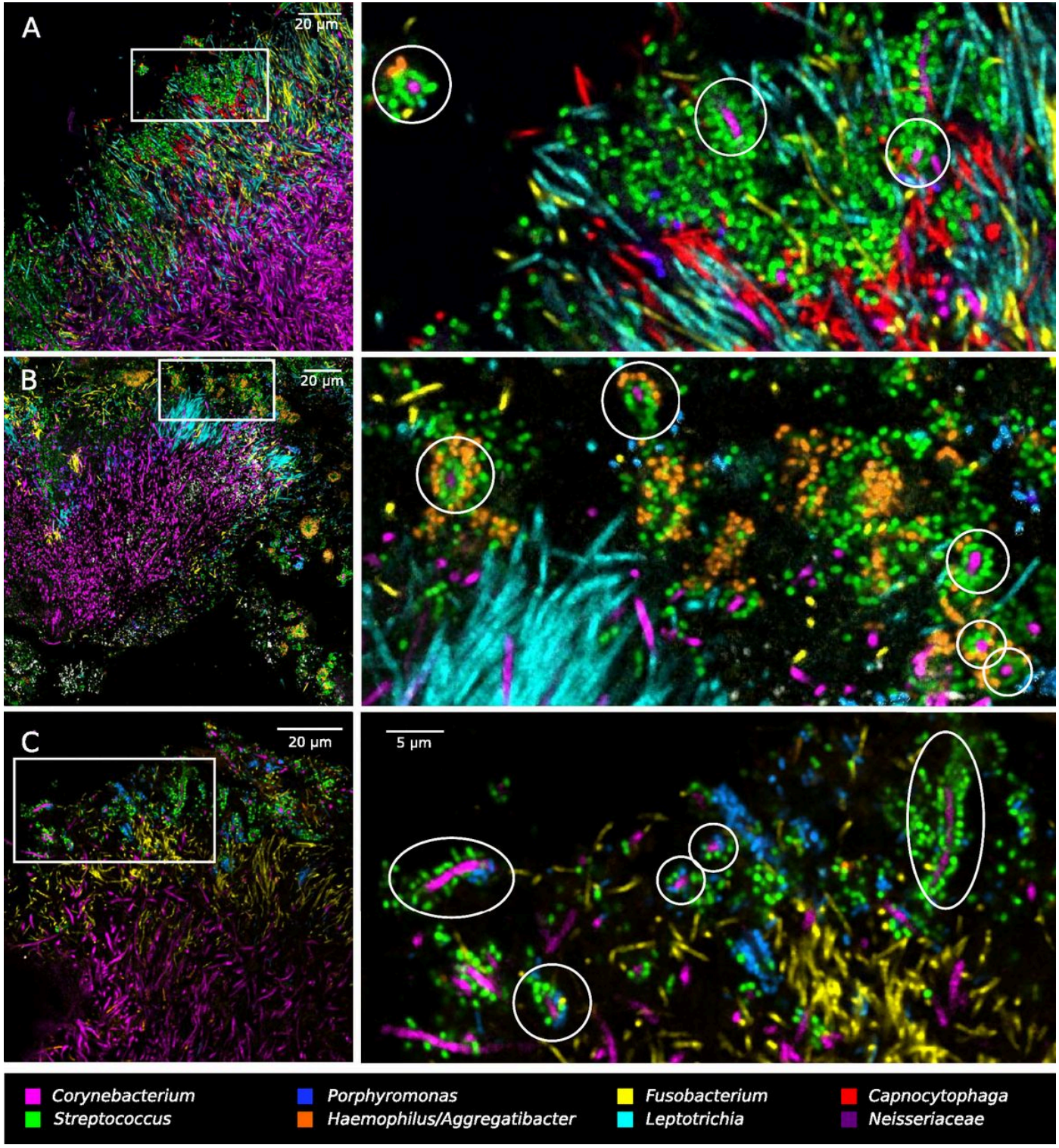


FIG. 4. DGGE gel of PCR-amplified 16S rDNA genes from samples collected along the salinity gradient on 28 September 2000. Bands from which DNA was sequenced are marked and numbered, corresponding to band numbers in Table 3.

rRNA Oligonucleotide Probes => Spatial context



J. Mark Welch et al. (2016) PNAS



J. Mark Welch et al. (2016) PNAS

Environmental Microbiology (2007) 9(1), 131–142 doi:10.1111/j.1462-2920.2006.01122.x

Diversity and abundance of sulfate-reducing microorganisms in the sulfate and methane zones of a marine sediment, Black Sea

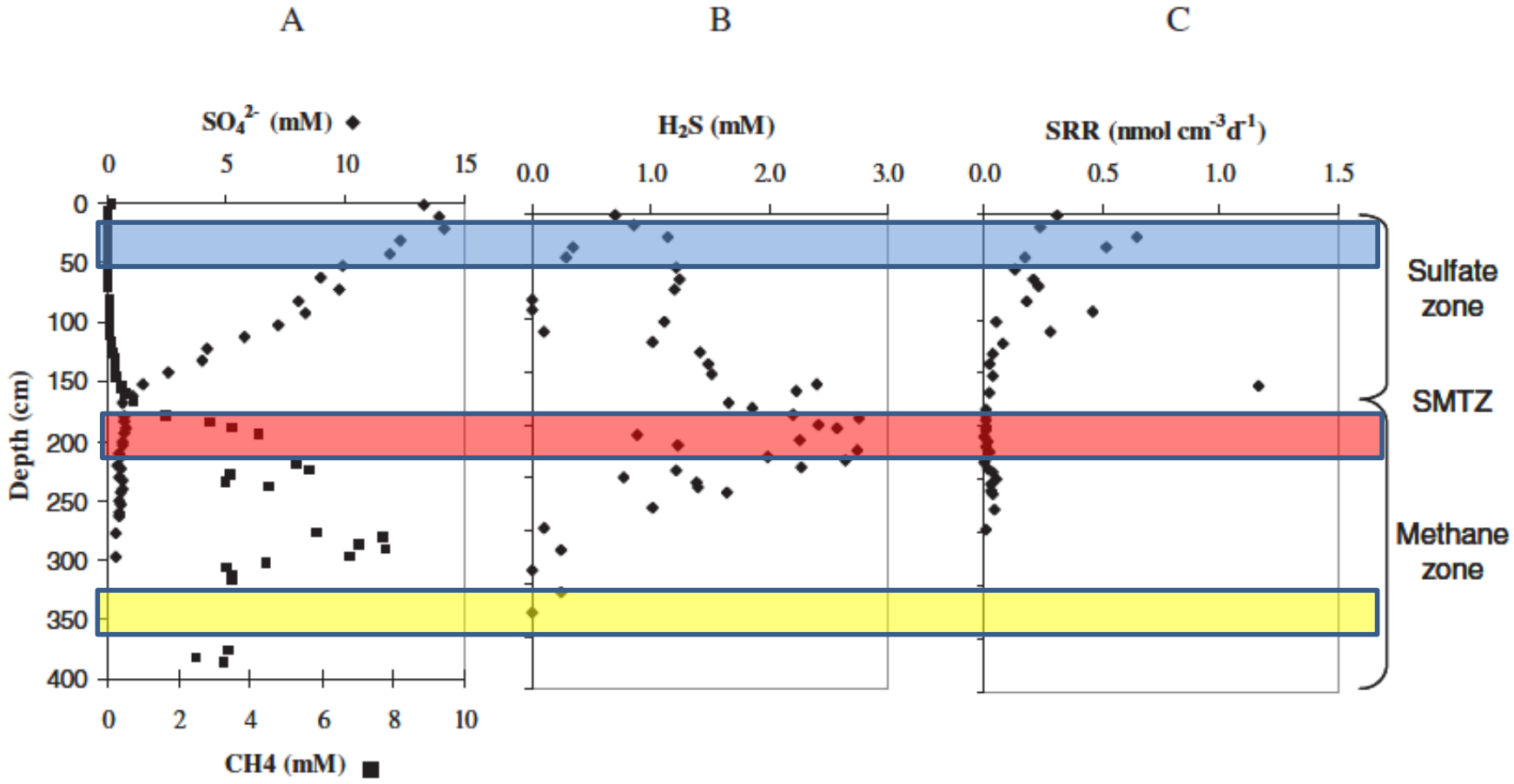
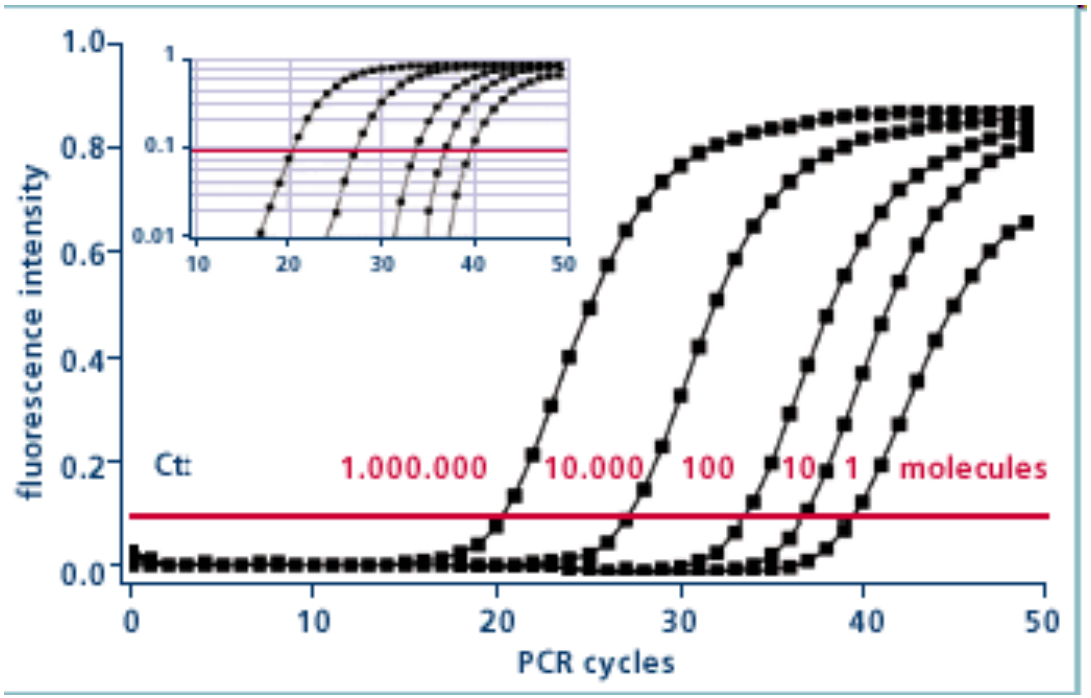


Fig. 2. Biogeochemical zonation and data from the Black Sea sediment core P824-GC.
A. Sulfate and methane concentration.
B. Sulfide concentration.
C. Sulfate reduction rate (SRR).

Quantitative PCR (aka qPCR, Real Time PCR)

qPCR monitors the fluorescence emitted during the reactions as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection



Fluorescent dye intercalates into dsDNA

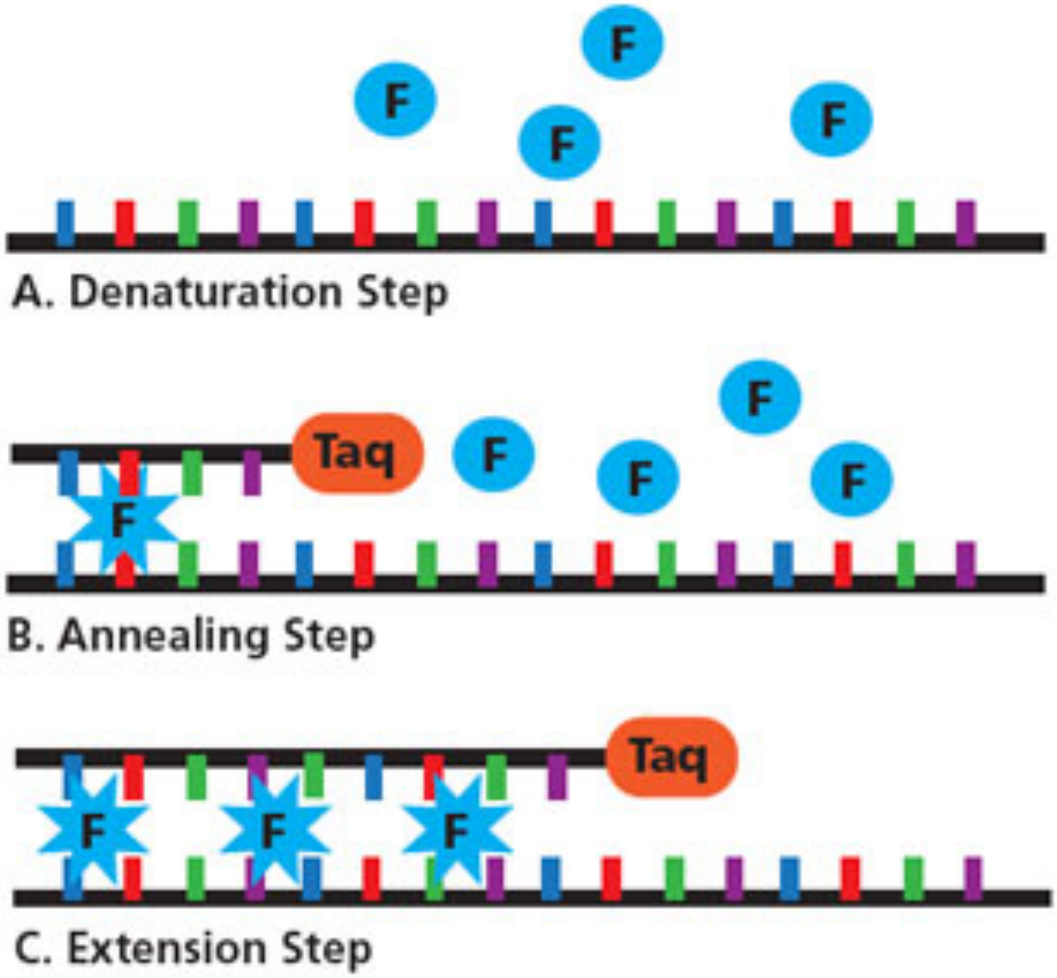
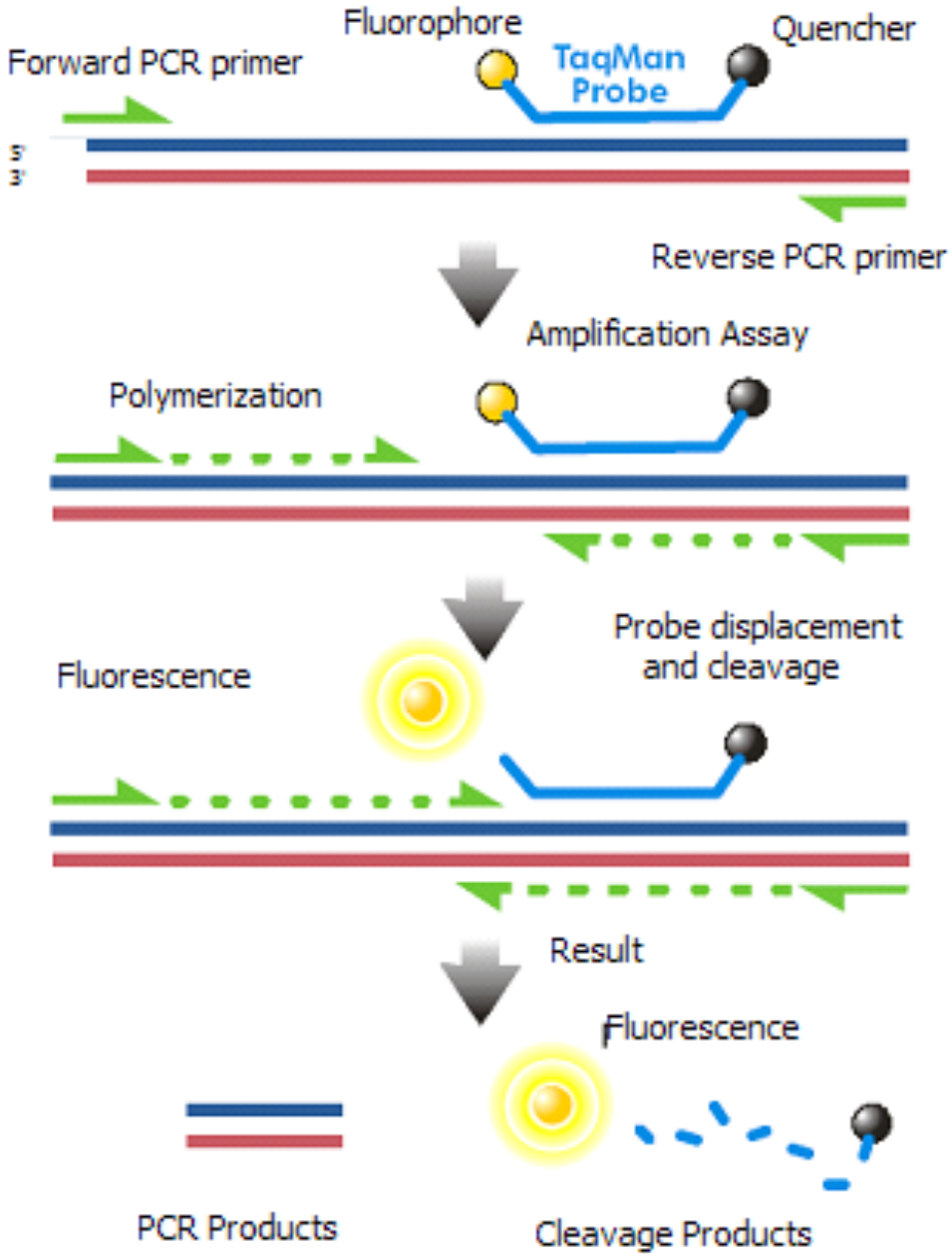


Figure 2: Fluorescent Dyes in qPCR

Probe-based qPCR



Quantitative (Real Time) PCR

- Detection of “amplification-associated fluorescence” at each cycle during PCR
- No gel-based analysis
- Computer-based analysis
- Compare to internal standards
- Must ensure specific binding of probes/dye

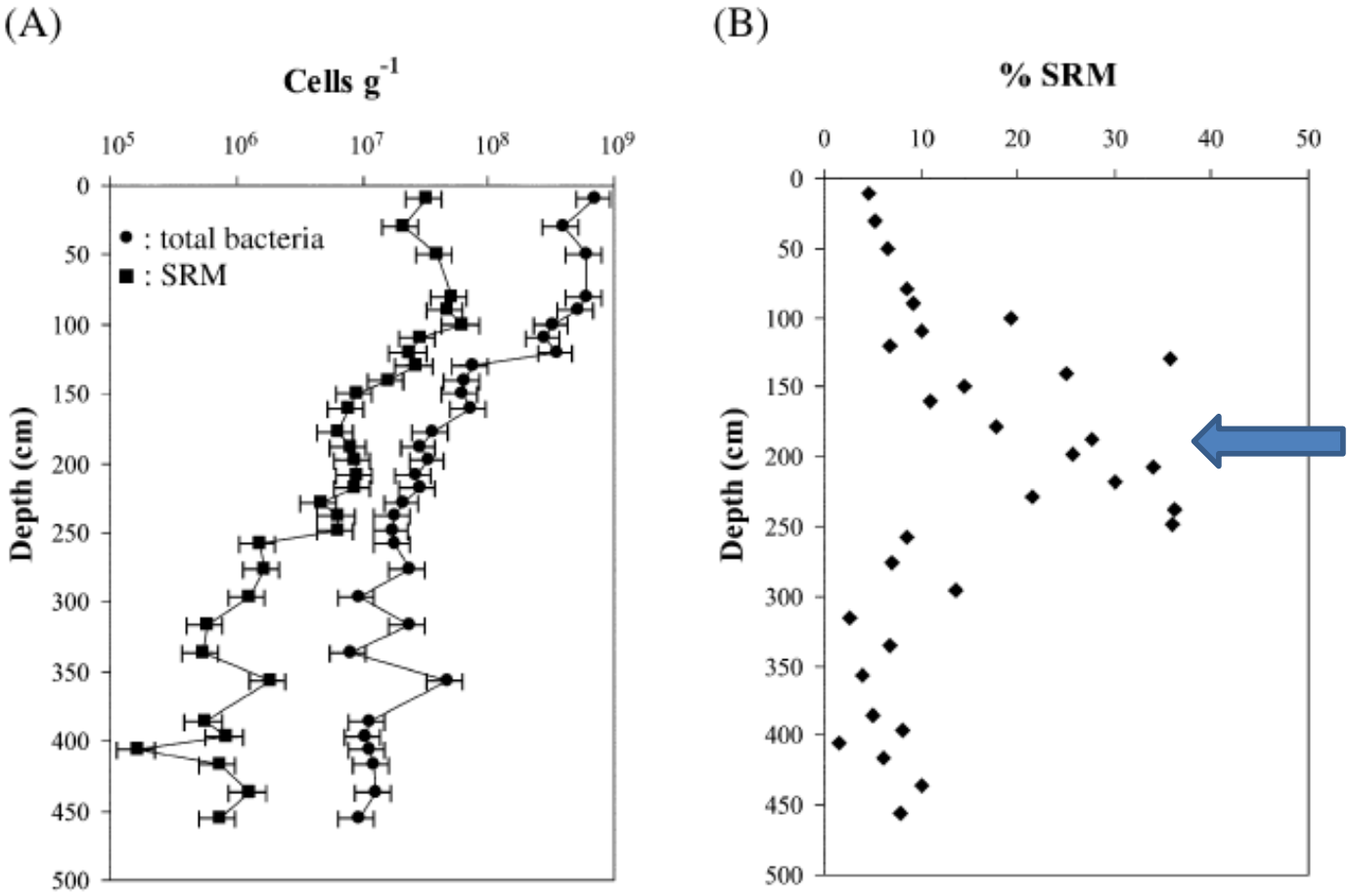
Environmental Microbiology (2007) 9(1), 131–142 doi:10.1111/j.1462-2920.2006.01122.x

Diversity and abundance of sulfate-reducing microorganisms in the sulfate and methane zones of a marine sediment, Black Sea

- Used qPCR to quantify total bacteria (16S rRNA) and total sulfate reducers (*dsr*)

Environmental Microbiology (2007) 9(1), 131–142 doi:10.1111/j.1462-2920.2006.01122.x

Diversity and abundance of sulfate-reducing microorganisms in the sulfate and methane zones of a marine sediment, Black Sea



Sulfate reducing bacteria (*dsr* gene) peaks at SMTZ

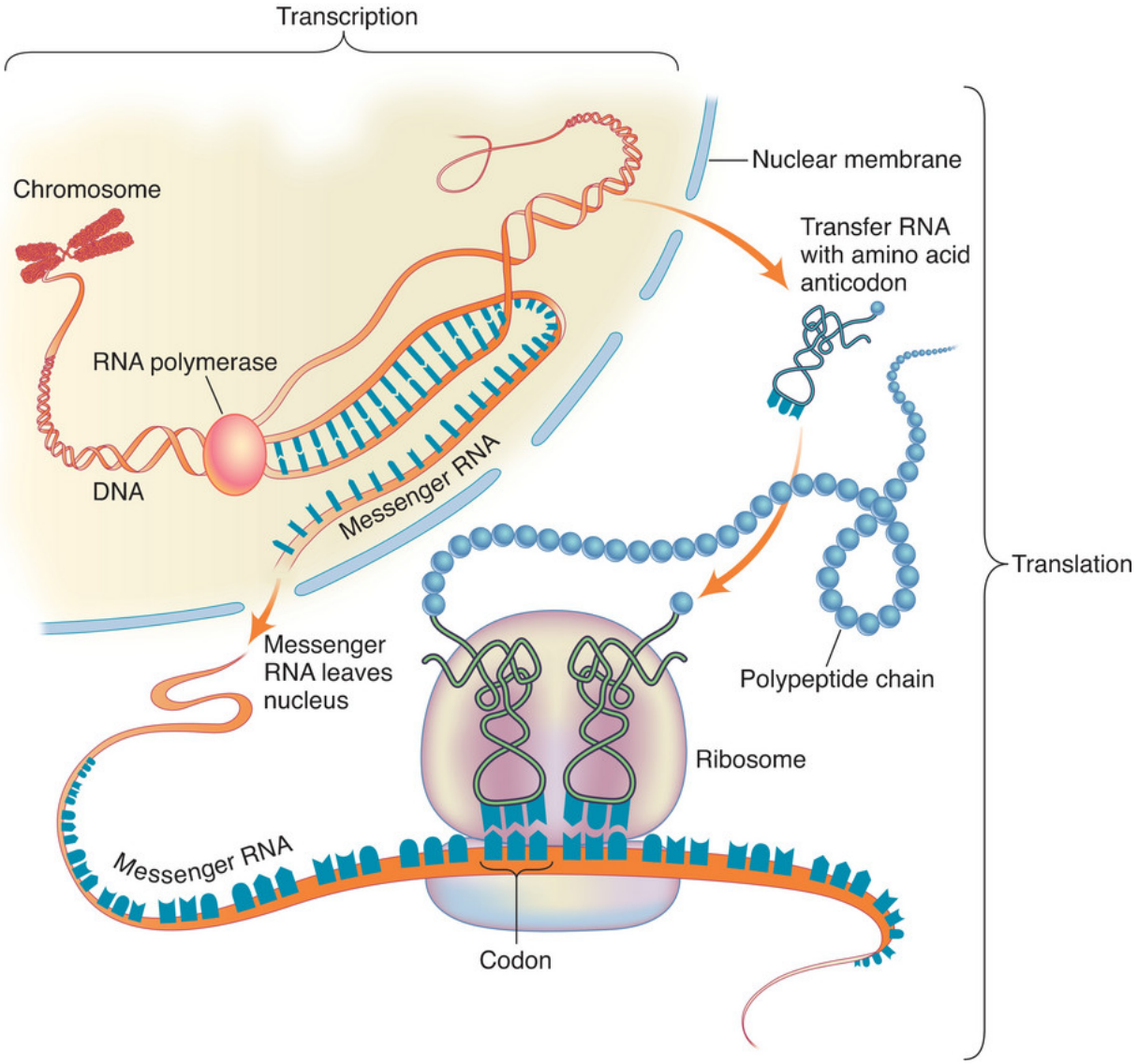
Fig. 3. Abundance of total bacteria and SRM in the Black Sea sediment core P824-GC.
A. Total bacteria and SRM as inferred from real-time PCR data. Values are given as mean ± standard deviation of triplicates. • total bacterial cells; ■ sulfate-reducing cells.
B. Depth profile of the relative contribution of SRM to the total bacterial cells as calculated from the data in (A).

Environmental Microbiology (2007) 9(1), 131–142 doi:10.1111/j.1462-2920.2006.01122.x

Diversity and abundance of sulfate-reducing microorganisms in the sulfate and methane zones of a marine sediment, Black Sea

Future studies will reveal whether these yet unidentified microorganisms with new *dsrAB* variants are active in the environment and which life strategies they employ to thrive in low-sulfate habitats that are apparently inhospitable for SRM.

The Central Dogma



DNA

Transcription

RNA

Translation

Protein

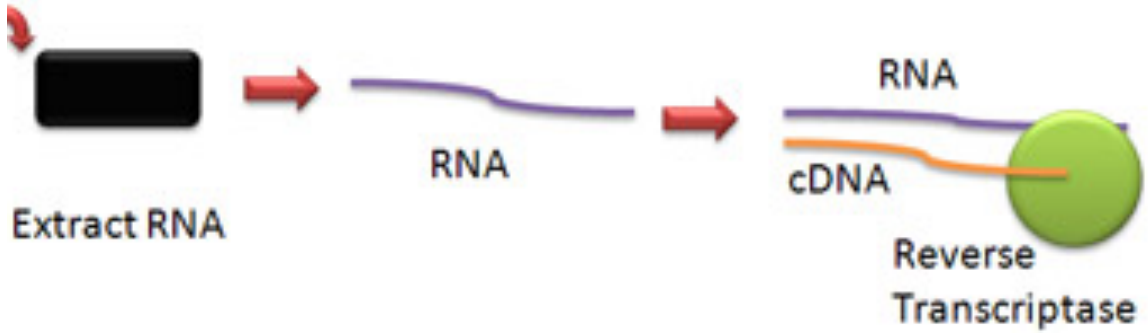
Moving from "who is there?" to "who is active?"

Reverse Transcription PCR (RT-PCR)

- Looks at gene expression in the environment or experimental treatment
- Isolate mRNA
- Reverse transcribe mRNA to produce complementary DNA (cDNA)
- Amplify cDNA by PCR or qPCR

RT-PCR

- RNA + Reverse Transcriptase + dNTPs ➡ cDNA



- cDNA + Primers + Taq + dNTPs ➡ gene of interest

- Who is active? What genes are active?

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2007, p. 3612–3622 Vol. 73, No. 11
0099-2240/07/$08.00+0 doi:10.1128/AEM.02894-06

Diversity and Abundance of Nitrate Reductase Genes (*narG* and *napA*), Nitrite Reductase Genes (*nirS* and *nrfA*), and Their Transcripts in Estuarine Sediments[▽]

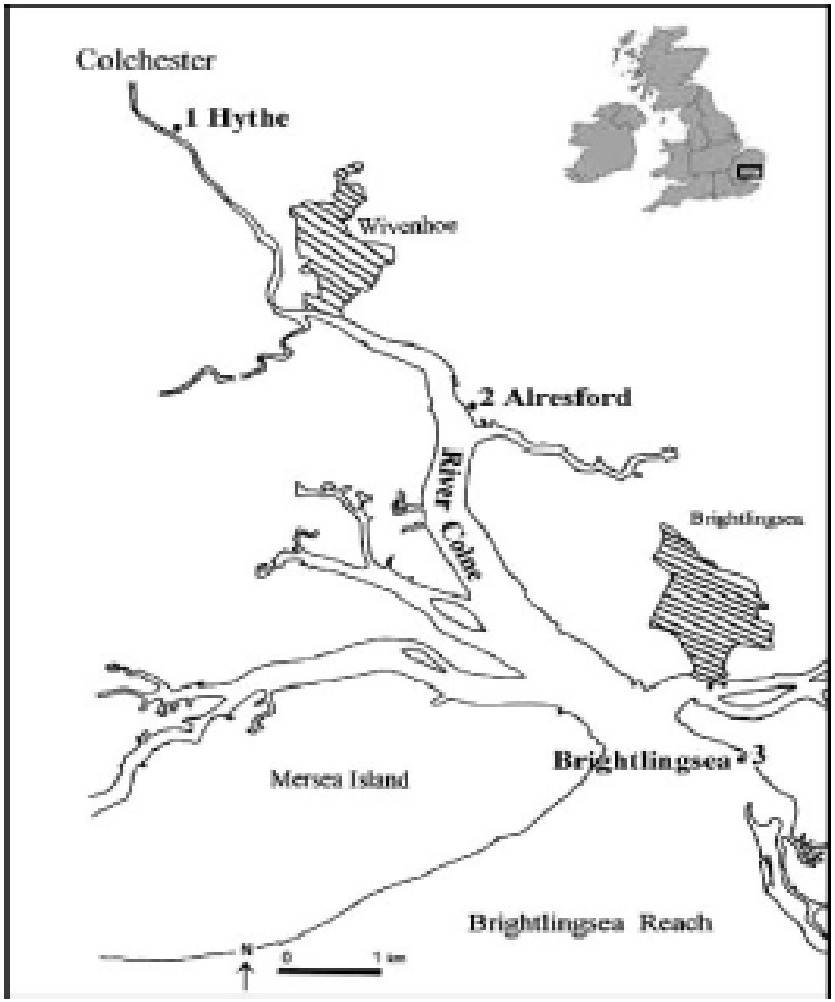
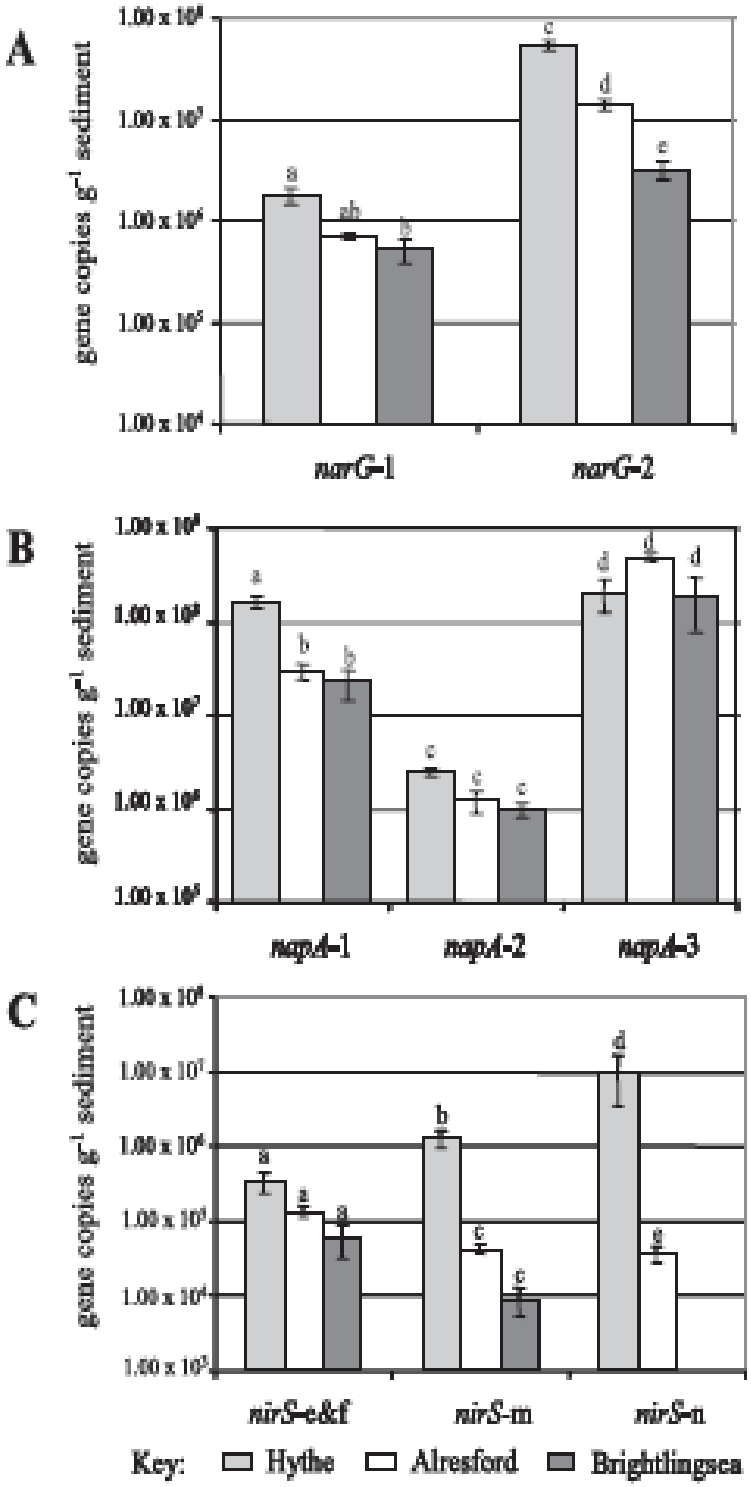


FIG. 1. Map of the Colne estuary, Essex, United Kingdom, showing the locations of the three sampling sites (Hythe, Alresford, and Brightlingsea).

TABLE 1. Primer and probe sets used for Q-(RT)-PCR

Target gene	Phylotype	Amplicon size (bp)	Primer or probe		Q-PCR cycle annealing temp (°C)
			Name[a]	Sequence (5′→3′)	
napA	*napA*-1	111	*napA*-1F	GTY ATG GAR GAA AAA TTC AA	55
			napA-1R	GAR CCG AAC ATG CCR AC	
			napA-1 (TM-MGB)	AAC ATG ACC TGG AAG	
	napA-2	76	*napA*-2F	GAA CCK AYG GGY TGT TATG	55
			napA-2R	TGC ATY TCS GCC ATR TT	
			napA-2 (TM-MGB)	CTT TGG GGT TCA A	
	napA-3	130	*napA*-3F	CCC AAT GCT CGC CAC TG	60
			napA-3R	CAT GTT KGA GCC CCA CAG	
			napA-3 (TM-MGB)	TGG GTT GTT ACG A	
narG	*narG*-1	69	*narG*-1F	GAC TTC CGC ATG TCR AC	60
			narG-1R	TTY TCG TAC CAG GTG GC	
			narG-1 (TM-MGB)	TAY TCC GAC ATC GT	
	narG-2	89	*narG*-2F	CTC GAY CTG GTG GTY GA	55
			narG-2R	TTY TCG TAC CAG GTS GC	
			narG-2 (TM-MGB)	AAC TTC CGC ATG GA	
nrfA	*nrfA*-2	67	*nrfA*-2F	CAC GAC AGC AAG ACT GCC G	60
			nrfA-2R	CCG GCA CTT TCG AGC CC	
			nrfA-2 (TM-MGB)	TTG ACC GTC GGC A	
nirS	*nirS*-e	172	*nirS*-efF	CAC CCG GAG TTC ATC GTC	60
			nirS-efR	ACC TTG TTG GAC TGG TGG G	
			nirS-ef (TM-MGB)	TGC TGG TCA ACT A	
	nirS-m	162	*nirS*-mF	GGA AAC CTG TTC GTC AAG AC	60
			nirS-mR	CSG ART CCT TGG CGA CGT	
			nirS-m (TM)	TCT GGG CCG ACG CGC CGA TGA AC	
	nirS-n	140	*nirS*-nF	AAG GAA GTC TGG ATY TC	55
			nirS-nR[b]	CGT TGA ACT TRC CGG T	
			nirS-n (TM-MGB)	ATC CGA AGA TSA	

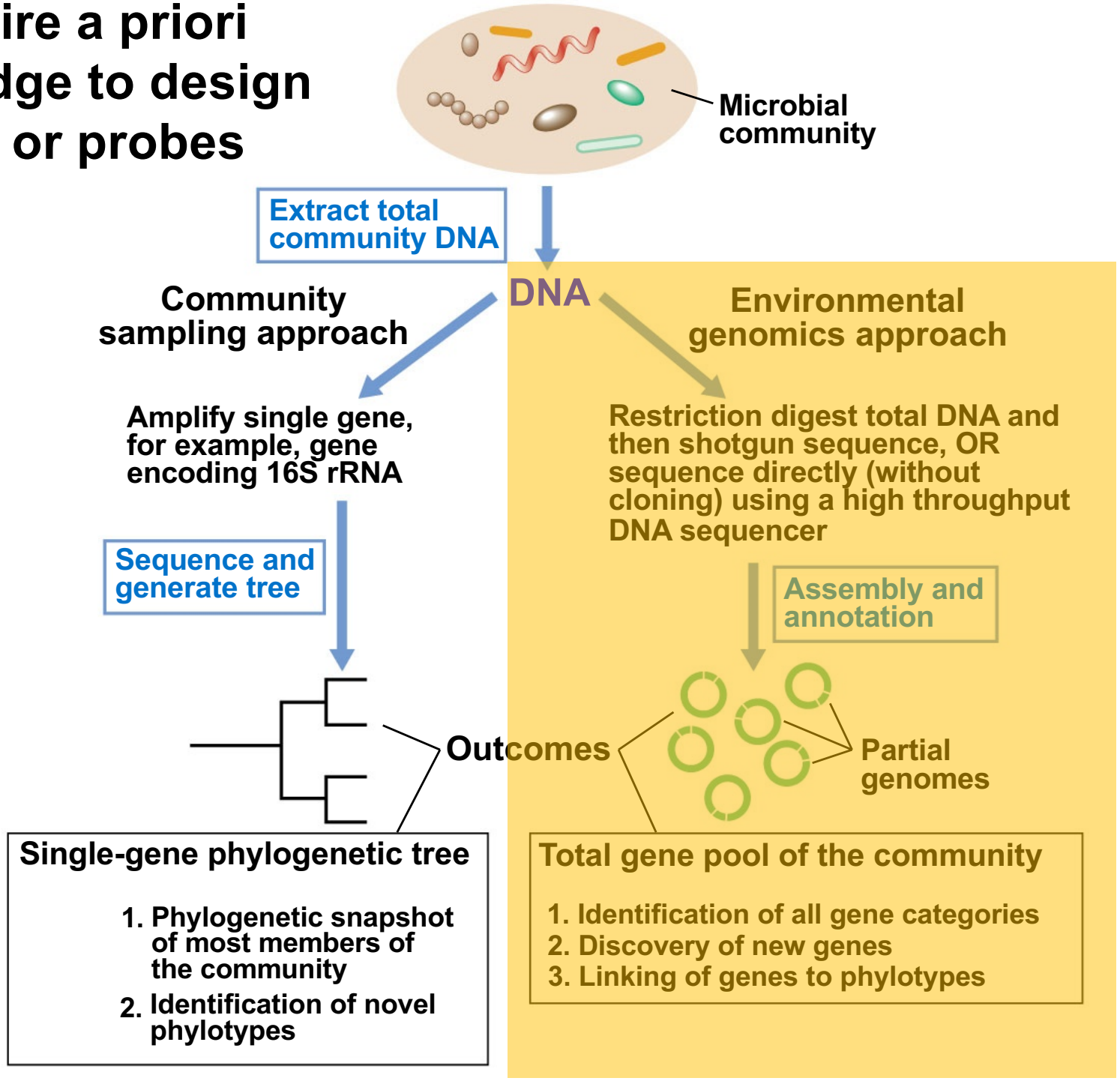
[a] For probes: TM-MGB, TaqMan minor groove binding; TM, TaqMan.
[b] Also known as nirS6r (6).



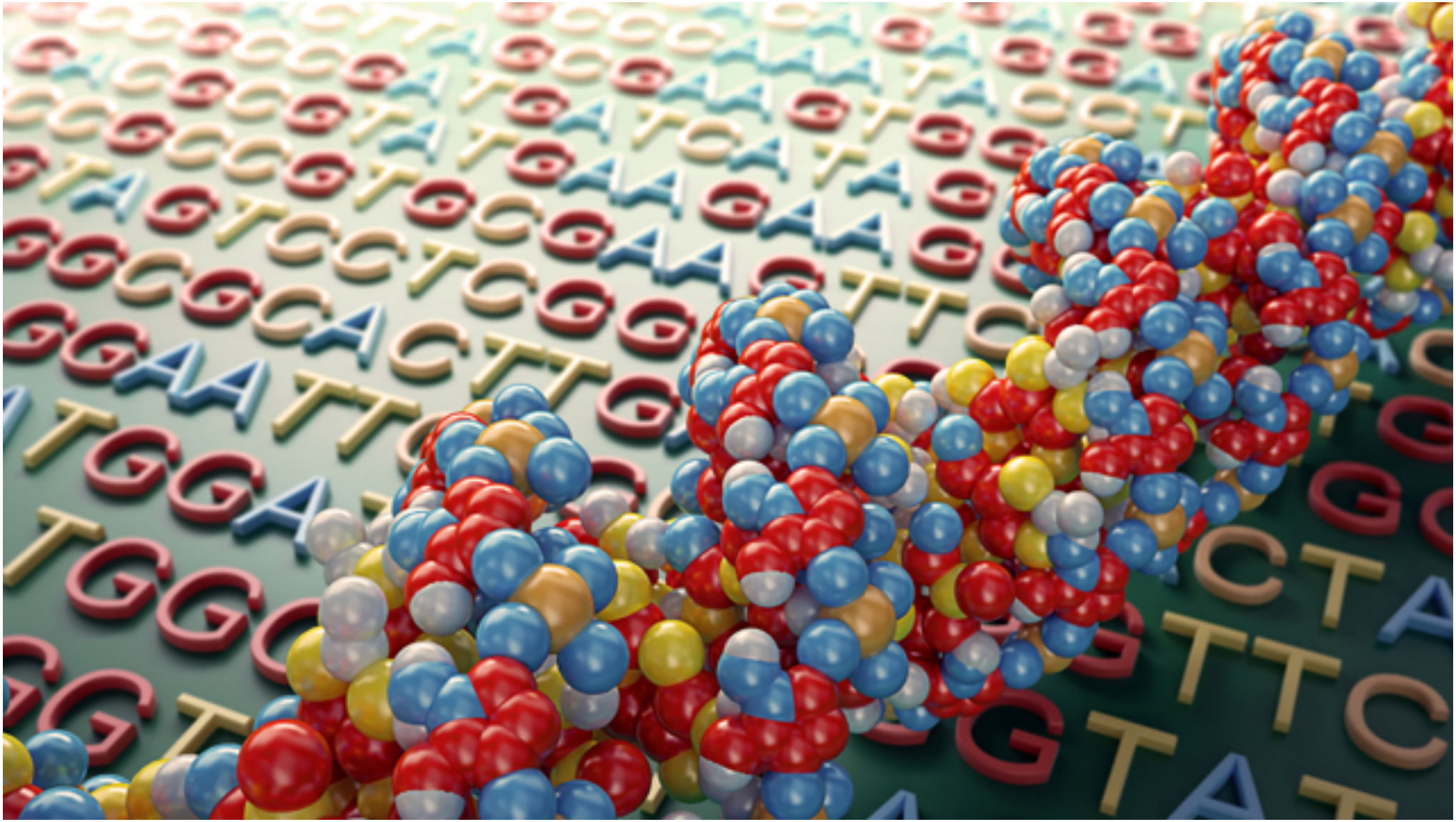
qRT-PCR

Gene copy and transcript numbers are greatest at the estuary head (Hythe), where the rates of denitrification/DNRA are highest.

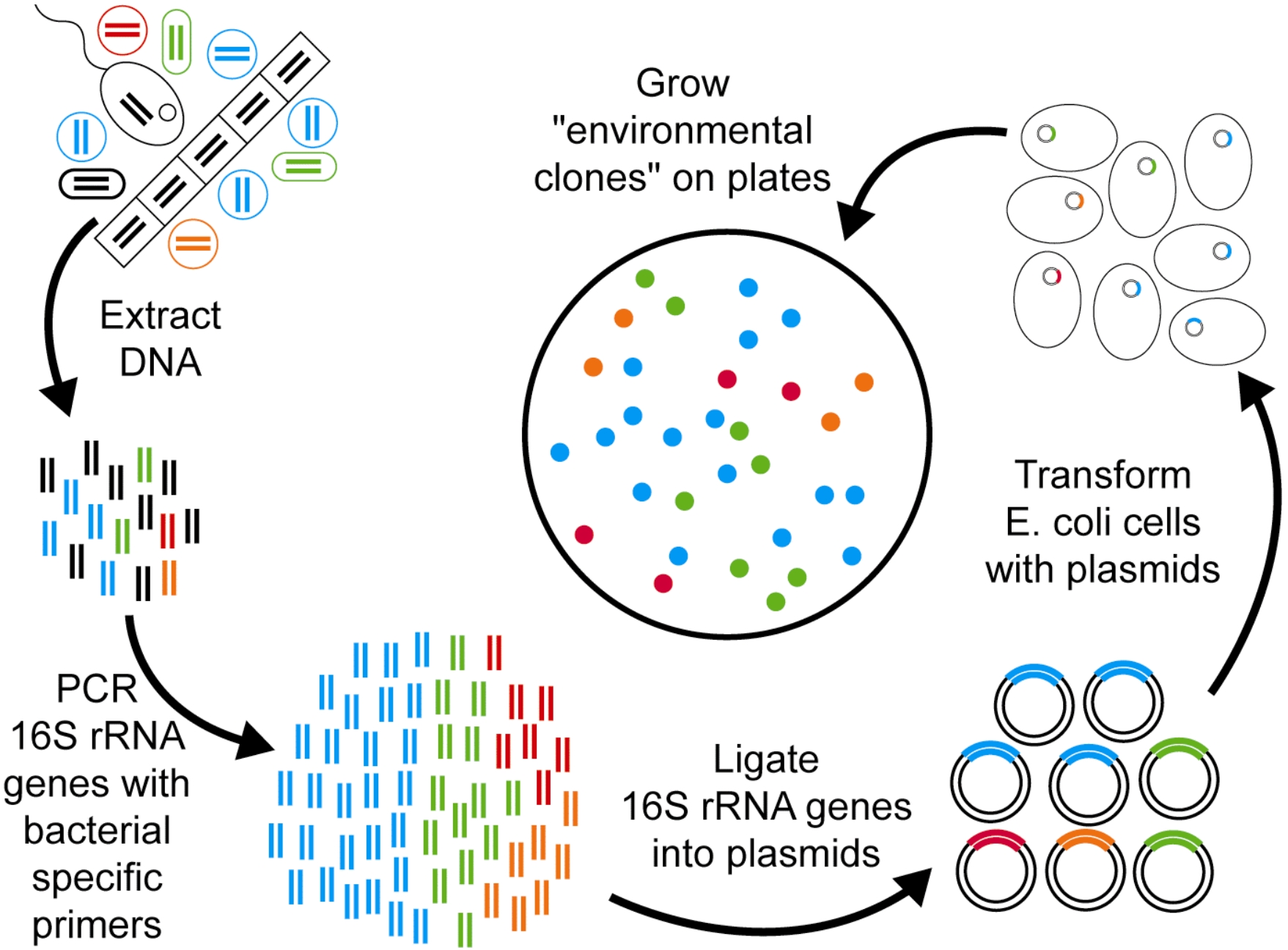
All require a priori knowledge to design primers or probes



Sequencing Revolution

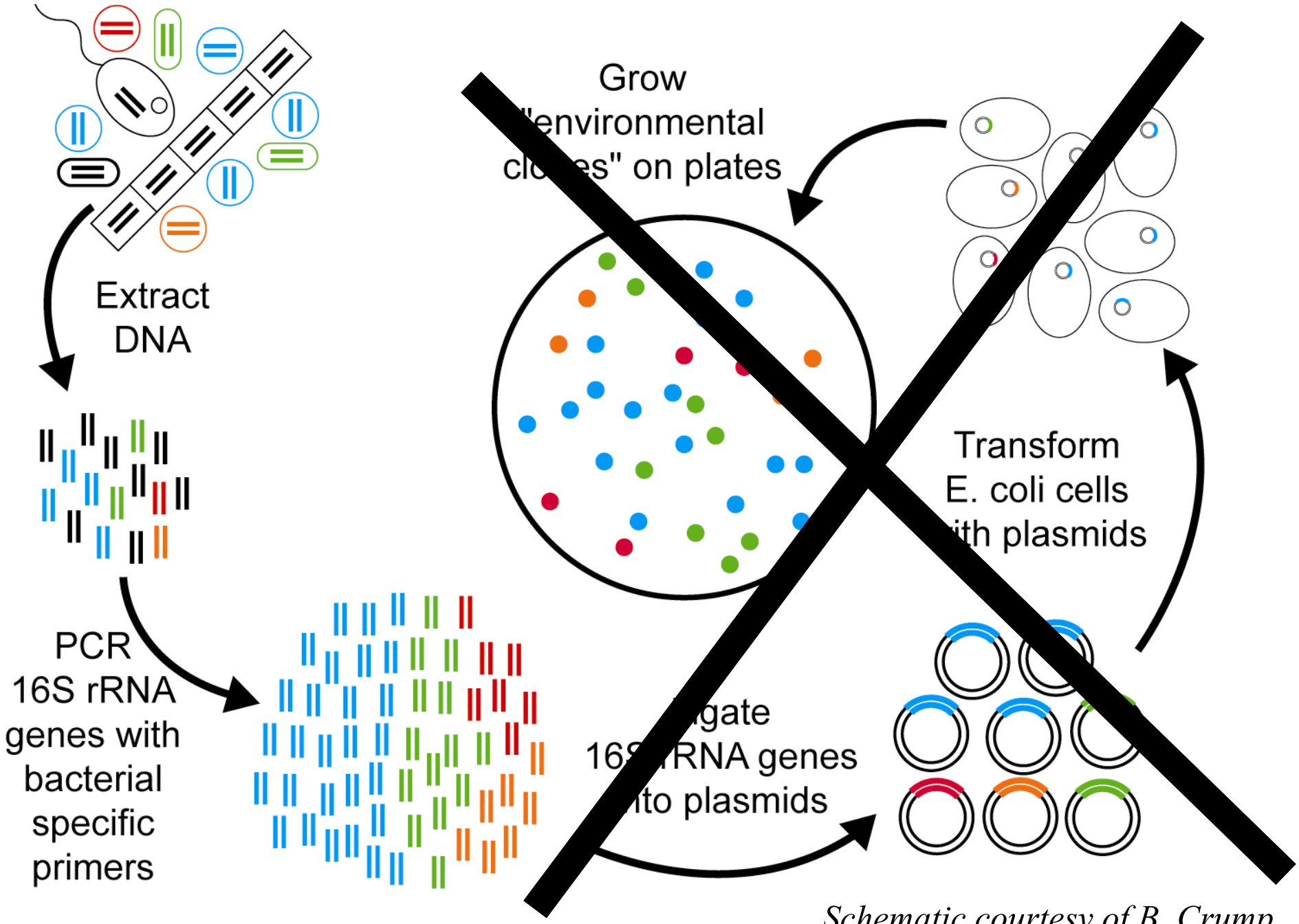


Traditional Gene Cloning

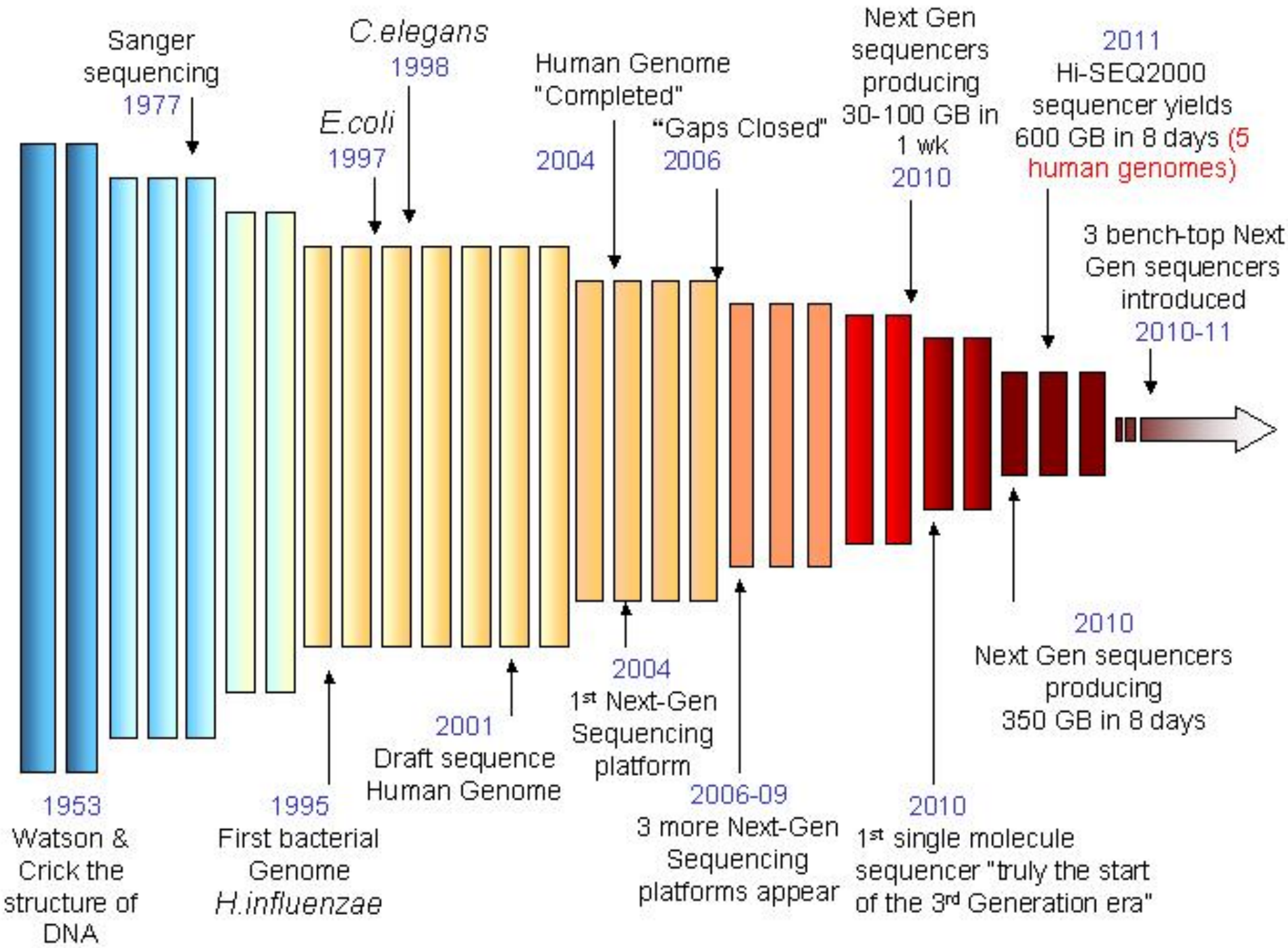


Schematic courtesy of B. Crump

NextGen Approaches



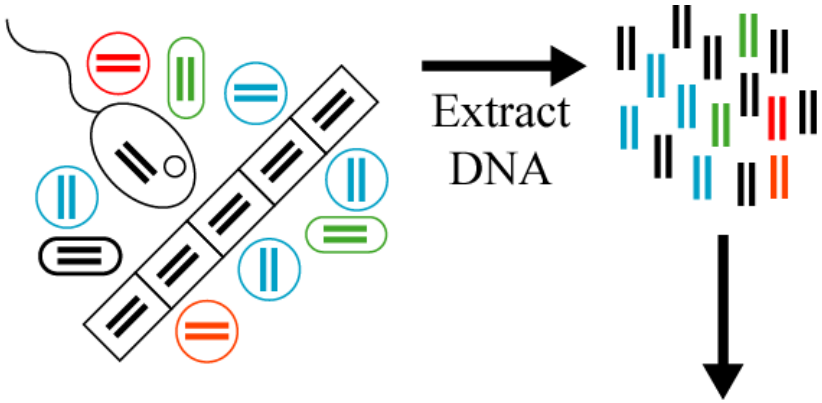
Schematic courtesy of B. Crump



http://www.ipc.nxgenomics.org

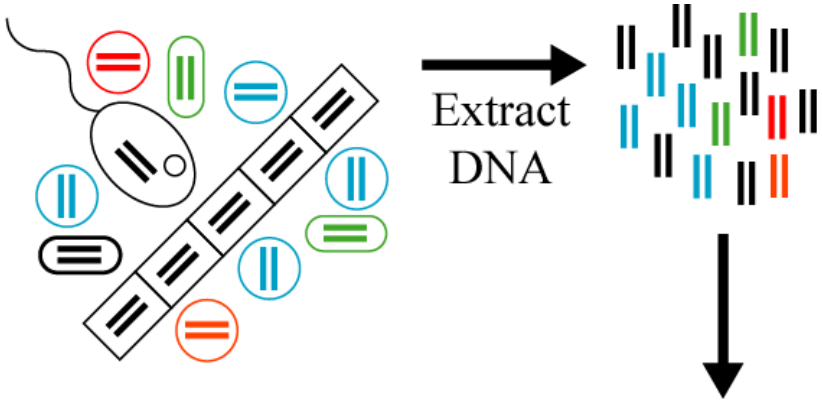
What is the difference between “standard” and “next-gen” sequencing?

Metagenomics



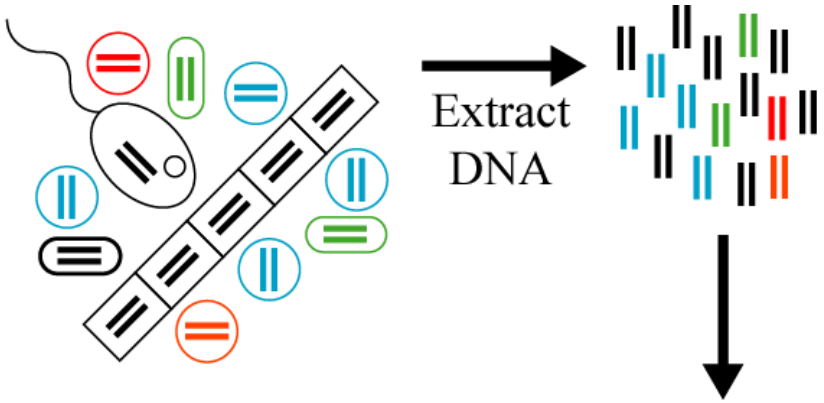
Clone (BAC, Fosmid or Small Insert)
or
Directly Sequence (Illumina, PacBio, 10X, Nanopore, etc)
Total Environmental DNA

Metagenomics



Clone (BAC, Fosmid or Small Insert) or
Directly Sequence (Illumina)
Total Environmental DNA

Metagenomics



Clone (BAC, Fosmid or Small Insert) or
Directly Sequence (Illumina)
Total Environmental DNA

Access genomes of uncultured microbes:

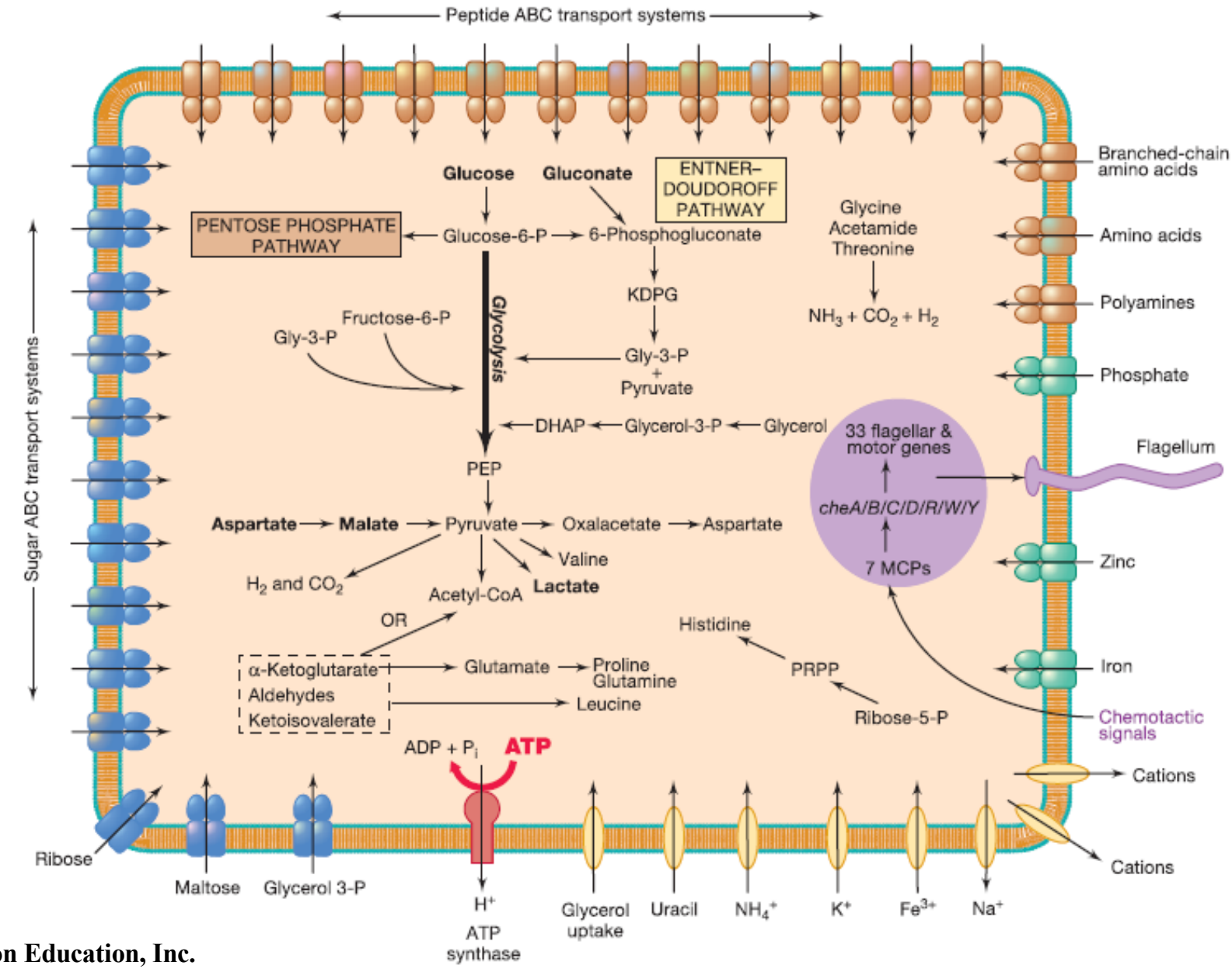
Functional Potential

Metabolic Pathways

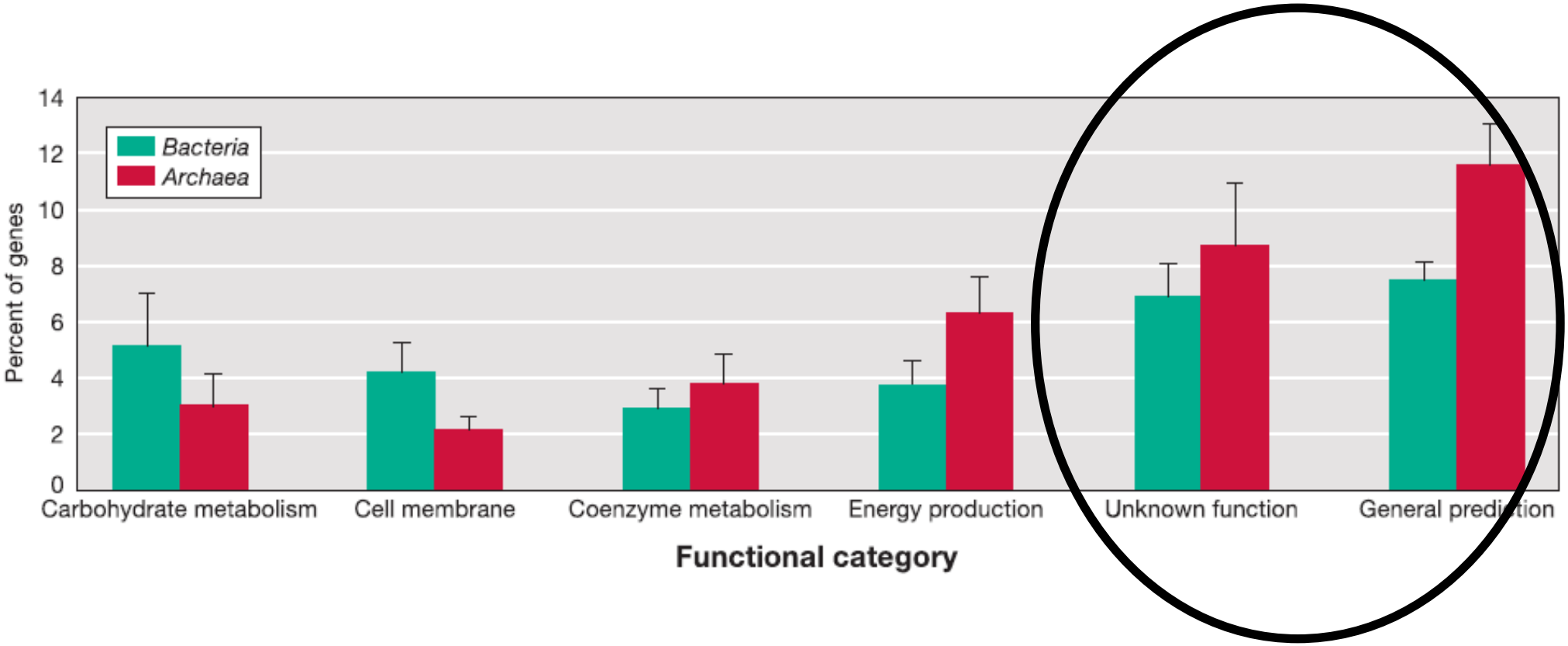
Horizontal Gene Transfer

…

Reconstruct Genomes



Categorize Functions



Proteorhodopsin phototrophy in the ocean

Oded Béjà*†, Elena N. Spudich†‡, John L. Spudich‡, Marion Leclerc*
& Edward F. DeLong*

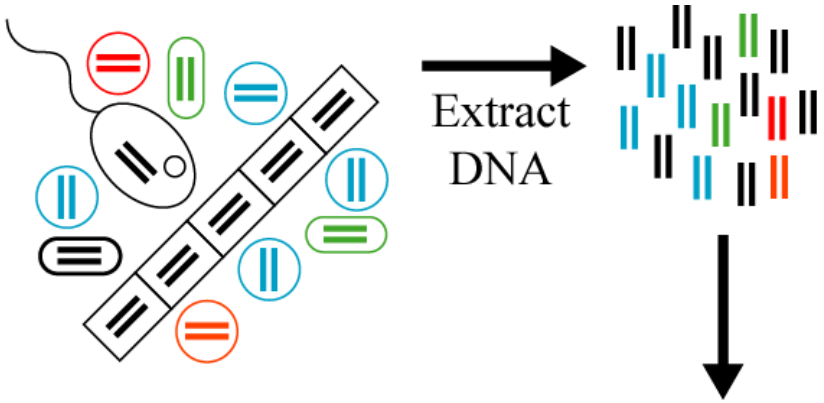
Bacterial Rhodopsin: Evidence for a New Type of Phototrophy in the Sea

Oded Béjà,[1] L. Aravind,[2] Eugene V. Koonin,[2]
Marcelino T. Suzuki,[1] Andrew Hadd,[3] Linh P. Nguyen,[3]
Stevan B. Jovanovich,[3] Christian M. Gates,[3] Robert A. Feldman,[3]
John L. Spudich,[4] Elena N. Spudich,[4] Edward F. DeLong[1]*

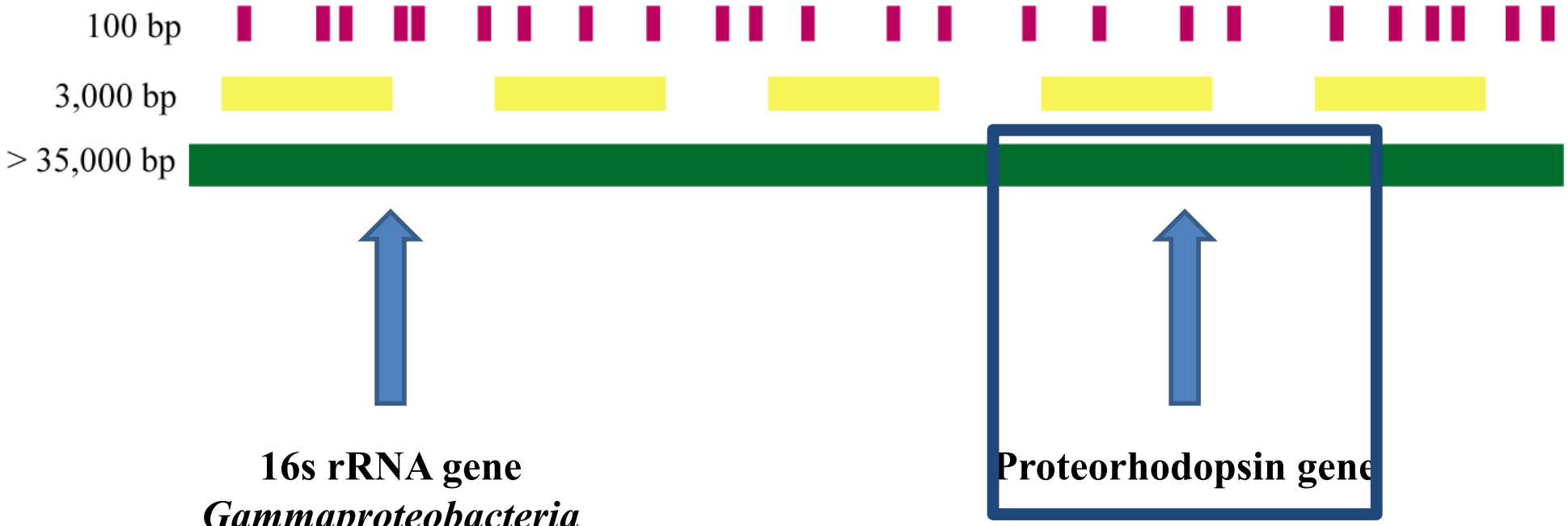
Proteorhodopsin genes are distributed among divergent marine bacterial taxa

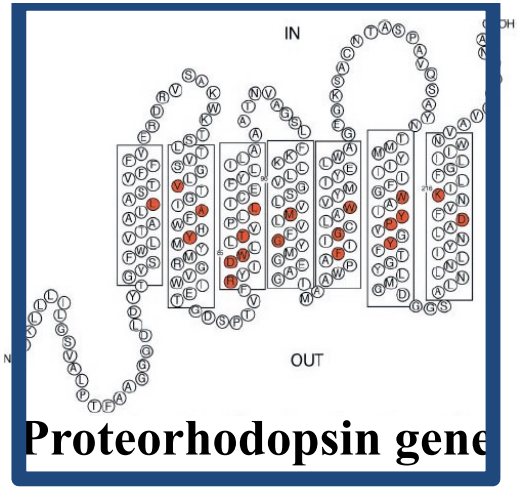
José R. de la Torre[†‡], Lynne M. Christianson[†], Oded Béjà[†§], Marcelino T. Suzuki[†¶], David M. Karl[‖], John Heidelberg[**],
and Edward F. DeLong[†,††]

Metagenomics

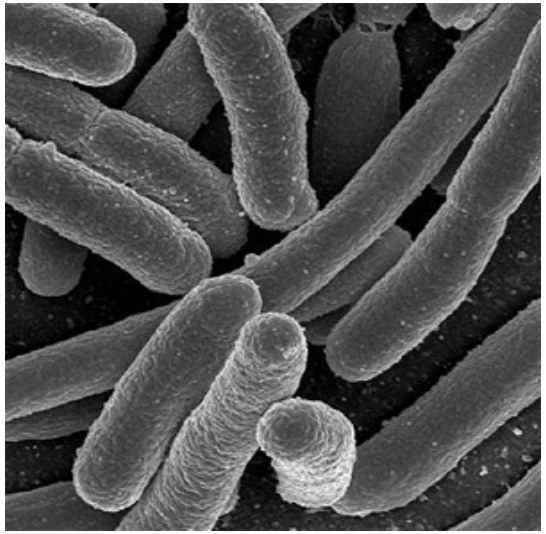


Clone (BAC, Fosmid or Small Insert) or
Directly Sequence (Illumina)
Total Environmental DNA



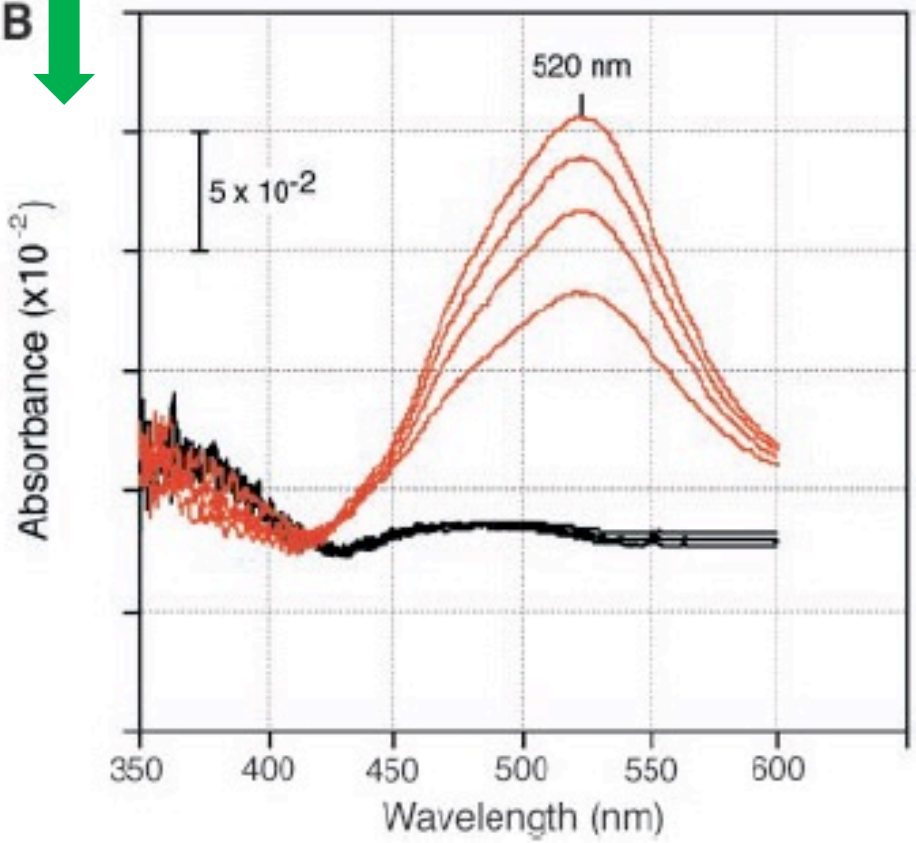


Proteorhodopsin gene

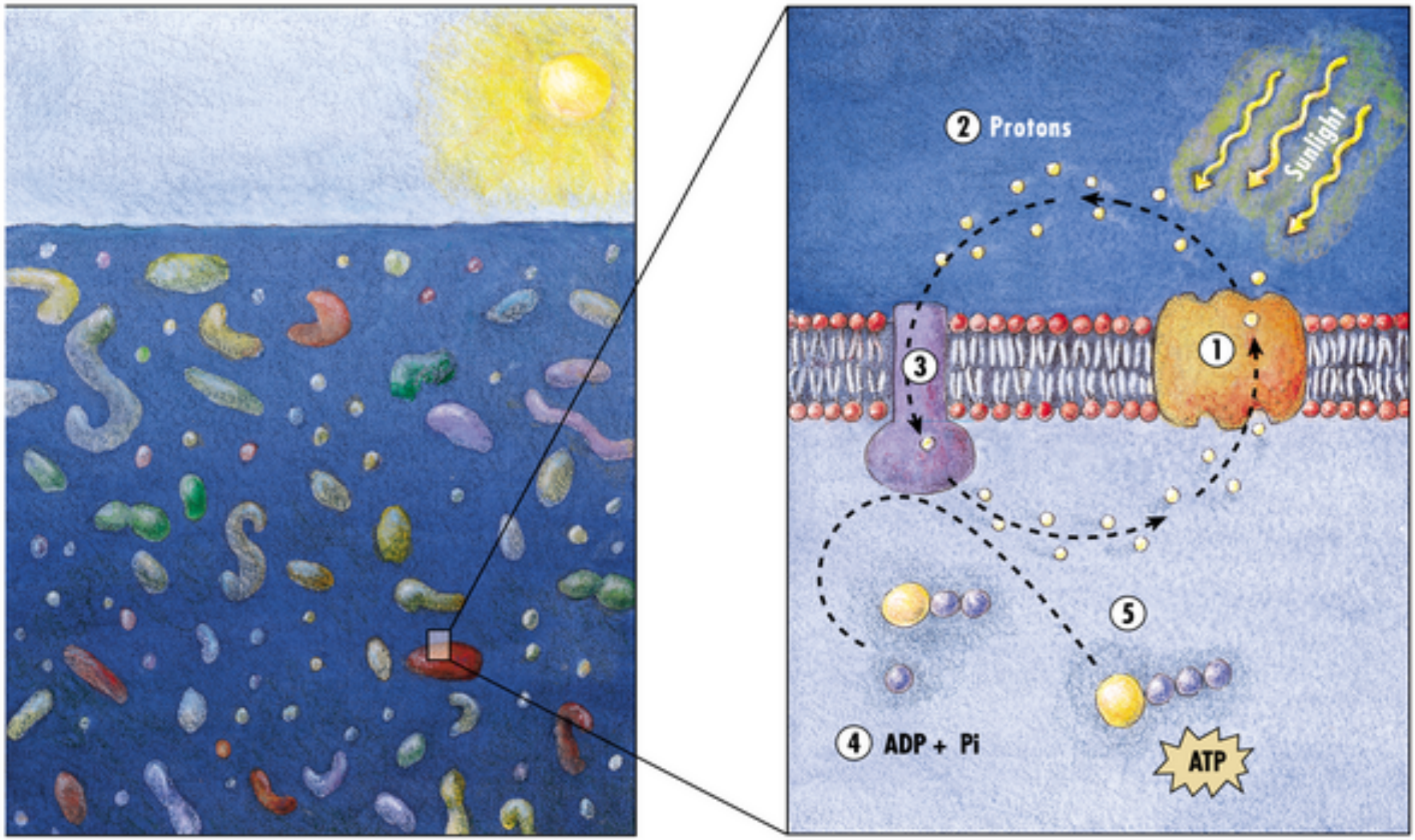


A

B



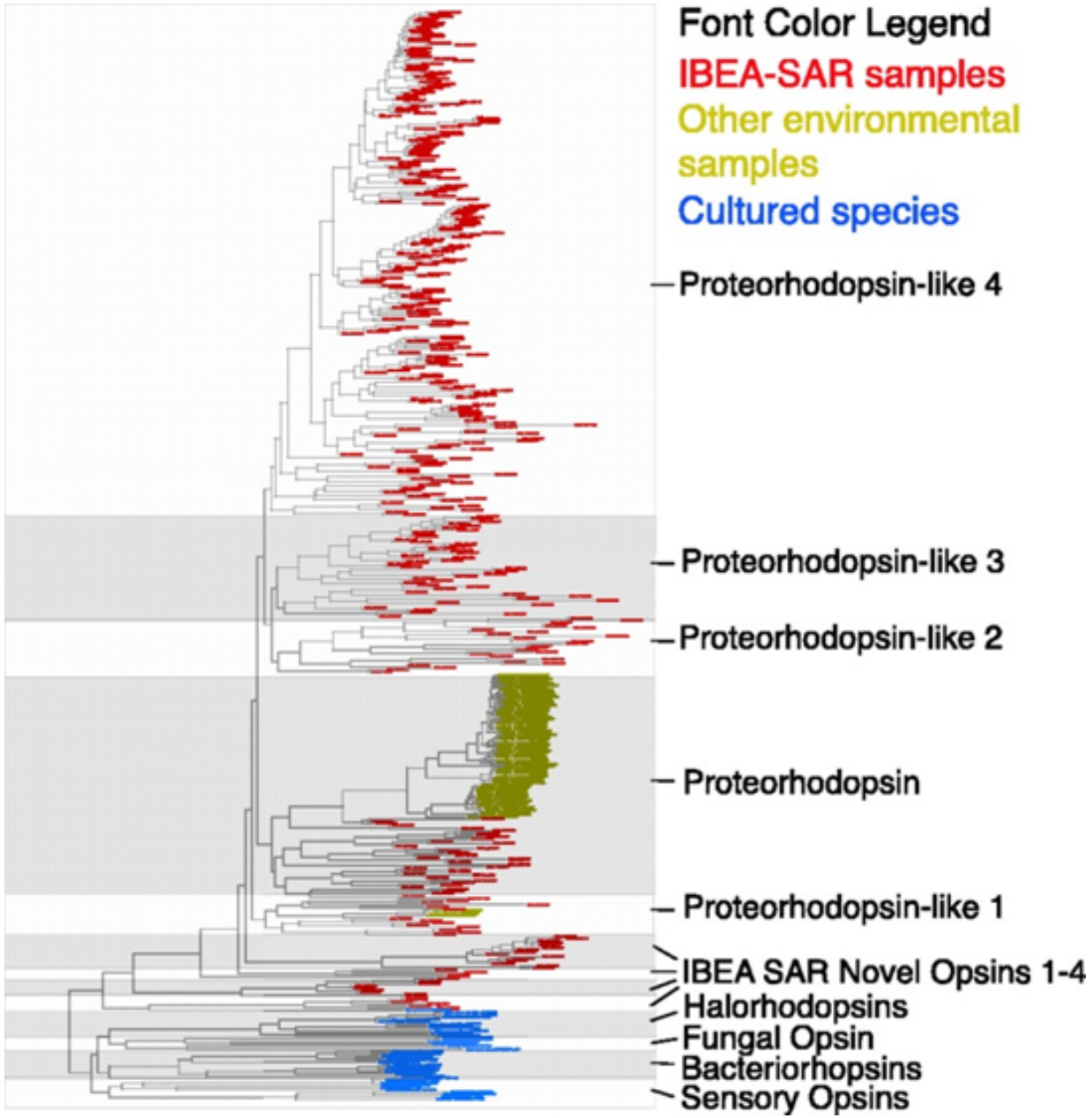
A new way of using sunlight in the surface ocean



DeLong EF, Béjà O (2010) The Light-Driven Proton Pump Proteorhodopsin Enhances Bacterial Survival during Tough Times. PLoS Biol 8(4): e1000359. doi:10.1371/journal.pbio.1000359
http://www.plosbiology.org/article/info:doi/10.1371/journal.pbio.1000359

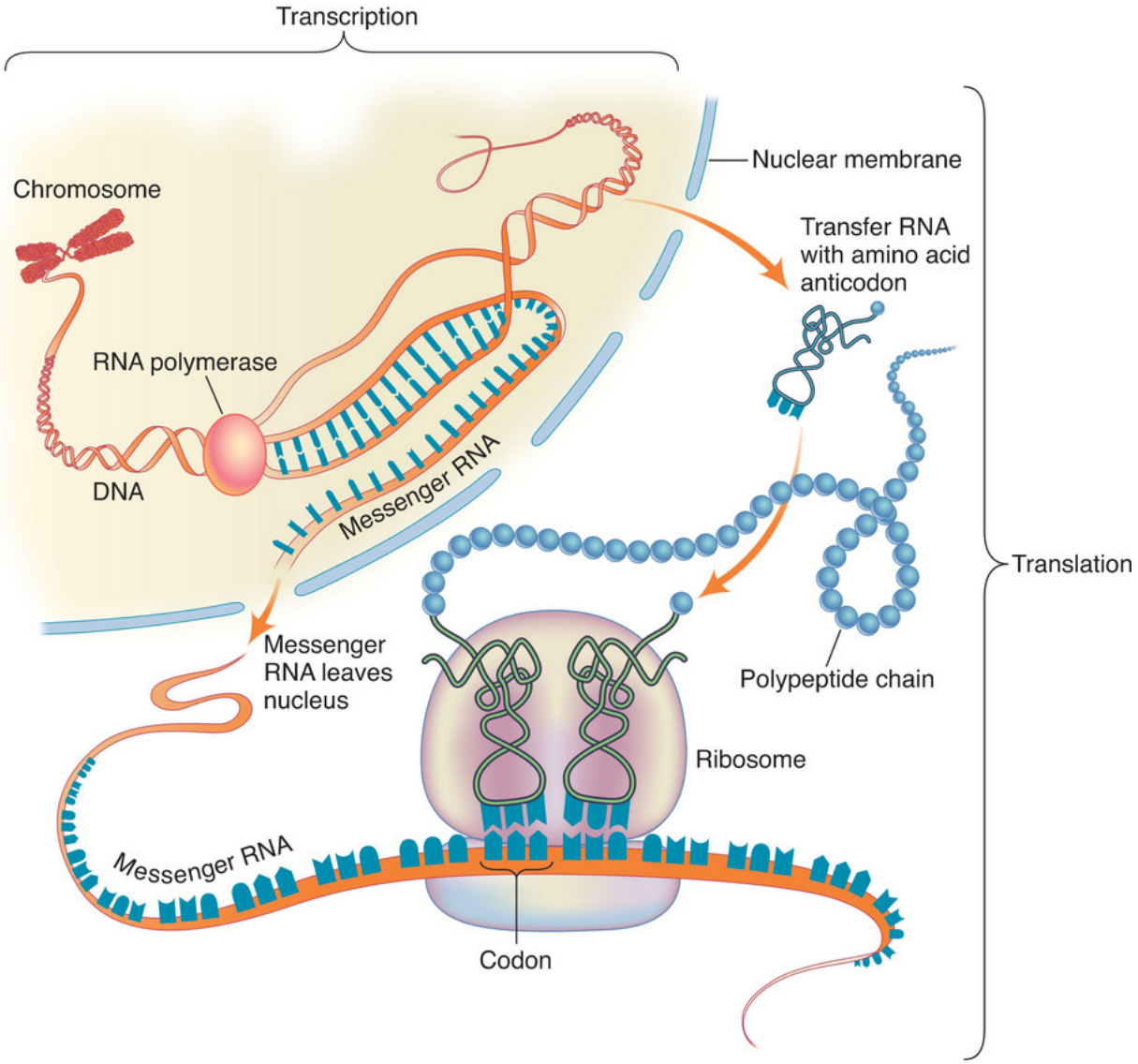
PLoS BIOLOGY

Proteorhodopsins occur in 13%-80% of marine bacteria and archaea in oceanic surface waters



Venter *et al*. 2004

The Central Dogma



DNA

Transcription

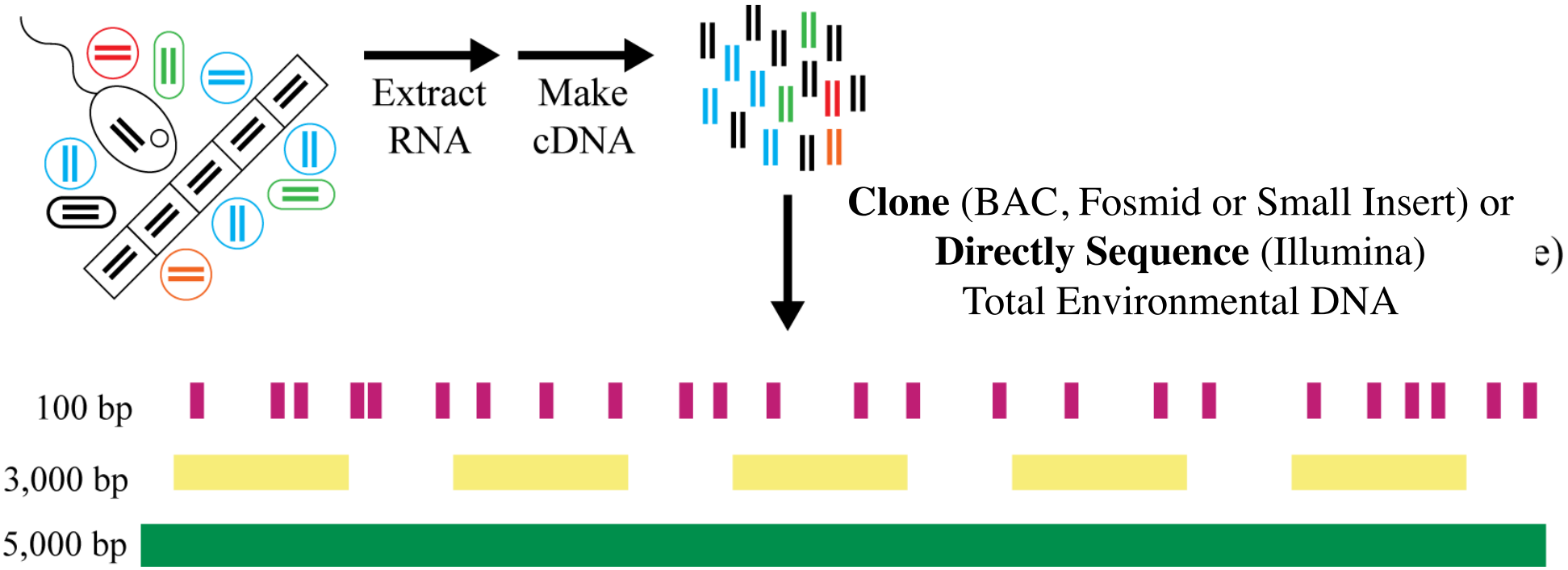
RNA

Translation

Protein

Moving from "who is there?" to "who is active?"

Metatranscriptomics



Access expressed genes of uncultured microbes
Looking at expression of defined genes via PCR
GeoChip-type analyses with RNA
Etc.

Stable Isotope Probing (SIP)

- Links specific metabolic activity to diversity using a stable isotope

- Microorganisms metabolizing stable isotope (e.g., ^{13}C) incorporate it into their DNA/RNA/Lipids

- Characterization of DNA/RNA/Lipids with ^{13}C can then be used to identify the organisms that metabolized the ^{13}C

RNA SIP

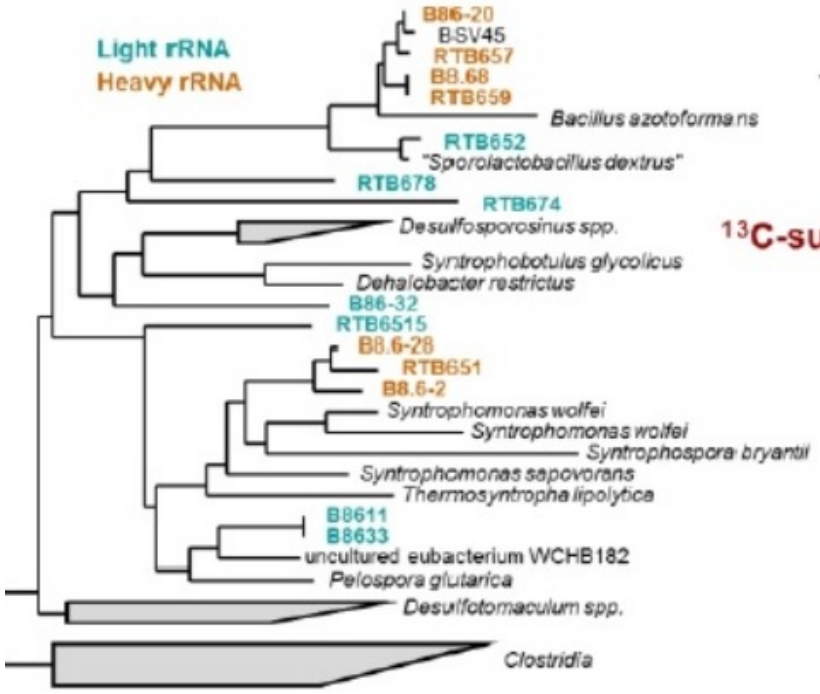
Labelling-based process query (^{13}C, ^{15}N- or ^{18}O-substrates)

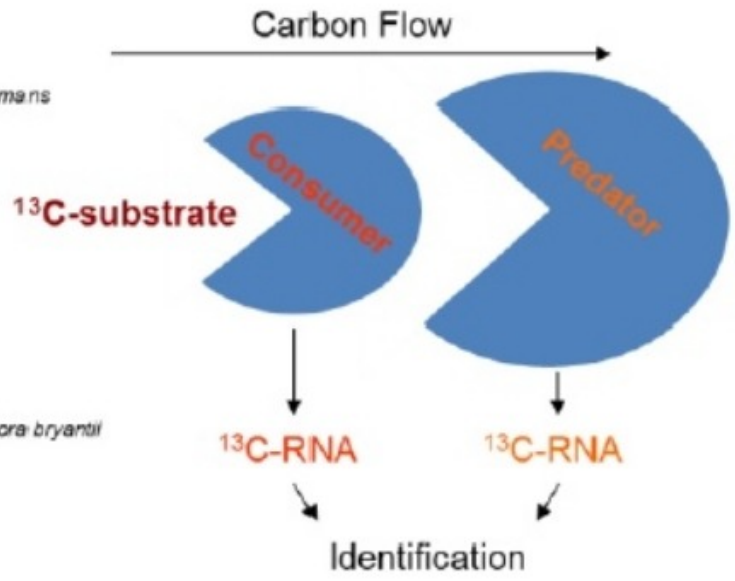
Physical RNA fractionation

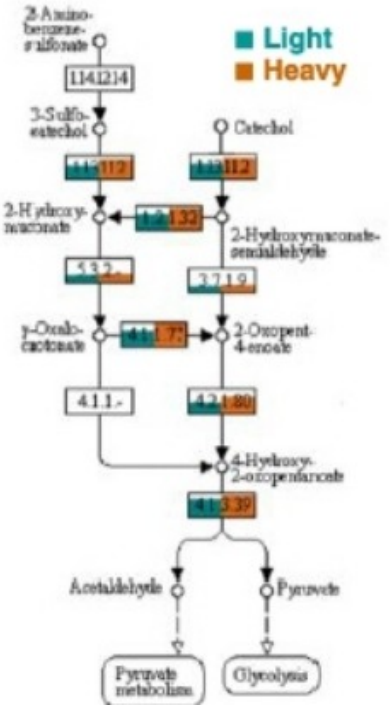
Identification of specifically active microbes

Carbon flow, interactions, biotechnology

Process-targeted transcriptomics

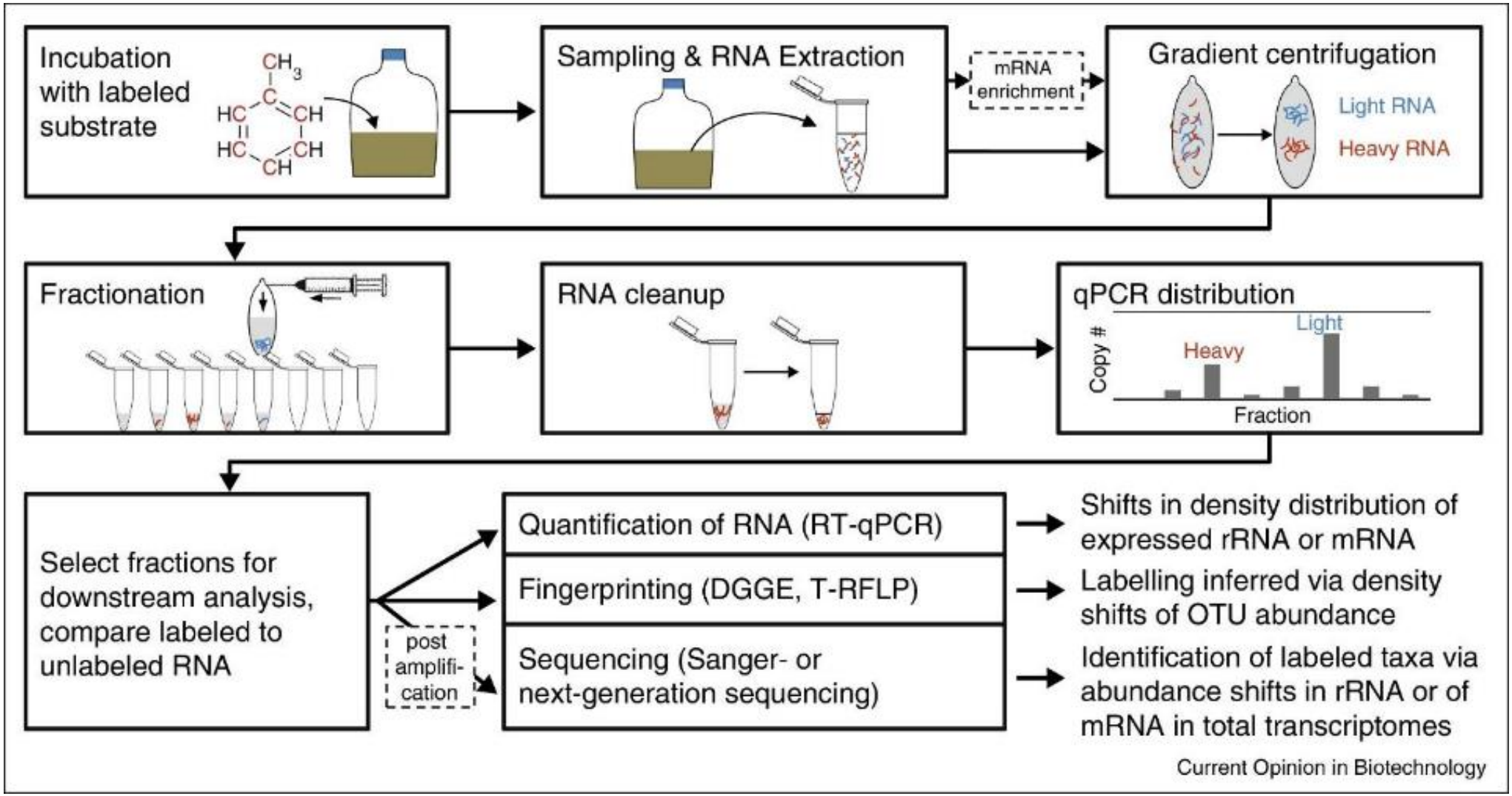






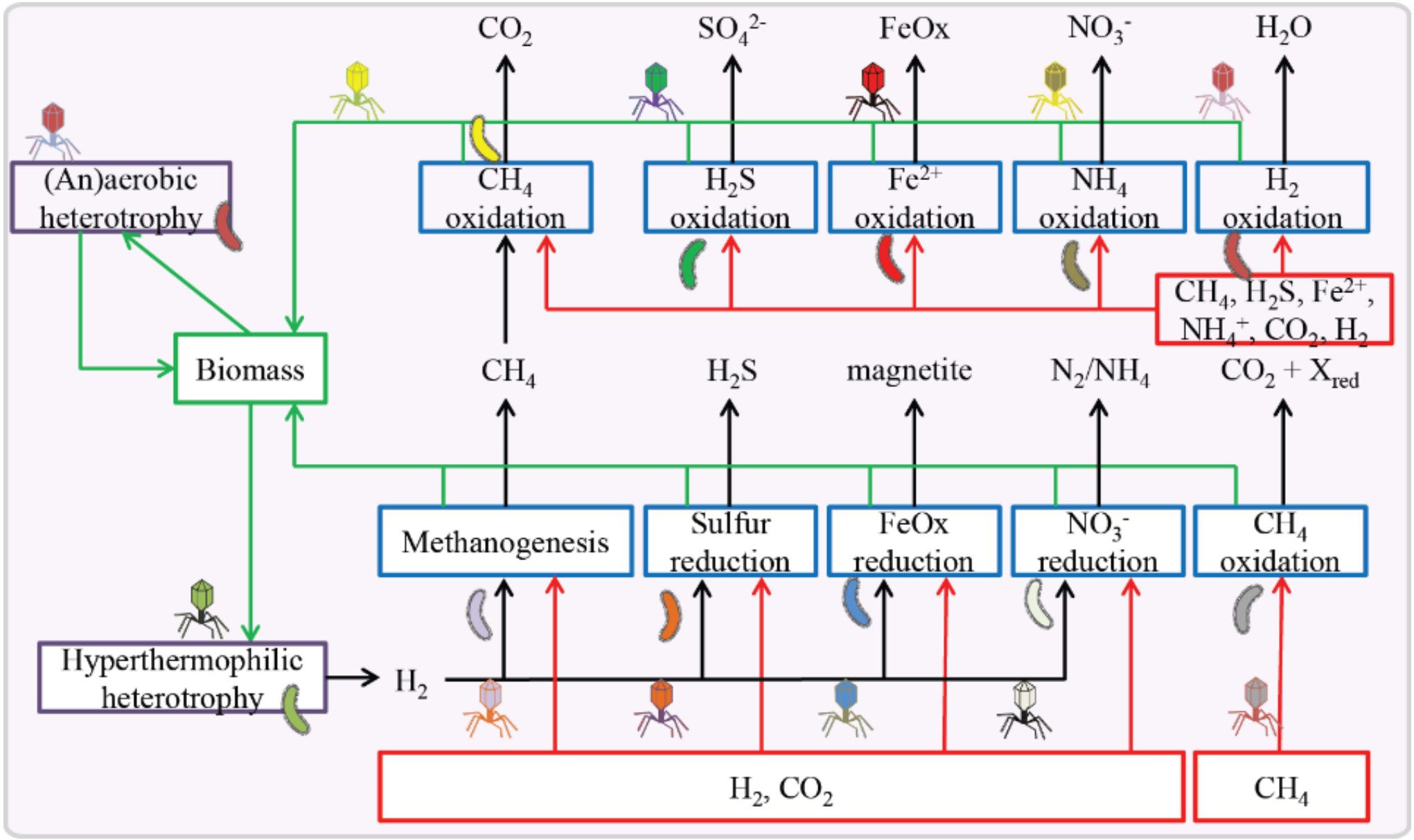
Lueders et al. 2016

RNA SIP



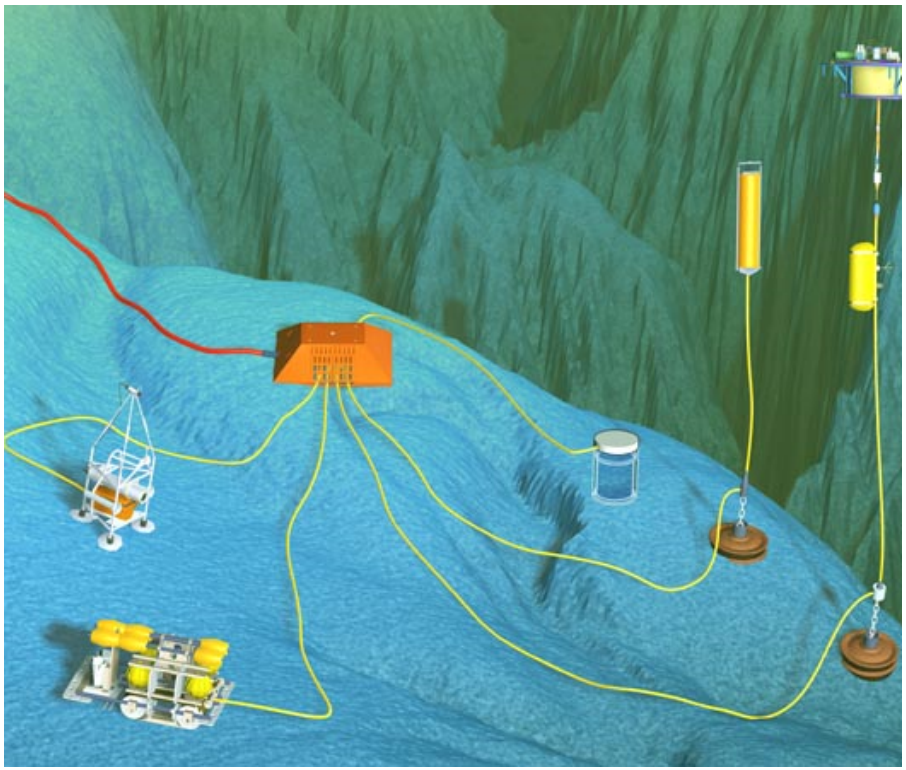
Lueders et al. 2016

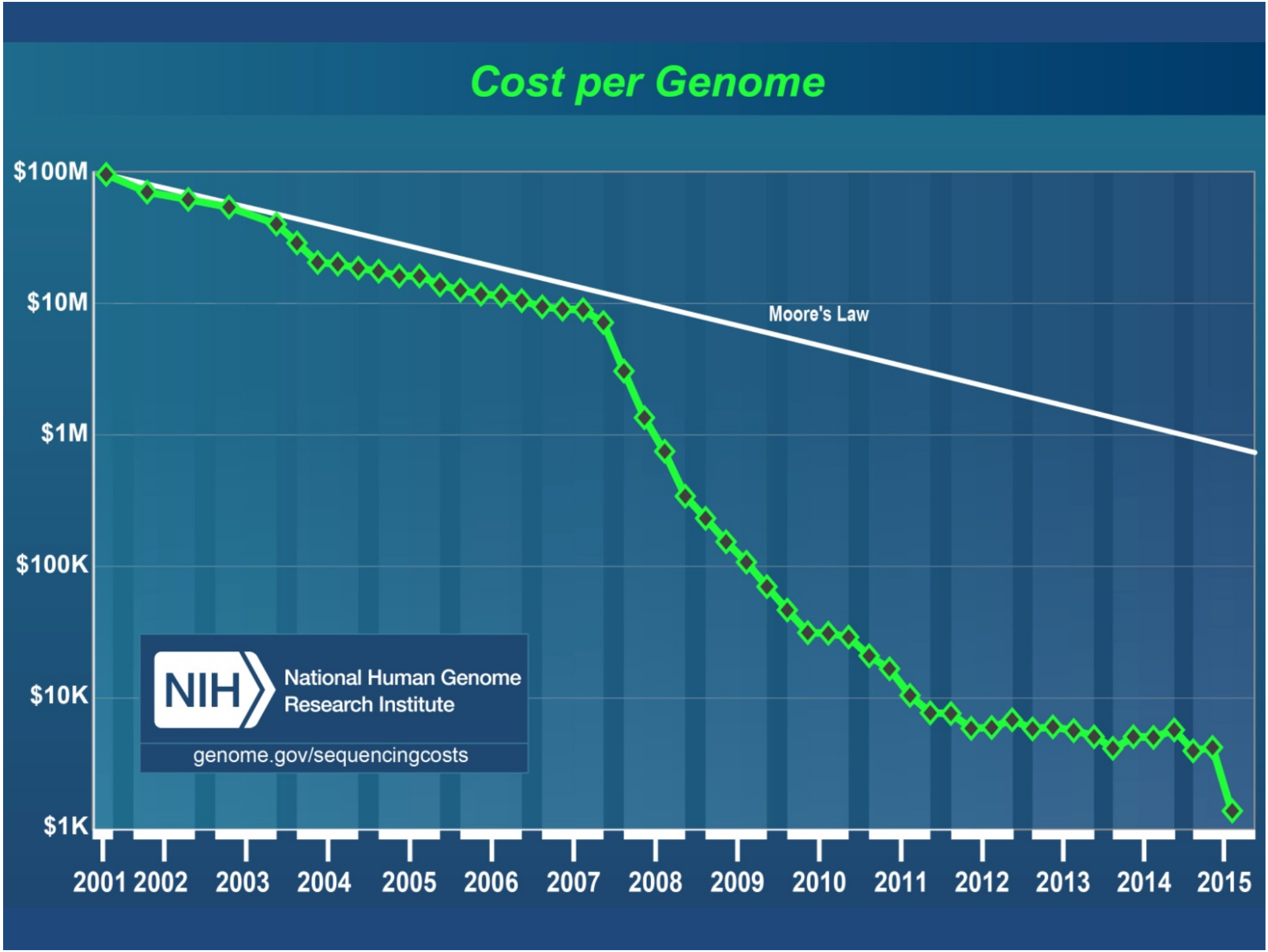
Diverse Metabolisms



And the list goes on…

- Optical tweezers
- Single cell genomics
- Meta-proteomics
- Microarrays
- Flow Cytometry
- Nano-SIMS FISH
- In-situ PCR and FISH
- …




Cost per Genome
$100M
$10M
$1M
$100K
$10K
$1K
Moore's Law
NIH
National Human Genome Research Institute
genome.gov/sequencingcosts
2001
2002
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2015