Topic: *大肠杆菌单基因敲除突变体库的构建*

Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection

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Report Outline:

- Introduction
- Experiment
- Result
- Discussion and application
- Summary

1. Introduction

- Escherichia coli K-12 has been one of the best-characterized organisms in molecular biology -----Whole genome sequences are now available.
- Yet, many key resources for functional genomics and systems biology studies of E. coli are still lacking.

This E. coli K-12 functional genomics project is aim to:

- (1) create new experimental resources;
- (2) establish new analysis methods;
- (3) develop new computational approaches;
- (4) improve databases;
- (5) analyze gene function through experimentation by using these resources, methods, approaches, and databases.

Question:how to make mutants?

- Using a PCR gene replacement method

(PCR products encoding kanamycin resistance and containing 45-nt flanking homologous sequences for adjacent chromosomal regions.)

----*Saccharomyces cerevisiae* functional genomics project

- By inactivating each gene with a gene-specific plasmid clone

----Genome-scale disruption of *Bacillus subtilis* genes

- Transposon mutagenesis by generating a large set (30100) of sequence-defined mutants-laborious,incomplete disruption, requiring additional testing…

----*Pseudomonas aeruginosa(绿脓假单胞菌)*

Then,how to do it in this project?

- The method is analogous to the one that has been used in yeast, except by use of cells carrying an easily curable, low-copy-number plasmid expressing the λ Red recombinase.
- Advantages : complete deletion, to design deletions arbitrarily and precisely, and to easily eliminate the antibiotic resistance marker subsequently.
- E. coli K-12 single-gene knockout mutants

= the Keio collection

There are two closely related K-12 strains:MG1655 and W3110

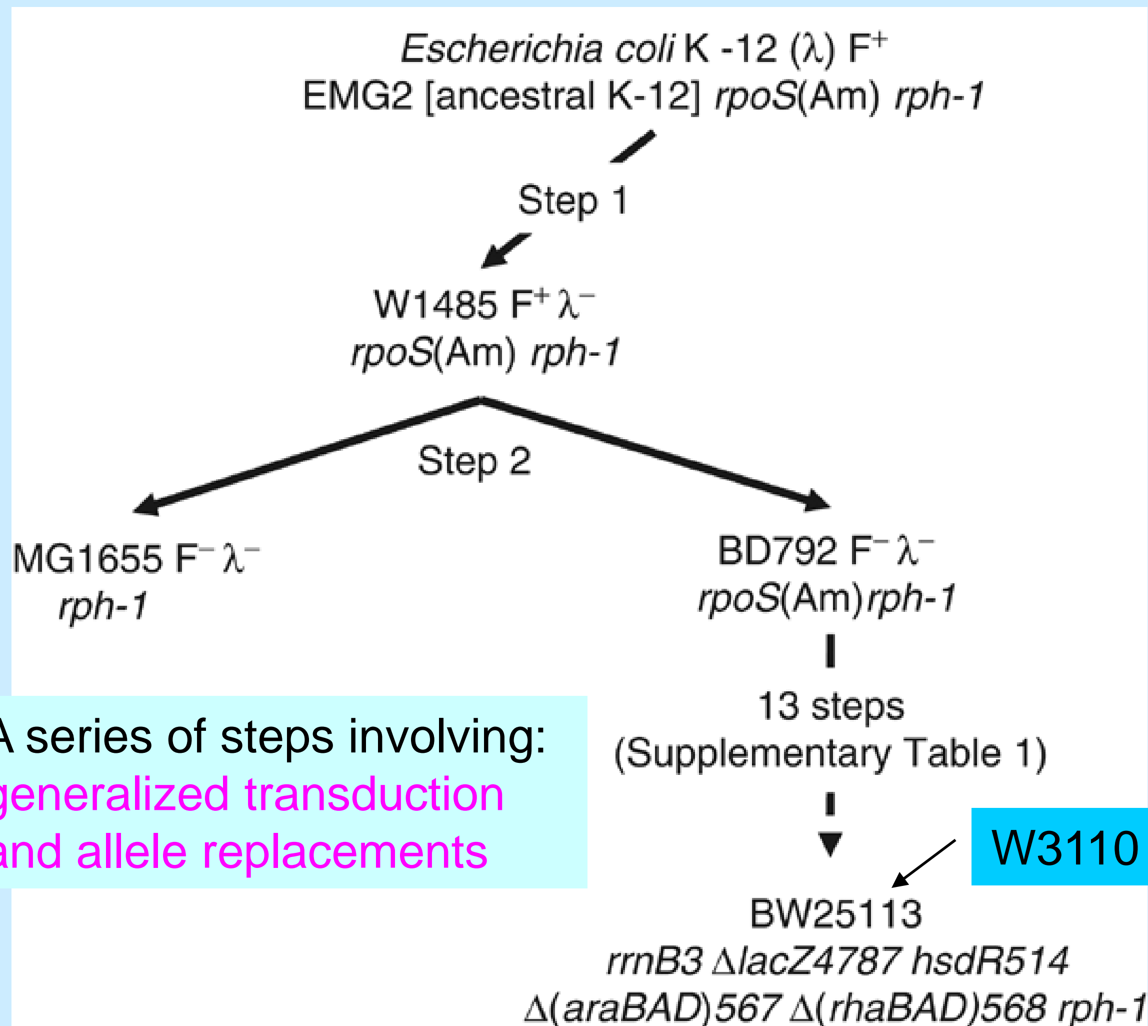
- The composite K-12 genome has 4453 genes, encoding 4296 ORFs (including 74 pseudogenes), 156 RNAs, and one annotated feature (oriC).
- Major differences between the MG1655 and W3110 genomes are the 12 additional sites of an insertion sequence (IS) in W3110, and one additional IS site and the defective CPZ-55 phage (seven prophage genes) only in MG1655.

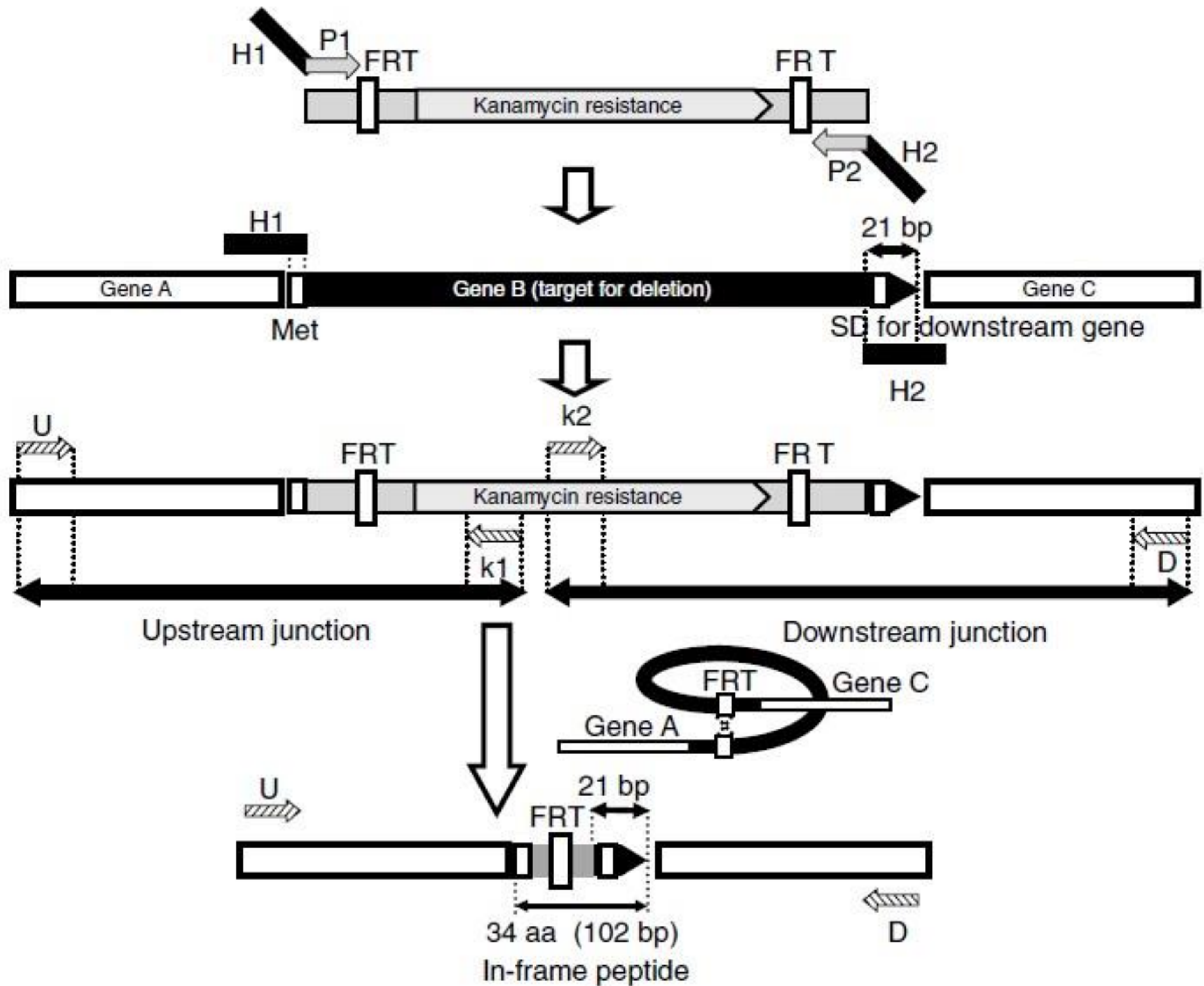
Genome datas are updating…

- Thus, on the basis of the 2005 annotation snapshot, MG1655 has a total of 4464 genes and W3110 has 4474 (Hayashi et al, 2006).
- In addition to updating annotations of gene functions, start sites were changed for 682 MG1655 ORFs (Riley et al, 2006). An additional 76 ORFs that have been predicted in W3110 have been targeted, for a total of 4550 genes encoding 4390 ORFs (Hayashi et al, 2006), although these have not been recognized as ORFs in the recent K-12 annotation workshops.

2. Experiment

- Material: E. coli K-12 strain BW25113





Primer design and construction of single-gene deletion mutants

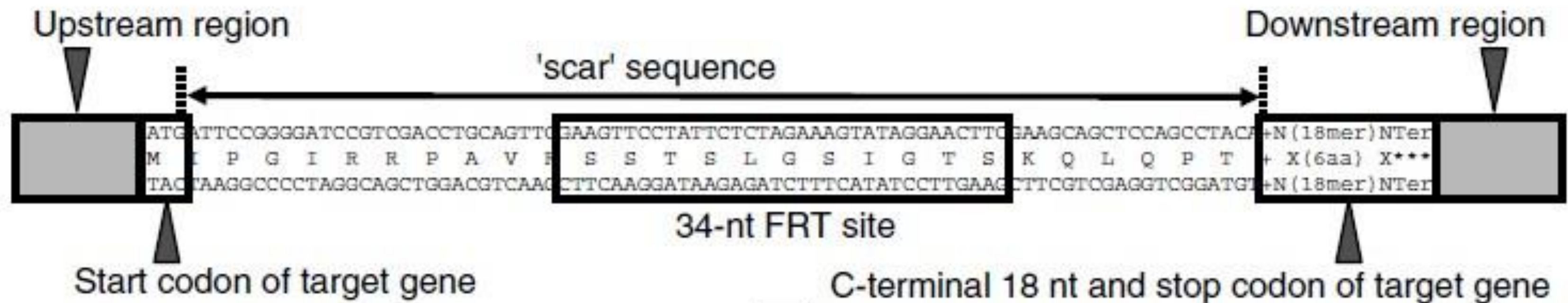
What is FRT (FLP recombinase target) sequence?

- The FLP recombinase is active at a particular 34 base pair DNA sequence, termed the FRT (FLP recombinase target) sequence.

- When two of these FRT sites are present, the FLP enzyme creates double-stranded breaks in the DNA strands, exchanges the ends of the first FRT with those of the second target sequence, and then reattaches the exchanged strands. This process leads to inversion or deletion of the DNA which lies between the two sites. Whether there is an inversion or deletion depends on the orientation of the FRT sites:

  if the sites are in the same direction, the intervening DNA will be deleted, but if the sites are in opposite orientation, the DNA is inverted.

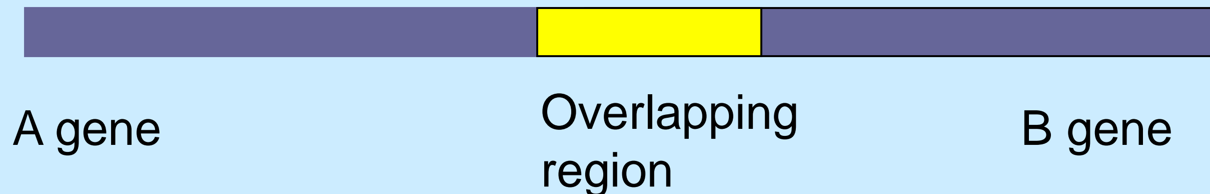
Structure of in-frame deletions



FLP-mediated excision of the FRT-flanked resistance gene is expected to produce : 34-residue scar peptide with an N-terminal Met, 27 scar-specific residues, and six C-terminal, gene B-specific residues

Overlapping genes?

- According to its latest genome annotation, E. coli K-12 has 742 overlapping genes.



- Solution:PCR product that was synthesized with an N-terminal primer that was redesigned to prevent altering the next coding region.

Verification of deletion mutants

- Our standard protocol usually yielded 10–1000 KmR colonies when cells were incubated aerobically at 37℃ on Luria broth (LB) agar containing 30 mg/ml kanamycin.
- The most critical step was preparation of highly electrocompetent cells (4×10^9 transformants per 1 μg of plasmid DNA under standard conditions).
- From every gene deletion experiment, four or eight KmR colonies were chosen and checked for ones with the correct structure by PCR using a combination of locus- and kanamycin-specific primers.

3. Result

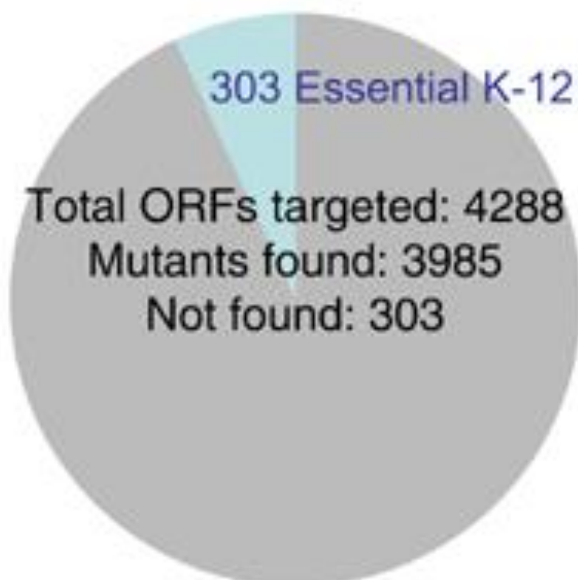
- Systematically made a set of precisely defined, single-gene deletions of

  all nonessential genes in Escherichia coli K-12

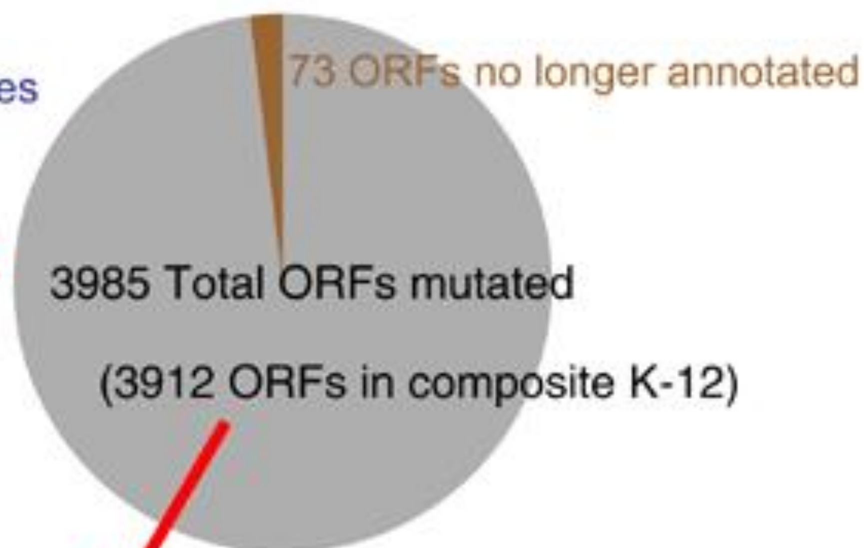
- Of 4288 genes targeted, deletions were obtained for 3985 ORFs. Our ORF deletions include 3912 genes annotated in both E. coli K-12 MG1655 and W3110 and 73 previously annotated genes.

- The 3912 composite K-12 ORF deletions include 2157 characterized genes and 1755 genes of uncharacterized or unknown function.

K-12 ORF mutagenesis



Nonessential K-12 ORF candidates



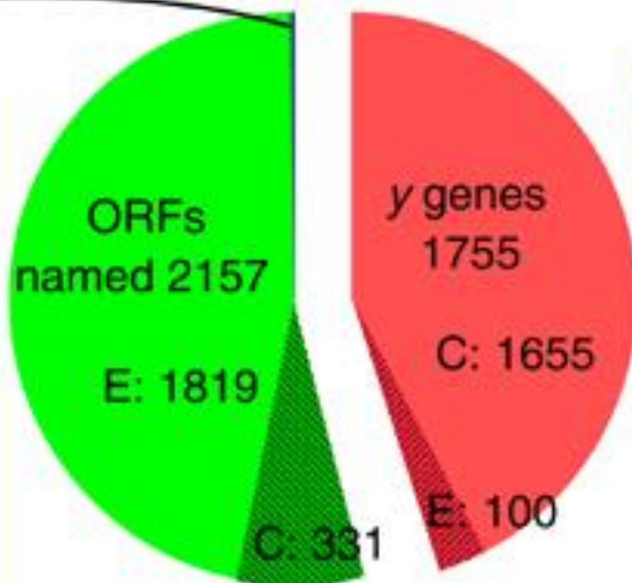
W3110-specific pseudogenes

dcuC; ECK0614
(1) JW0613, (2) JW0616 fragments

gatA; ECK2087
(3) JW2078, (4) JW2081 fragments

rcsC; ECK2211
(5) JW2206, (6) JW5370 fragments

tnaB; ECK3702
(7) JW5619



Composite ORF categories

1) Characterized ORFs: Named
2) Uncharacterized ORFs: *y* genes
3) ORF evidence:
C, computational
E, experimental

Mutant Summary

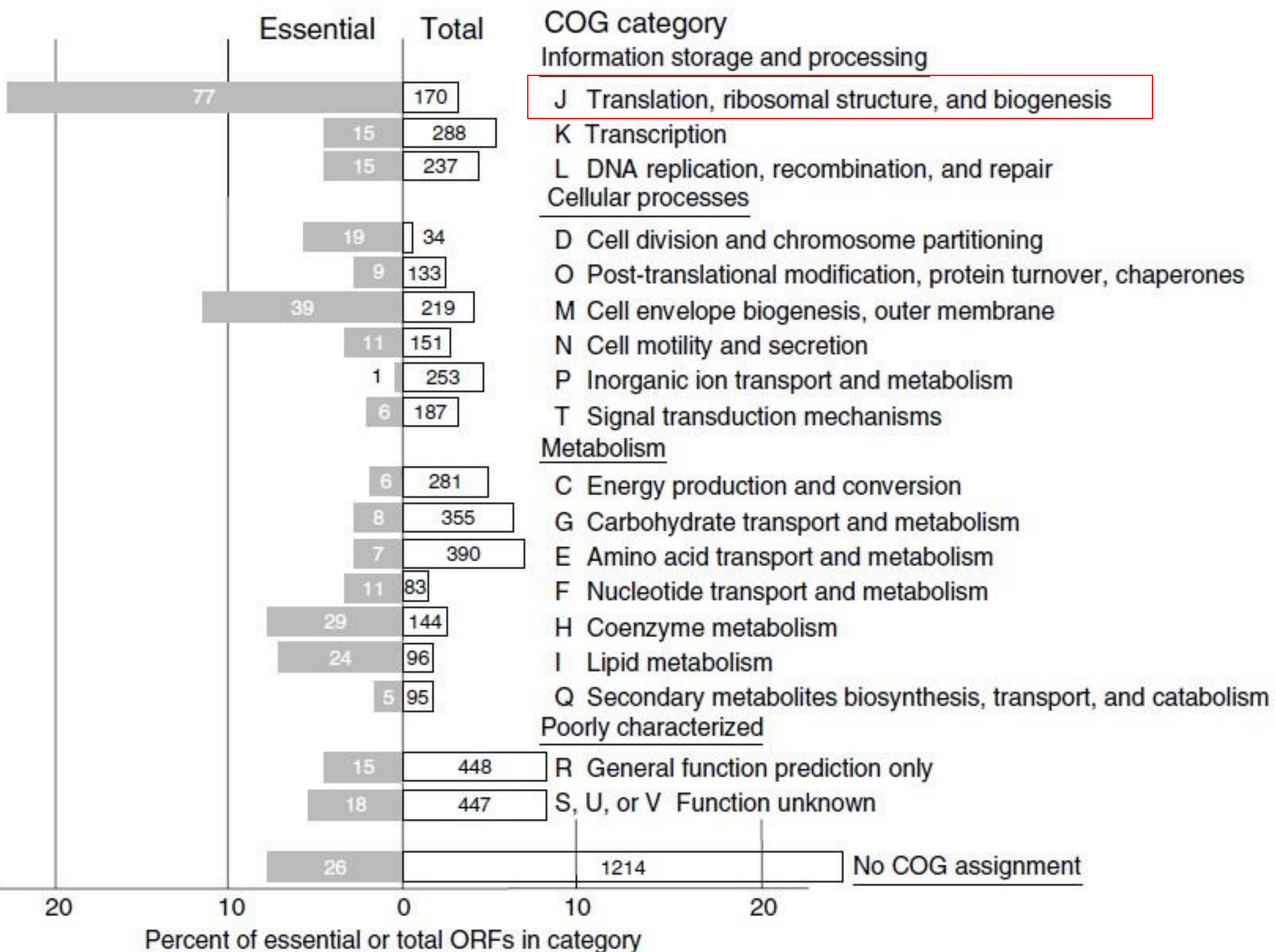
Table I Mutant summary

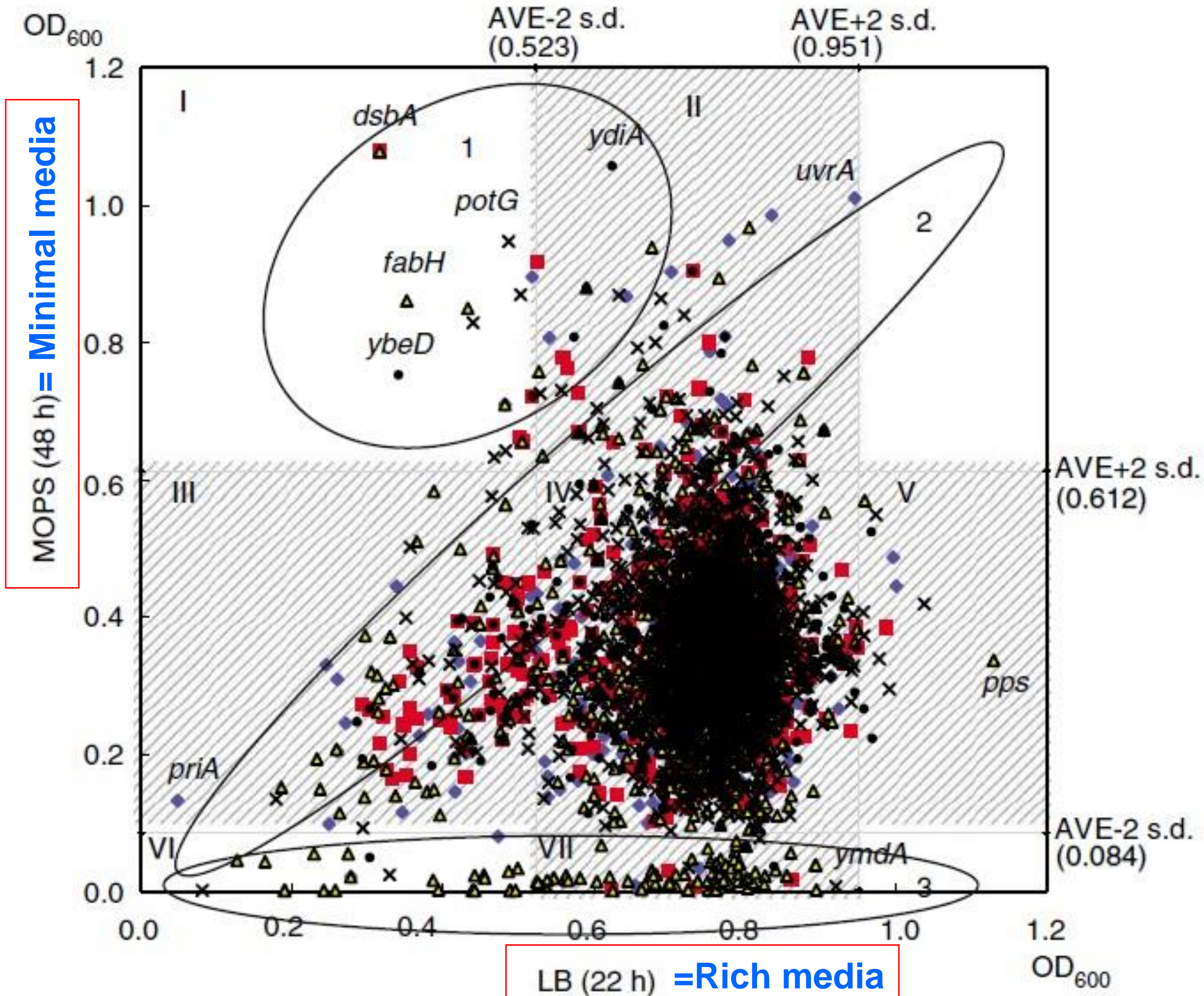
Class	ORFs
Total	4390
Targeted[a]	4288
Nonessential	3985
Essential	303
Not targeted[b]	102

Categories of ORFs

- ORFs can be classified into clusters of orthologous groups(COGs) belonging to different functional categories

- It is natural for multidomain proteins to be comprised of more than one COG. Some COGs also belong to more than one functional class.


Essential
Total
COG category
Information storage and processing
77
170
J Translation, ribosomal structure, and biogenesis
15
288
K Transcription
15
237
L DNA replication, recombination, and repair
Cellular processes
19
34
D Cell division and chromosome partitioning
9
133
O Post-translational modification, protein turnover, chaperones
39
219
M Cell envelope biogenesis, outer membrane
11
151
N Cell motility and secretion
1
253
P Inorganic ion transport and metabolism
6
187
T Signal transduction mechanisms
Metabolism
6
281
C Energy production and conversion
8
355
G Carbohydrate transport and metabolism
7
390
E Amino acid transport and metabolism
11
83
F Nucleotide transport and metabolism
29
144
H Coenzyme metabolism
24
96
I Lipid metabolism
5
95
Q Secondary metabolites biosynthesis, transport, and catabolism
Poorly characterized
15
448
R General function prediction only
18
447
S, U, or V Function unknown
26
1214
No COG assignment
20
10
0
10
20
Percent of essential or total ORFs in category



OD600
AVE-2 s.d.
(0.523)
AVE+2 s.d.
(0.951)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
I
II
III
IV
V
VI
VII
1
2
3
dsbA
ydiA
uvrA
potG
fabH
ybeD
pps
priA
ymdA
AVE+2 s.d.
(0.612)
AVE-2 s.d.
(0.084)
MOPS (48 h)= Minimal media
0.0
0.2
0.4
0.6
0.8
1.0
1.2
OD600
LB (22 h) =Rich media

Evaluation of gene essentiality

- 1) Mutants with suppressors

----for suppressors allow viability of mutants with the respective deletions.

='quasi-essential' genes

For example:

secM, has a translational arrest sequence within its C-terminus that is required for expression of the downstream secA, encoding an essential preprotein translocase SecA subunit .Thus, it is reasonable to suggest that the sole secM mutant arose because it acquired a suppressor allowing secA expression.

- 2) Functional redundancy or duplication can hide gene essentiality.

- 3) Polar effect of genes:

----Mutants described here are initially nonpolar because downstream genes can be expressed from the resistance gene promoter, and from the upstream native promoter upon elimination of the resistance cassette.

Definition of polar effect :

Many bacterial genes are located in operons in which they are transcribed in a single polycistronic mRNA. A mutation that prevents the transcription or translation of one gene can prevent the transcription of promoter-distal genes in the same operon.

- 4) Media/Culture media:

----In absence of some substance which is involved in central metabolic pathways, particular mutants can’t recover.

- 5) Occasional technical problems

----In a few instances, PCR products failed to target a gene due to the presence of IS elements at sites that were previously unrecognized.

Solution: Redesign primers

- 6) Toxin–antitoxin (TA) systems

----Deletion of a single gene can lead to aberrant behavior in certain gene contexts.

For example:

RelE and MazF are toxins that cleave mRNA in response to a nutritional stress. Under nonstress conditions, a specific antitoxin (RelB or MazE) prevents cleavage, allowing normal growth.

4. Discussion and Application

- Distribution is being handled via GenoBase(http://ecoli.aist-nara.ac.jp/)

- Together with supporting data and other key resources, including the ASKA

(A complete Set of E. coli K-12 ORF Archive) clone sets

Contributions…

- The Keio collection should provide not only a basic resource for systematic functional genomics but also experimental data source for systems biology approaches. The mutants can serve as fundamental tools for a number of reverse genetics approaches, permitting analysis of the consequences of the complete loss of gene function.
- The authors hope to link the worldwide efforts towards a comprehensive understanding of the E. coli K-12 model cell.
- Because many E. coli gene products are well conserved in nature, the Keio collection is likely to be useful not only for studying E. coli and other bacteria but also for examining properties of genes from a wide range of living organisms.

Summary

The Keio collection

- E. coli K-12 single-gene knockout mutants, they are create in-frame (nonpolar) deletions upon elimination of the resistance cassette.
- Obtained 3985 nonessential ORFs
- Clusters of Orthologous Groups(COGs)

----rich and minimal media

- Evaluation of gene essentiality

And so on…

Thanks for your attention!