E. coli host engineering for efficient enzyme discovery

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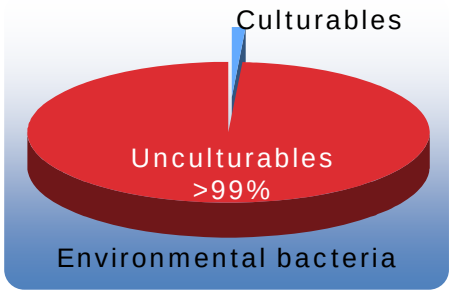
AIST

Bacteria as a source for industrial enzymes

More than 99% of microorganisms are difficult to culture

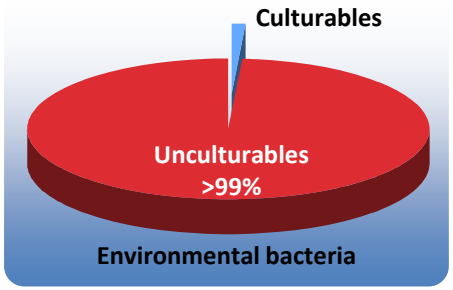


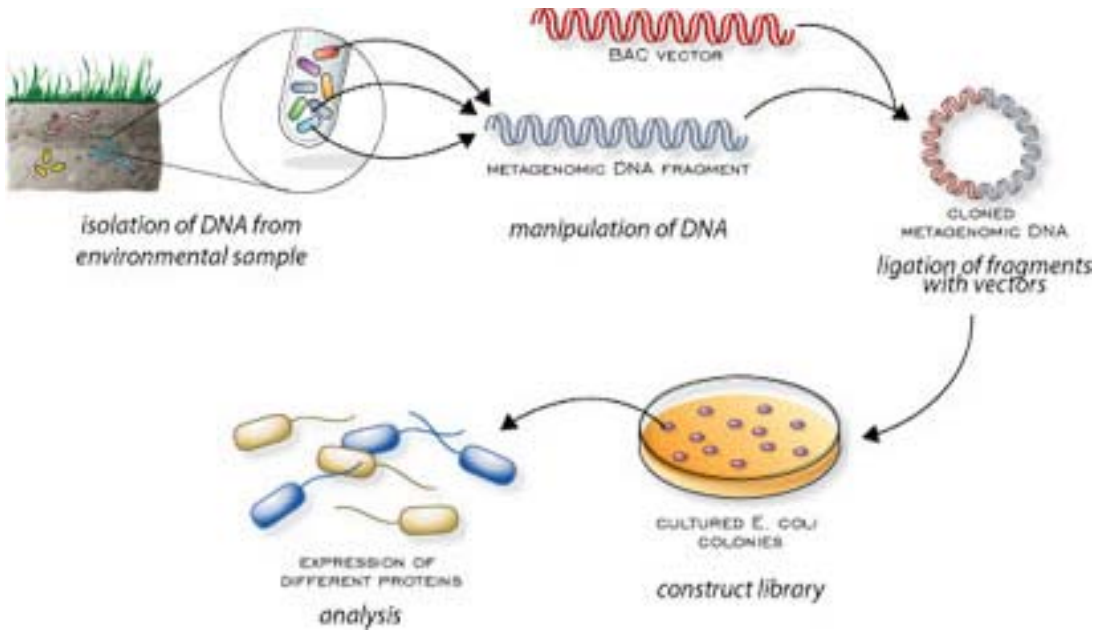
Culturability determined as a % of culturable bacteria (measured as CFU) in comparison with total cell counts

Habitat	Culturability (%)
Seawater	0.001-0.1
Freshwater	0.25
Mesotrophic lake	0.1.1
Unpolluted estuarine waters	0.1-3
Activated sludge	1-15
Sediments	0.25
Soil	0.3

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev*. 59(1):143-169.

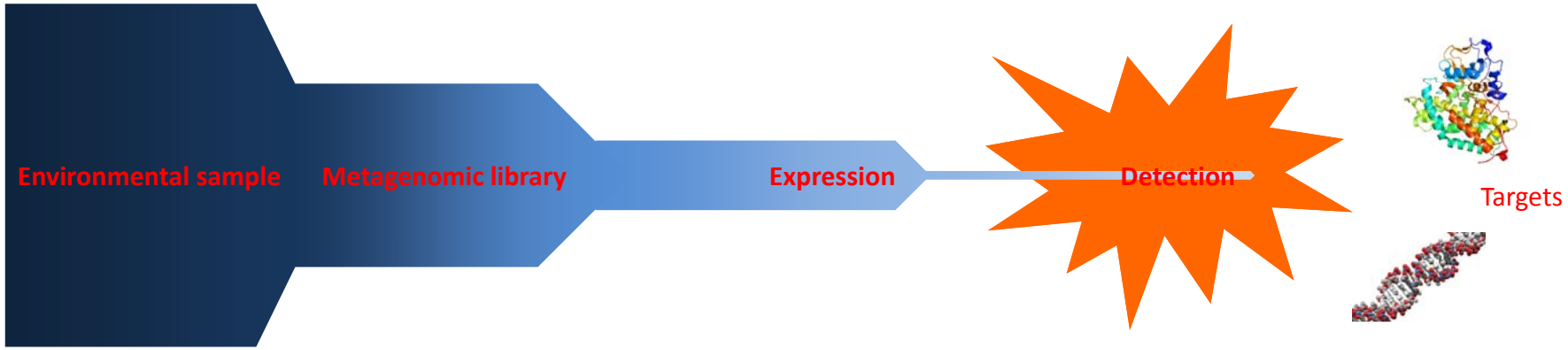
Functional metagenomics: Approach to discovering novel enzymes





- **Can avoid culturability problem.**
- **Heterologous gene expression problem.**

Bottlenecks in metagenomic library screening



- ✓ Accessibility to unique natural environments

- ✓ Extraction bias
- ✓ Yield, quality of DNA
- ✓ Cloning efficiency

- ✓ Gene expression
  - ✓Transcription
  - ✓Translation
  - ✓Protein folding
  - ✓Secretion
- ✓ Host-vector system

- ✓ Sensitivity
- ✓ Throughput
- ✓ Assay system

Low hit-rate problem

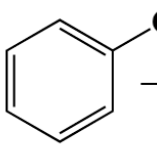
Case study: Functional screening of microbial degradation pathways for aromatic compounds

Activated sludge

Common intermediate

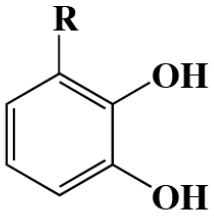
EDO

Phenol



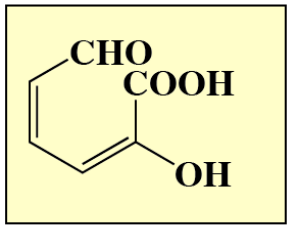
phenol hydroxylase

Catechol compound

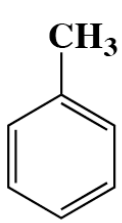


extradiol dioxygenase

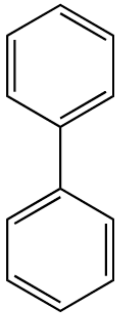
***meta*-cleavage yellow compound**



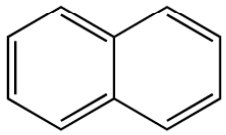
→→→ **TCA**



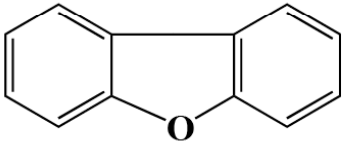
Toluene



Biphenyl



Naphthalene



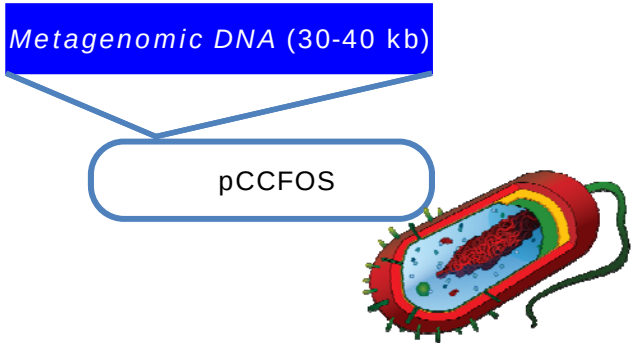
Dibenzofuran

Metagenomic library construction

Coke plant

Activated sludge treating Industrial wastewater polluted with phenolic compounds.

Metagenomic DNA



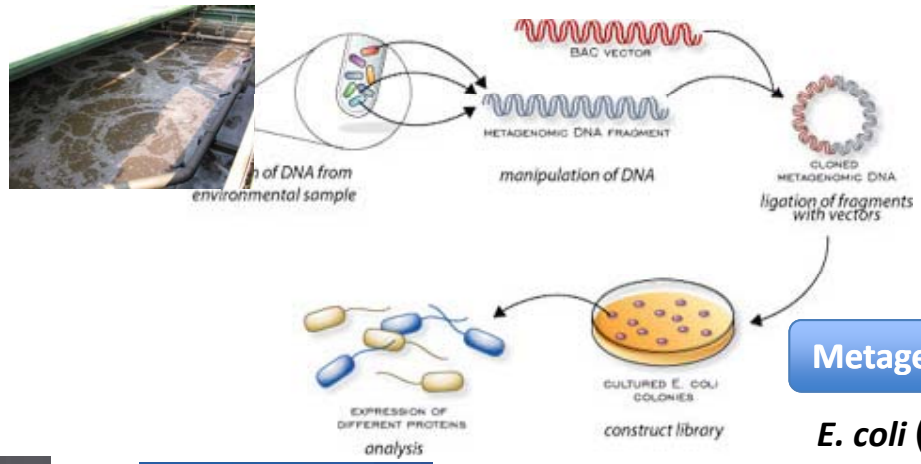
Metagenomic library

- Host, *E. coli*
- Vector, pCCFOS
- ~35 kb insert
- Total number of independent clones: ~10^5/library

HTS of metagenomic library

Environmental sample

Activated sludge treating wastewater from coke plant (rich in phenolic compounds)

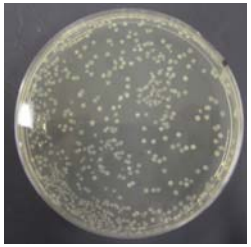


Metagenomic DNA

~35 kb DNA in a fosmid vector

Metagenomic library

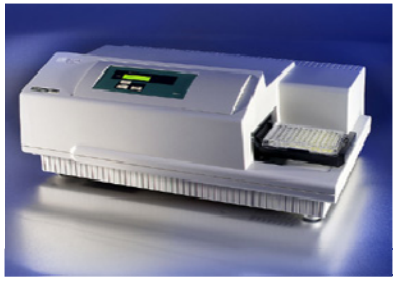
***E. coli* (host)**



Colony picker

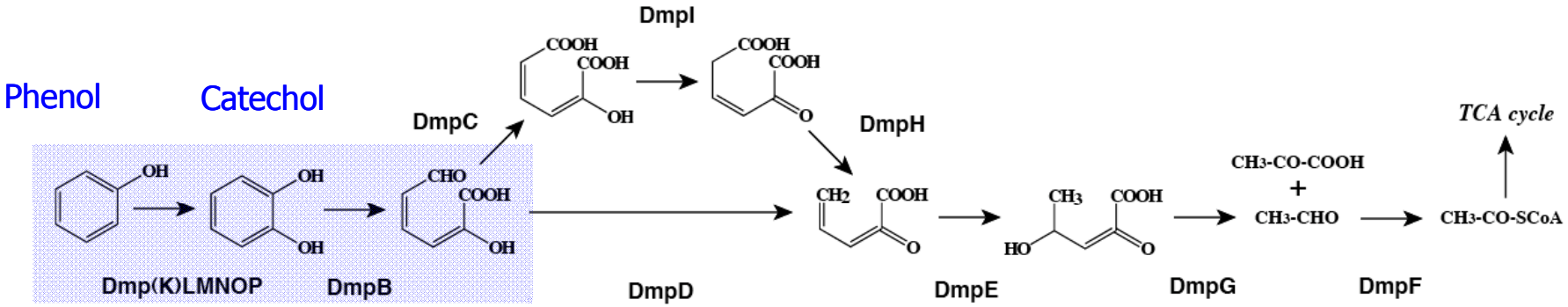
66 well plate shaker

Liquid handling machine



Microplate reader

Optimization of screening

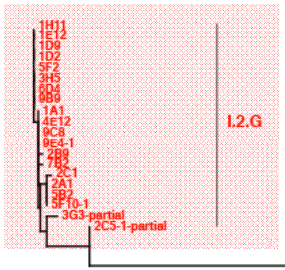


Liquid assay

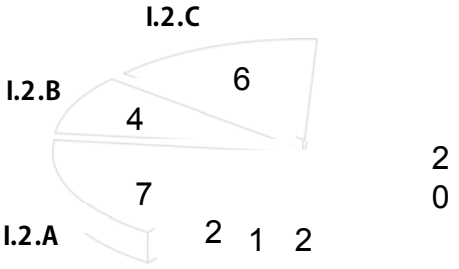
Agar-plate assay

Negative | Positive (slow) | Positive (rapid) | Positive standard

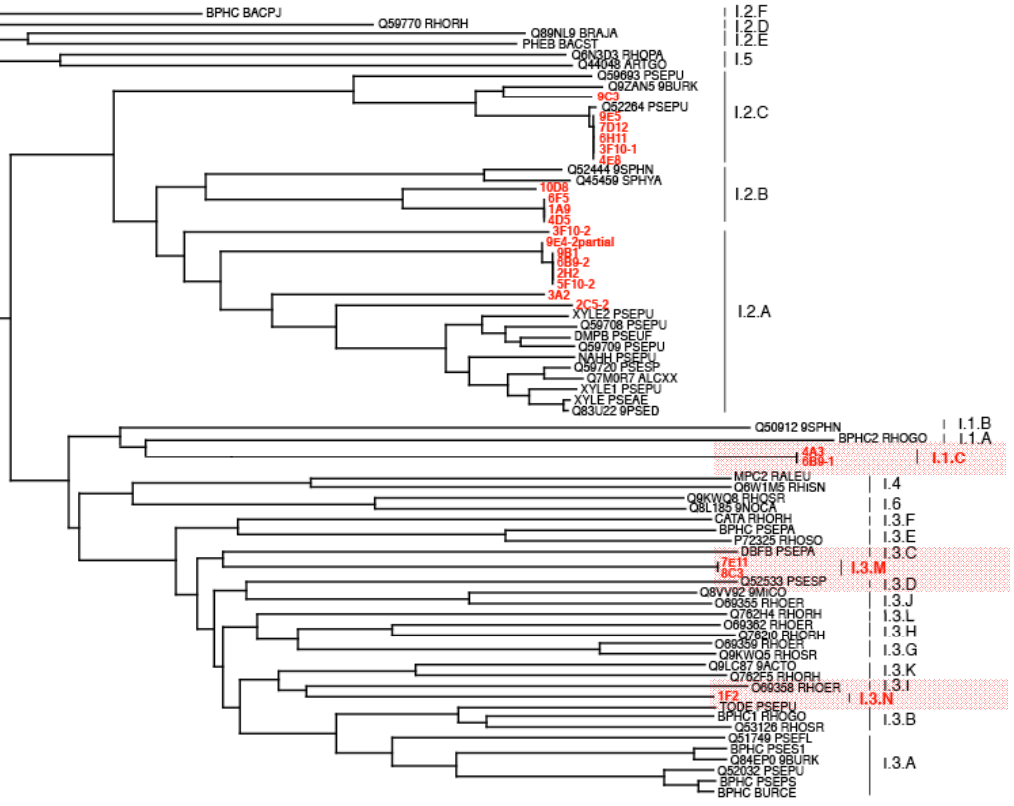
Novel EDOs retrieved from the metagenome



Total 43 EDO genes



I.2.G



- ❖ Functional metagenomics is effective for discovering novel class of enzymes.
- ❖ Proposed four new subfamilies (I.1.C, I.3.M, I.3.N, I.2.G).
- ❖ Genes belonging to new subfamily I.2.G over-represented the community.

Suenaga et al., Environ Microbiol (2007)
Suenaga et al., ISME J (2009)

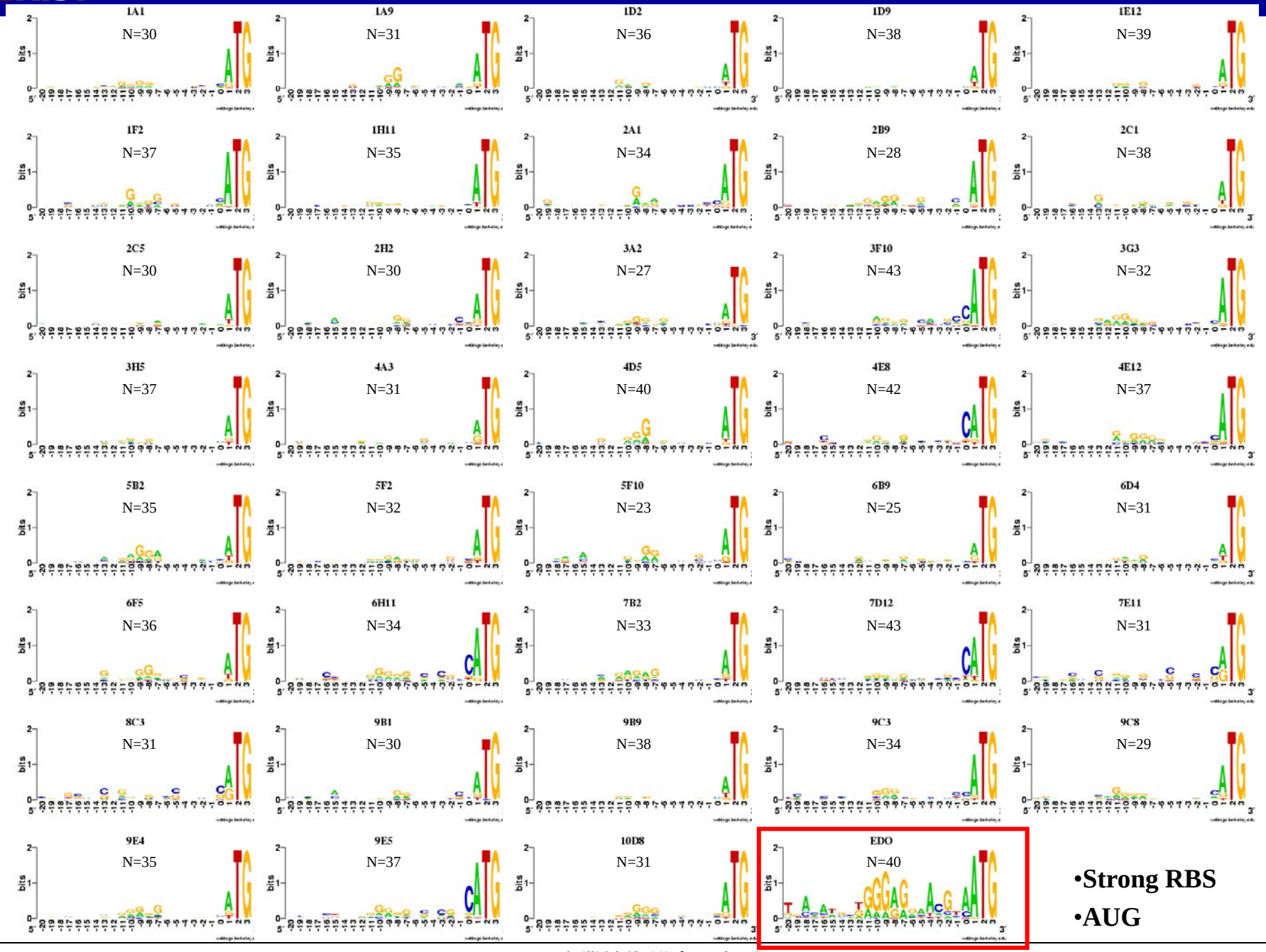
Sequence analysis of positive clones

Analysis of 43 fosmid's insert (~35 kb, ~30 ORFs)

- ✓ **EDO (target)**
- ✓ **Others (non-target)**

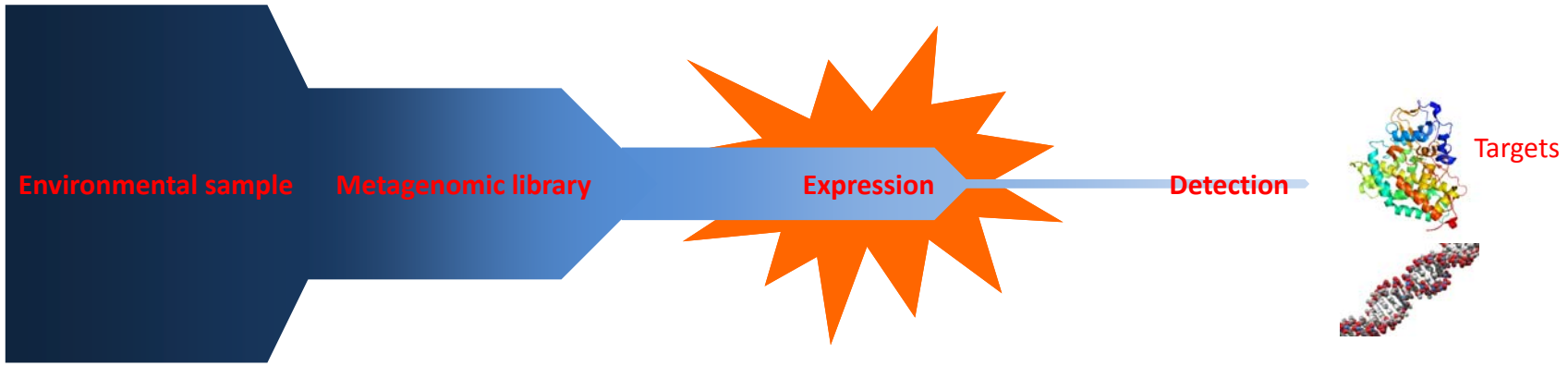
5'-UTR

- **Ribosome binding site (RBS).**
- **Initiation codon.**



- **Strong RBS**
- **AUG**

Expression problems in functional screening of metagenomic libraries



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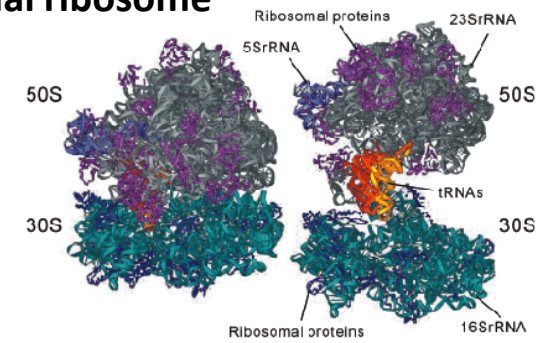
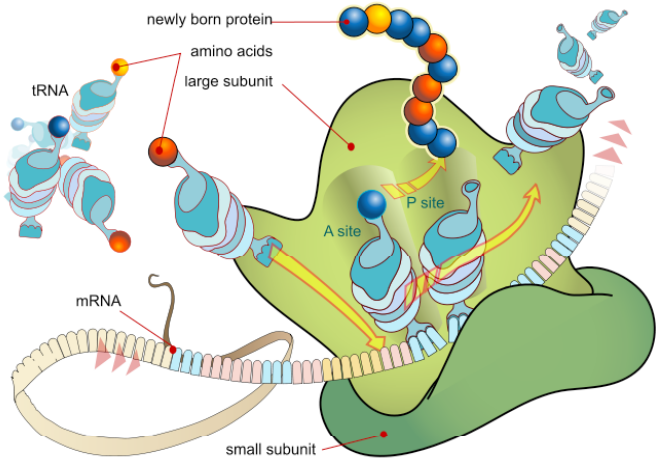
- Uchiyama & Miyazaki (2008) Functional metagenomics for enzyme discovery: challenges to efficient screening. *Curr. Opi. Biotechnol*. 20(6):616-22
- Hosokawa-Okamoto R, Miyazaki K (2011) *Escherichia coli* host engineering for efficient metagenomic enzyme discovery. *in* "Metagenomics: Current Innovations and Future Trends" (ed. Diana Marco, pp. 241-252, Horizon Scientific Press)

Translational machinery (Ribosome) engineering approach to solving heterologous gene expression problems

Bacterial ribosome





Factors affecting translational efficiency

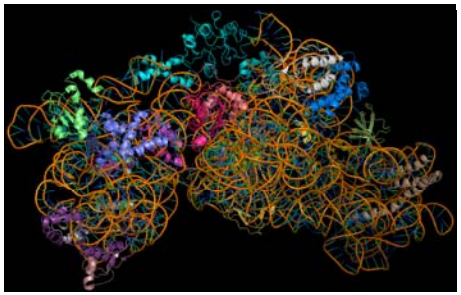
- **Ribosome binding site (RBS)**
- **Initiation codon**
- **Secondary structure of mRNA**
- **Rare codons**

and more

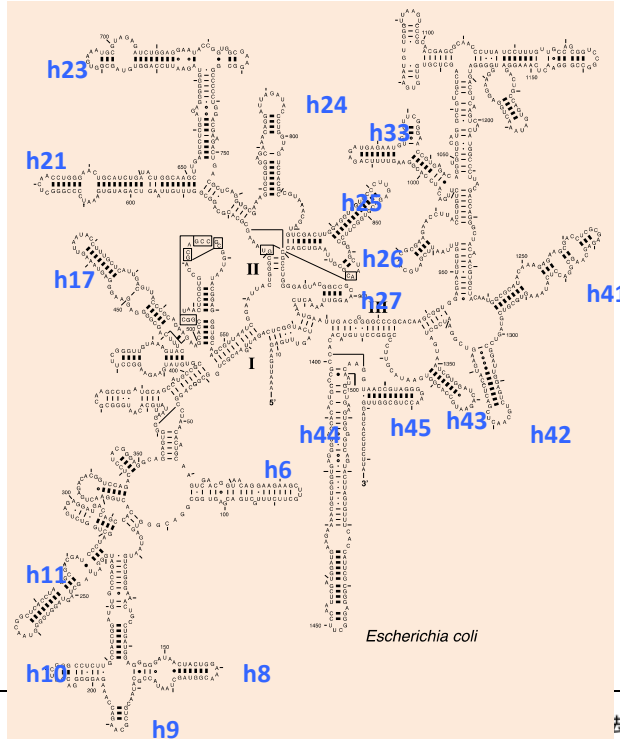
- ✓ **3D structure available, but the structure is extremely complex (3 ribosomal RNA plus >50 proteins).**
- ✓ **Functions of each components are not well understood.**
- ➢ **Target rRNA to induce perturbation into the whole ribosome molecule.**
- ➢ **Relax the specificity of *E. coli* ribosome to recognize mRNA's expression signal of diverse bacteria.**

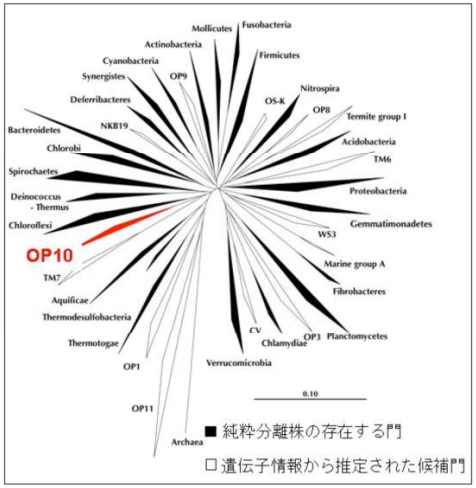
Ribosome engineering may be extremely difficult (?)

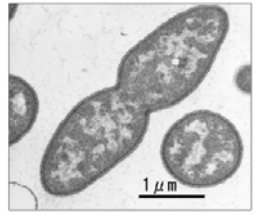
30S subunit
16S rRNA + 21 ribosomal proteins



Secondary structure map of *E. coli* 16S rRNA



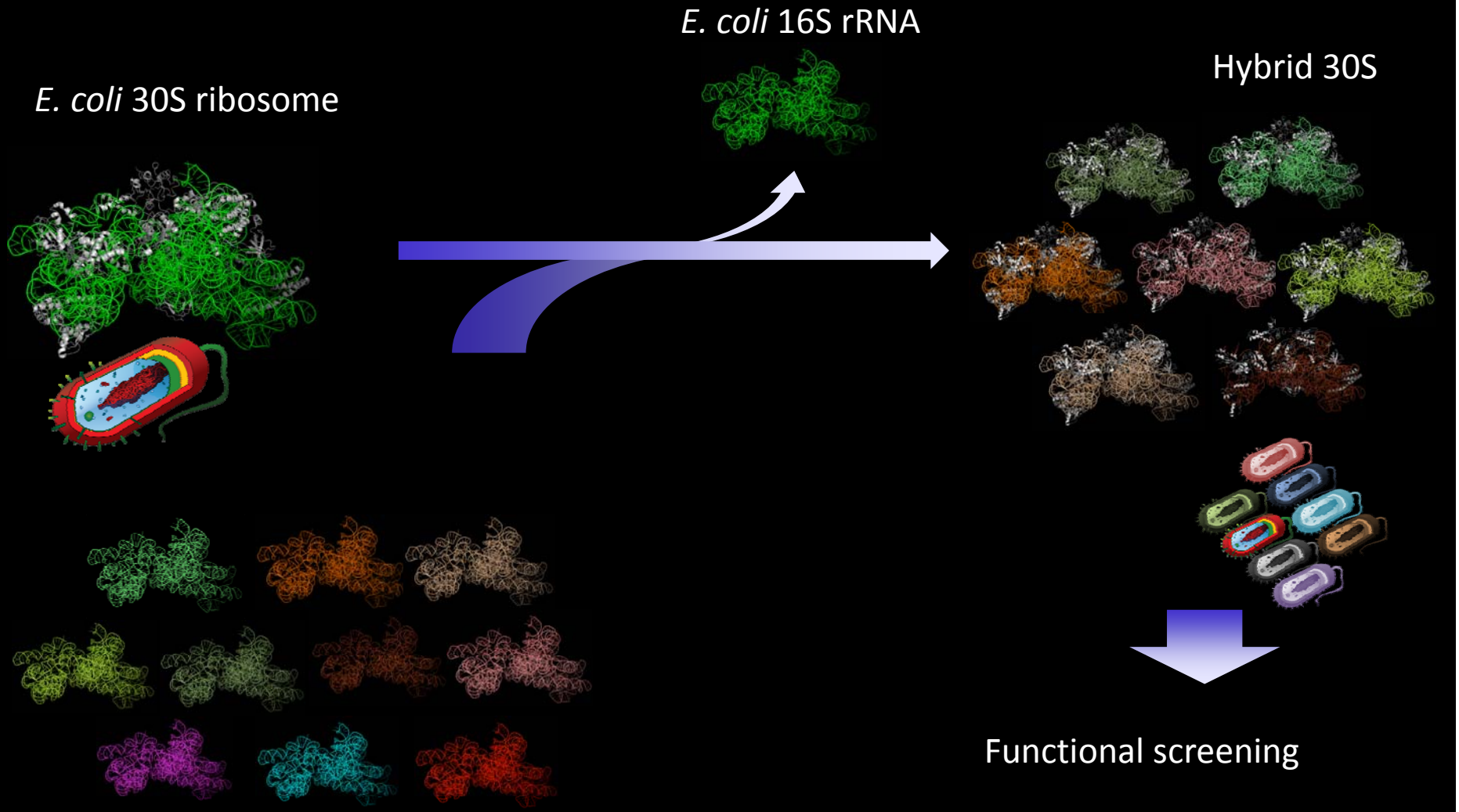




16S rRNA

- ✓ **Structural scaffold of 30S subunit**
- ✓ **Molecular clock (indigenous to the host organism)**
- ✓ **Susceptible to mutations?**
- ✓ **Mutations, if accepted, should induce functional perturbation (i.e., altered translational profile).**

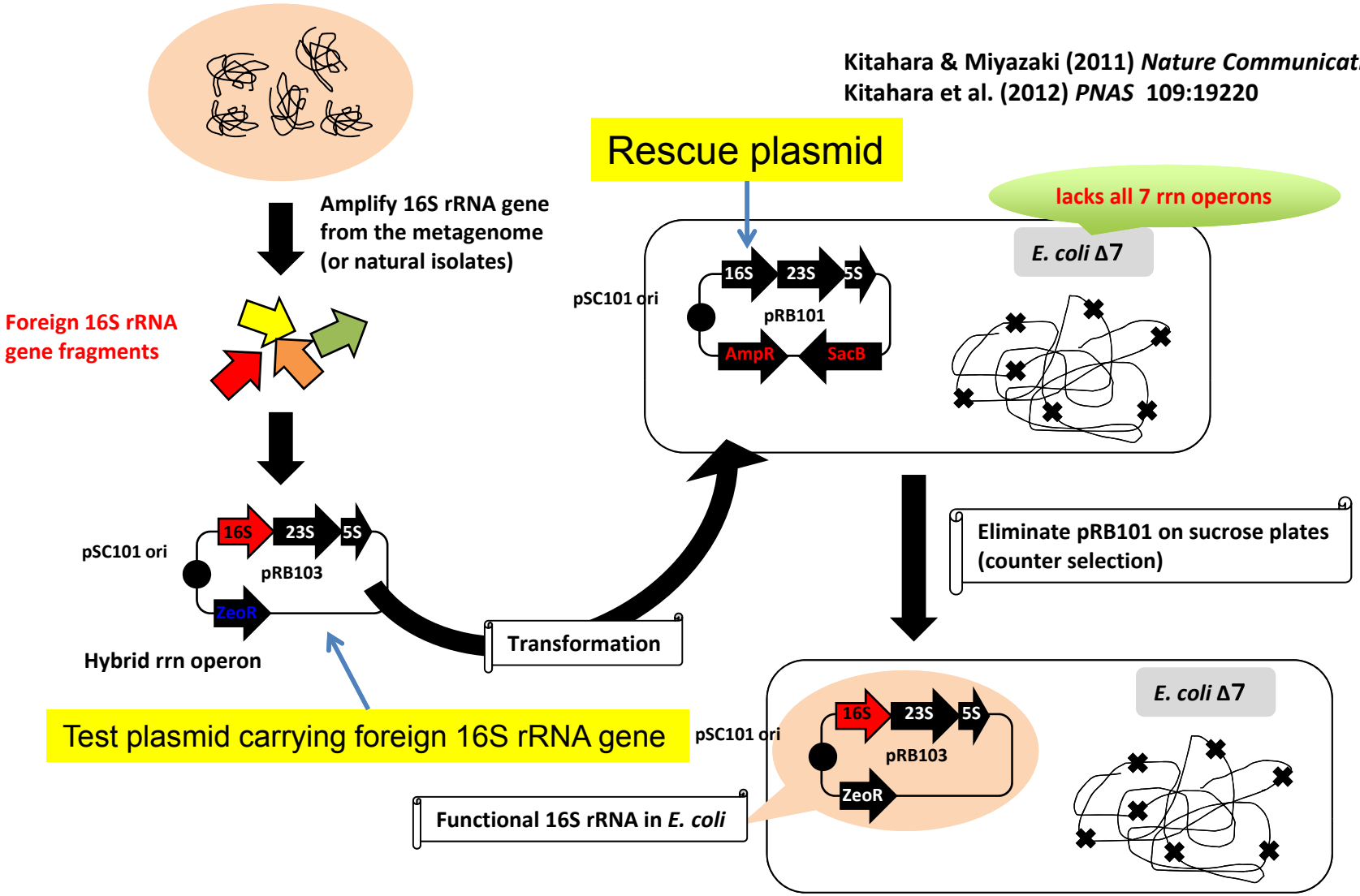
Systematic replacement of 16S rRNA in *E. coli* 30S ribosome



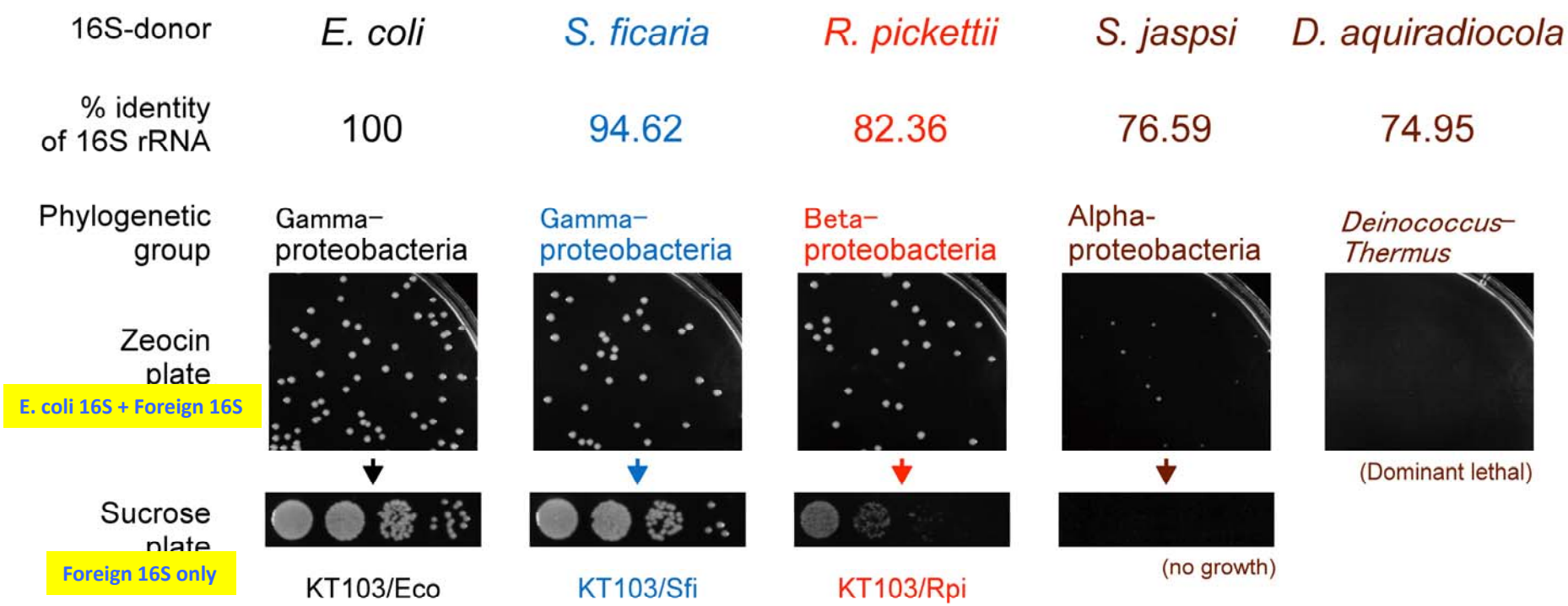
Kitahara & Miyazaki (2011) *Nature Communications* 2:549
Kitahara et al. (2012) *PNAS* 109:19220

“Horizontal gene transfer” test of 16S rRNA

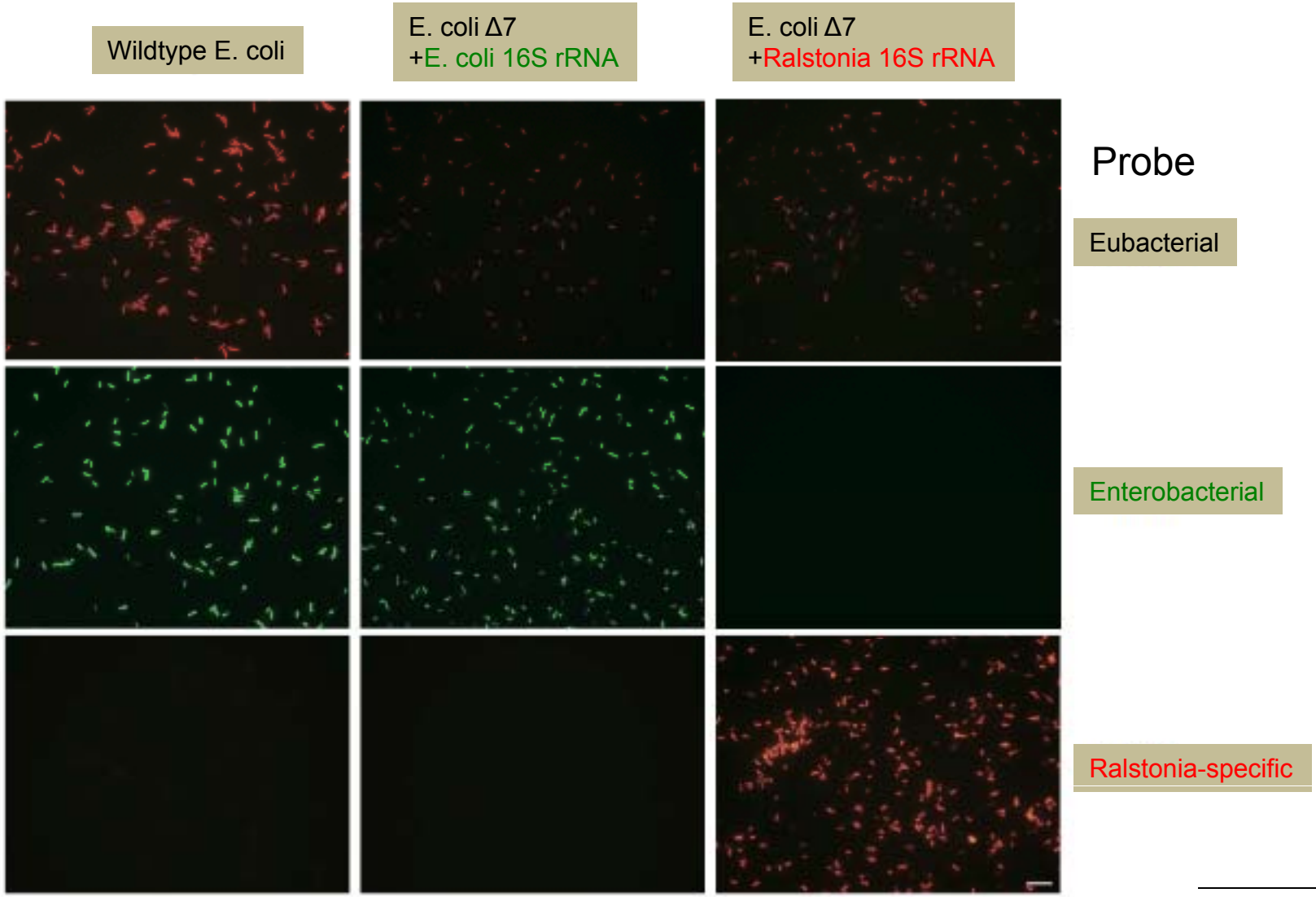
Kitahara & Miyazaki (2011) *Nature Communications* 2:549
Kitahara et al. (2012) *PNAS* 109:19220



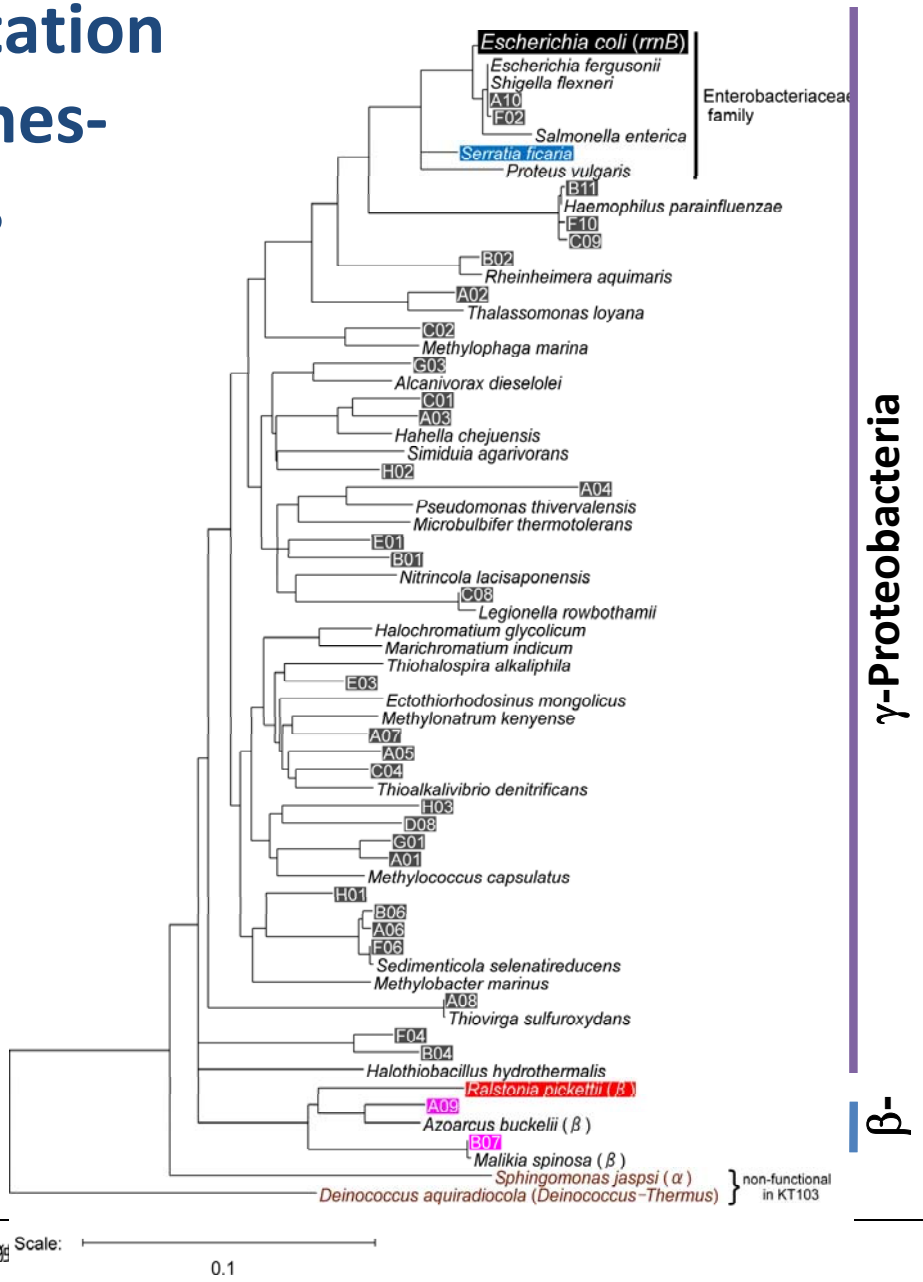
Functional complementation test of foreign 16S rRNA genes in *E. coli* Δ7



FISH analysis of 16S rRNA-substituted *E. coli*

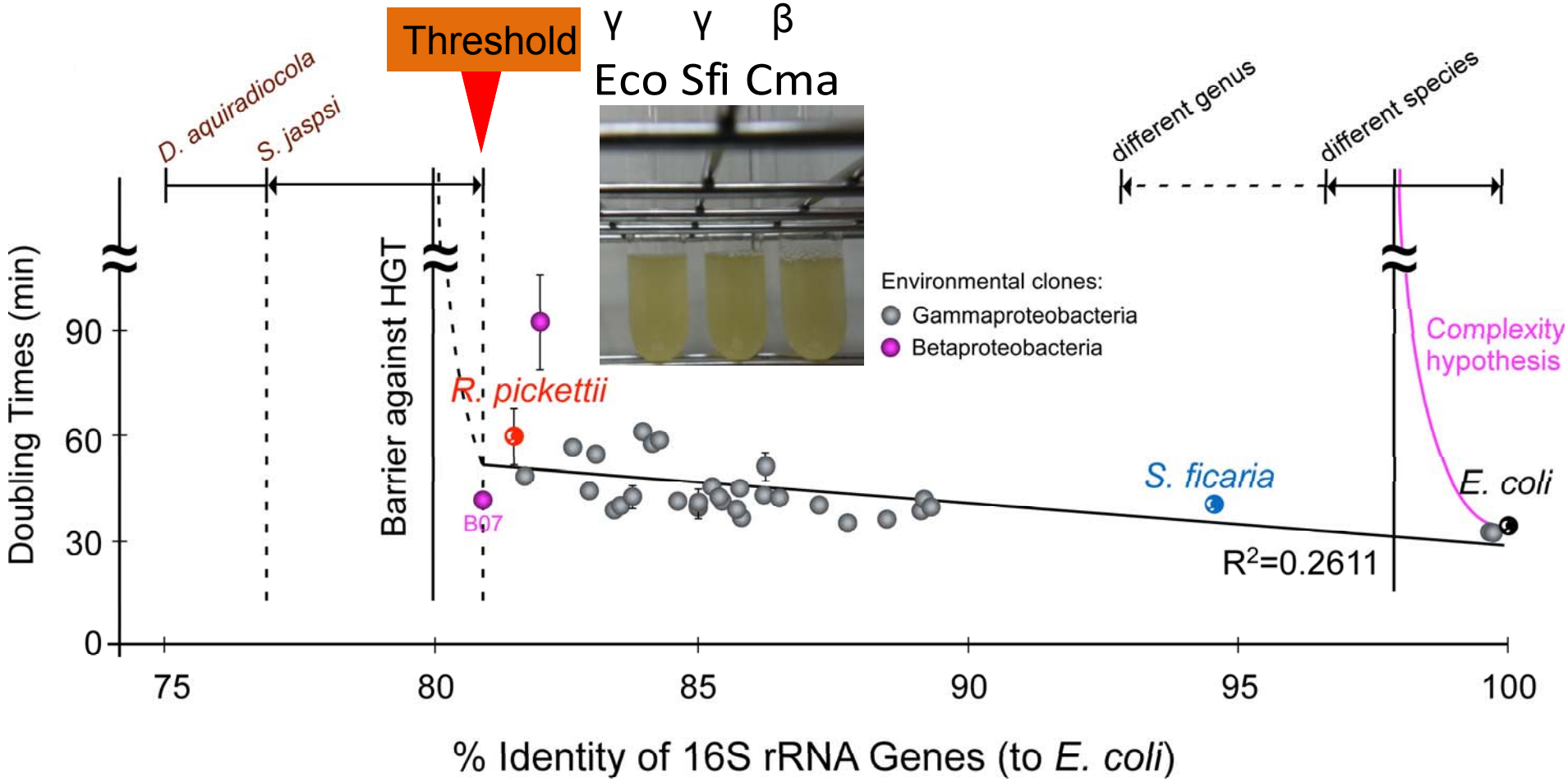


Functional complementation test using metagenomes-derived 16S rRNAs



Kitahara et al. (2012) *PNAS* 109:19220

Doubling times



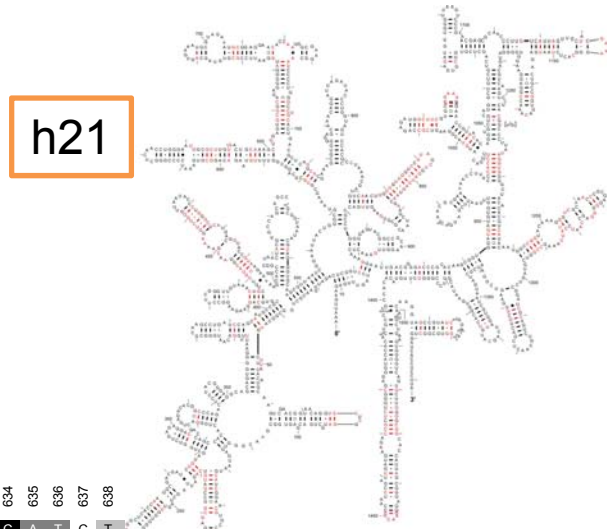
Kitahara et al. (2012) *PNAS* 109:19220

Ave. increase in DT = 4.6 sec/mutation

16S rRNA is transferable beyond classes

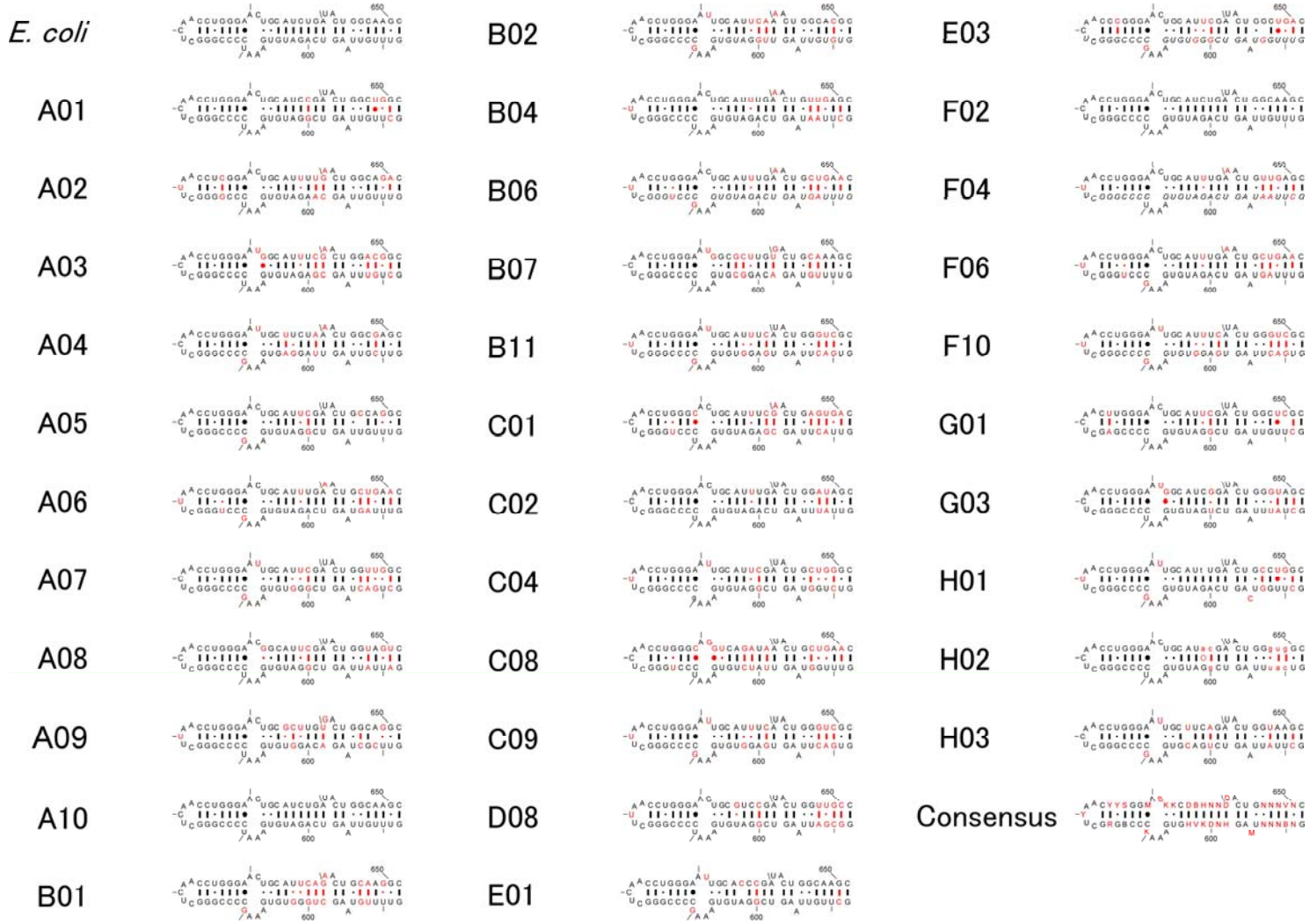
Lineage	
Bacteria;	Superkingdom
Proteobacteria;	Phylum
Gammaproteobacteria;	Class
Enterobacteriales;	Order
Enterobacteriaceae;	Family
Escherichia	Genus
Escherichia coli	Species

Basis for the functional compatibility -1

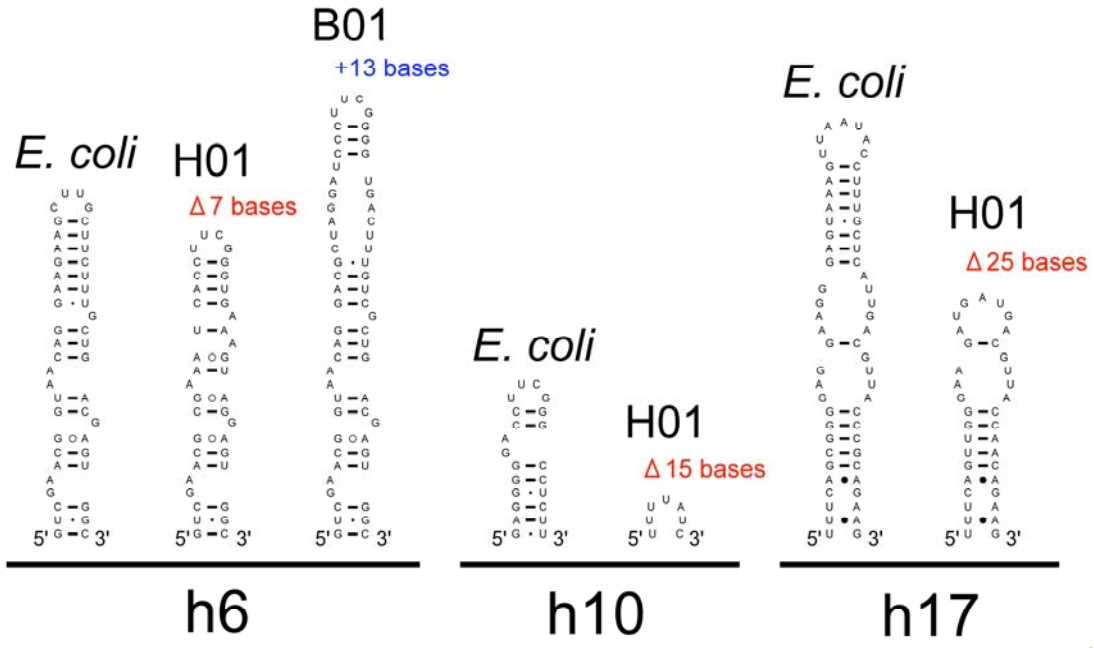


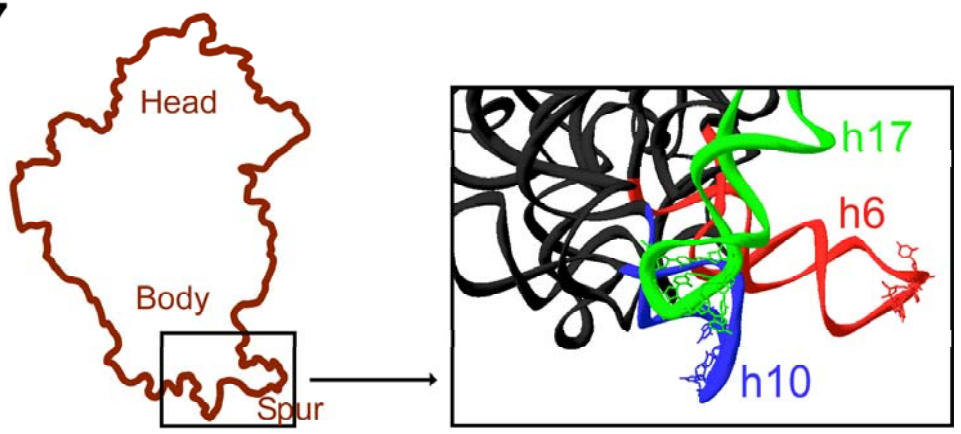
Conserved secondary structure

h21



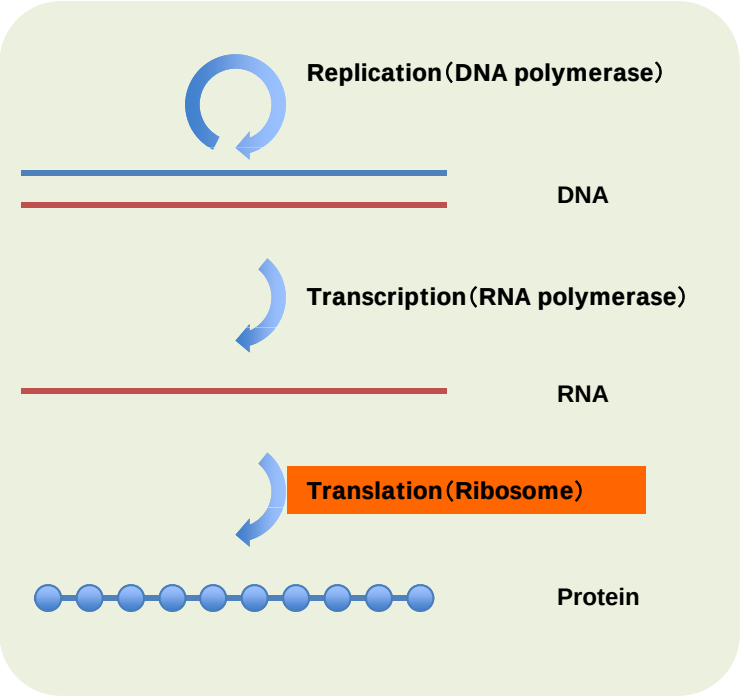
Basis for the functional compatibility -2

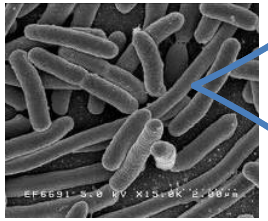


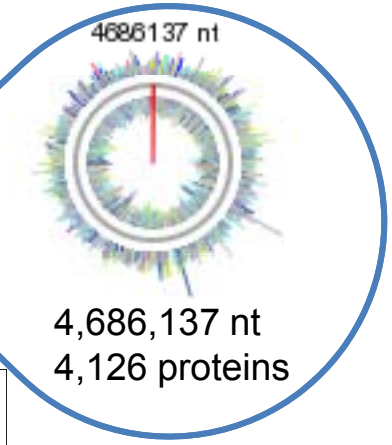


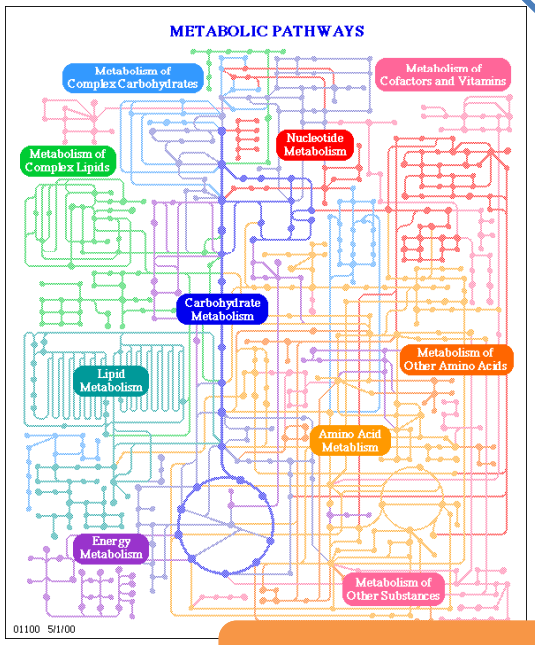
30S subunit
Interface view

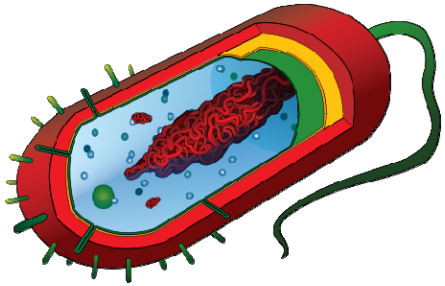
Diversifying cellular phenotype through translational engineering





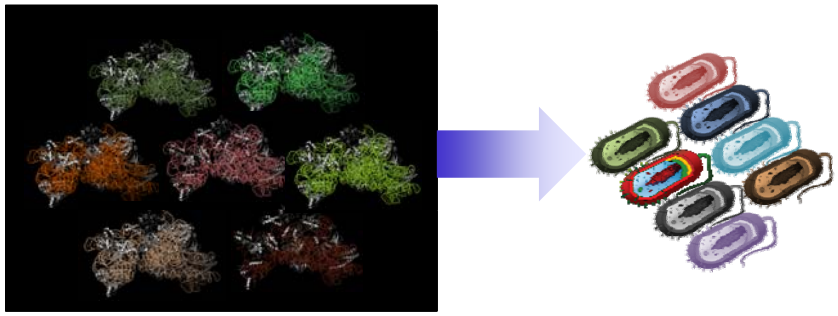


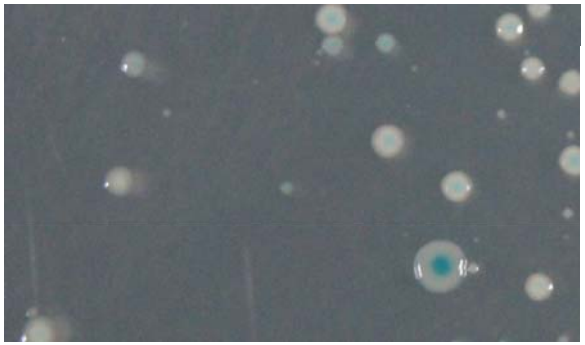




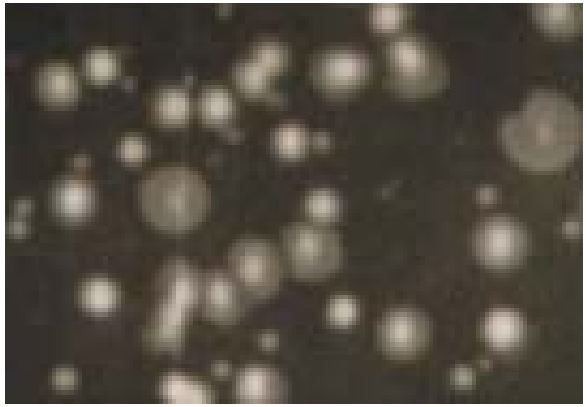
Rewired cellular network

Phenotypic diversity

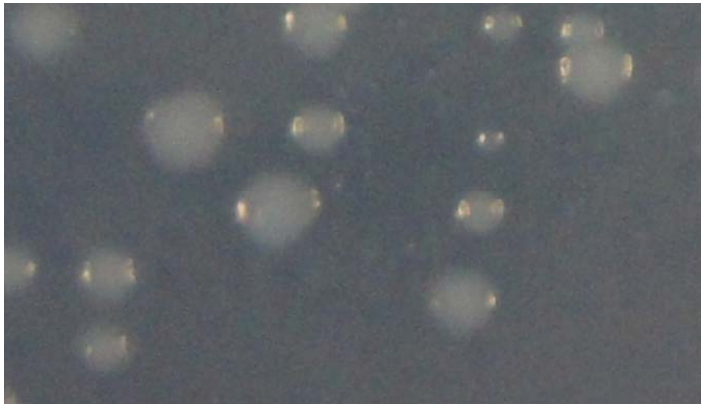




beta-Glucosidase



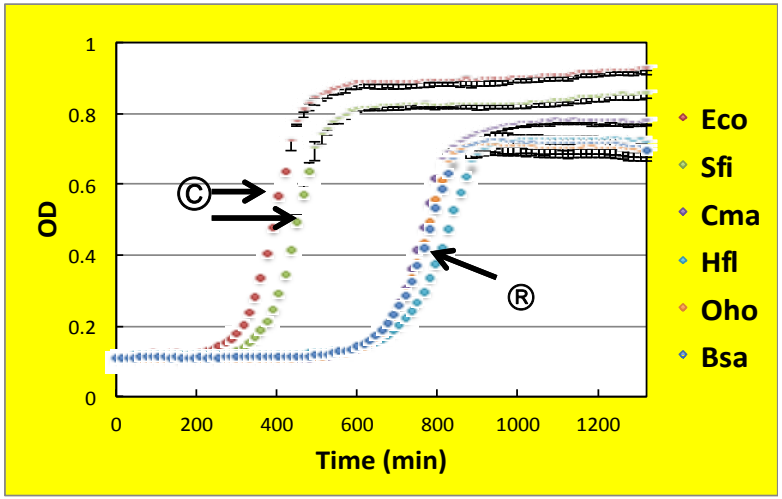
soft agar plates

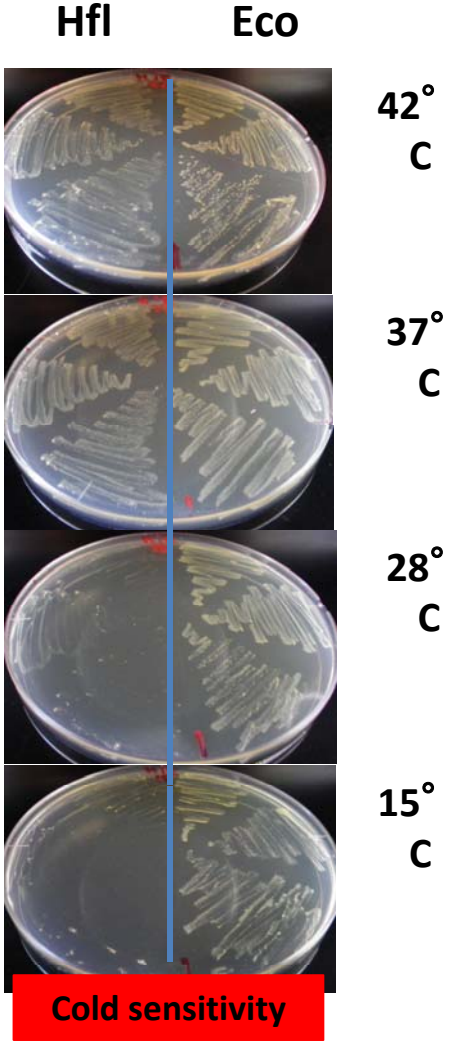


Standard agar plates

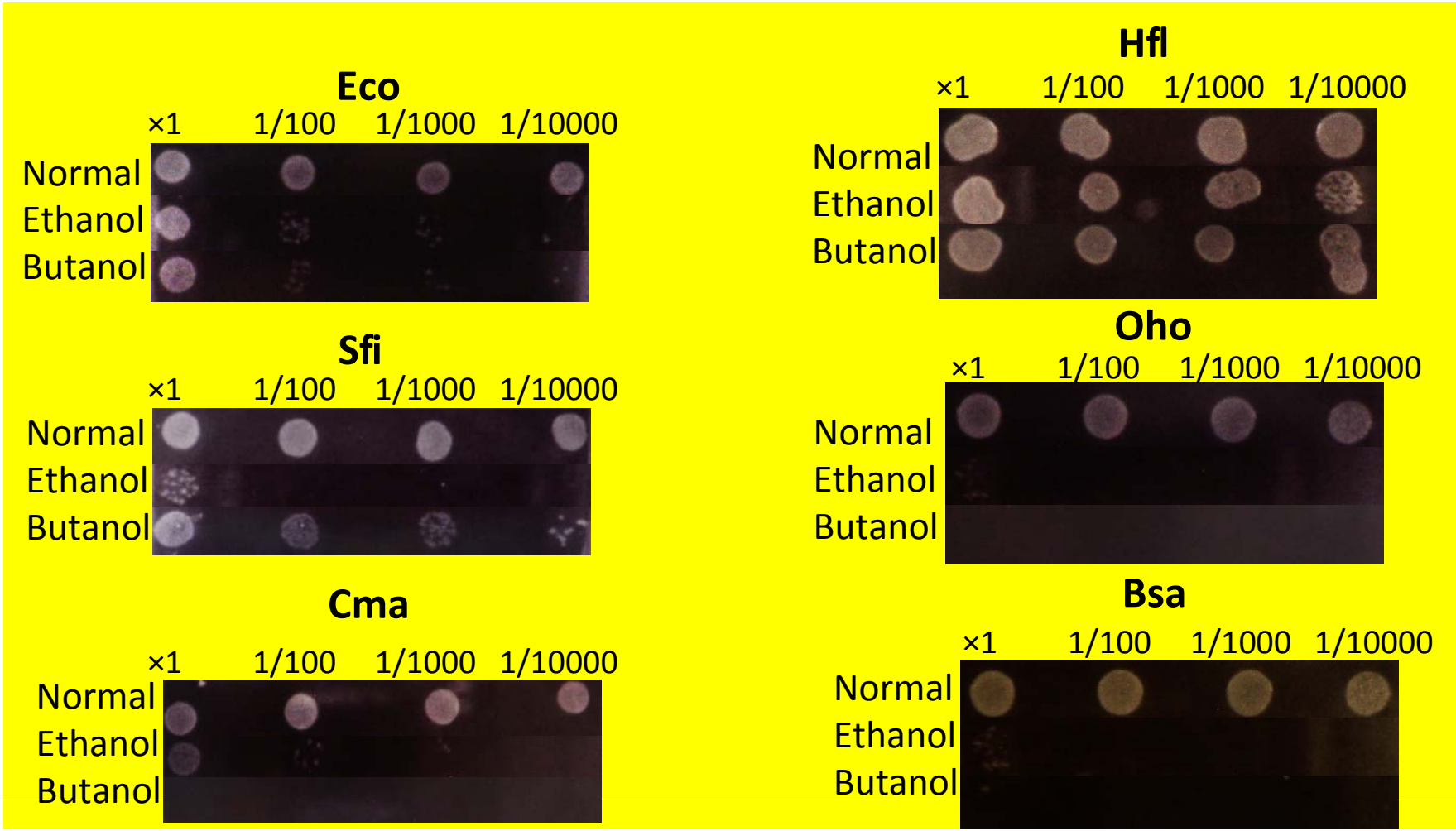
Phenotypic diversity obtained by ribosome engineering

Growth curve (37° C)



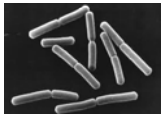


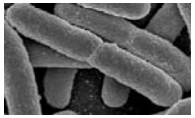
Phenotypic diversity – alcohol resistance (spot test)



Synonymous GFP genes as the model for metagenomic genes


Agaricus bisporus

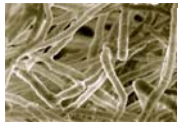

Bacillus subtilis

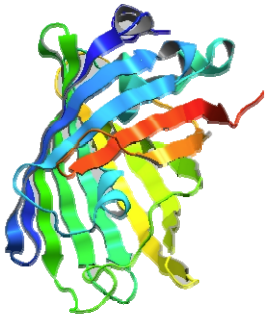

Escherichia coli

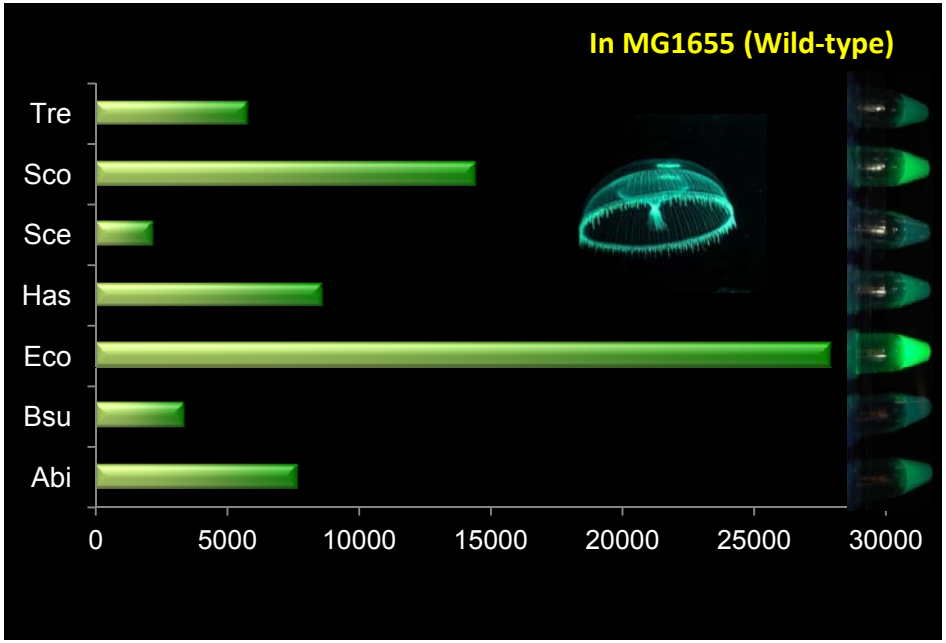

Homo sapiens


S. cerevisiae

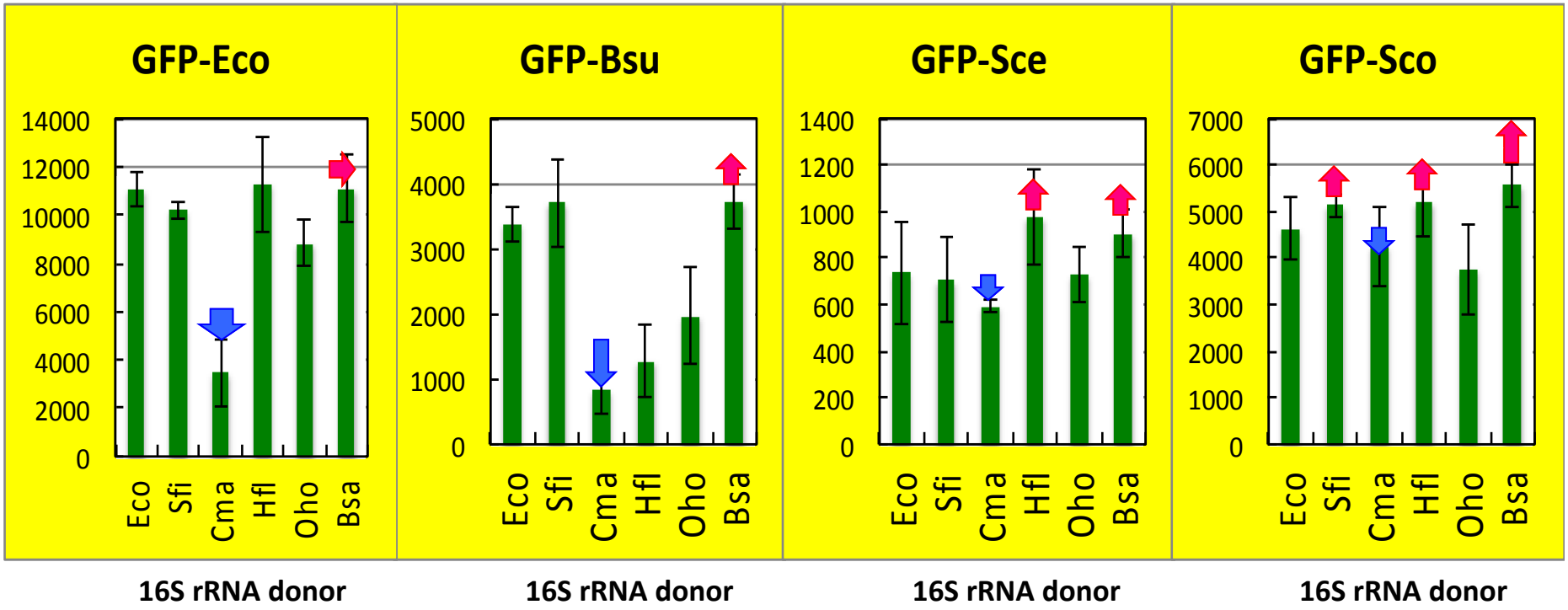

S. coelicolor


Trichoderma reesei



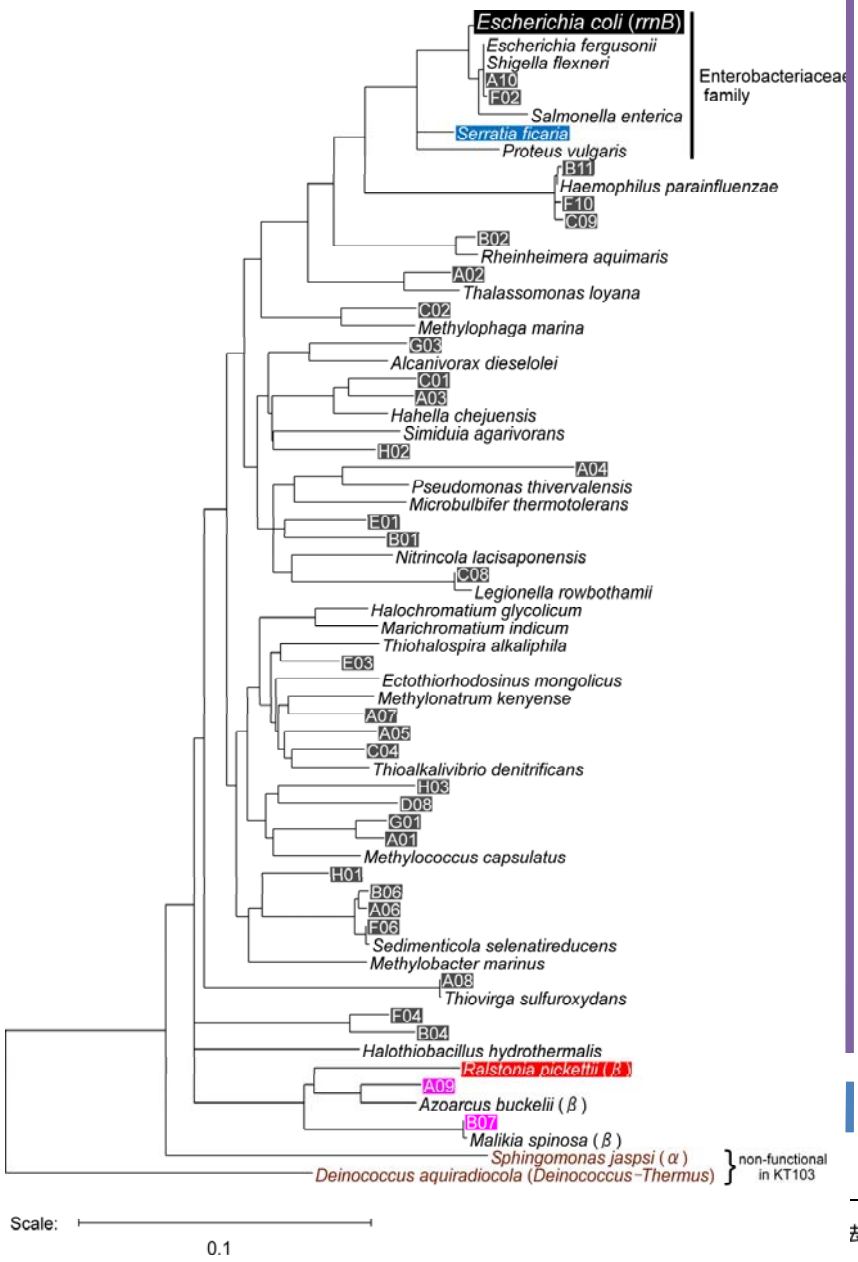


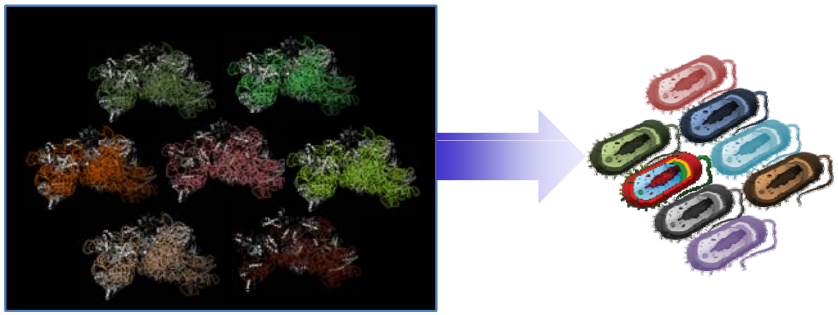
Phenotypic diversity - heterologous gene expression



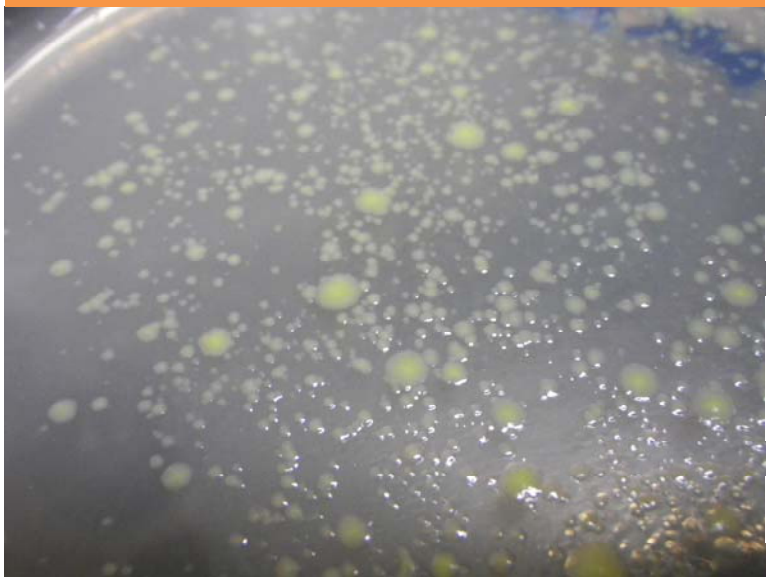
Improved expression in ribosome-engineered *E. coli* mutants

Functional complementation test using metagenomic 16S rRNAs

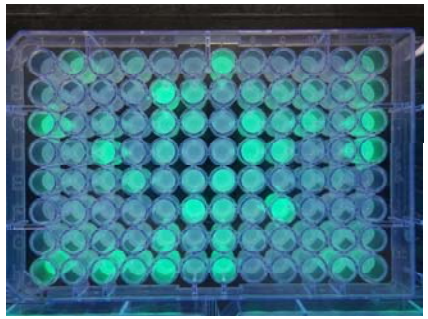




GFP-expressing *E. coli* mutants containing foreign 16S rRNA



Enhanced reporter gene expression in mutant *E. coli*



- ✓Types of 16S rRNA?
  - ✓Sequence?
  - ✓Secondary structure?
- ✓Reporter gene specificity?
- ✓Application to creating metagenomic library?

Creating chimera

Escherichia coli x *xxxxxxxxxxxxx*

```
[GENETYX-MAC: Multiple-Alignment]
Date        : 2013.02.19

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Sfi.seq       1 AAATTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAA     120

Eco.seq     121 TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATG     240
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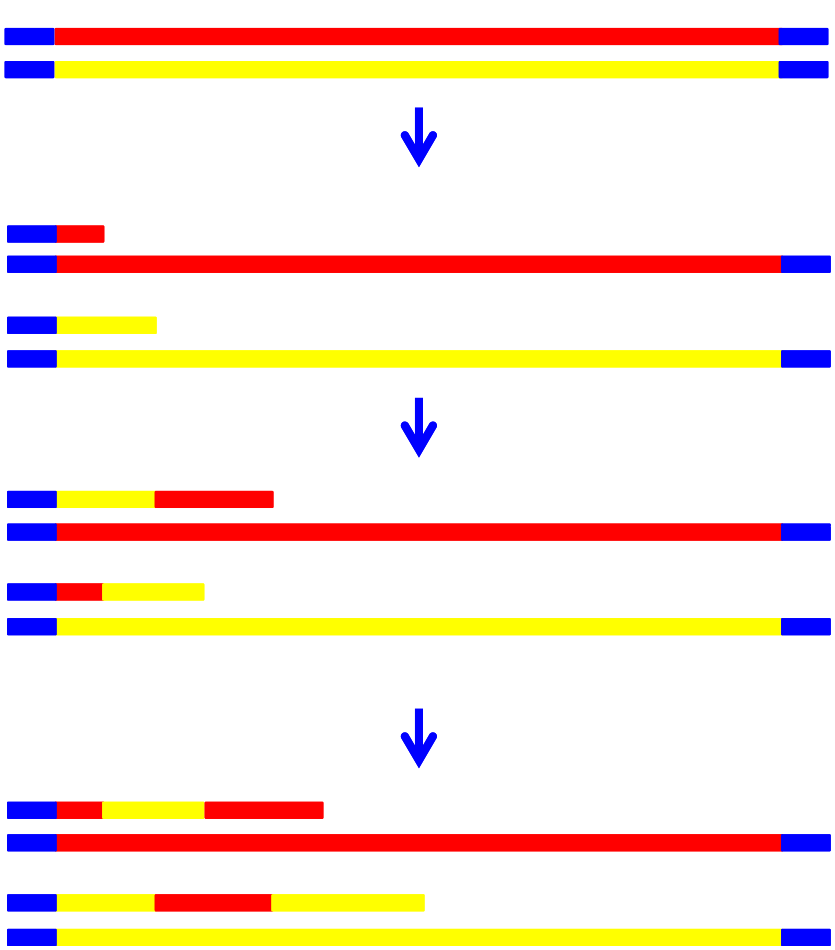
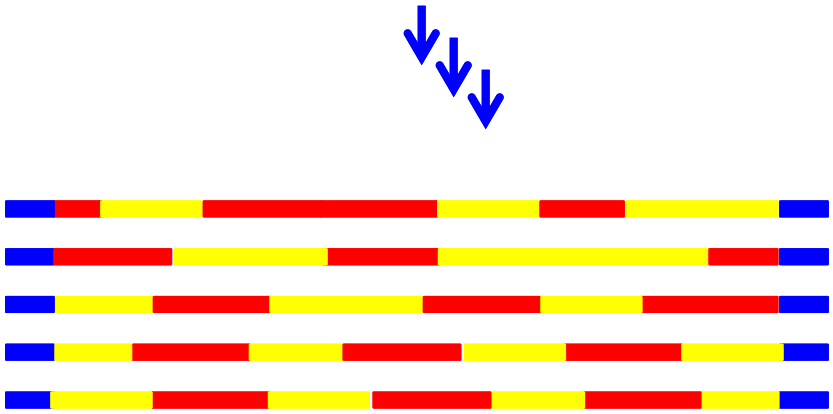
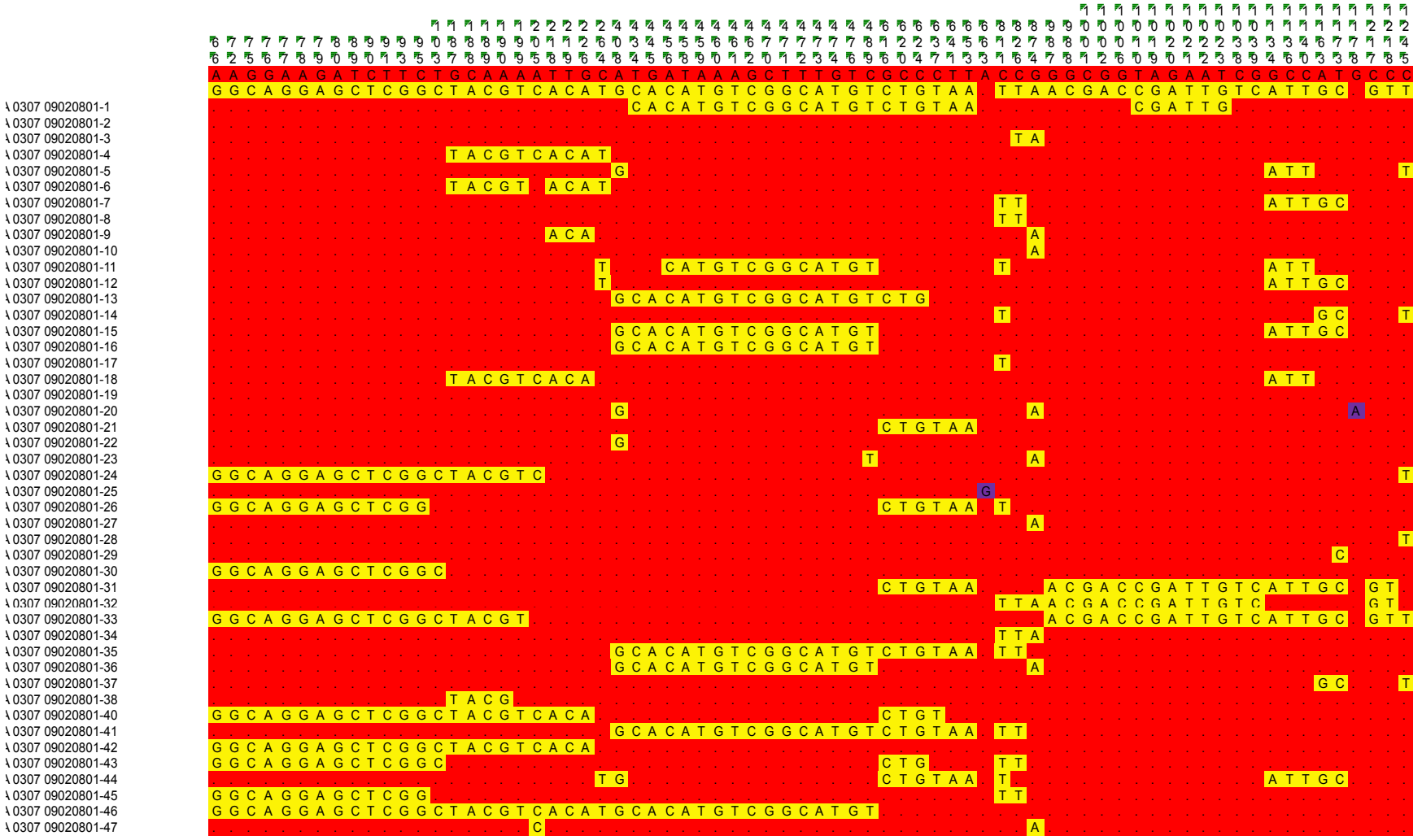
StEP DNA shuffling

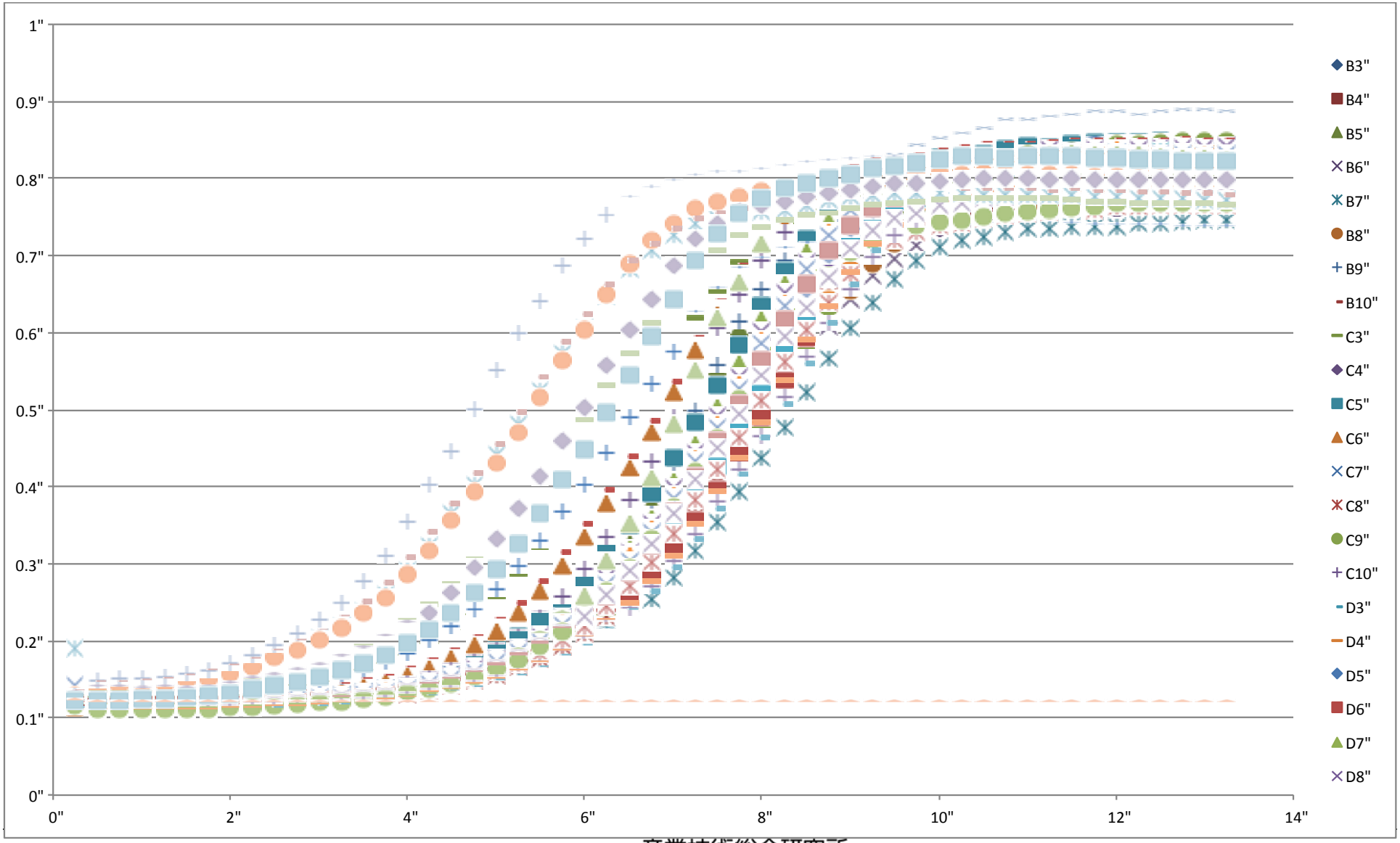




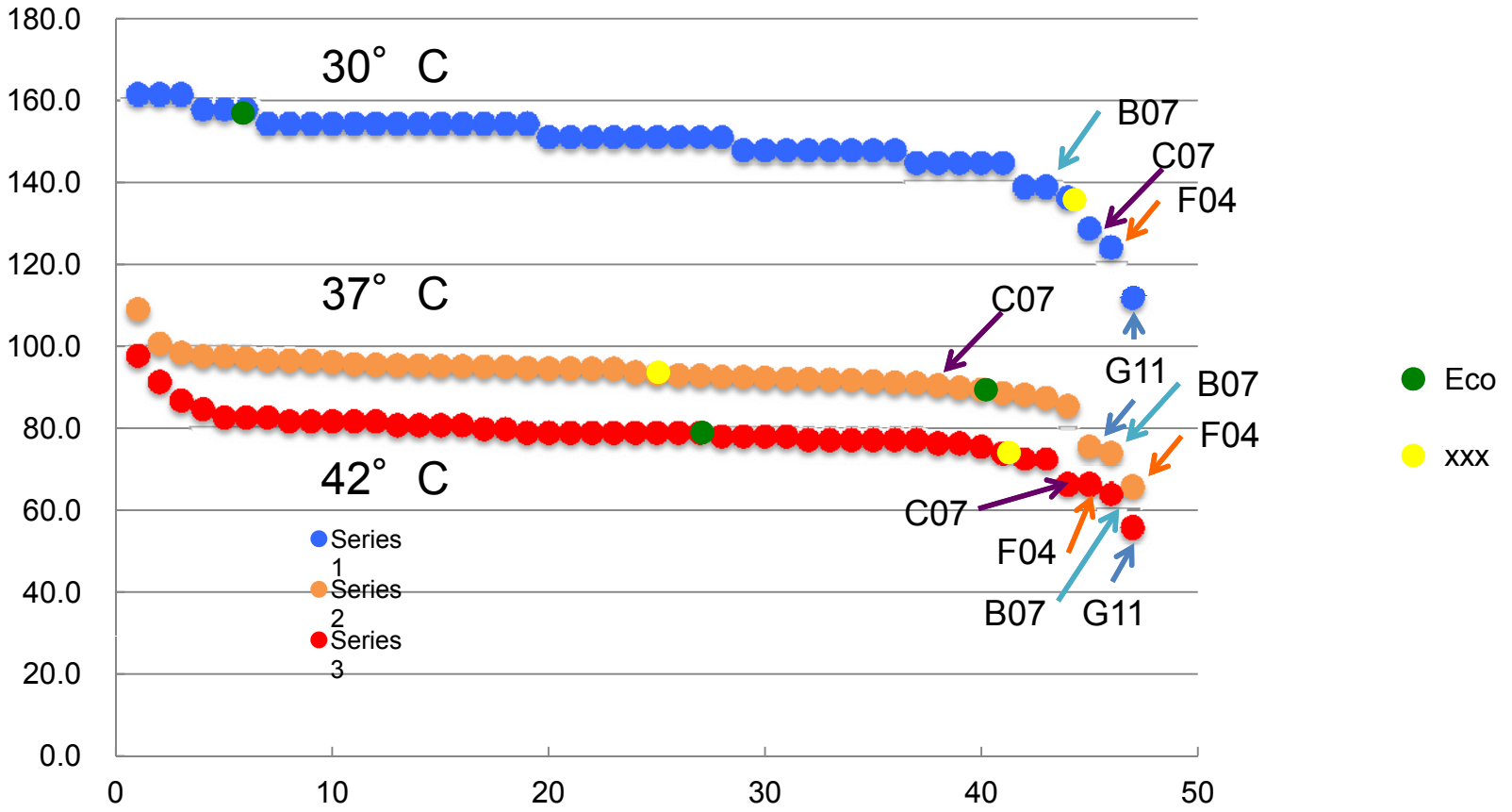
Functional DNA shuffling products



Growth curves



Fitness of the progeny



Conclusion & perspective

- *E. coli* ribosome can be engineered through interspecies exchange of 16S rRNA genes.
- Hybrid ribosomes carrying foreign 16S rRNAs showed diverse expression profiles.
- Some hybrid ribosomes showed enhanced expression relative to the wild type (*E. coli*) 16S rRNA.
- Ribosome engineering may be useful for expression of metagenomic genes.
- Ribosome engineering may be useful for bacterial cell breeding.

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Professor Tsutomu Suzuki (UT)

ขอบคุณ