厦门大学
生命科学学院
SCHOOL OF LIFE SCIENCES XIAMEN UNIVERSITY

MICROBIOLOGY
微生物学

Lecture 12

Yuan Jing

2017

厦门大学
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MICROBIOLOGY

Chapter 17
Recombinant DNA technology

Chapter X
Discovery of CRISPR/Cas9

Yuan Jing

Outline

- Genetic engineering by DNA recombination
- Genomics in Microbiology
- CRISPR/Cas9: History and Future

15.1 Key developments in recombinant DNA technology

- ✓ Restrictions enzymes
- ✓ DNA ligase
- ✓ PCR
- ✓ DNA Recombination

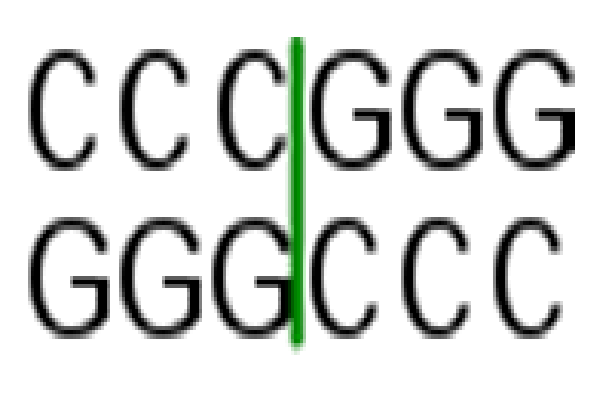
Restrictions enzymes

Restrictions enzymes

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts DNA at or near specific recognition nucleotide sequences known as restriction sites.

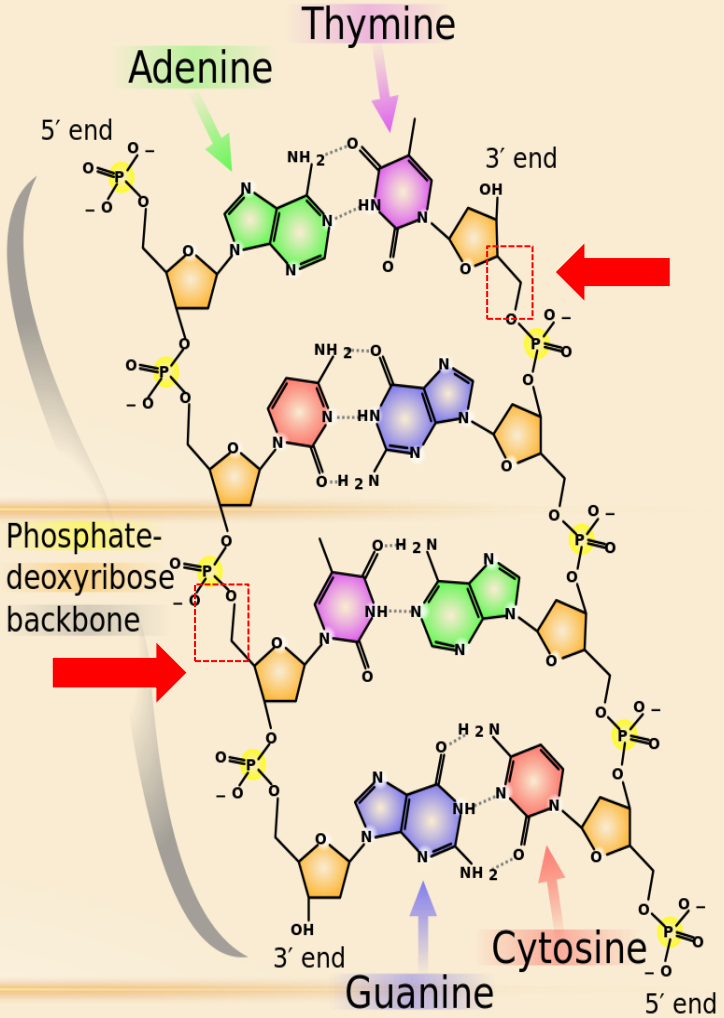
EcoRI

SmaI



palindromic 回文序列 recognition site

Restriction Enzyme(green) in a complex with its substrate DNA



Restrictions enzymes

Enzyme	Source	Recognition Sequence	Cut
*Eco*RI	*Escherichia coli*	5’GAATTC 3’CTTAAG	5’---G AATTC---3’ 3’---CTTAA G---5’
*Eco*RII	*Escherichia coli*	5’CCWGG 3’GGWCC	5’--- CCWGG---3’ 3’---GGWCC ---5’
*Bam*HI	*Bacillus amyloliquefaciens* 芽孢杆菌	5’GGATCC 3’CCTAGG	5’---G GATCC---3’ 3’---CCTAG G---5’
*Hind*III	*Haemophilus influenzae* 流感嗜血杆菌	5’AAGCTT 3’TTCGAA	5’---A AGCTT---3’ 3’---TTCGA A---5’

The Nobel Prize in Physiology or Medicine 1978

Restrictions enzymes

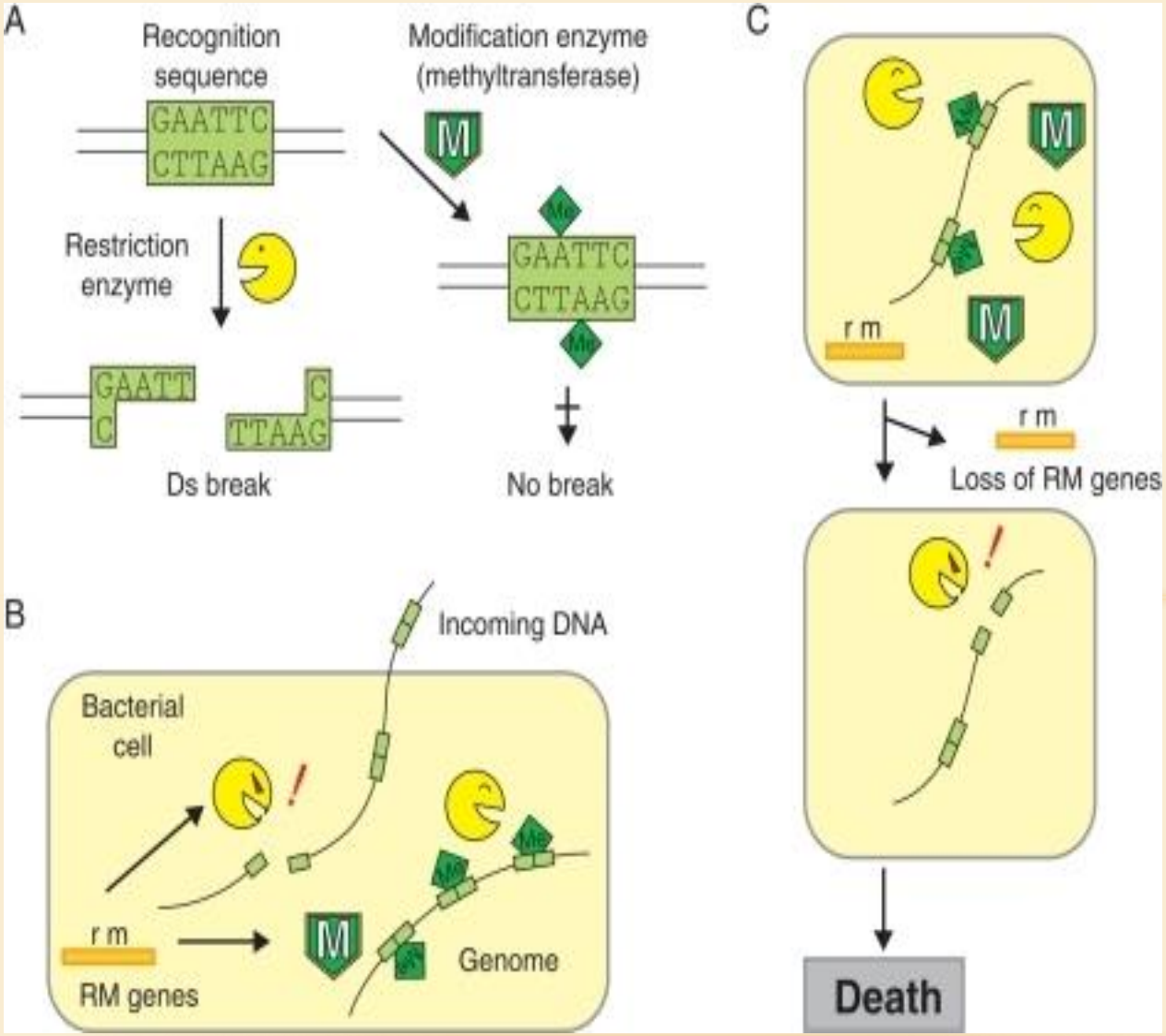
Werner Arber

Daniel Nathans

Hamilton O. Smith

The Nobel Prize in Physiology or Medicine 1978 was awarded jointly to Werner Arber, Daniel Nathans and Hamilton O. Smith *"for the discovery of restriction enzymes and their application to problems of molecular genetics"*.

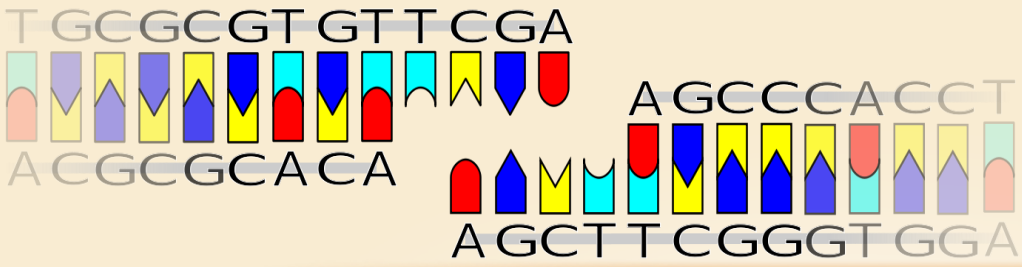
Bacterial Restriction–Modification Systems



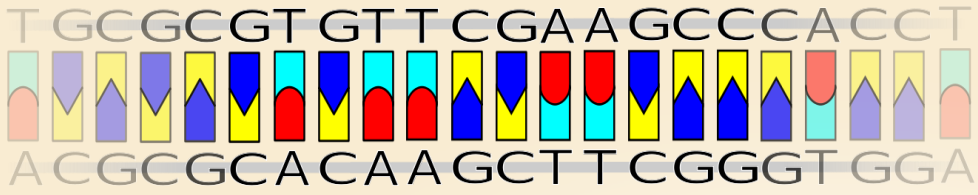
Restriction–modification systems allow bacterial cells to distinguish between their own DNA and any foreign DNA entering the cell, and to destroy the latter. They operate through two enzyme activities: a restriction endonuclease that cleaves the foreign DNA, and a modification methyltransferase that protects the host DNA.

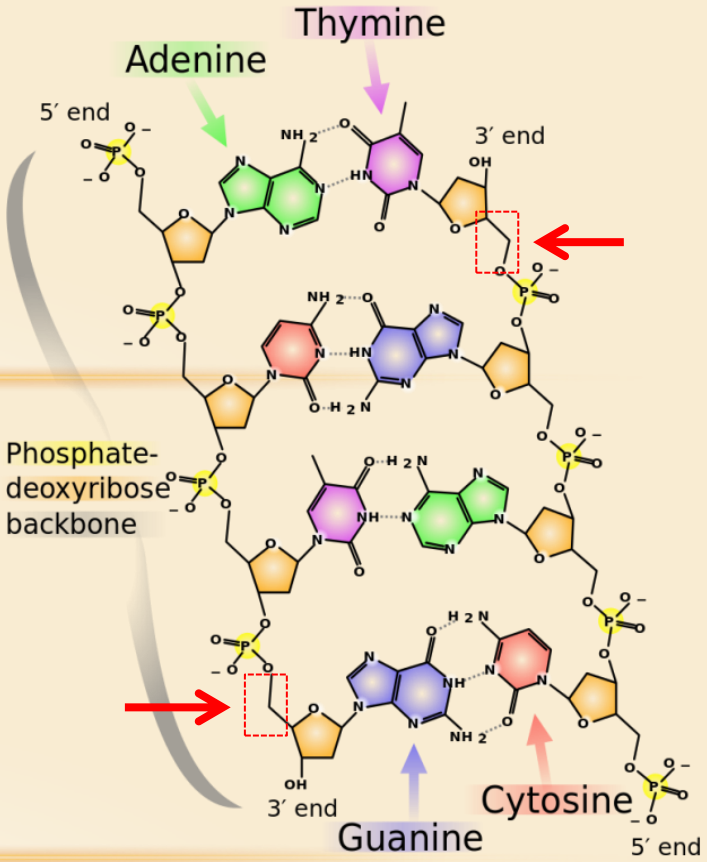
DNA Ligase DNA 连接酶

DNA ligase is an enzyme that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond.



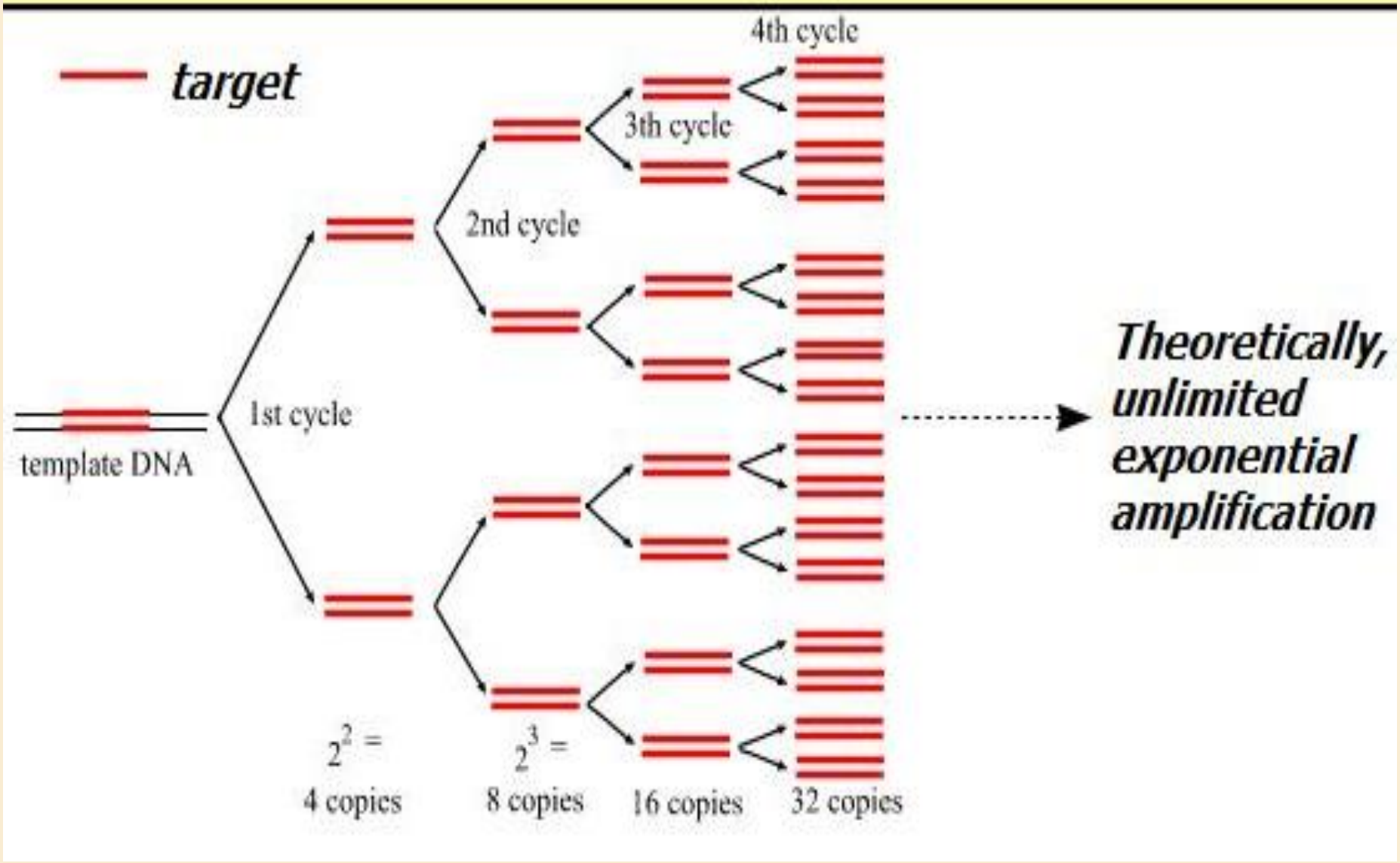
DNA Ligase



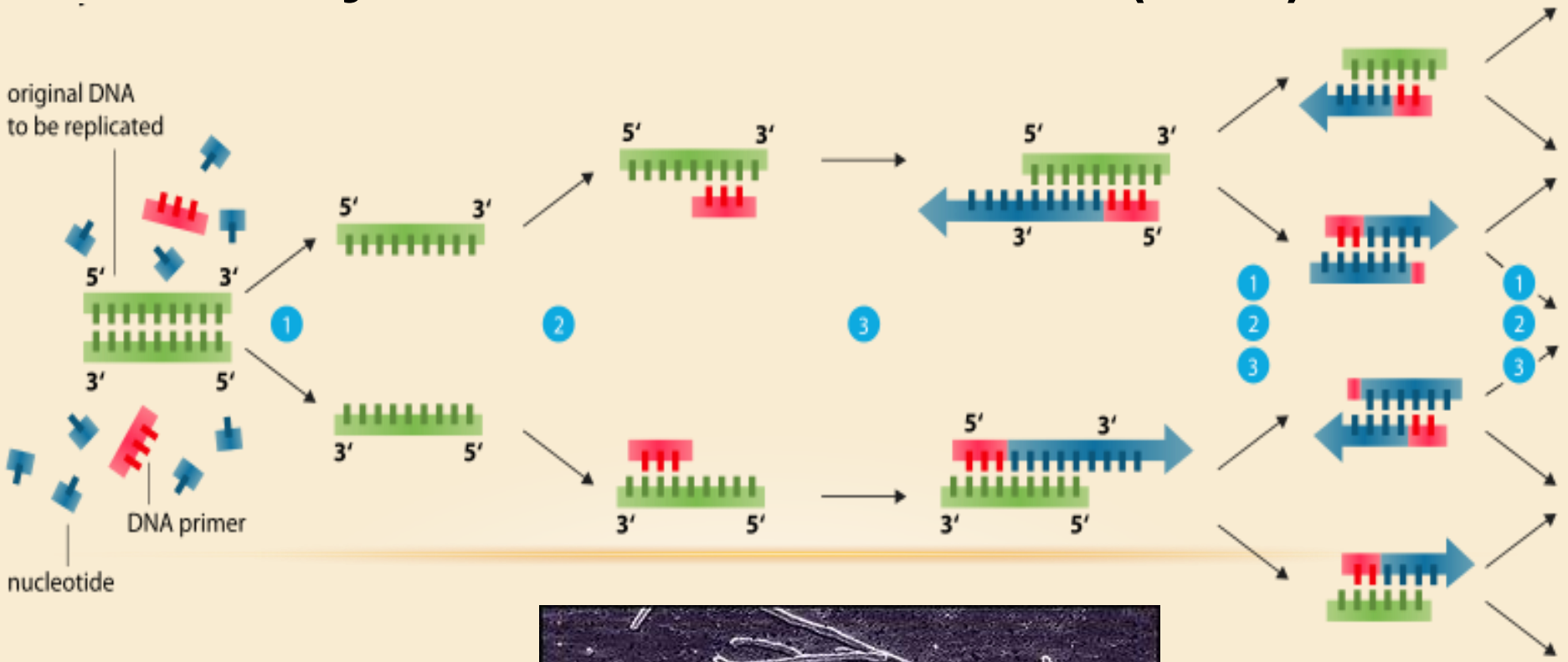



DNA Recombination

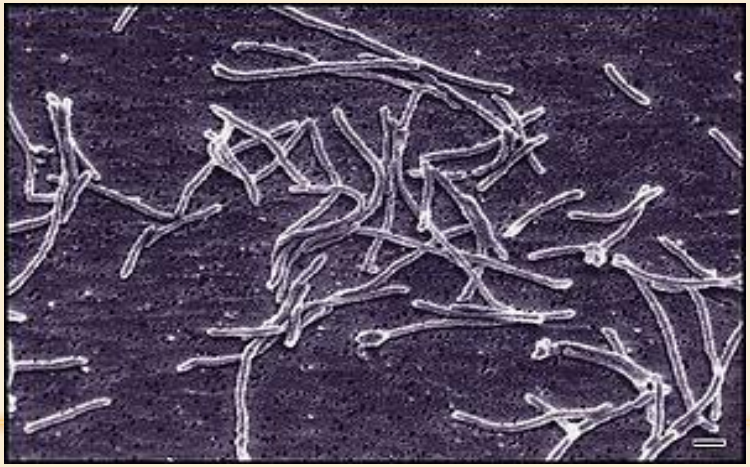
Polymerase Chain Reaction (PCR)



Polymerase Chain Reaction (PCR)



1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C



DNA polymerase from
Thermus aquaticus

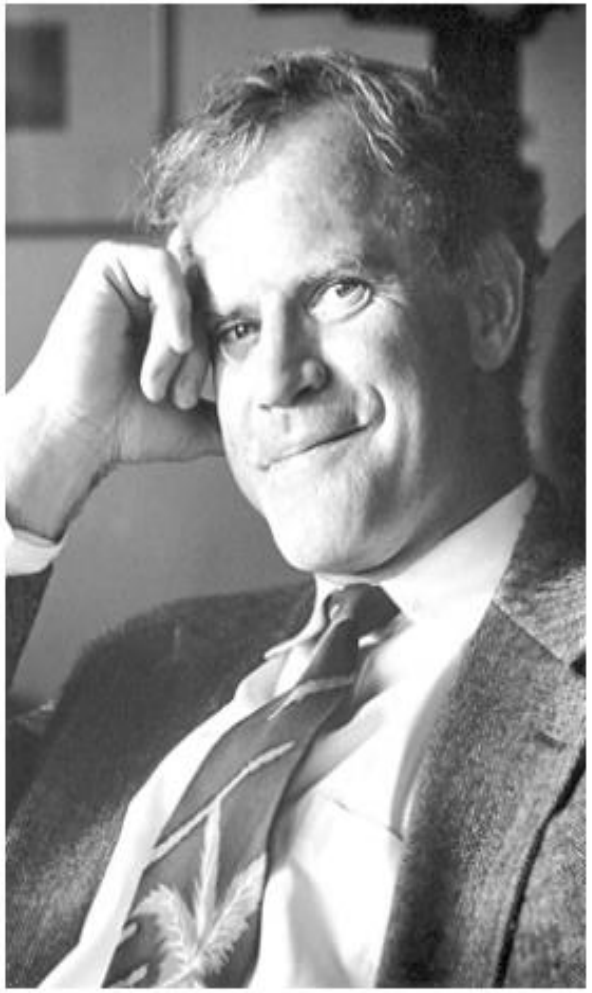
PCR

- **Primer**
- **Polymerase**
- **Buffer**
- **Single Strand**

Polymerase Chain Reaction (PCR)

The Nobel Prize in Chemistry 1993

Kary B. Mullis - Facts



Kary B. Mullis

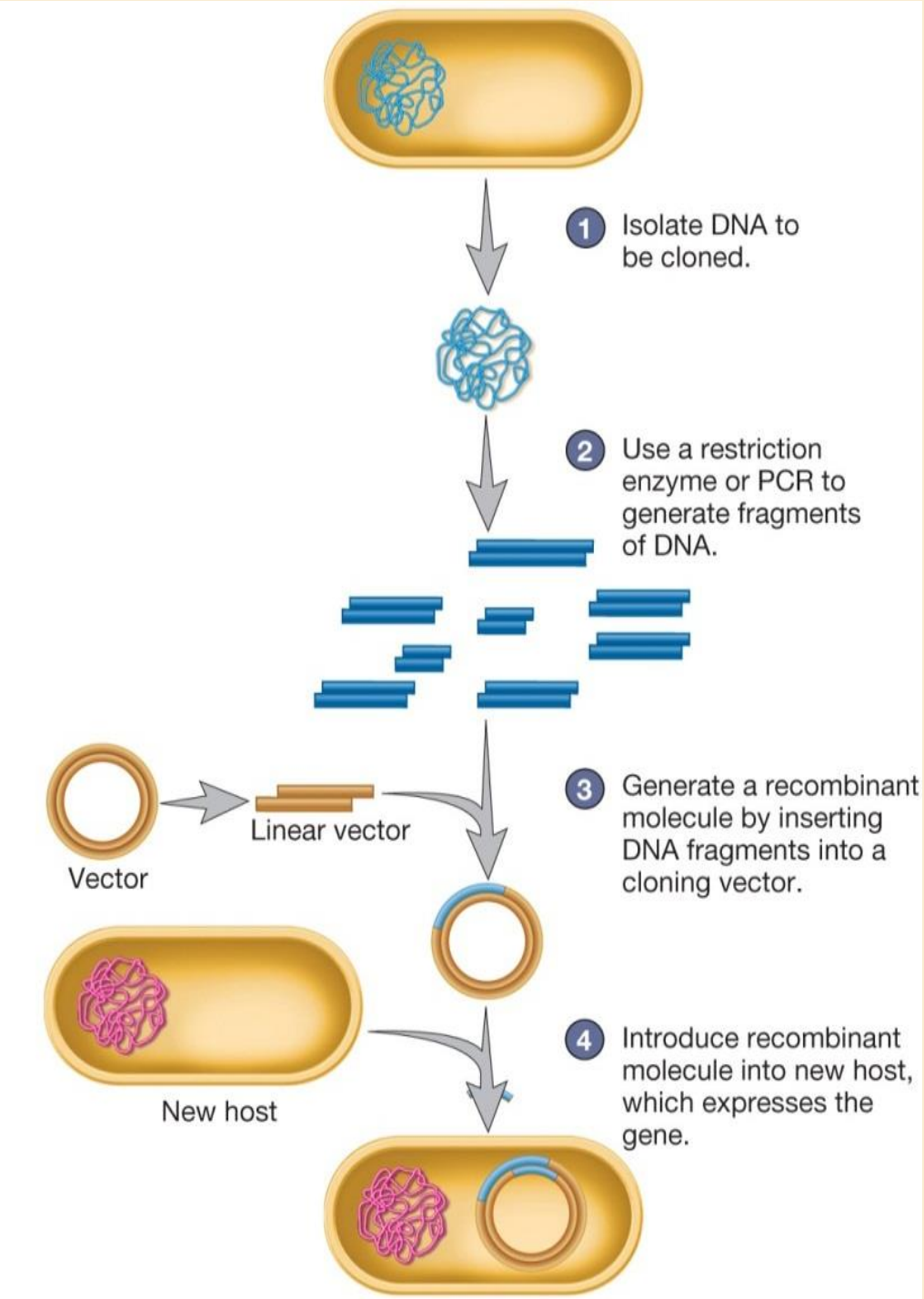
Born: 28 December 1944, Lenoir, NC, USA

Prize motivation: "for his invention of the polymerase chain reaction (PCR) method"

Field: biochemistry

- **Genetic engineering**
  - **deliberate modification of organism's genetic information by directly changing the sequence of nucleic acids in its genome**
- **Recombinant DNA technology**
  - **procedures used to carry out genetic engineering**

Flowchart of Genetic Engineering



Cloning Vectors

Key

- there are four types of cloning <u>vectors</u>
  - Plasmids (most commonly used) 质粒
  - Phages and viruses
  - Cosmids (artificial)
  - Artificial chromosomes

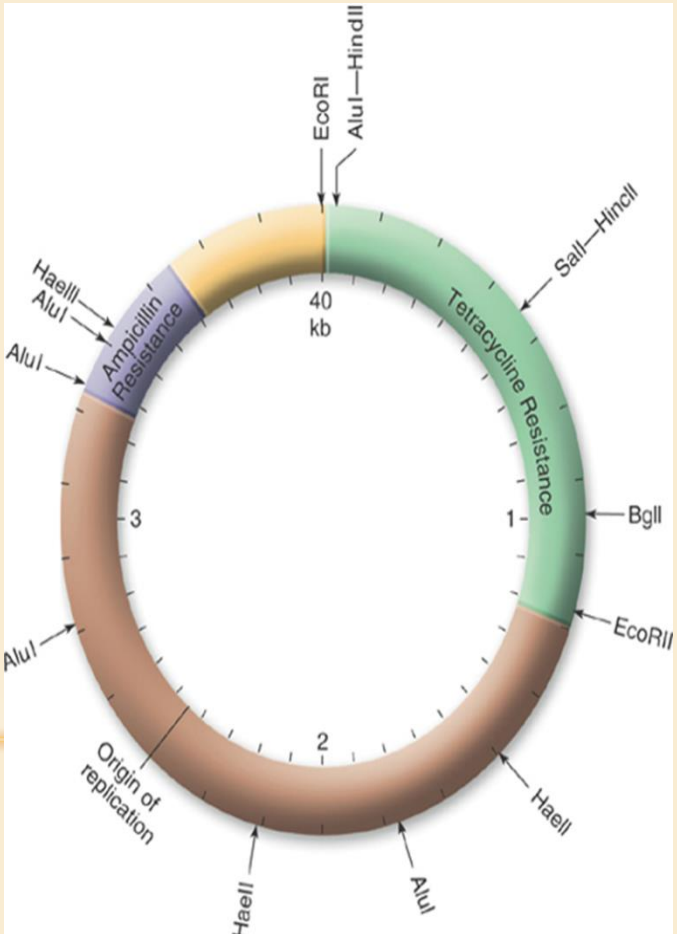


Table 15.3 Recombinant DNA Cloning Vectors

Vector	*Insert Size (kb, 1 kb = 1,000 bp)*	*Example*	*Features*
Plasmid	<20 kb	pBR322, pUC19	Replicates independently of microbial chromosome so many copies may be maintained in a single cell
Bacteriophage	9–25 kb	λ 1059, λ gt11, M13mp18, EMBL3	Packaged into lambda phage particles; single-stranded DNA viruses such as M13 have been modified (e.g., M13mp18) to generate either double- or single-stranded DNA in the host.
Cosmids	30–47 kb	pJC720, pSupercos	Can be packaged into lambda phage particles for efficient introduction into bacteria, then replicates as a plasmid
PACs (P1 artificial chromosomes)	75–100 kb	pPAC	Based on the bacteriophage P1 packaging mechanism
BACs (bacterial artificial chromosomes)	75–300 kb	pBAC108L	Modified F plasmid that can carry large DNA inserts; very stable within the cell
YACs (yeast artificial chromosomes)	100–1,000 kb	pYAC	Can carry largest DNA inserts; replicates in *Saccharomyces cerevisiae*

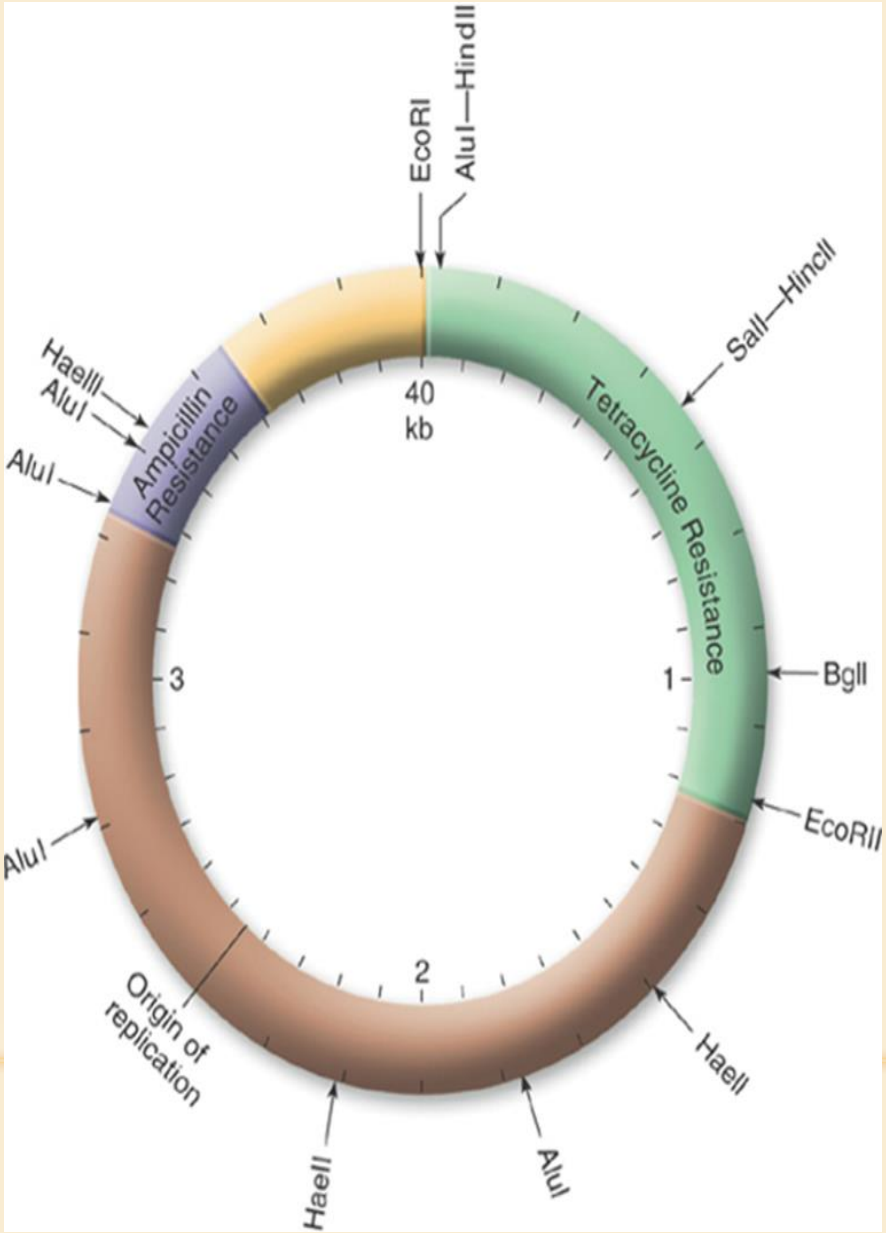
Key

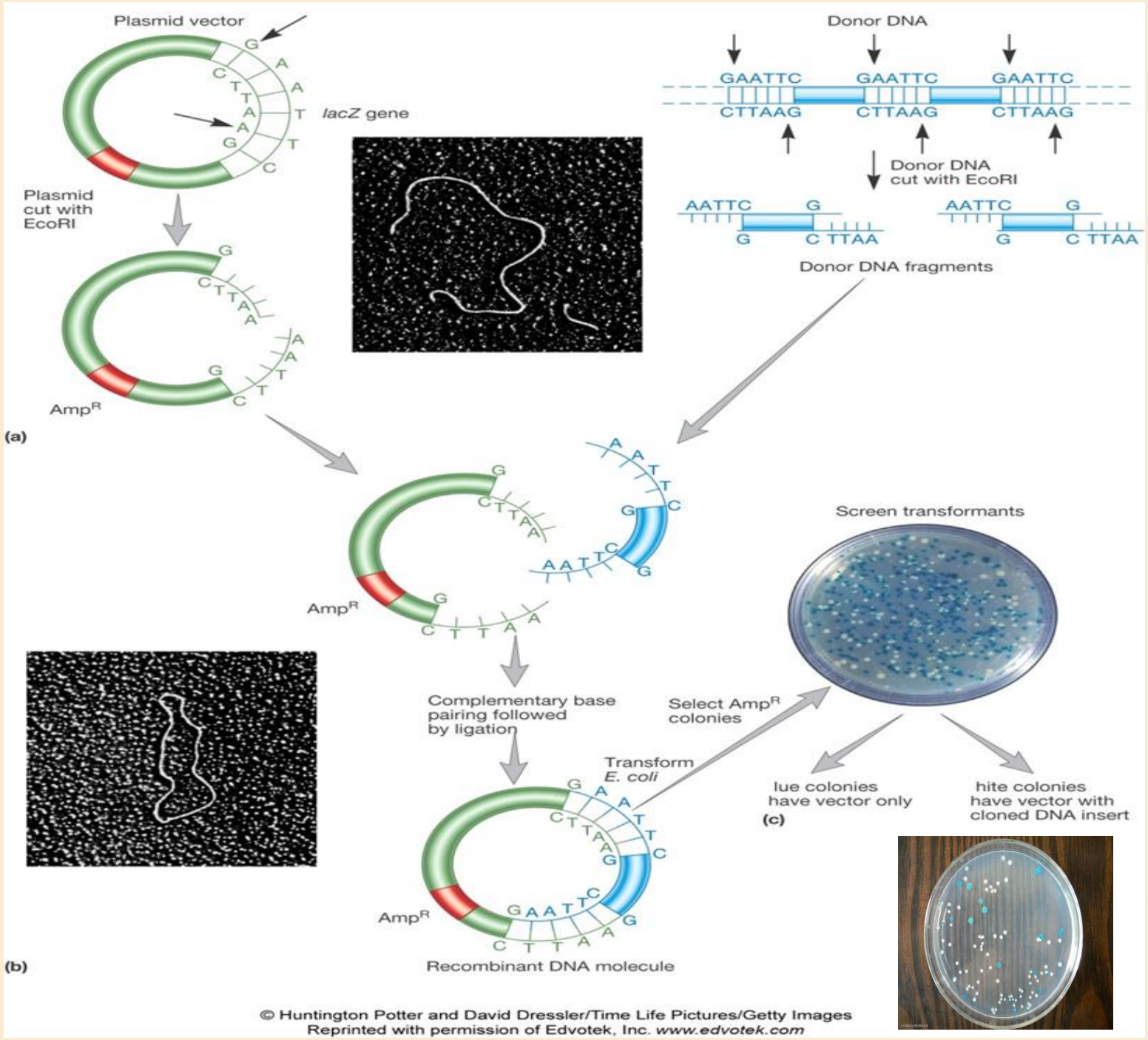
Plasmids

- Replicate autonomously and easy to purify
- Requirements for vectors
  - an Origin of Replication
  - a Selectable Marker
  - a Multicloning site
    - site that allows DNA to be inserted to the plasmid vector

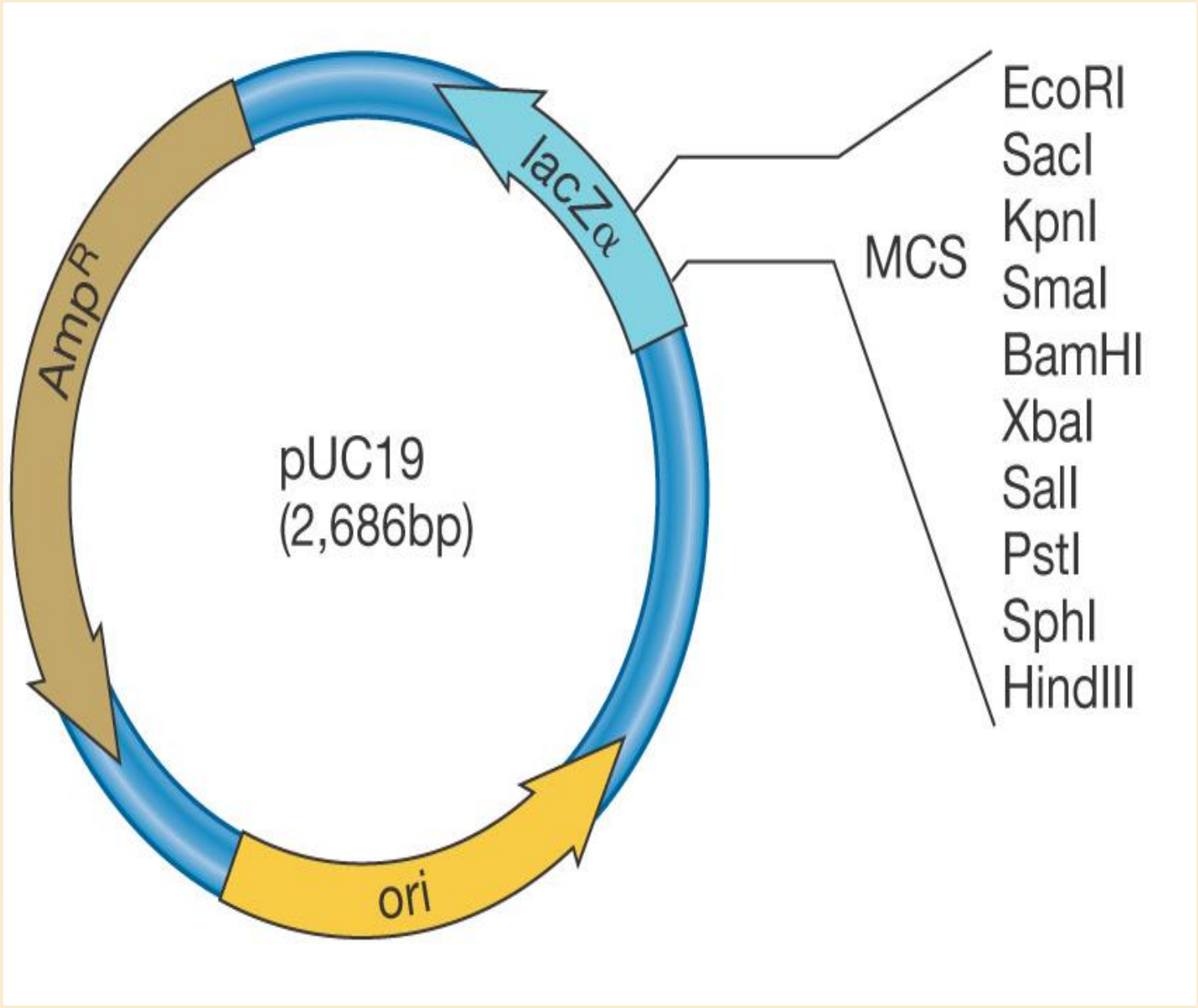
Plasmid as cloning vector

Key





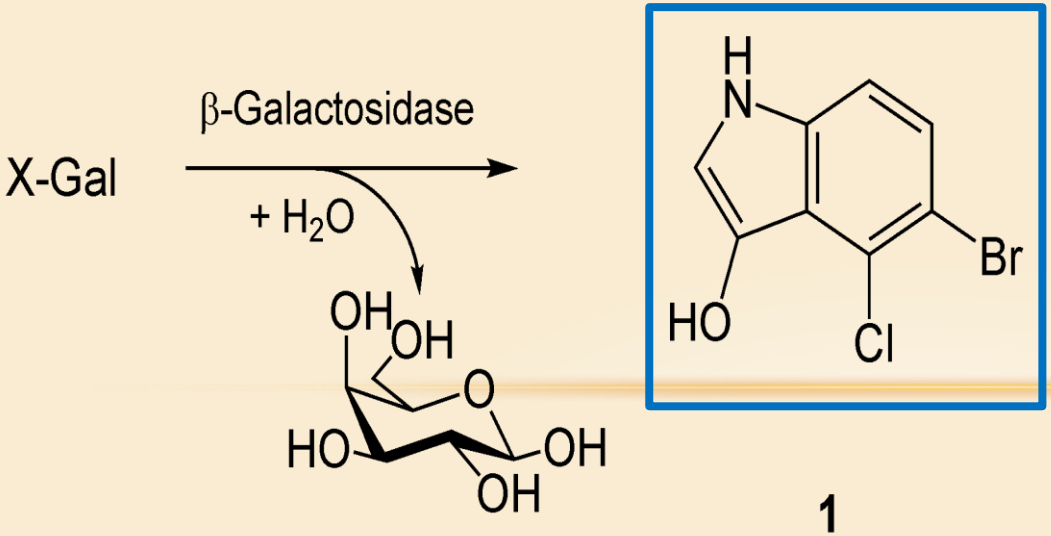
Clone vector for Recombinants Screening

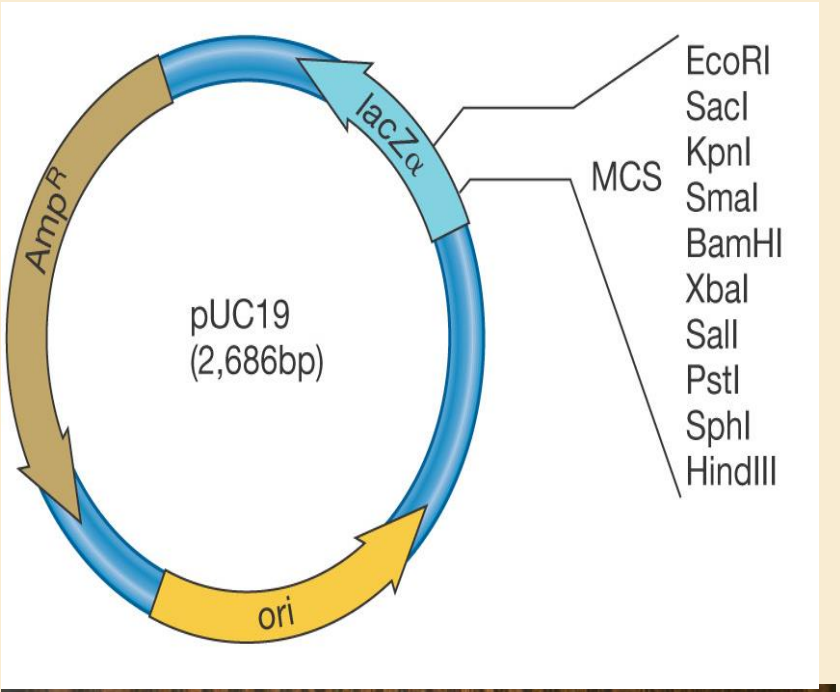


无色化合物(5-溴-4-氯-3-吲哚-β-D-半乳糖苷)

β-半乳糖苷酶

半乳糖 + 深蓝色化合物(5-溴-4-靛蓝)





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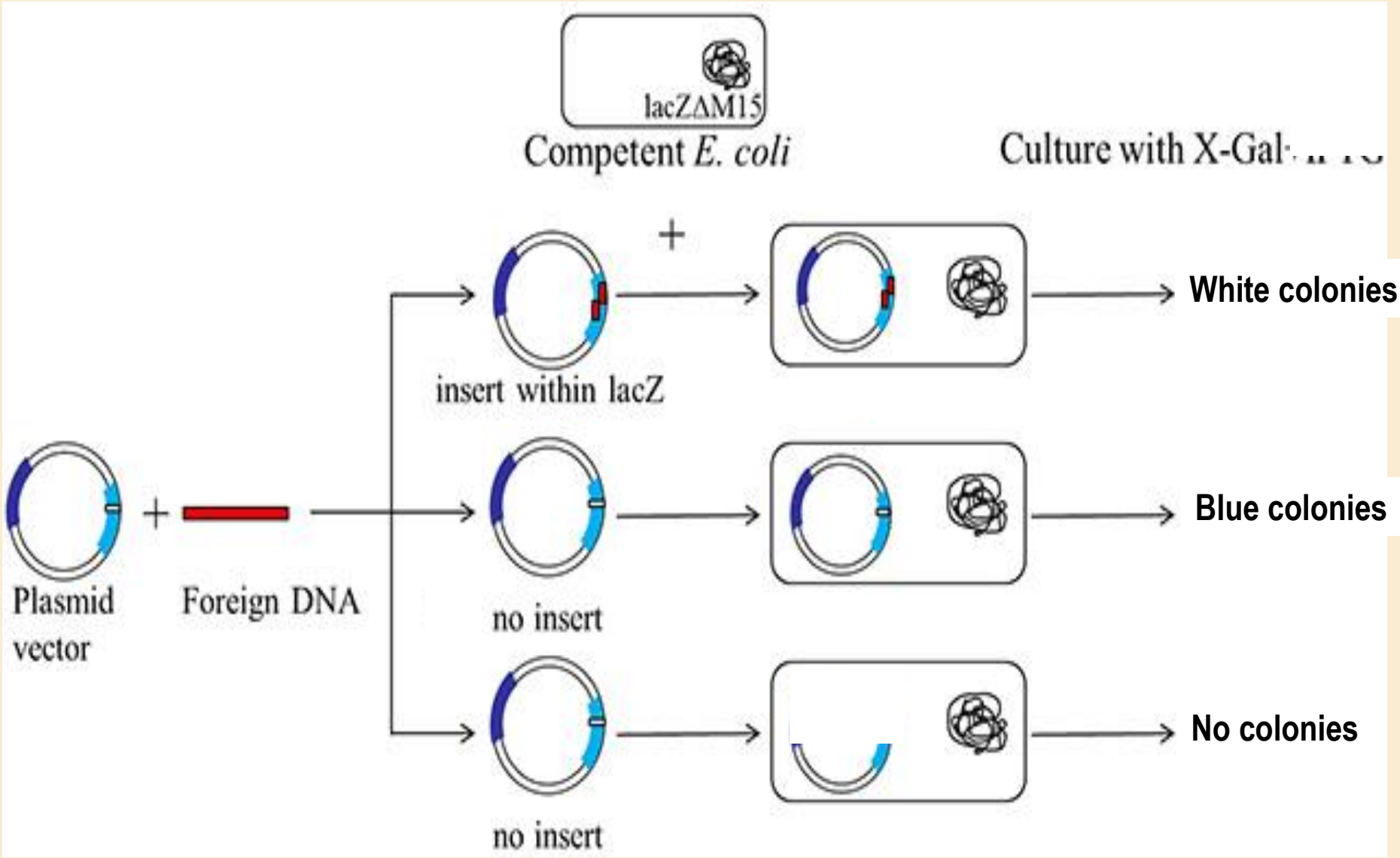
Blue White Screening for identifying the Recombinants



- **Most common used host cells**
  - ***E. coli* – bacteria**
  - ***S.cerevisiae* (酵母） – eukaryotic host**

Inserting recombinant DNA into eukaryotic host cells

- DNA introduction into microbes
  - transformation
  - electroporation 电击转化
  - gene gun 基因枪
  - Ti plasmid of *Agrobacterium tumefaciens* 农杆菌 (used to introduce foreign DNA into plant genomes)

Pioneer of Genetic Engineering

Stanley Cohen
Stanford University

Herbert Boyer
UCSF

Attempt to recombine gene from different bacteria into one DNA molecule

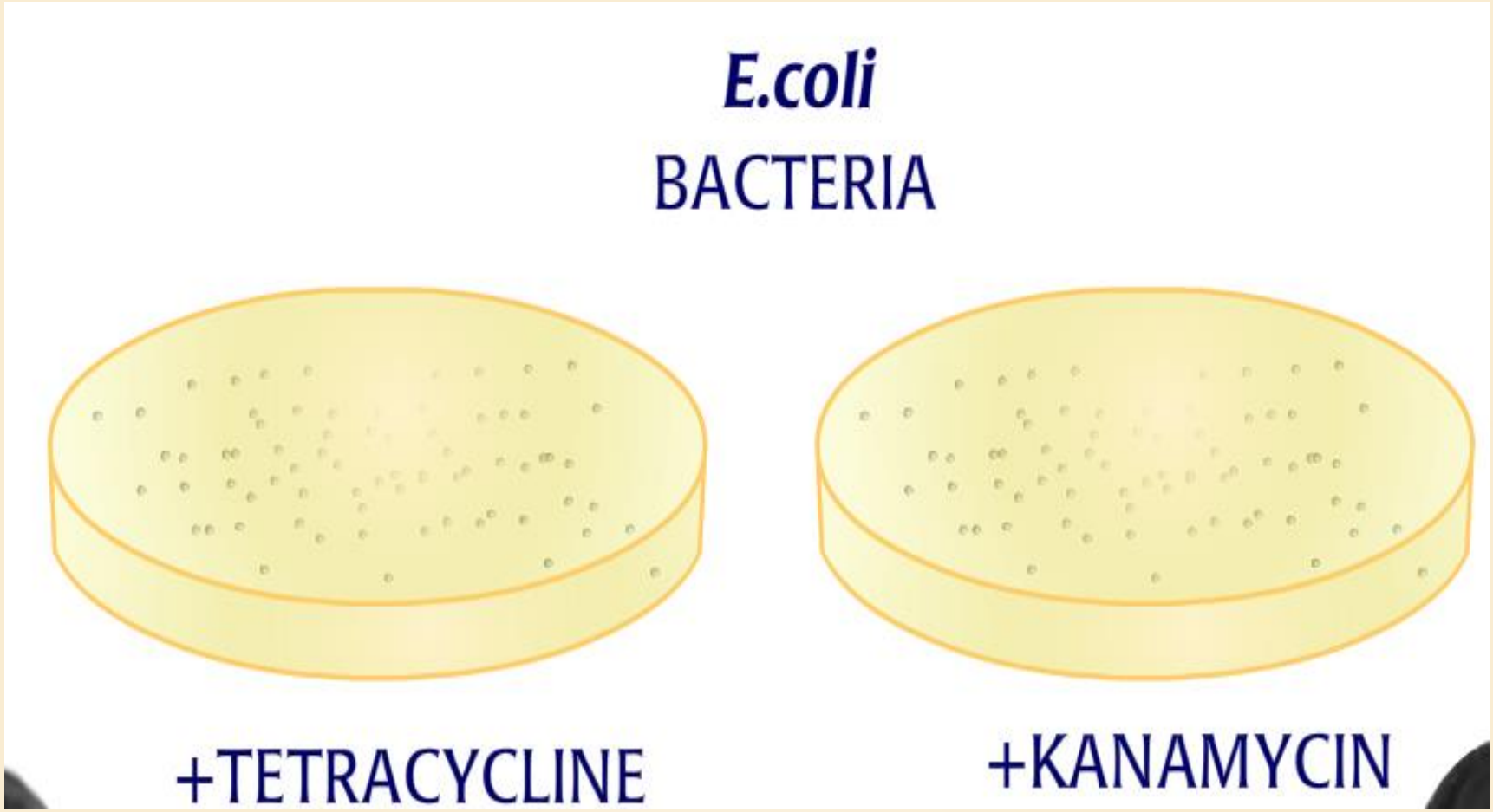
Stanley Cohen

Bacterial resistance to antibiotics

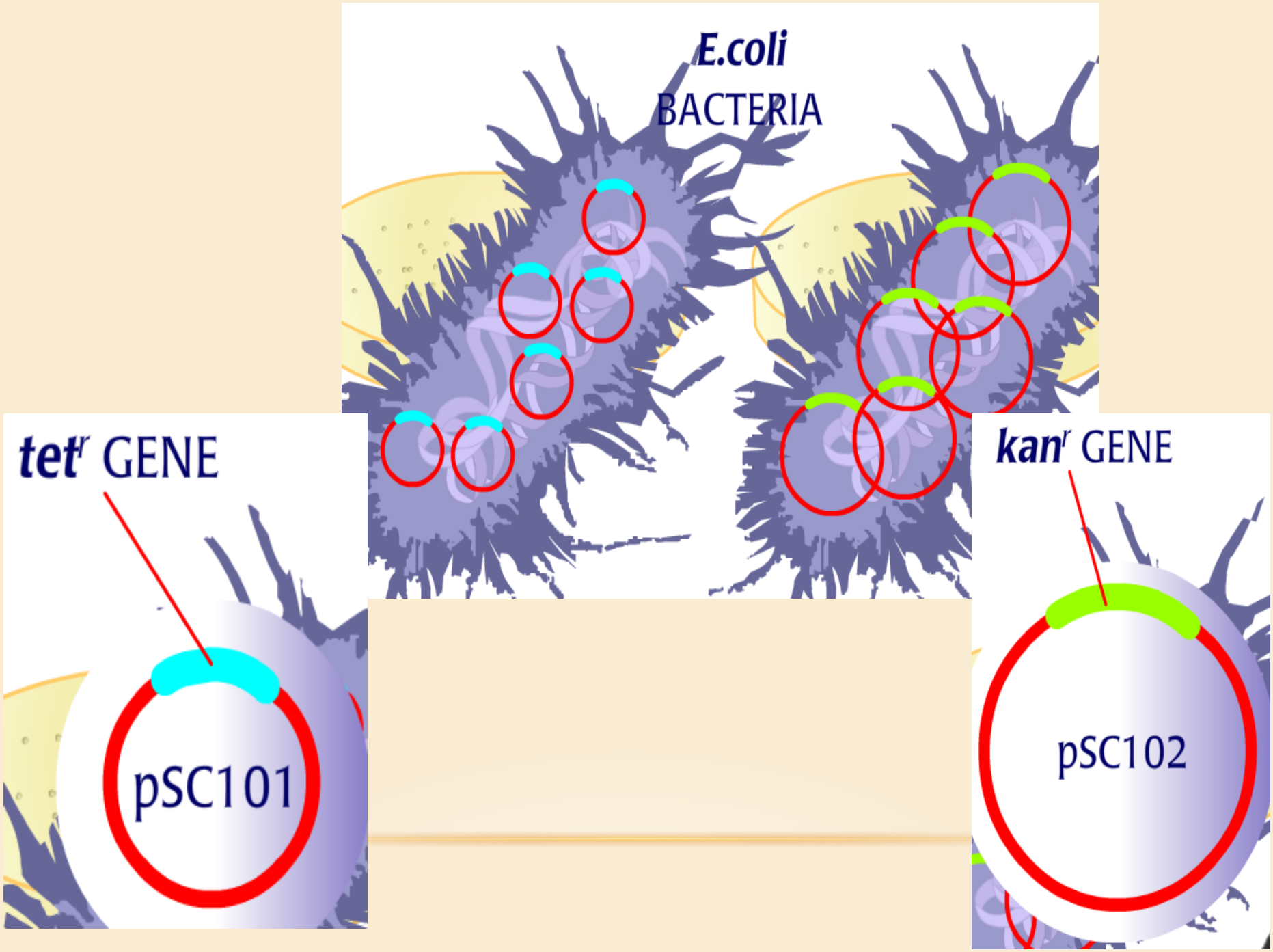
Herbert Boyer

Restriction Enzyme

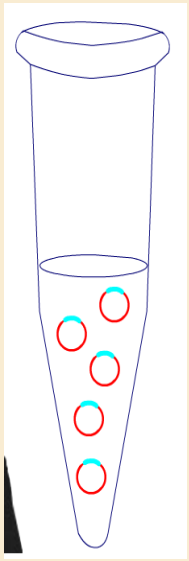
Hawaii, 1972

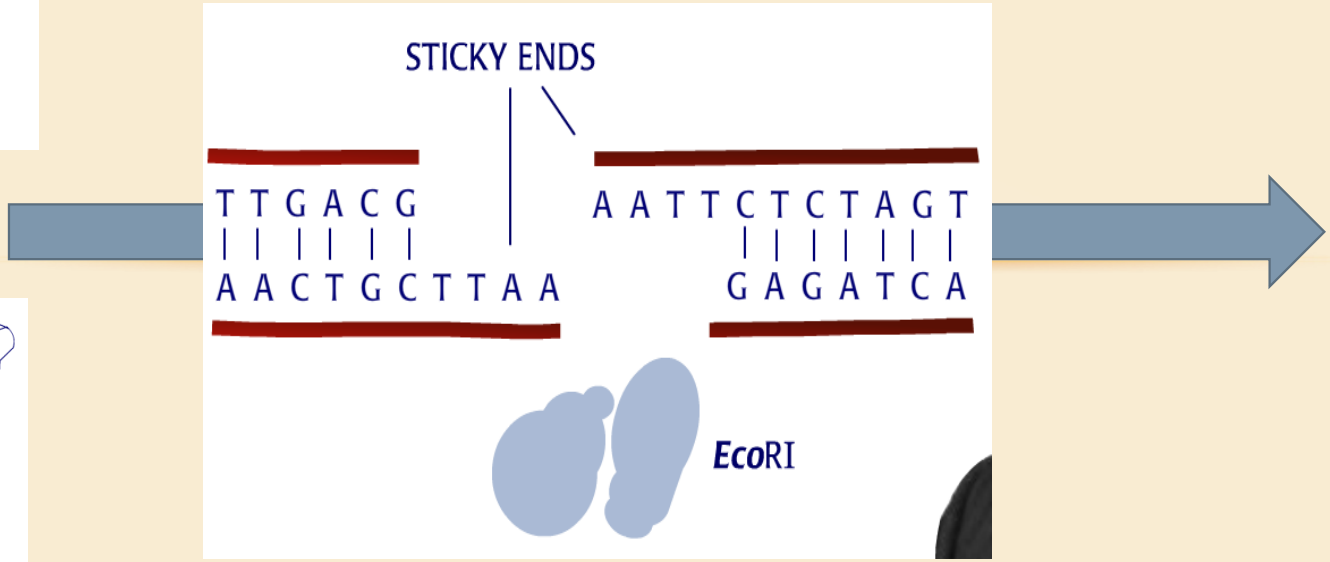


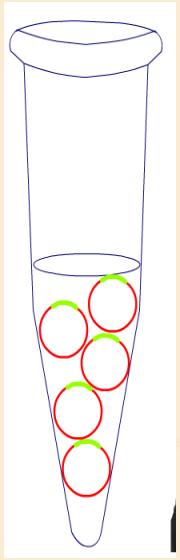
四环素　　卡那霉素


E.coli
BACTERIA
tetr GENE
pSC101
kanr GENE
pSC102

Plasmid digestion by Restriction enzyme
限制性内切酶酶切

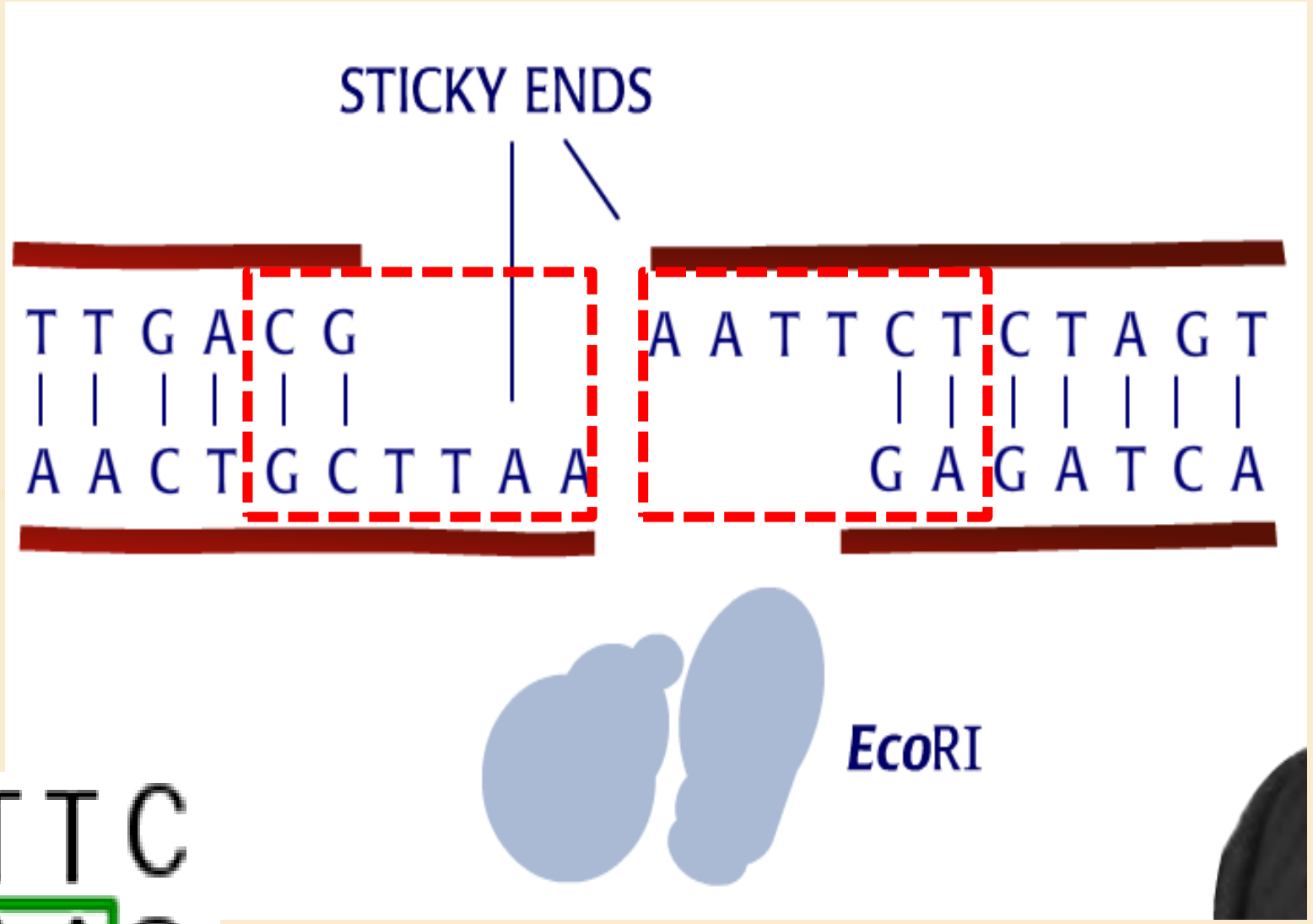






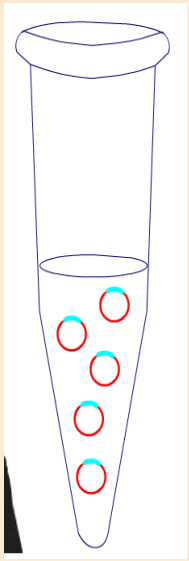
Restriction enzyme

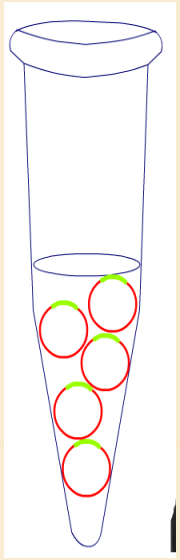
EcoRI

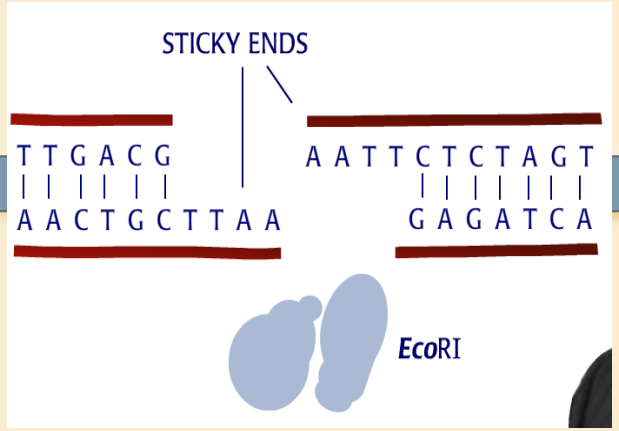


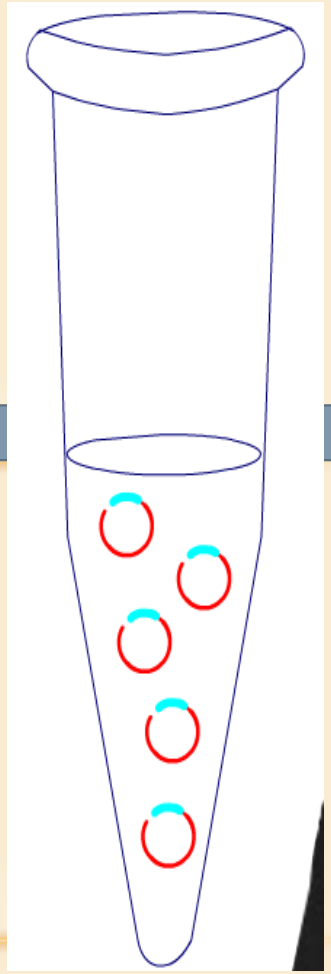
G A A T T C
C T T A A G

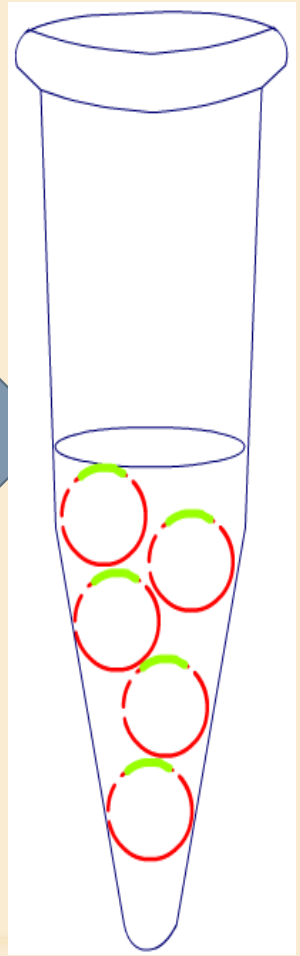
Plasmid digestion by Restriction enzyme
限制性内切酶酶切



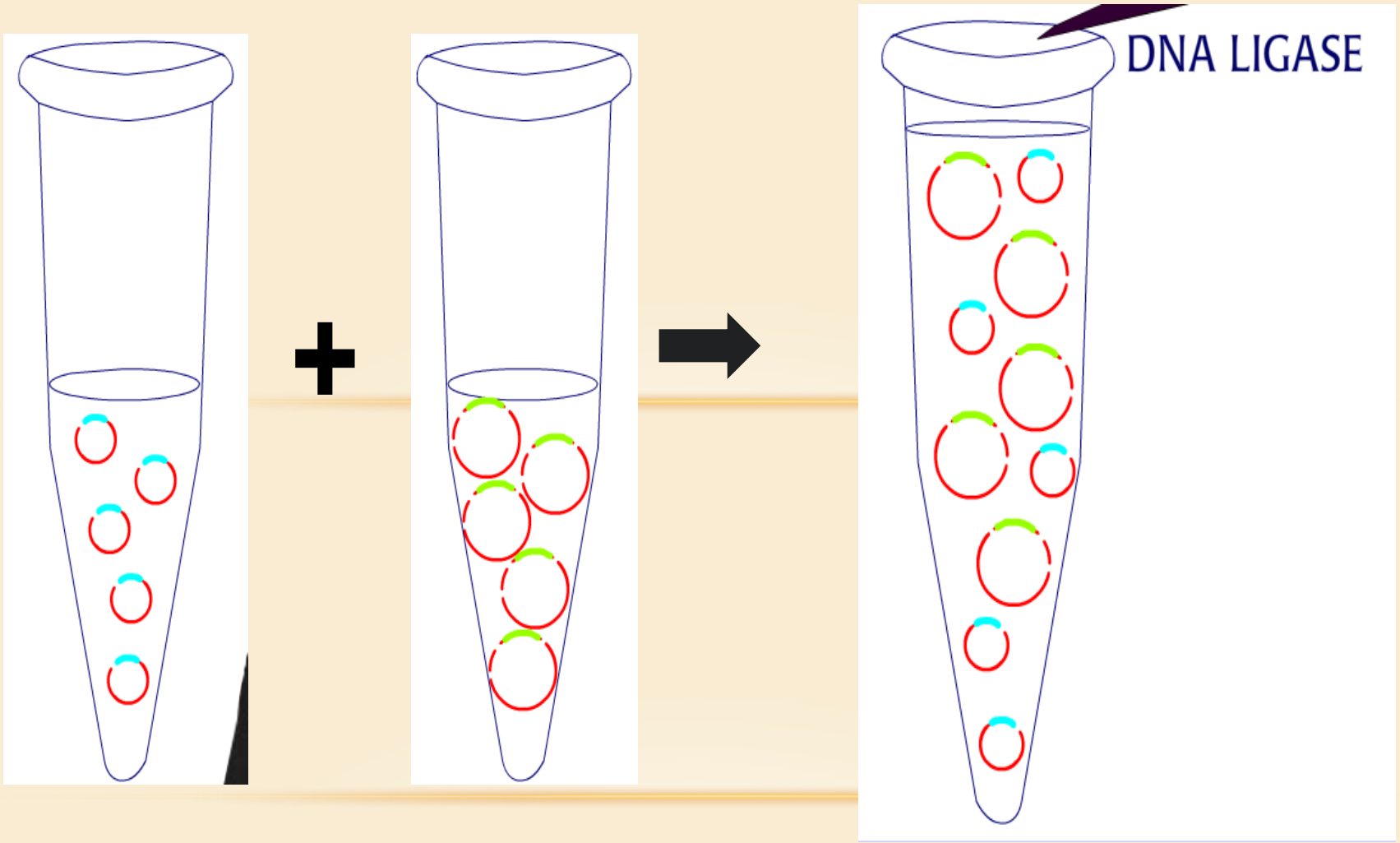








DNA Ligase DNA 连接酶



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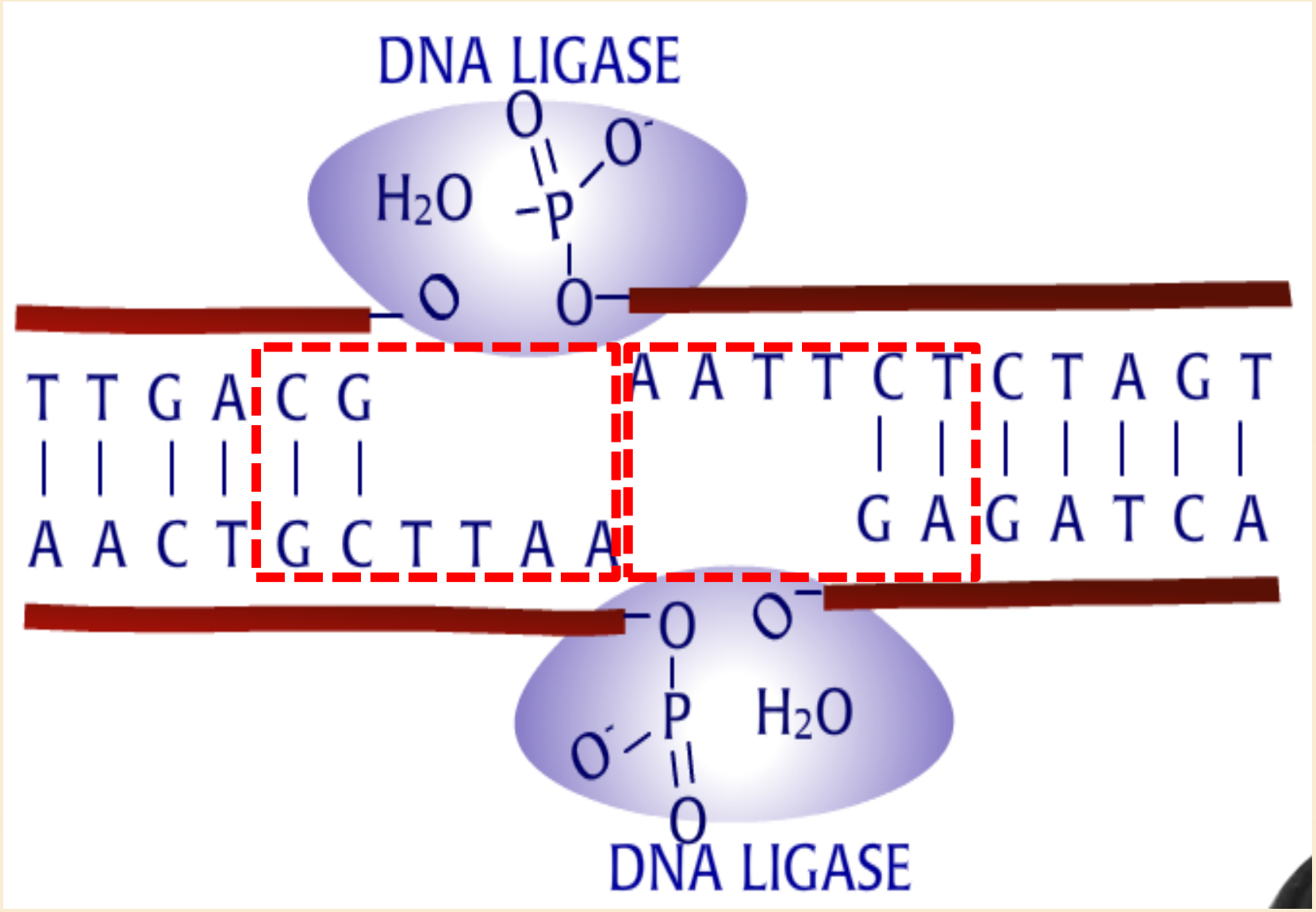
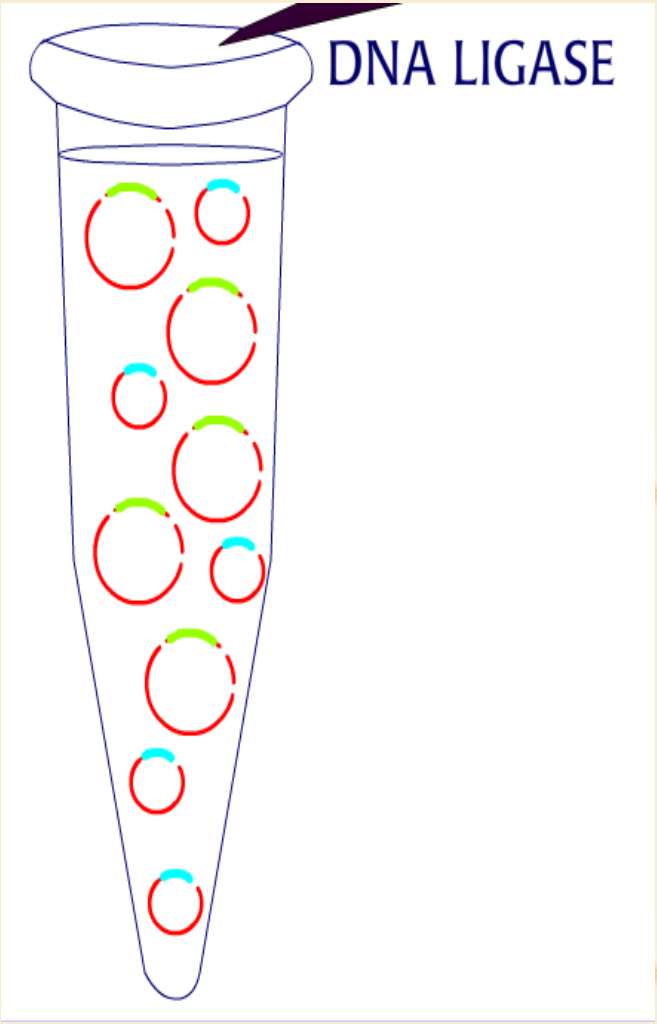
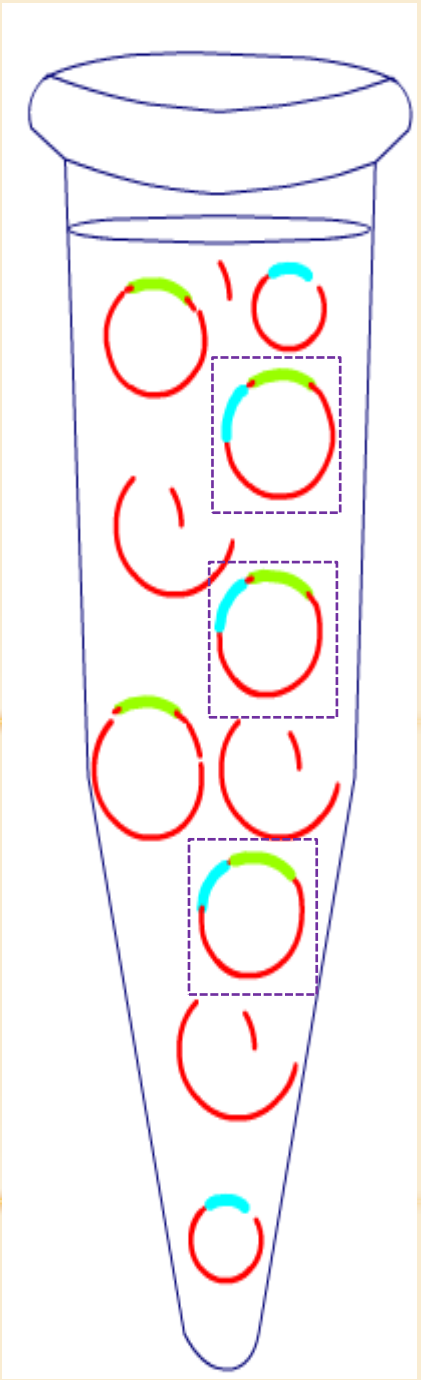




DNA Ligase

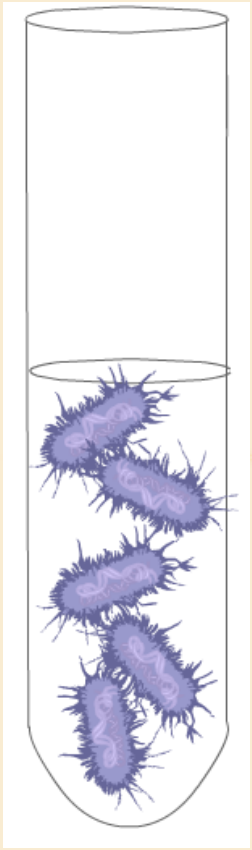


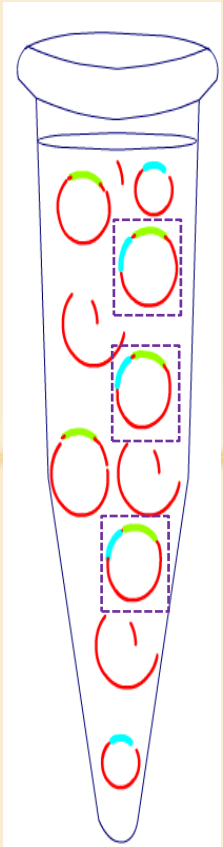
Tet+

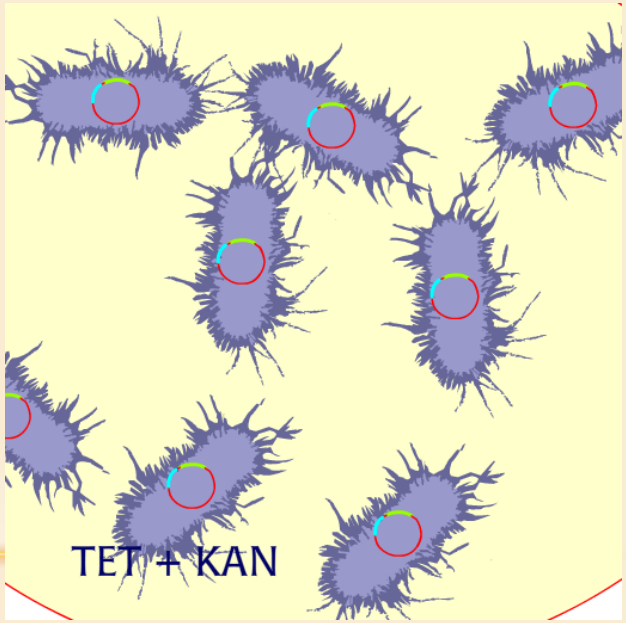
Kan+

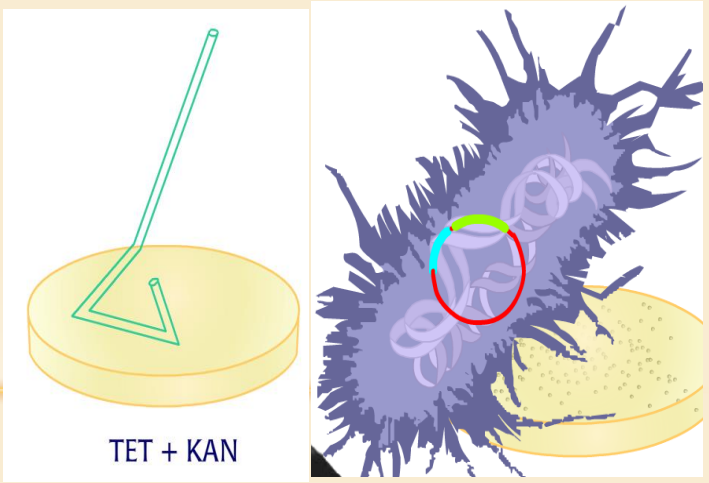
Kan+/Tet+

Plasmids transformed to Competent Cell



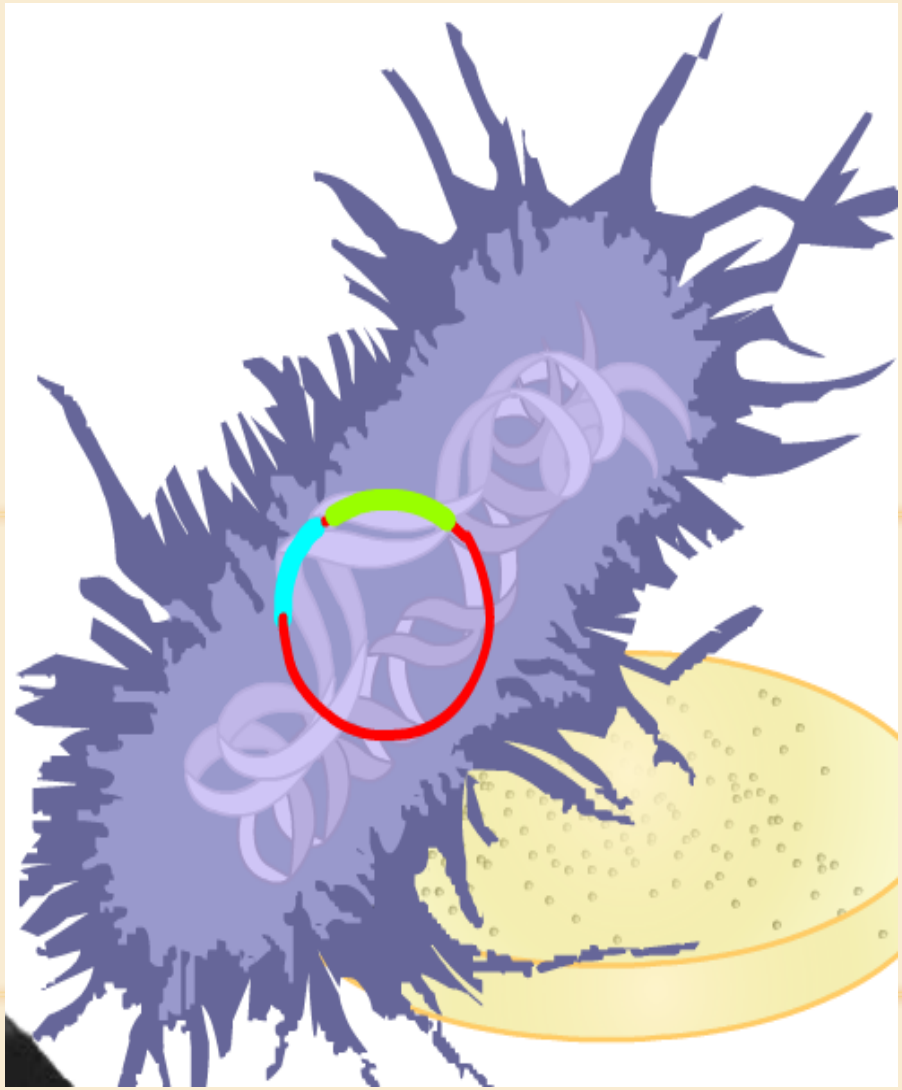




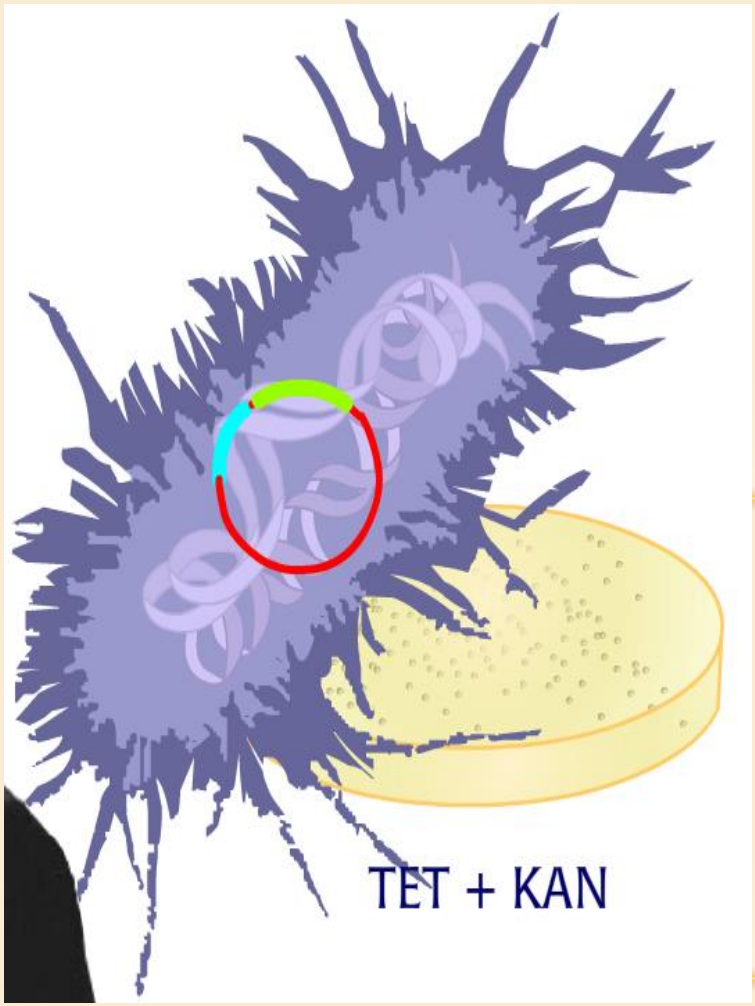


Doug Hanahan

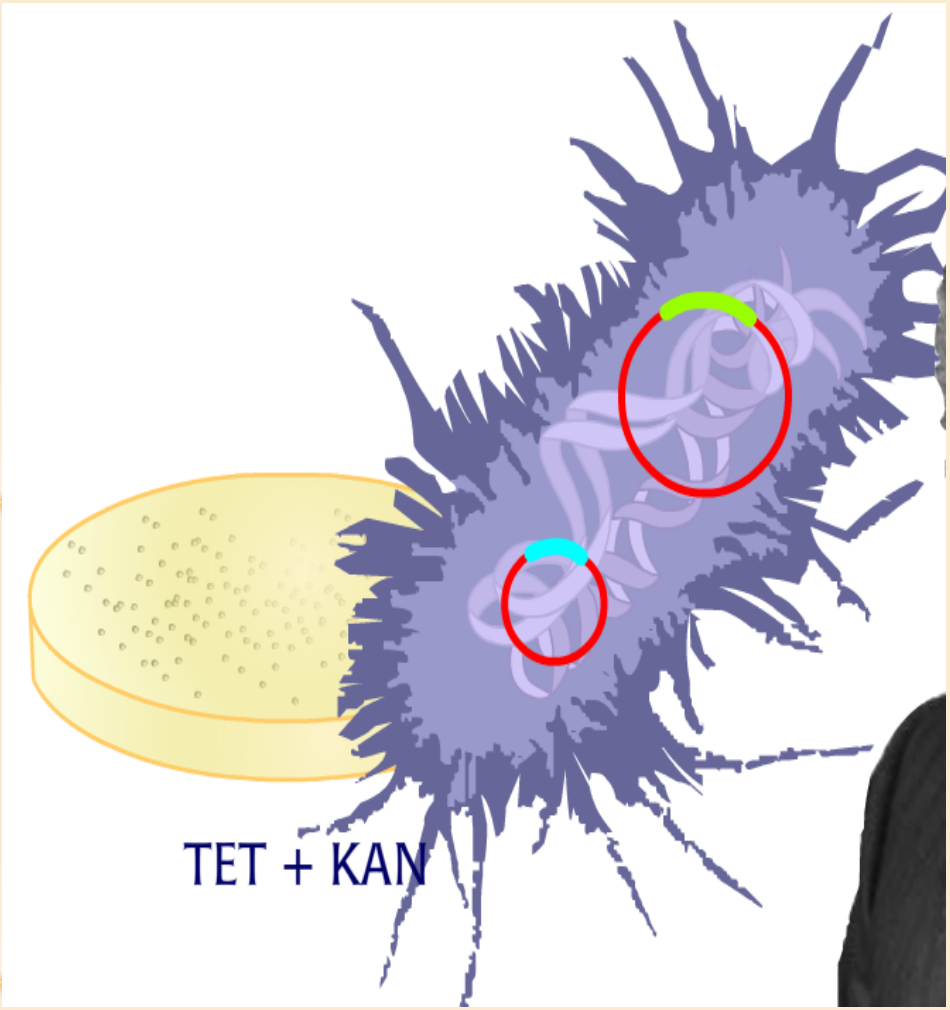
The First Recombinant Plasmid



One Possibility

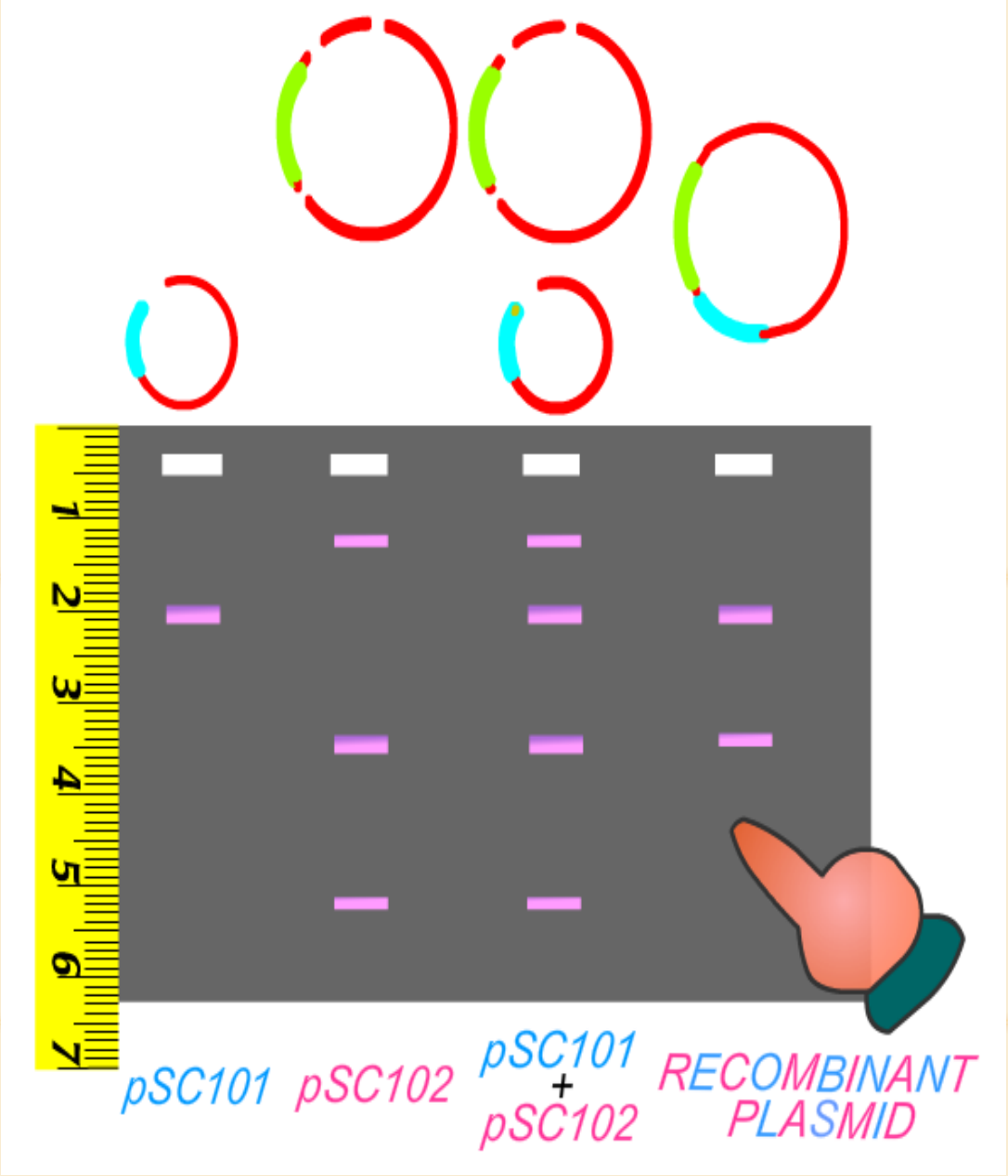


Recombinant Plasmid

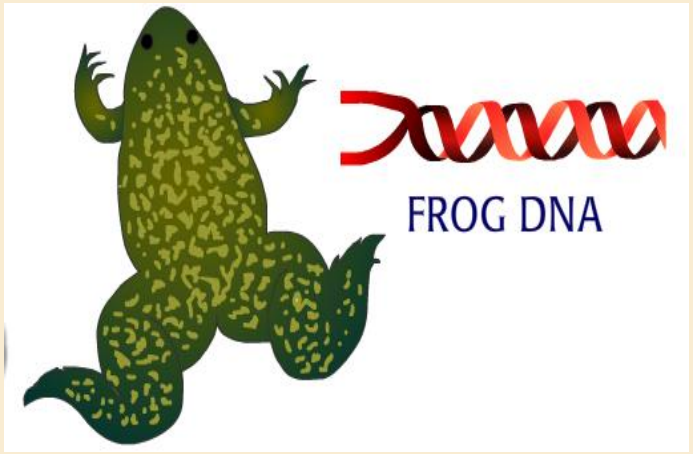


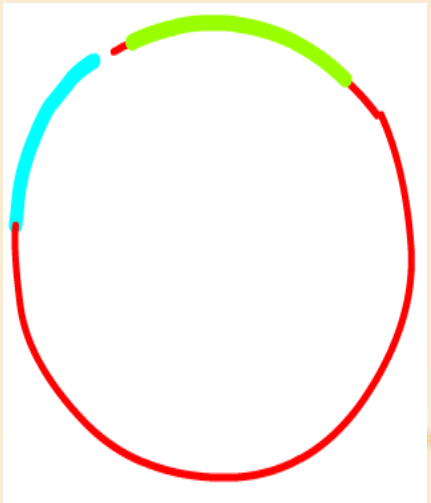
Twoe re-ligated plasmids

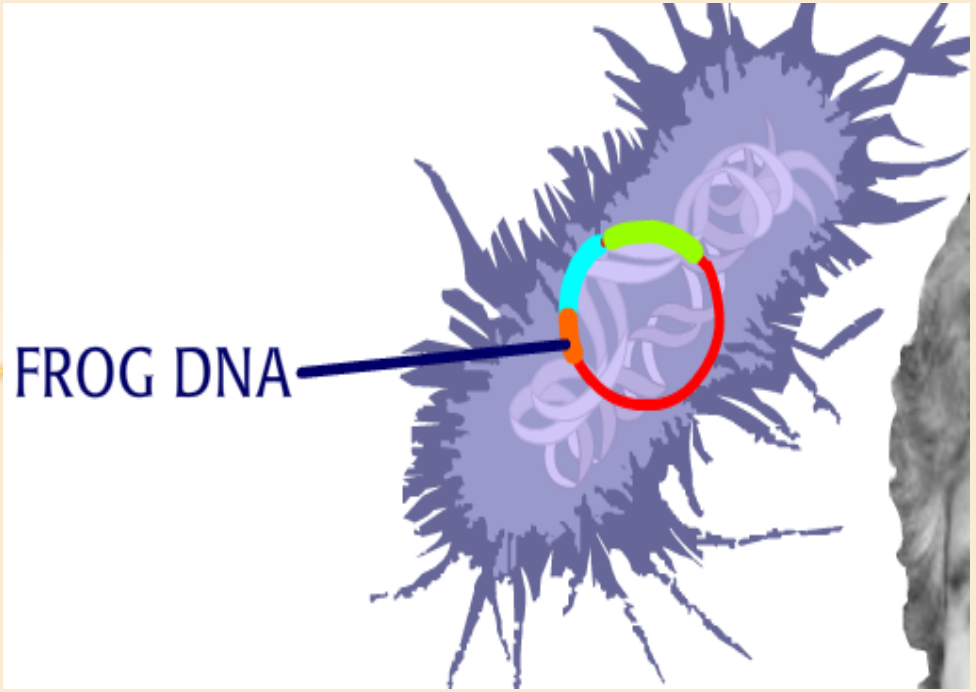
Recombinant Plasmid Characterization by Restriction Enzyme



Frog Gene recombined to the E.coli plasmid

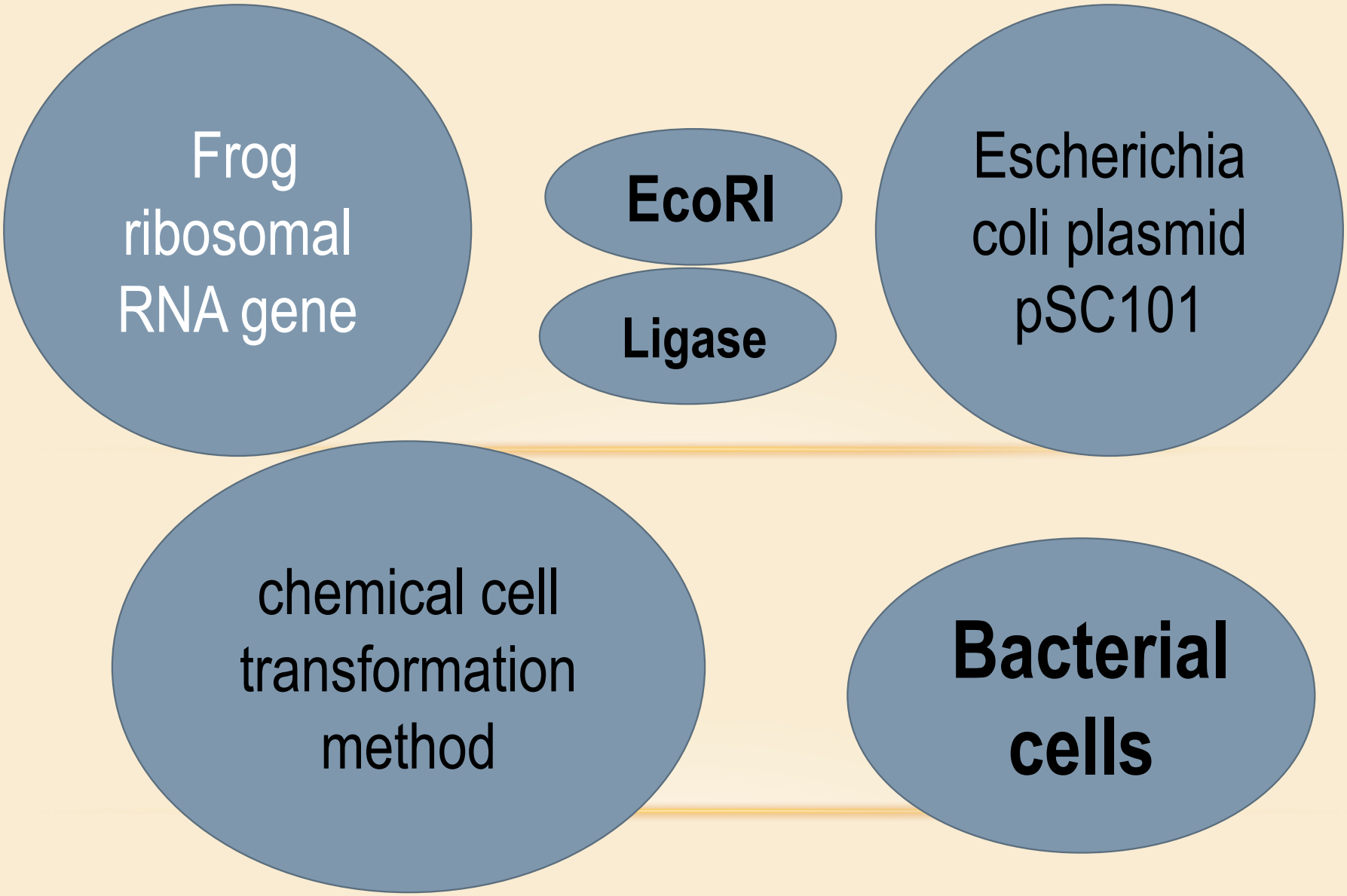






Frog rRNA gene

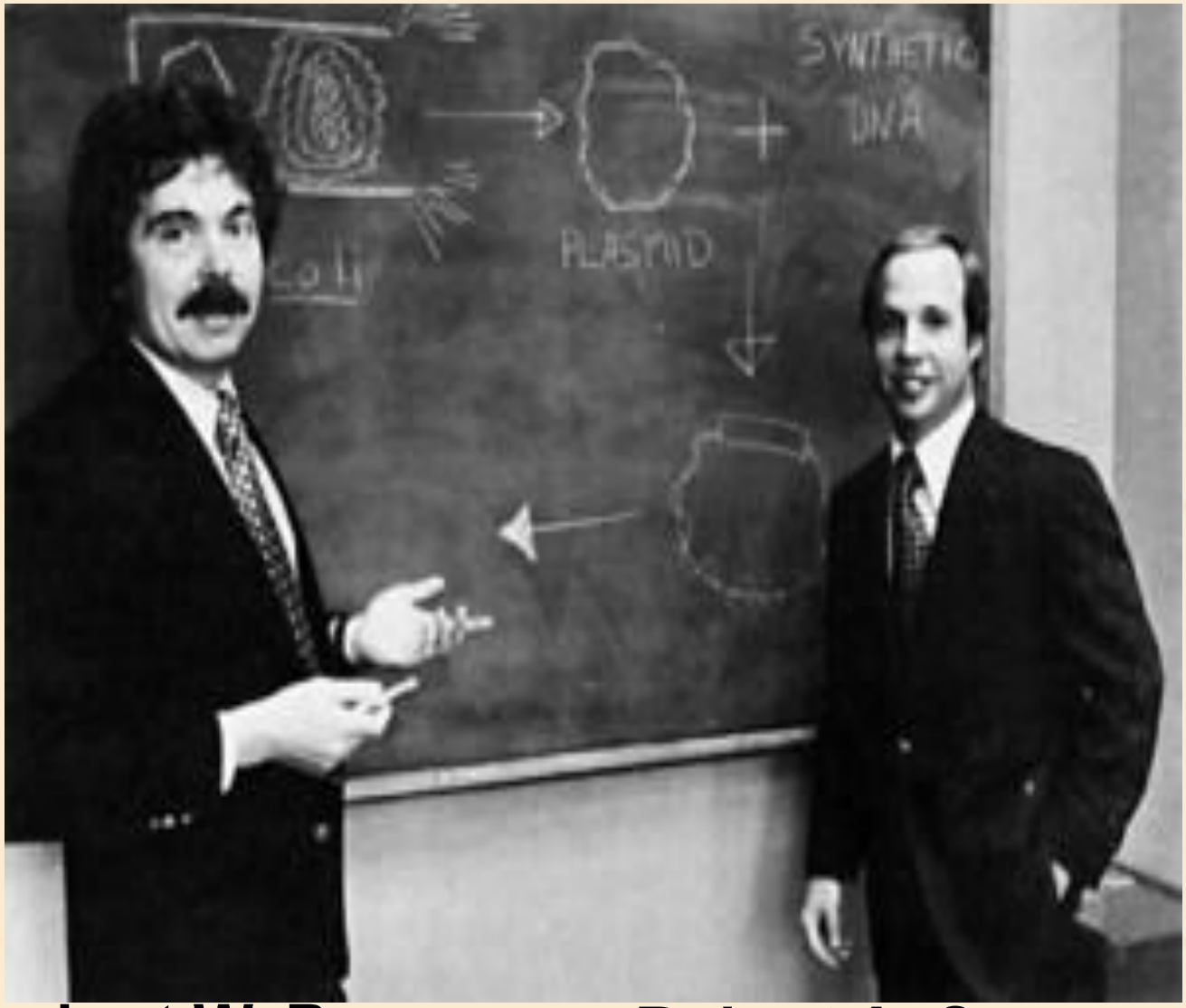
First Time Genetic Engineering



Genetic Engineering invented by "Boyer and Cohen" a quick and easy way to make chemicals like:

HGH (human growth hormone),
synthetic insulin,
factor VIII for hemophilia 血友病,
somatostatin 生长抑制素for acromegaly 肢端肥大症
clot-dissolving agent tissue plasmogen activator (tPA)
……
$$$$$$

Bio-Science and Technology Make Fortune



Herbert W. Boyer Robert A. Swanson

Founder in 1976

Genentech

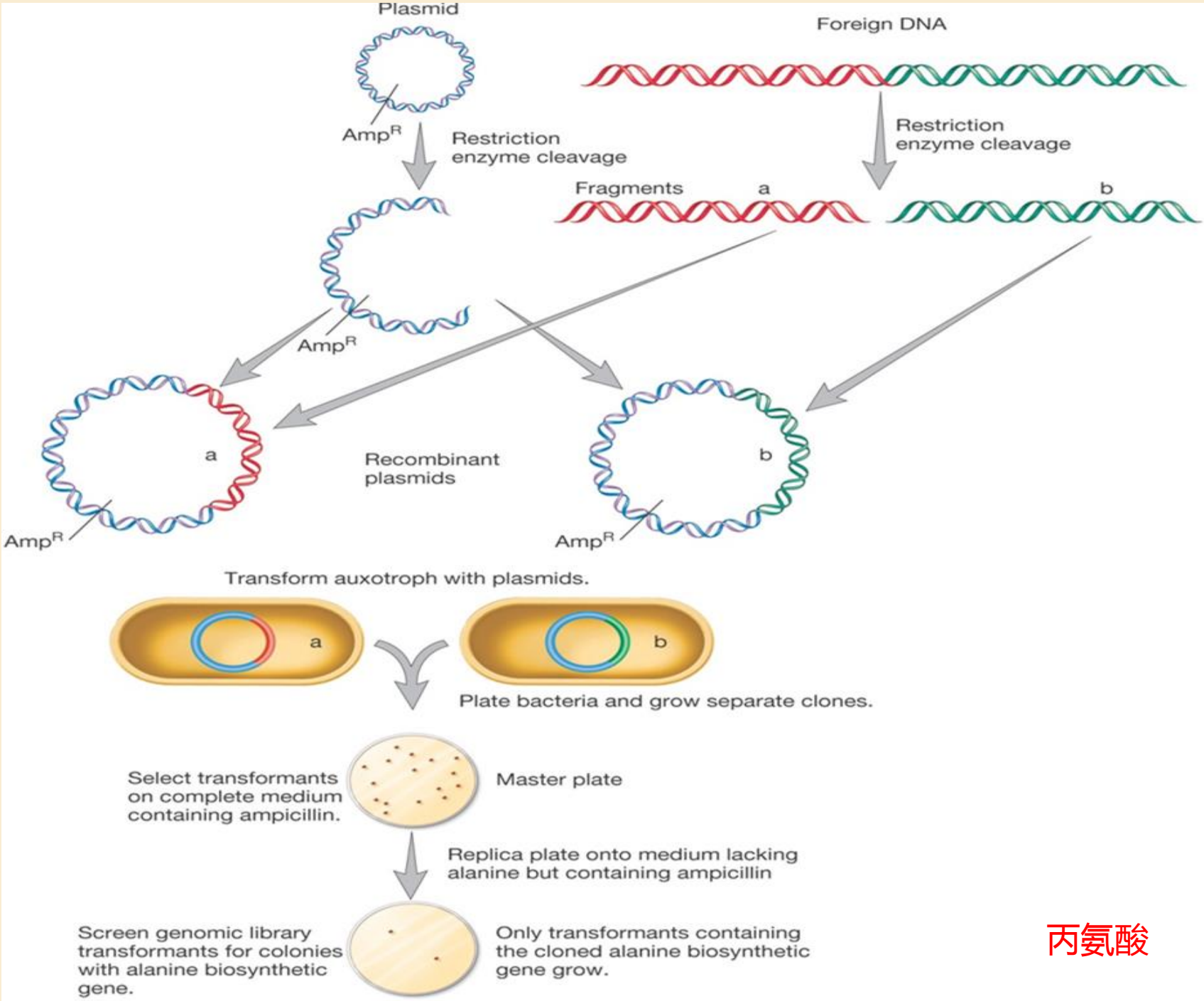
IN BUSINESS FOR LIFE

First Recombinant Insulin Protein

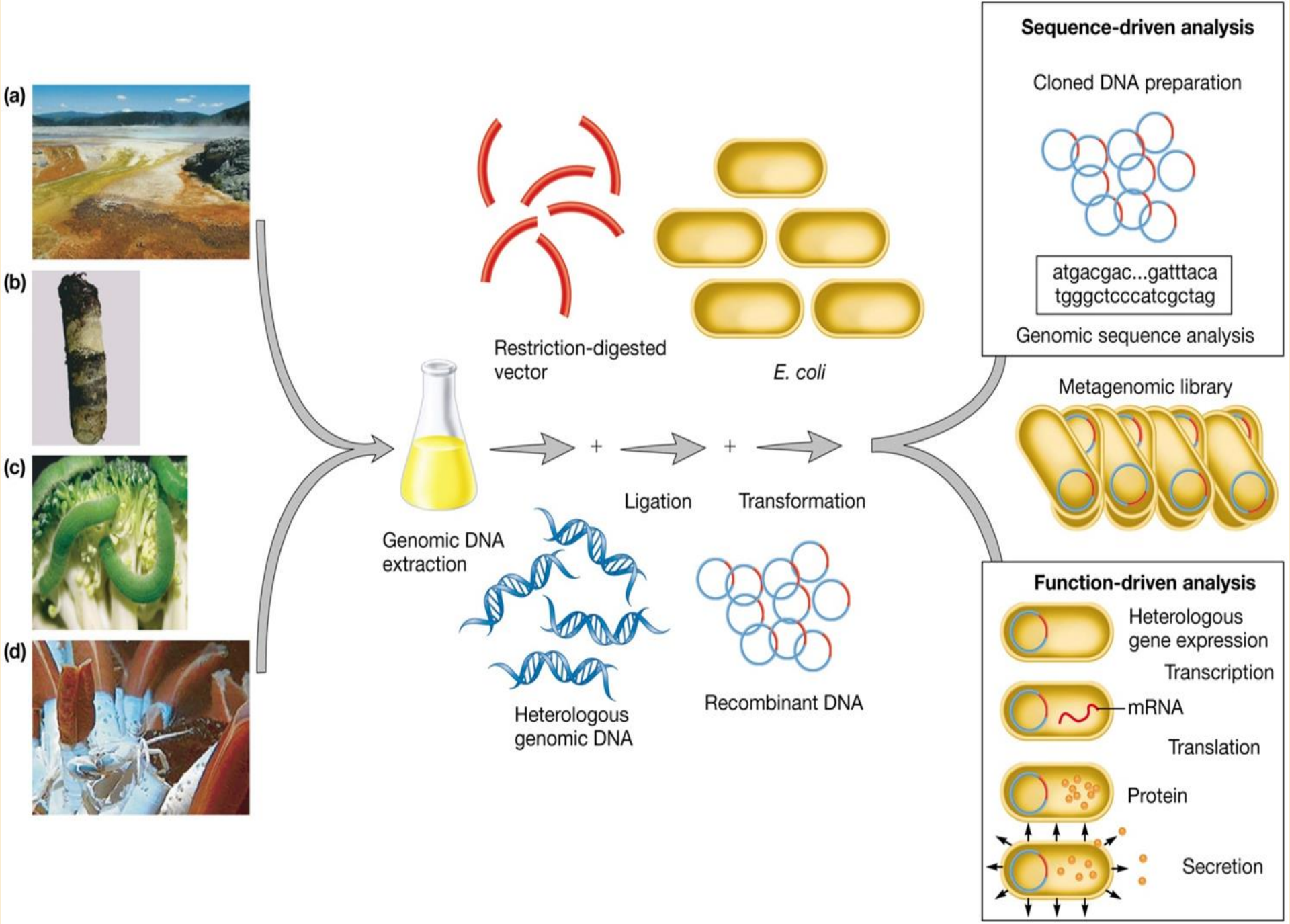
1978

Genentech produced the world's first genetically engineered human insulin. They engineered bacteria that produced human insulin whereas previous methods for obtaining insulin involved taking it from animals.

- **1978 genetically cDNA fragment coding for human insulin 胰岛素 recombined into *E. coli* vector**
- **produced new source of human insulin**
- **first commercial use for recombinant protein**


Plasmid
Foreign DNA
Amp^R
Restriction enzyme cleavage
Restriction enzyme cleavage
Fragments
a
b
Amp^R
a
Recombinant plasmids
b
Amp^R
Amp^R
Transform auxotroph with plasmids.
a
b
Plate bacteria and grow separate clones.
Select transformants on complete medium containing ampicillin.
Master plate
Replica plate onto medium lacking alanine but containing ampicillin
Screen genomic library transformants for colonies with alanine biosynthetic gene.
Only transformants containing the cloned alanine biosynthetic gene grow.

丙氨酸

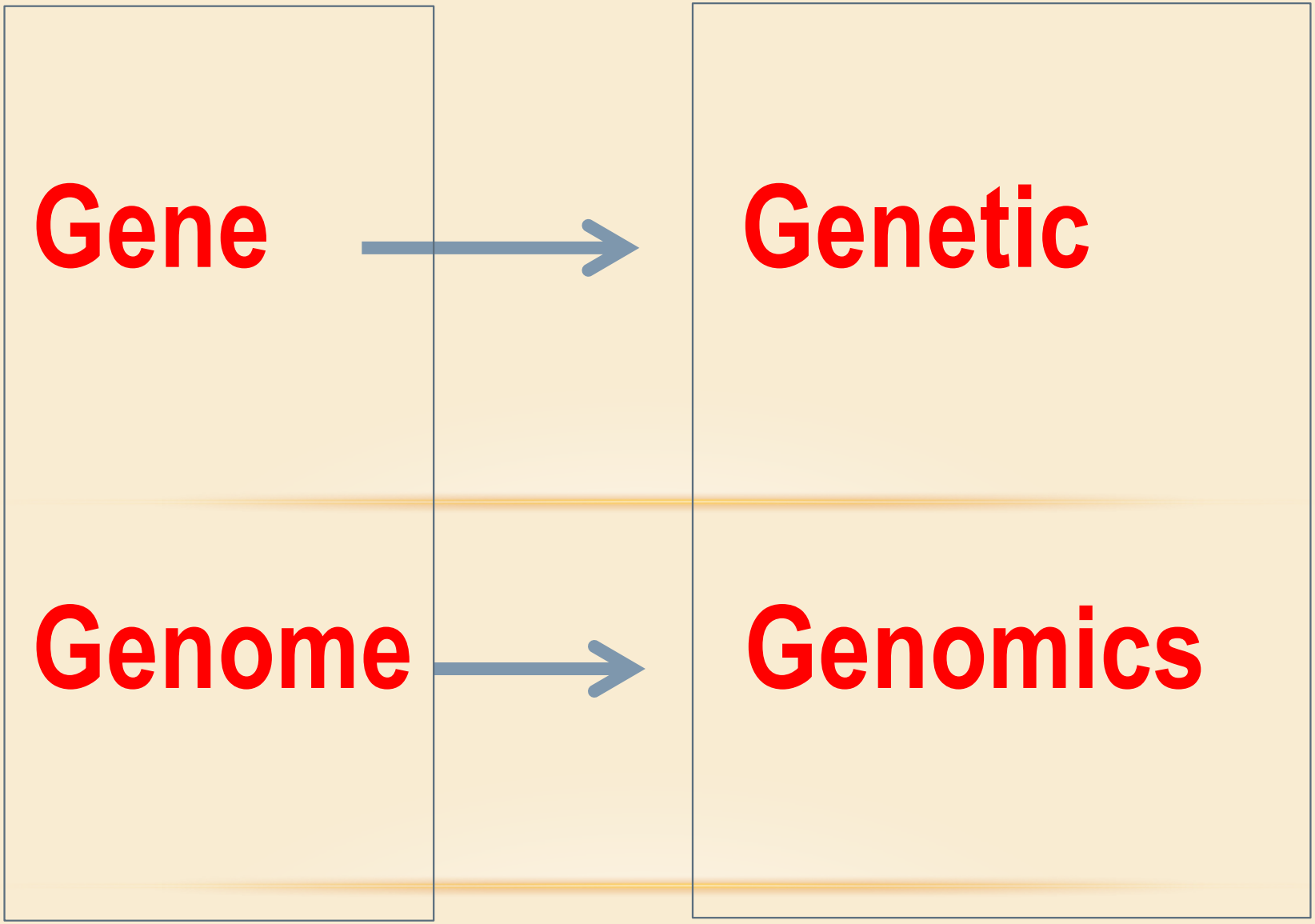


Jo Handelsman, *Metagenomics* MMBR, ASM Press Dec 2004, vol., 68, fi g. 3 page 674

Outline

- DNA/RNA are the genetic materials
- Transcription regulation of Operon
- DNA mutation
- DNA repair
- Horizantal Gene Transfer & Transposition
- Genetic engineering by DNA recombination

- Genomics in Microbiology


Gene
Genetic
Genome
Genomics

Genomic 基因组学 in Microbiology

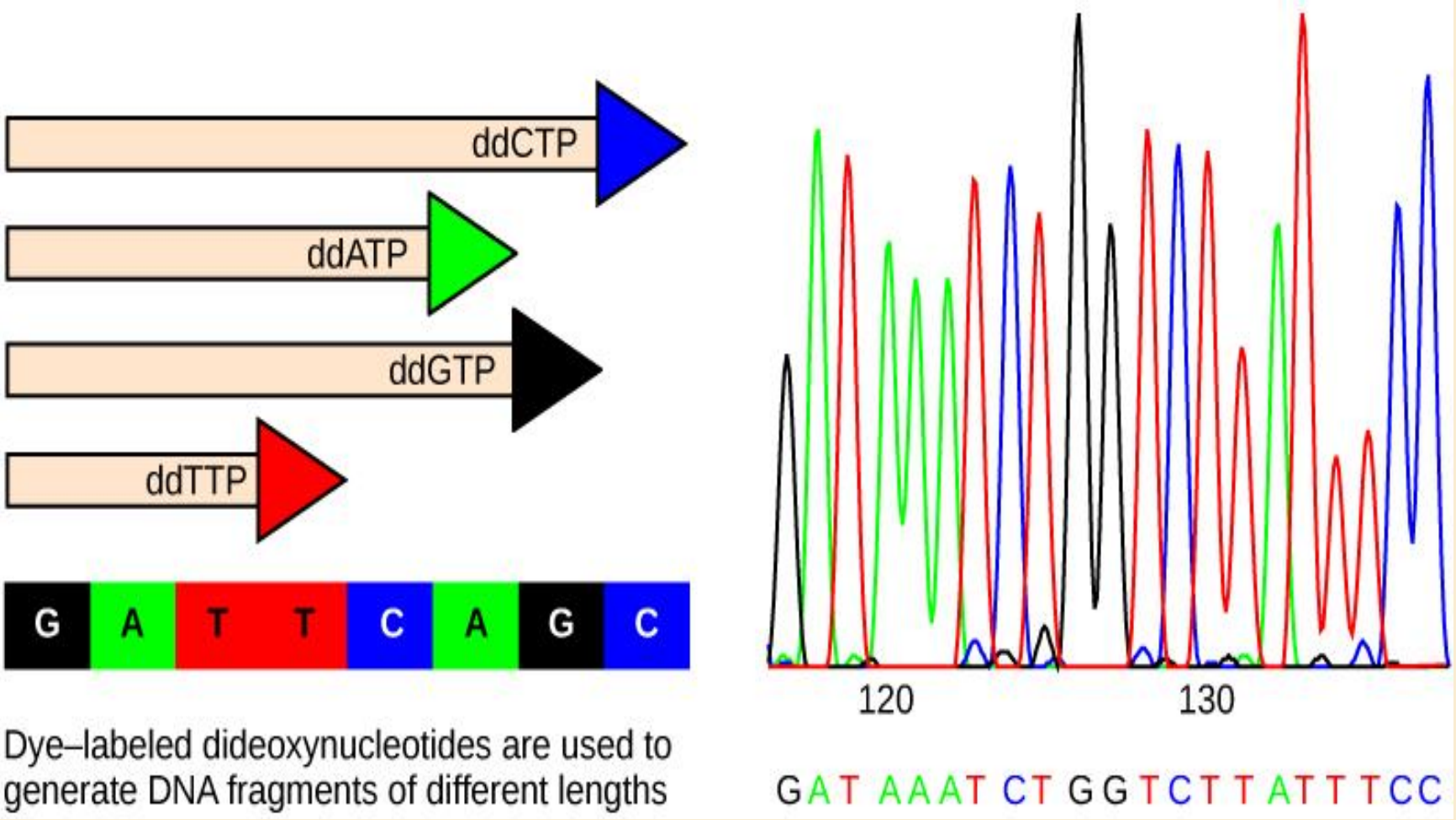
DNA sequencing methods:

Sanger DNA sequencing

Genome DNA sequencing

- Sanger Method
- Second generation and next-generation sequencing

Sanger Method for DNA sequencing



Dye–labeled dideoxynucleotides are used to generate DNA fragments of different lengths

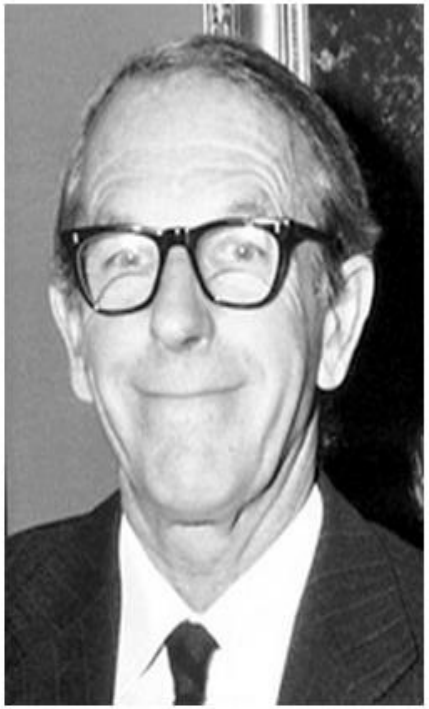
The Nobel Prize in Chemistry 1980

Paul Berg

Prize share:

Walter Gilbert

Prize share:



Frederick Sanger

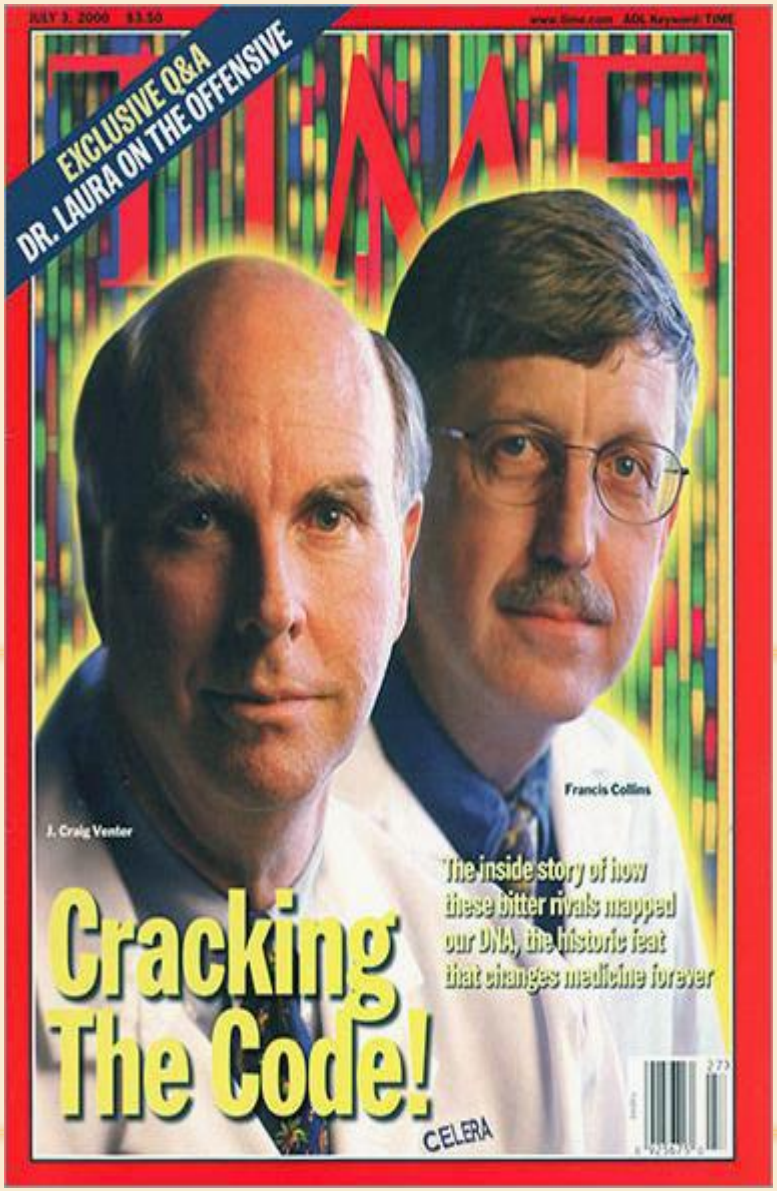
Prize share:

"for their contributions concerning the determination of base sequences in nucleic acids"

2000, Human Genome Project/HGP

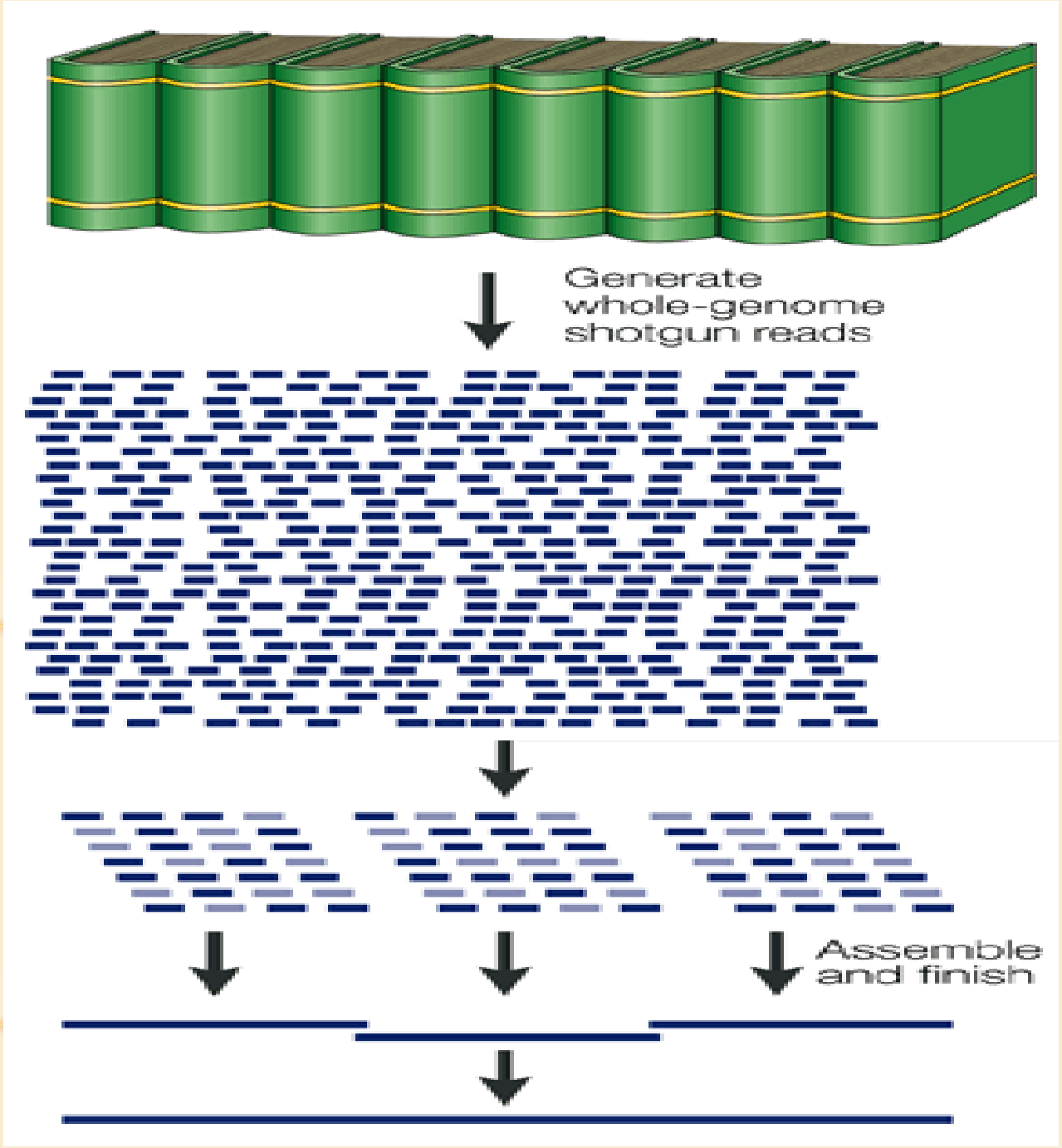
Craig Venter and Francis Collins

Craig Venter

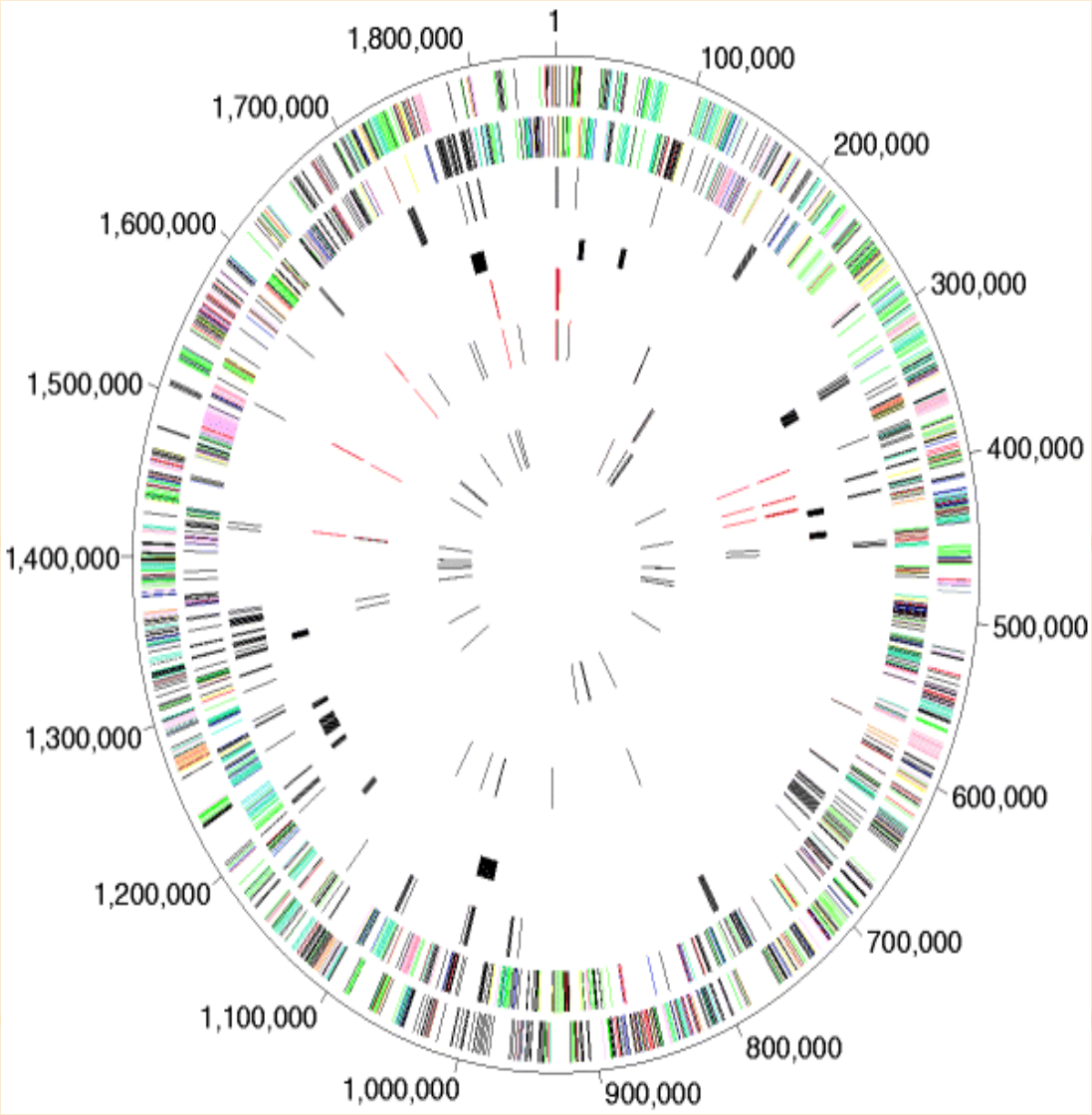


Francis Collins

Whole-genome shotgun sequencing

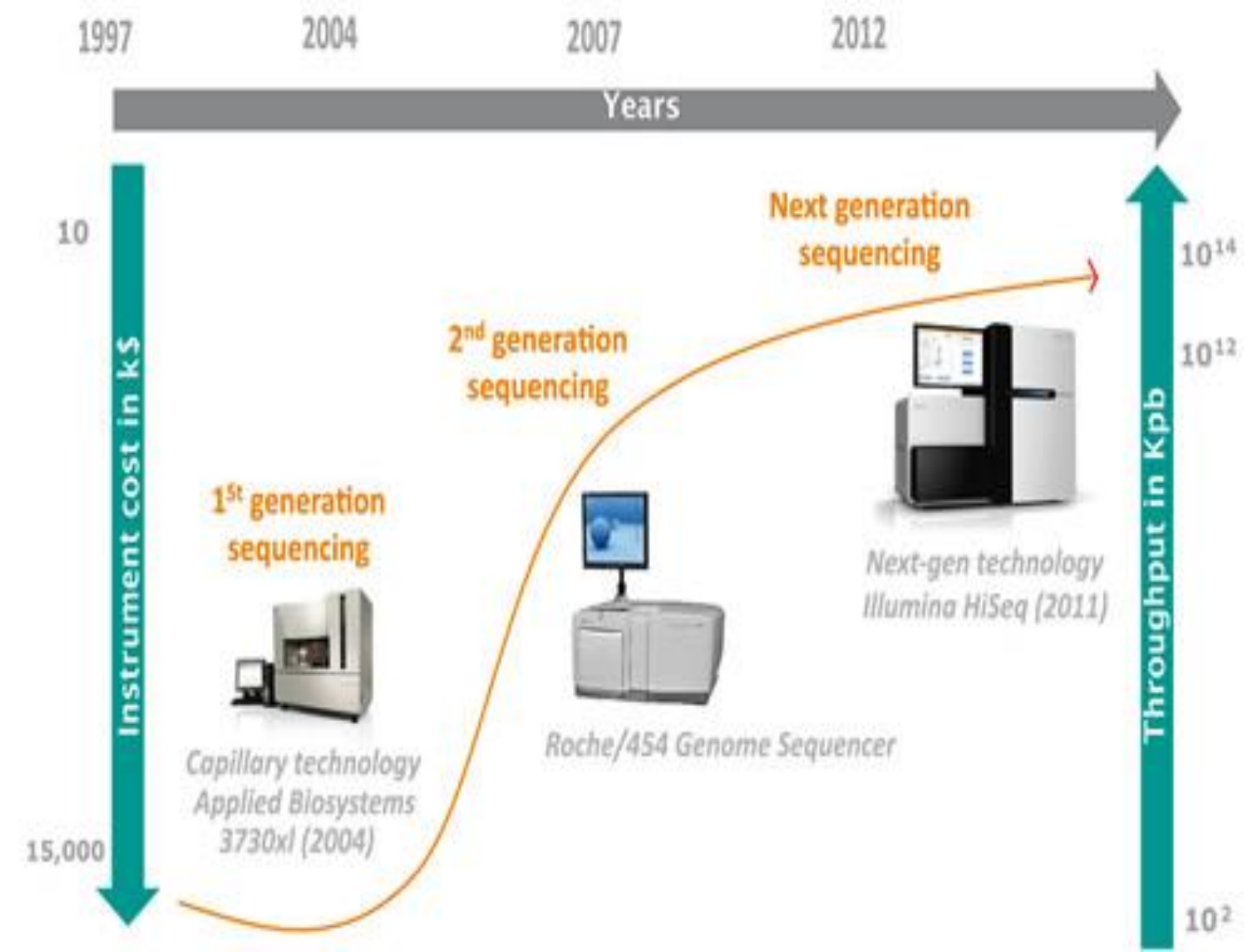


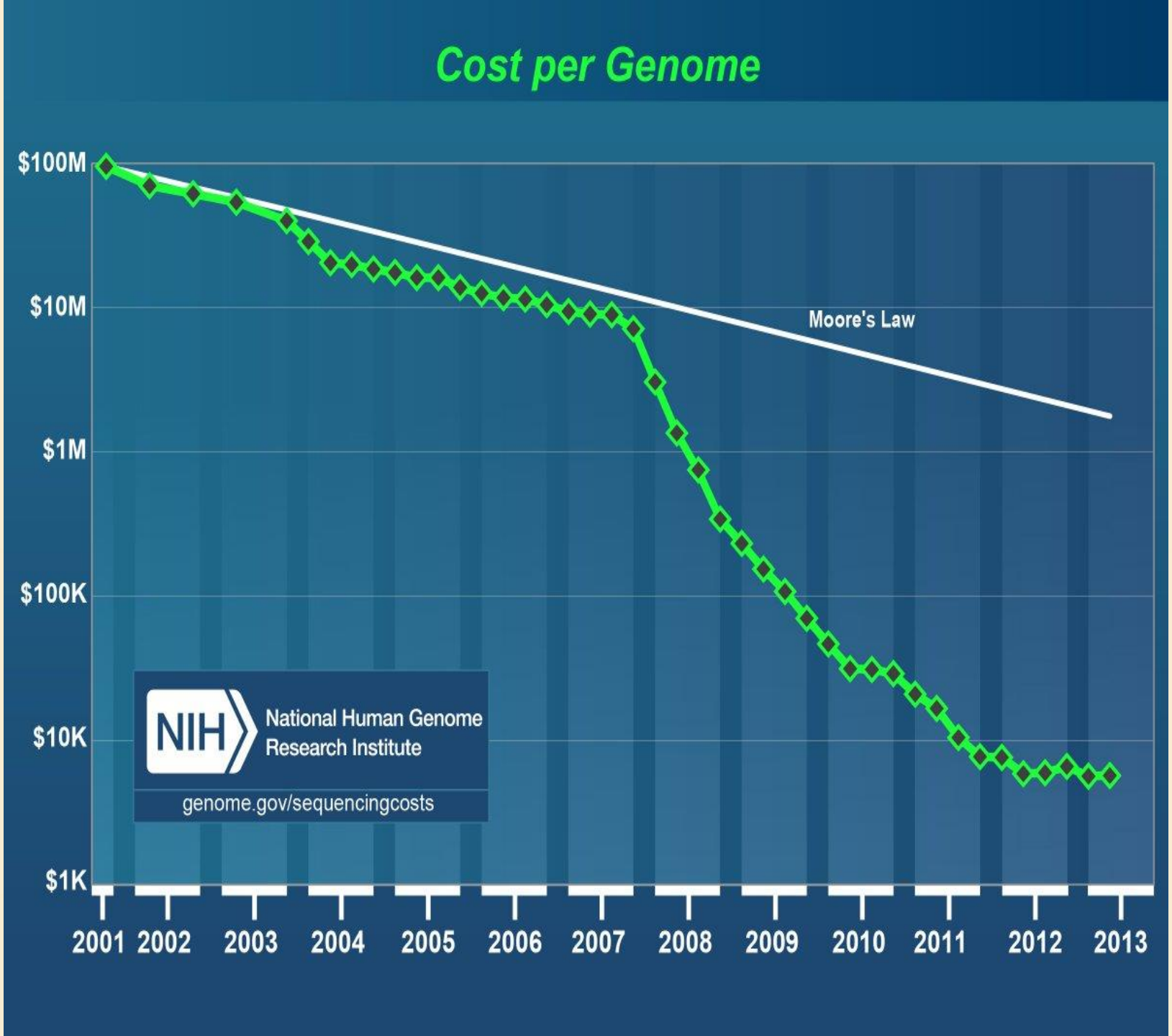
Haemophilus influenzae流感嗜血杆菌 genome sequencing finished at 1995 using Whole-genome shotgun sequencing

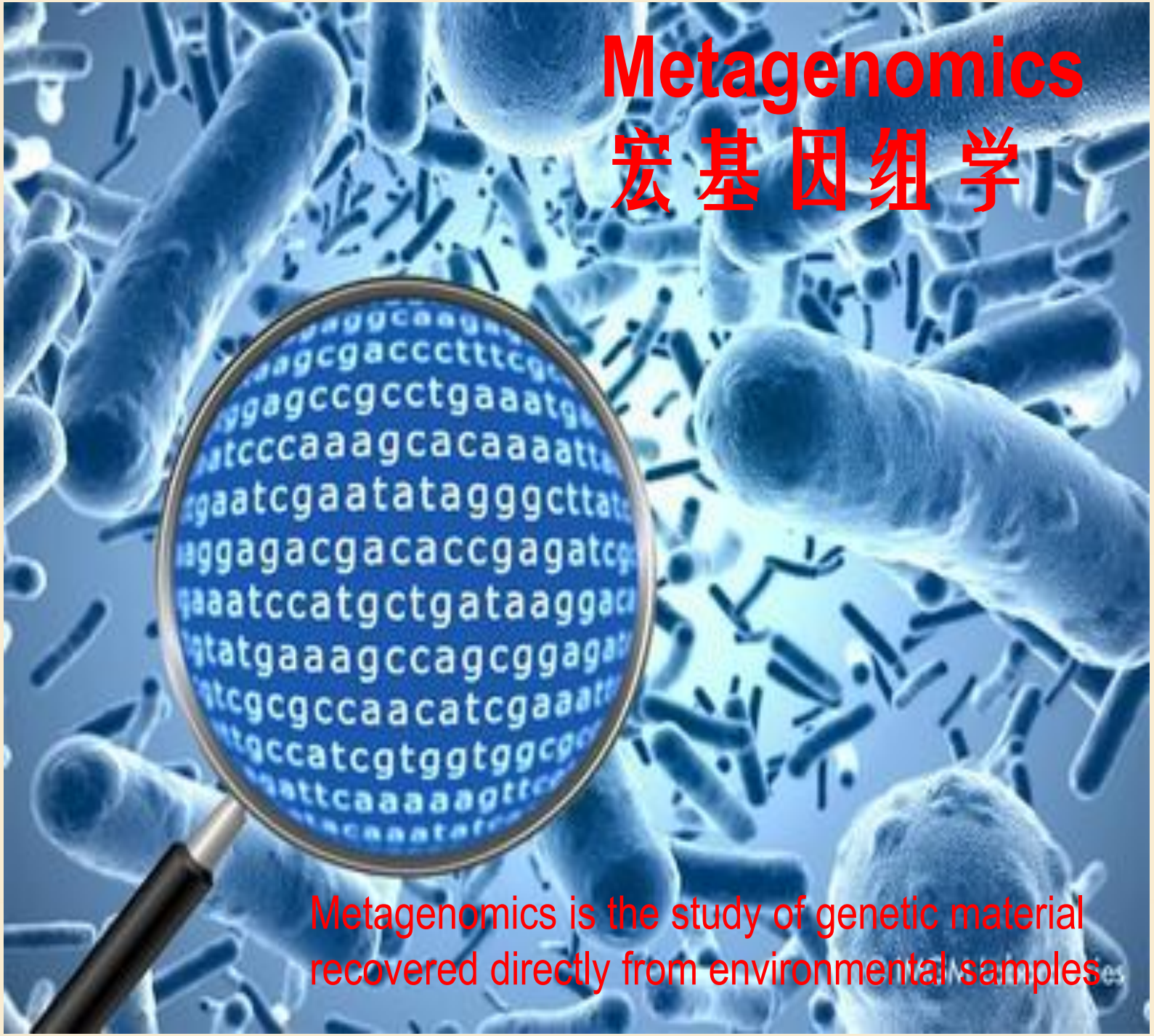


1.8 million bp, 1700 genes estimated

Evolution of sequencing instruments over time

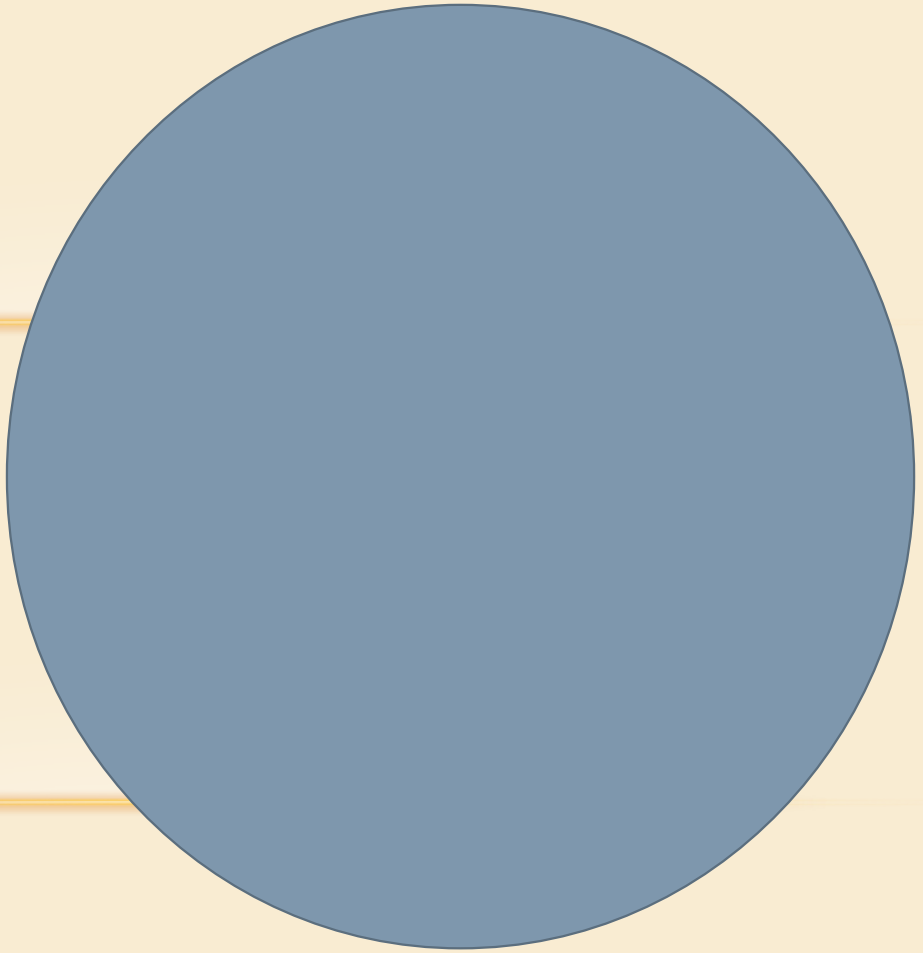


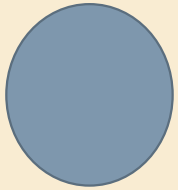

Cost per Genome
$100M
$10M
$1M
$100K
$10K
$1K
Moore's Law
NIH
National Human Genome Research Institute
genome.gov/sequencingcosts
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013


Metagenomics
宏基因组学
Metagenomics is the study of genetic material recovered directly from environmental samples

Why Metagenomics?

Microorganism that are not culturable



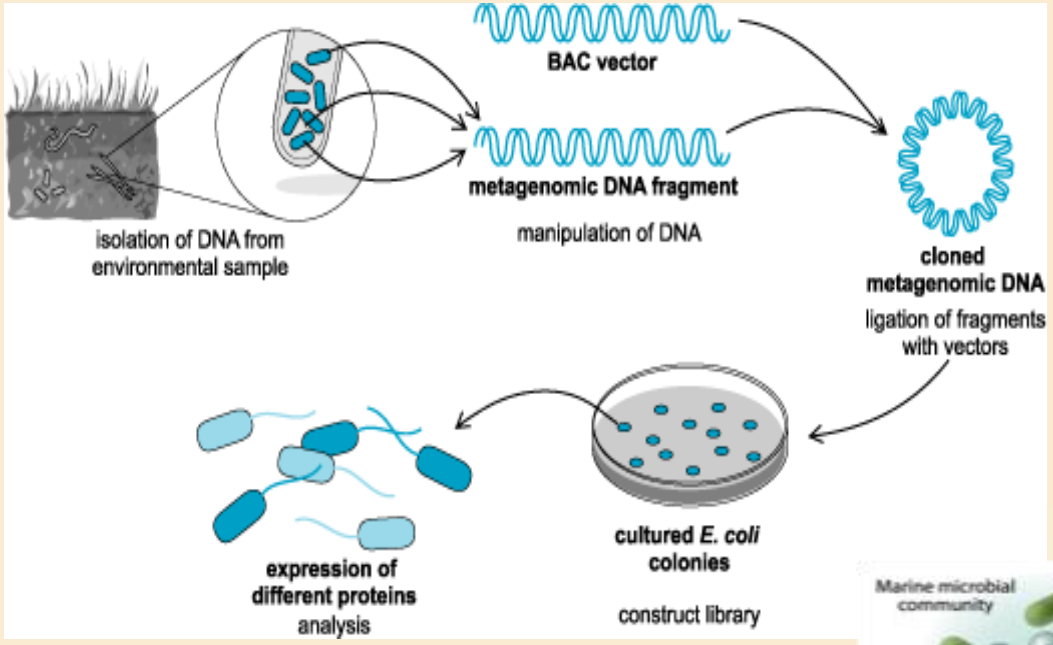


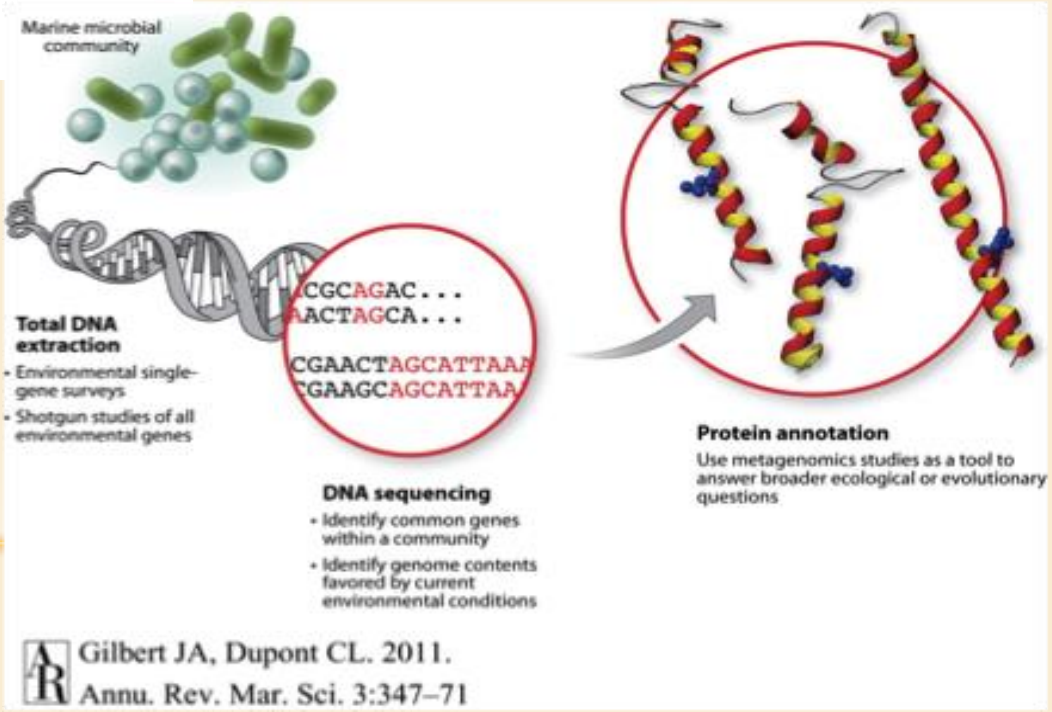
Microorganism that are culturable

Metagenomics

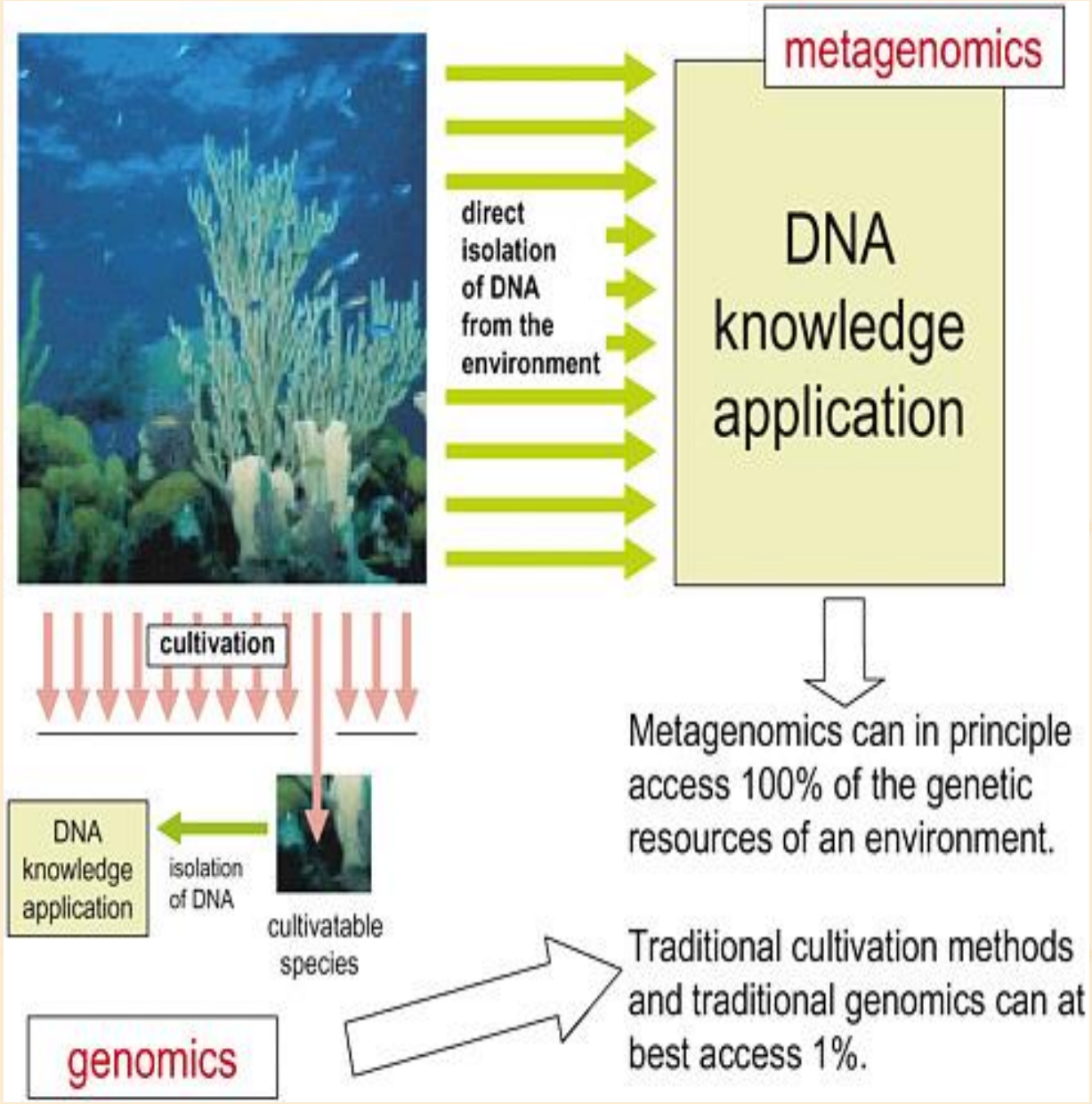
- High-throughput Sequencing
- Bioinformatics/Data analysis
- Applications
    - Biotechnology
    - Disease prevention and treatment
    - Agriculture
    - Environment

Discovery of Novel Protein from Non-culturable Microorganism

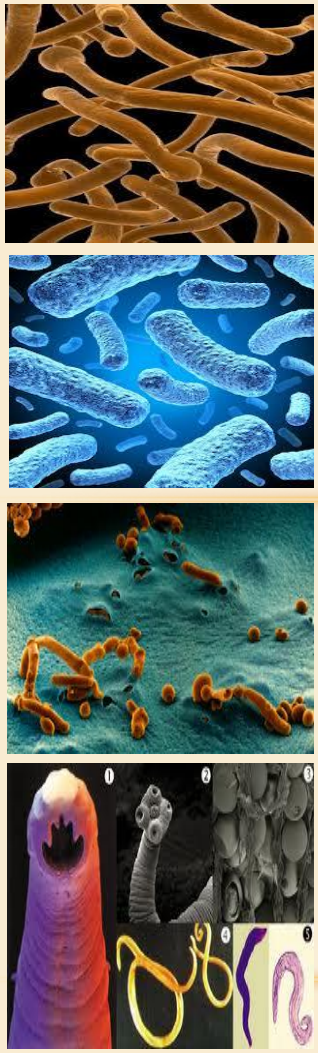




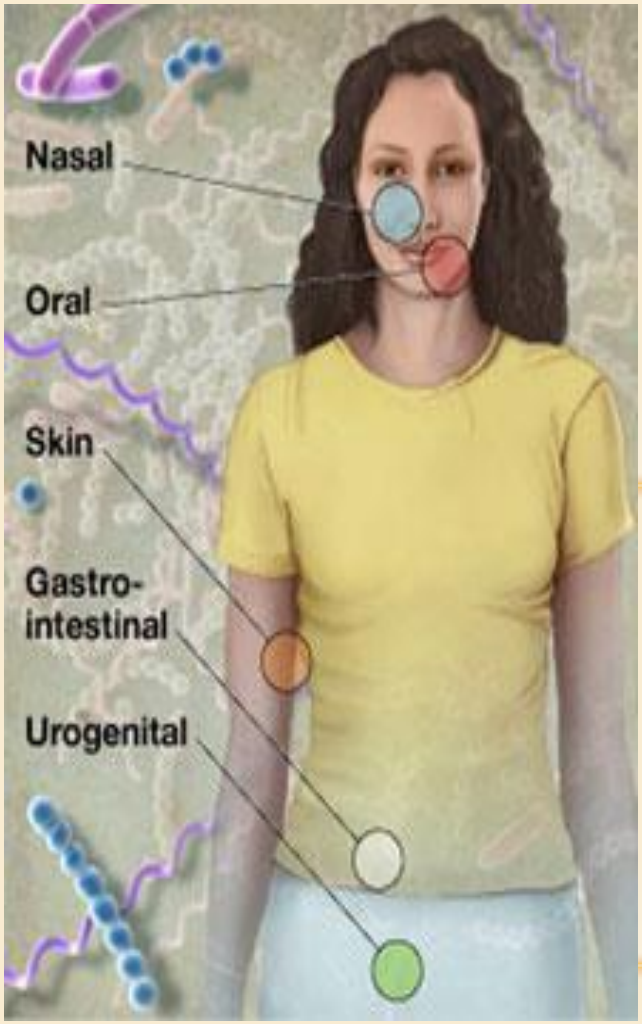
Treasury In The Deep Sea



Diagnosis of Pathogen infection by Genome Sequencing



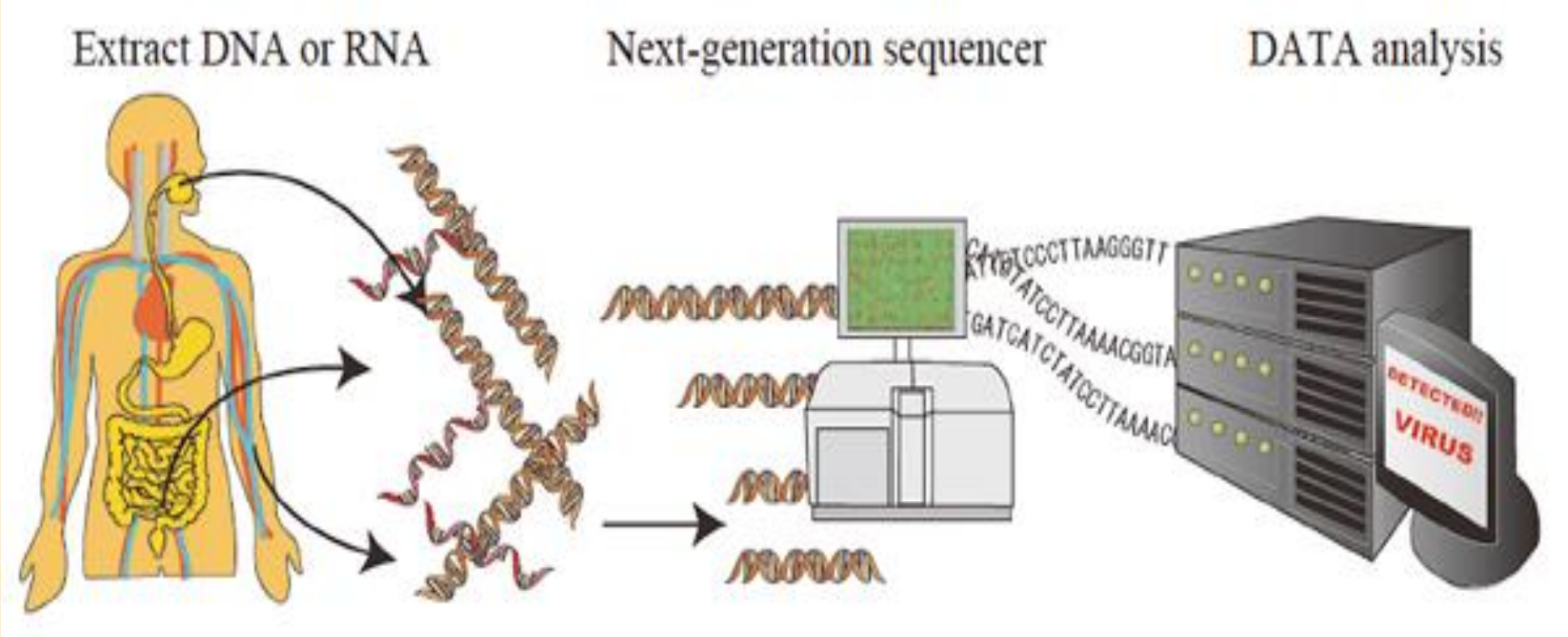
Human Microbiome Project

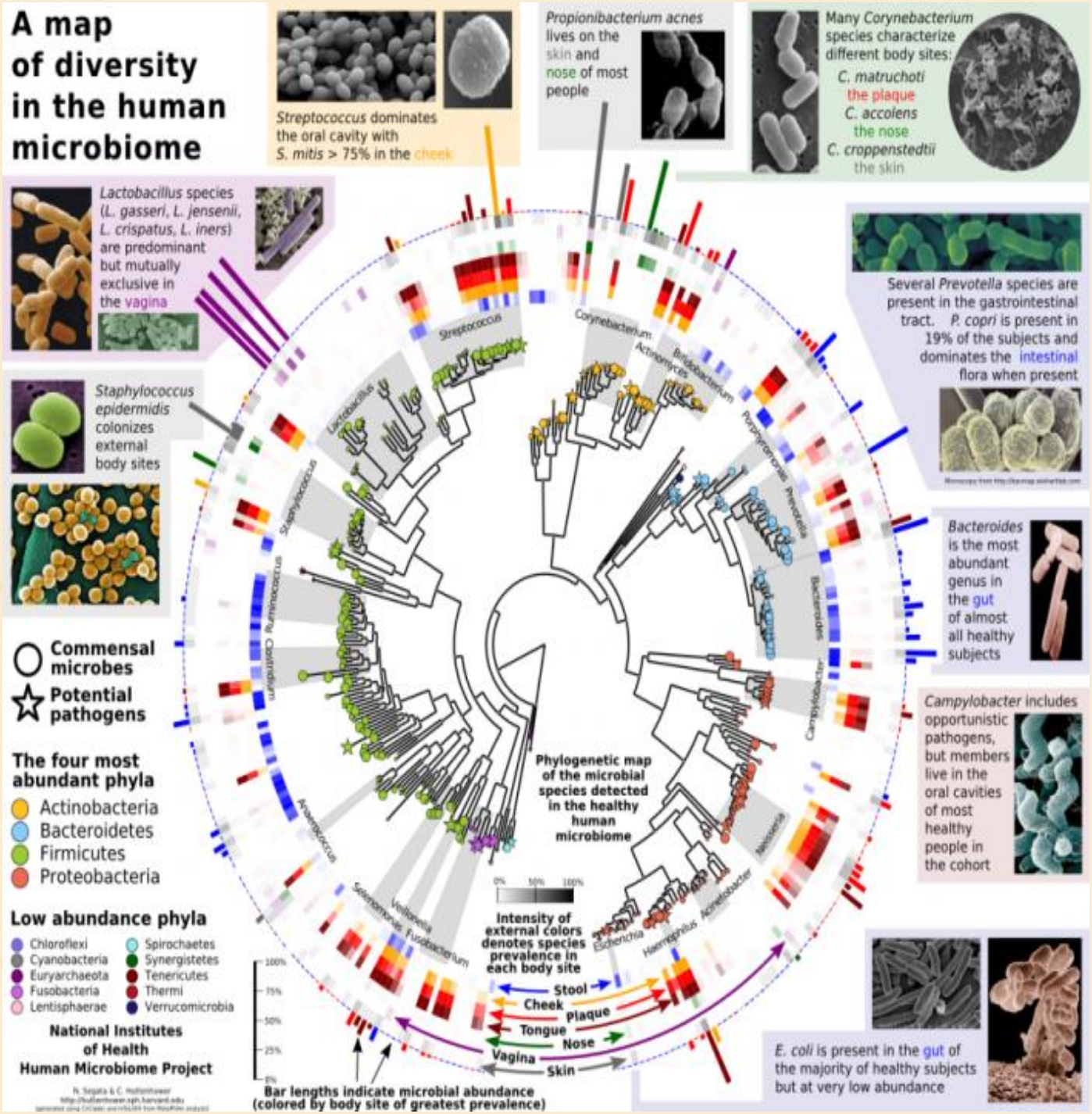


- Development of a reference set of microbial genome sequences and preliminary characterization of the human microbiome

- Elucidation of the relationship between disease and changes in the human microbiome

Metagenomics for Microorganism Resident in Human





放线杆菌 actinobacteria
bacteroidetes 拟杆菌
厚壁菌 firmicute
Proteobacteria 变形菌门

A healthy adult human harbors:

- about 100 trillion microbes outnumbering our own cells by a factor of ten;
- expanding our own gene repertoire by at least two orders of magnitude.

Science AAAS

- Population-level analysis of gut microbiome variation, *Science*. 2016

- Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity, *Science*. 2016

INTESTINAL MICROFLORA

10^{14} micro-organisms, >500 differentes species

Stomach 10^2 to 10^3

Lactobacilli

Duodenum $<10^{4-5}$

Streptococci
Lactobacilli

Jejunum

Enterobacteria
Enterococcus
Faecalis
Bacteroides
Bifidobacteria
Peptococcus
Peptostreptococc
Ruminococcus
Clostridia
Lactobacilli

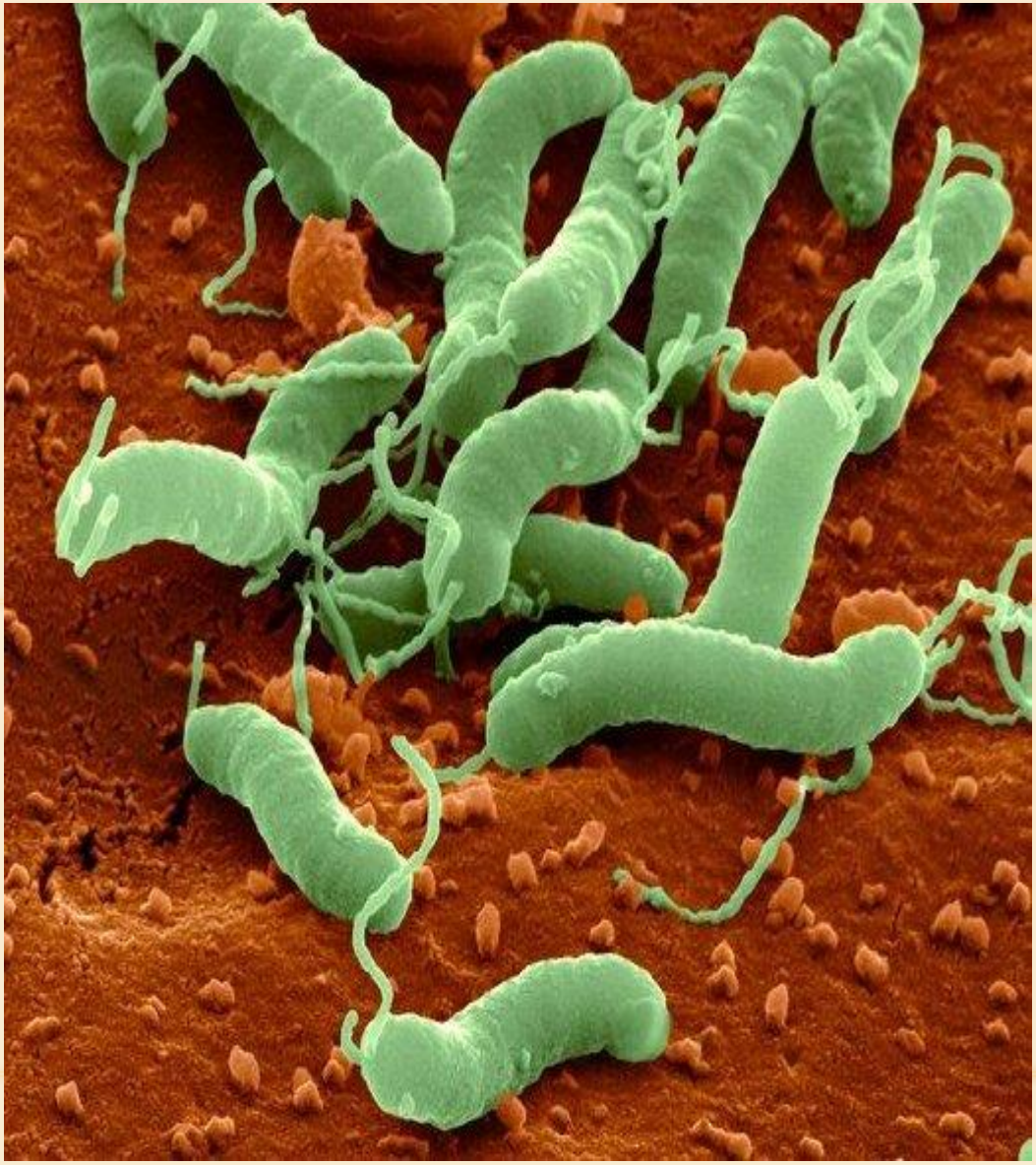
Ileum 10^3 to 10^7

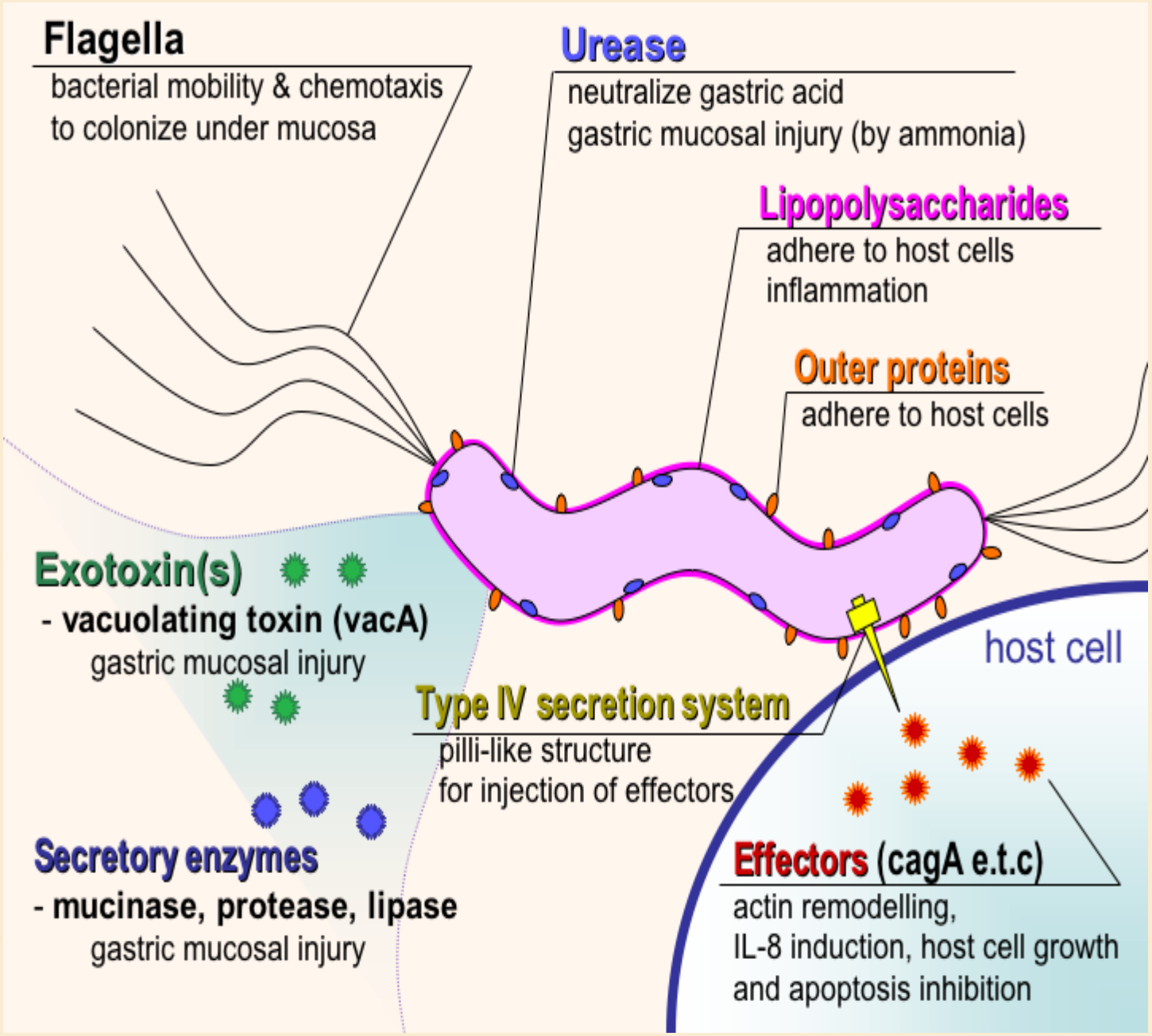
Colon with appendix 10^9 to 10^{12}

and....

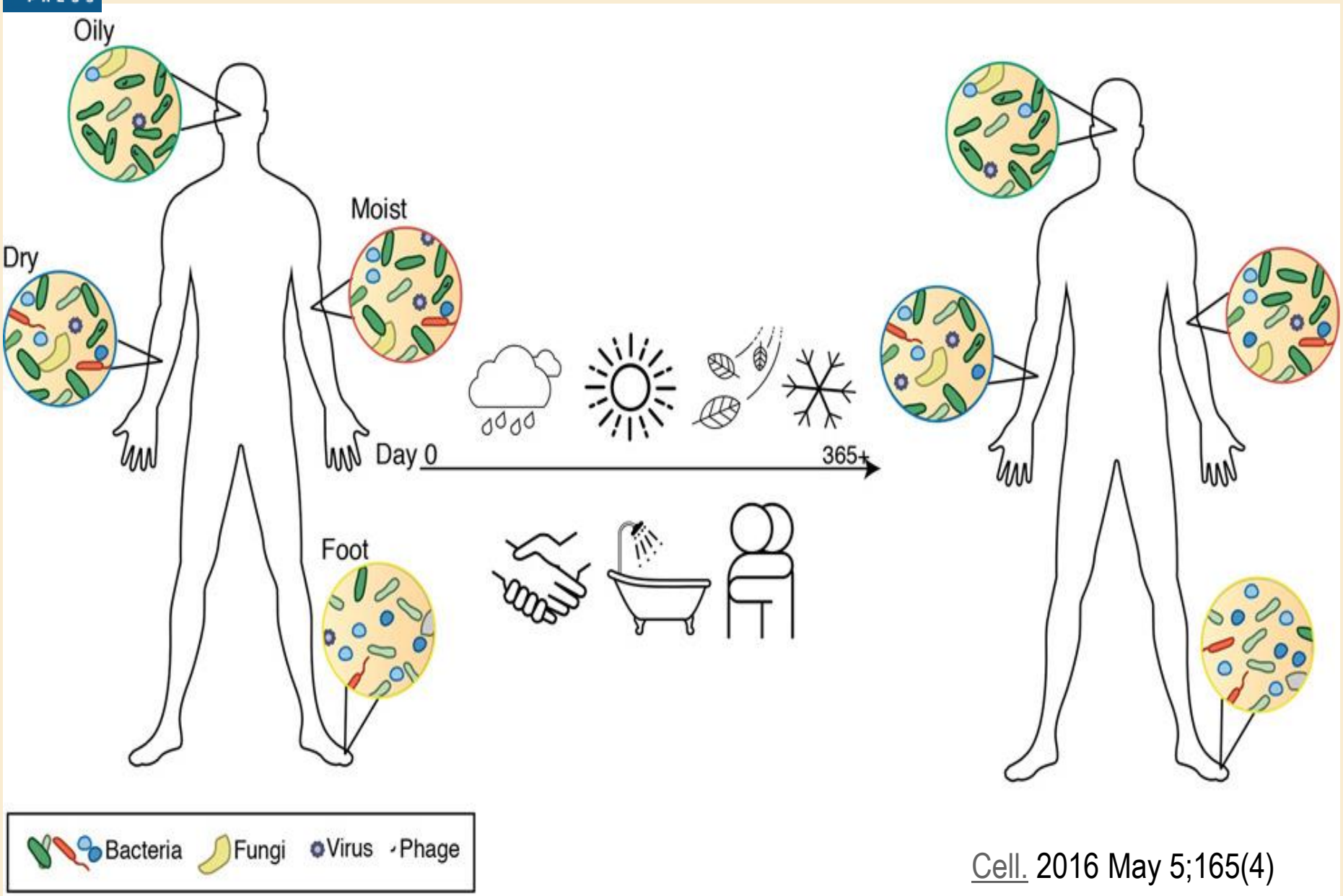
The Nobel Prize in Physiology or Medicine 2005

Barry J. Marshall, J. Robin Warren




Flagella
bacterial mobility & chemotaxis
to colonize under mucosa
Urease
neutralize gastric acid
gastric mucosal injury (by ammonia)
Lipopolysaccharides
adhere to host cells
inflammation
Outer proteins
adhere to host cells
Exotoxin(s)
- vacuolating toxin (vacA)
gastric mucosal injury
Type IV secretion system
pilli-like structure
for injection of effectors
host cell
Secretory enzymes
- mucinase, protease, lipase
gastric mucosal injury
Effectors (cagA e.t.c)
actin remodelling,
IL-8 induction, host cell growth
and apoptosis inhibition

Temporal Stability of the Human Skin Microbiome



Cell. 2016 May 5;165(4)

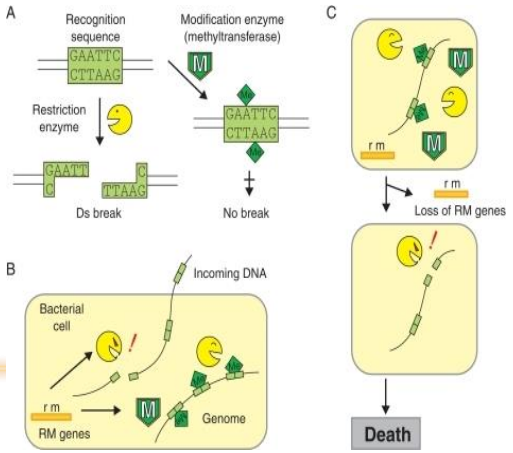
Discussion:

水土不服

夫妻相

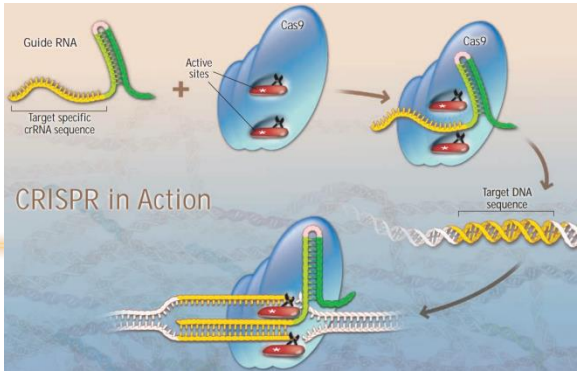
Evolve to distinguish cell own DNA and any foreign DNA entering the cell, and destroy the later.

- **Restriction–Modification Systems in archaea and bacterial**



- **CRISPR/Cas9 system in archaea and bacterial**

bacterial immune system



The Biology of CRISPR/Cas9

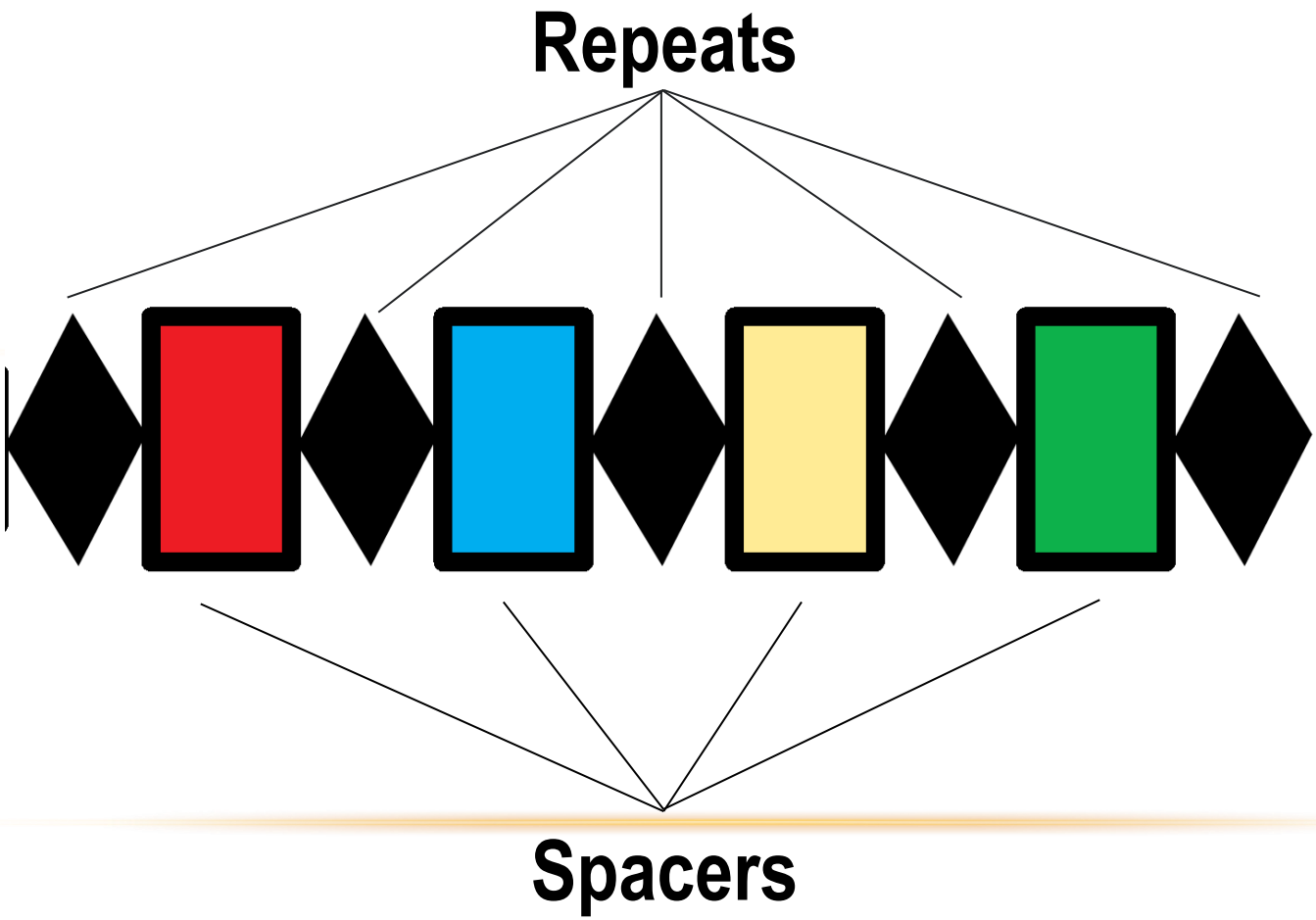
- CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)
  规律成簇间隔短回文重复
- CRISPR-associated (Cas) genes
- essential in adaptive immunity in select bacteria and archaea, enabling the organisms to respond to and eliminate invading genetic material.

An unusual structure was found in the 3′-end flanking region of *iap* (Fig. 5). Five highly homologous sequences of 29 nucleotides were arranged as direct repeats with 32 nucleotides as spacing. The first sequence was included in the putative transcriptional termination site and had less homology than the others. Well-conserved nucleotide sequences containing a dyad symmetry, named REP sequences, have been found in *E. coli* and *Salmonella typhimurium* (28) and may act to stabilize mRNA (18). A dyad symmetry with 14 nucleotide pairs was also found in the middle of these sequences (underlining, Fig. 5), but no homology was found between these sequences and the REP sequence. So far, no sequence homologous to these has been found elsewhere in procaryotes, and the biological significance of these sequences is not known.

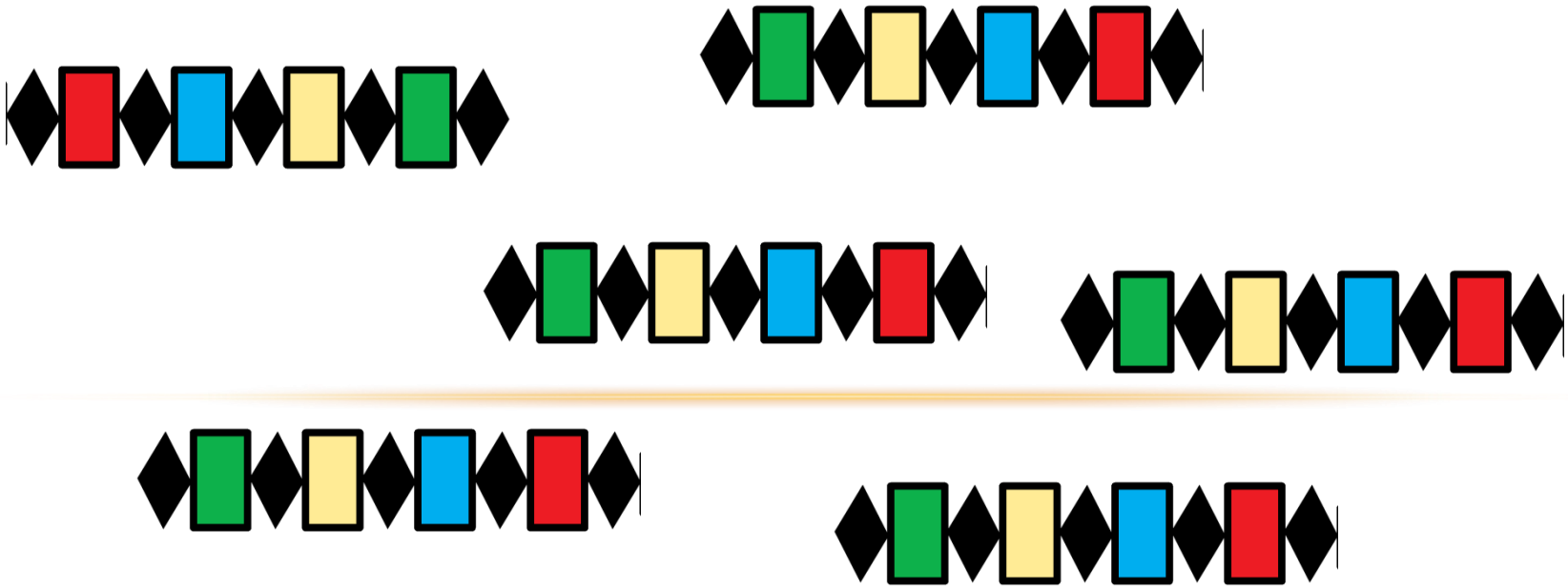
J Bacteriol. 1987 Dec;169(12):5429-33

Clustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeat sequences，

成簇的、规律间隔的短回文重复序列,即**CRISPR**序列



CRISPR: widespread occurrence in bacteria and archaea genomes



1.Jansen R, Embden JD, Gaastra W, Schouls LM. **Identification of genes that are associated with DNA repeats in prokaryotes**. *Mol Microbiol*. 2002; **43**:1565-75.

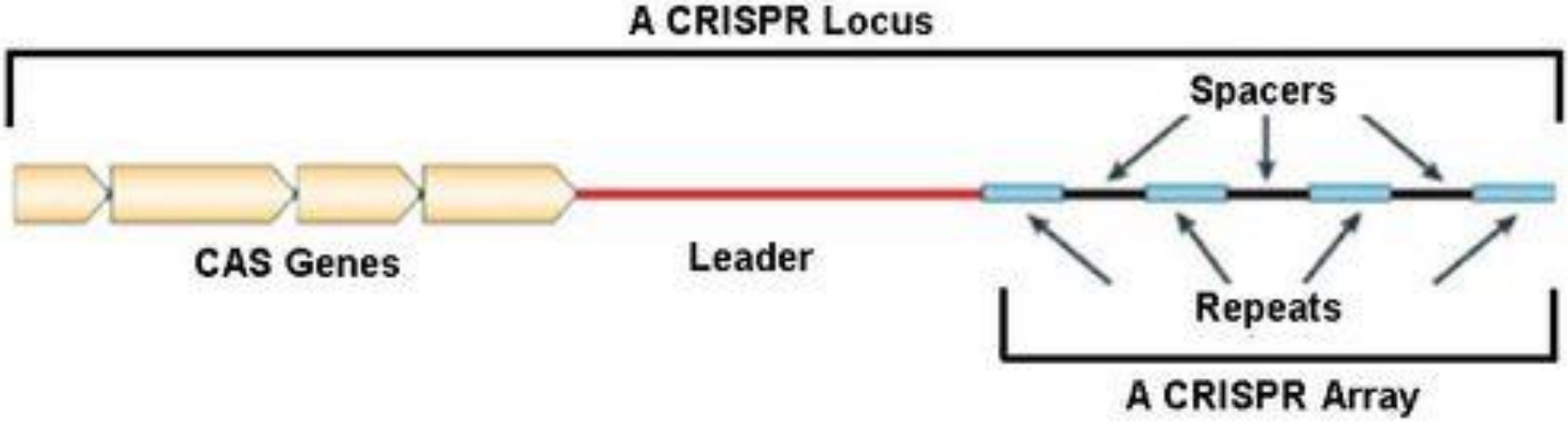
2. Mojica FJ, Diez-Villasenor C, Soria E, Juez G. **Biological significance of a family of regularly spaced repeats in the genomes of Archaea. Bacteria and mitochondria**.*Mol Microbiol*. 2000; **36**:244-6.

molecular microbiology

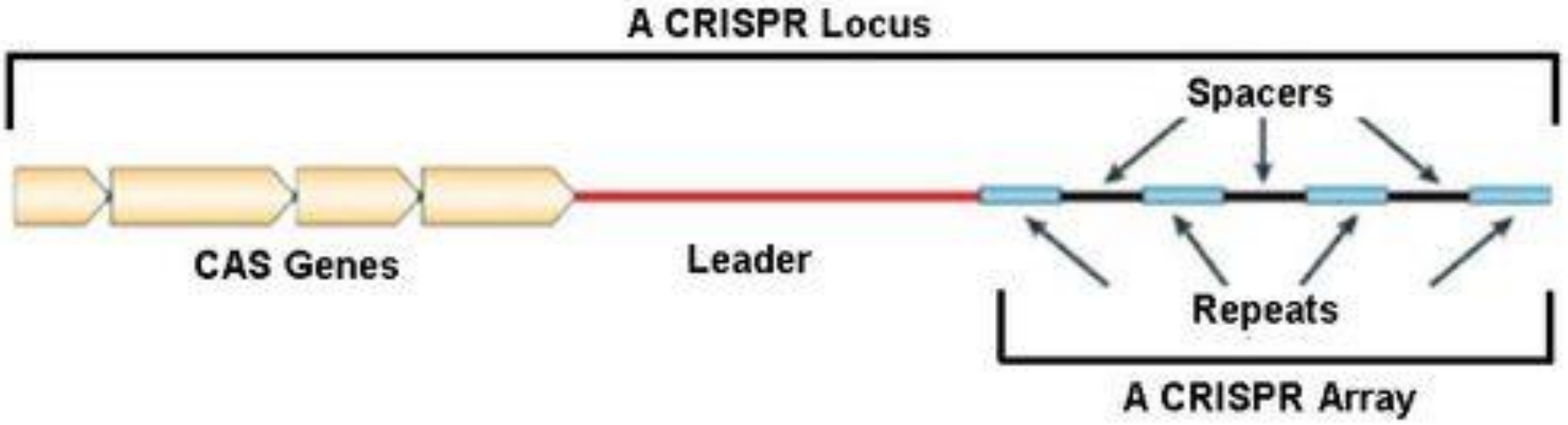
Identification of genes that are associated with DNA repeats in prokaryotes

Ruud. Jansen ✉, Jan. D. A. van Embden, Wim. Gaastra, Leo. M. Schouls

First published: March 2002 Full publication history


A CRISPR Locus
Spacers
CAS Genes
Leader
Repeats
A CRISPR Array

CRISPR: biological function?



The first functional clue emerged until 2005, with the observation that CRISPR spacers showed homology (序列同源) to viral sequences.

1.Pourcel C, Salvignol G, Vergnaud G. **CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies**. *Microbiology*. 2005; **151**:653-63.
耶尔森氏鼠疫杆菌

2.Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. **Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements**. *J Mol Evol*. 2005; **60**:174-82.

3.Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. **Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin**.*Microbiology*. 2005; **151**:2551-61.

Microbiology (2005), **151**, 653–663 DOI 10.1099/mic.0.27437-0

耶尔森氏鼠疫杆菌

CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies

C. Pourcel,[1] G. Salvignol[1] and G. Vergnaud[1,2]

[1]GPMS, Institut de Génétique et Microbiologie, Université Paris XI, 91405 Orsay cedex, France

[2]Centre d'Etudes du Bouchet, 5 rue Lavoisier, 91710 Vert le Petit, France

Correspondence
G. Vergnaud
Gilles.Vergnaud@igmors.u-psud.fr

2. **Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements**. *J Mol Evol*. 2005; **60**:174-82.

Table 2. Distribution of CRISPR-spacer homologs

序列同源

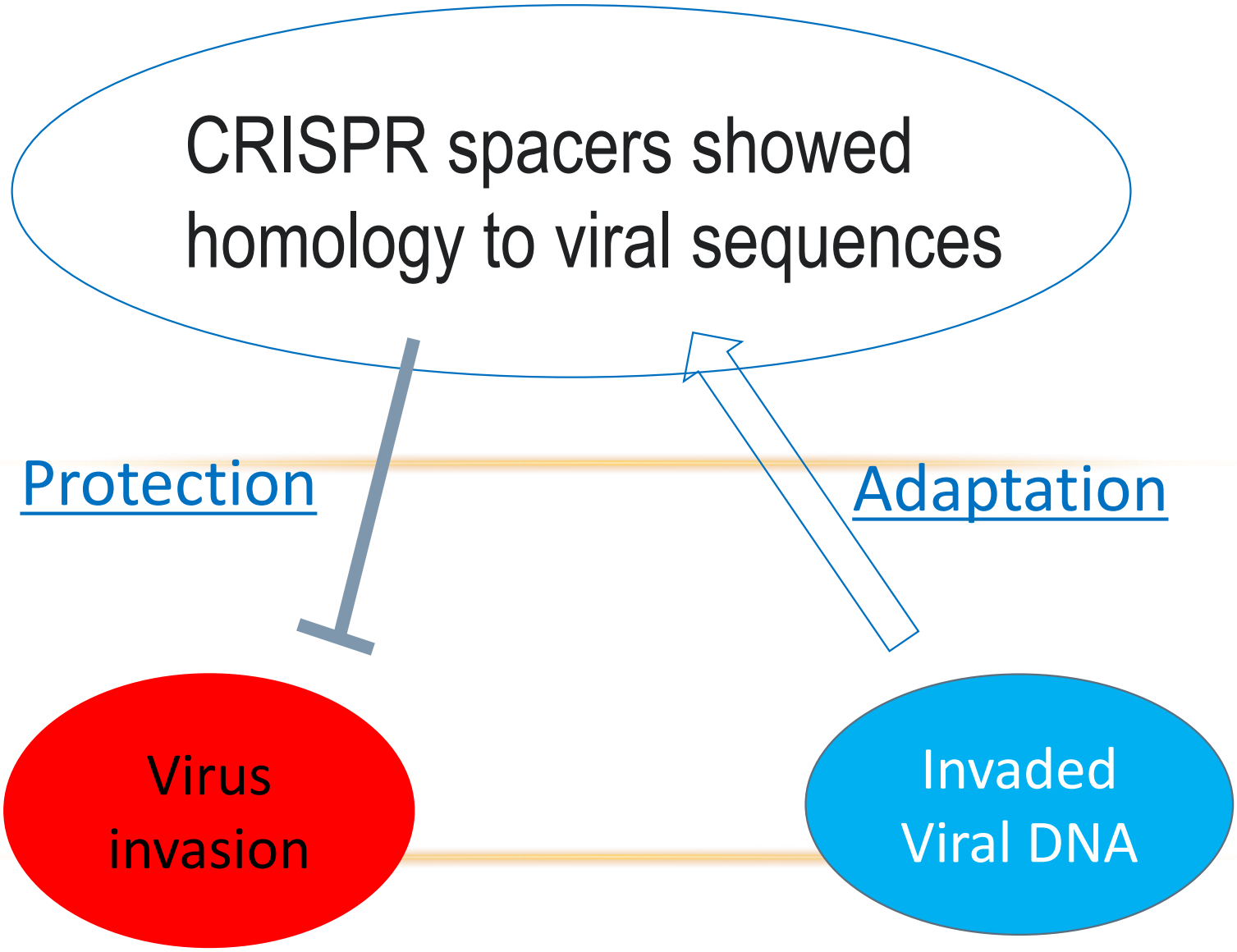
Strain	No. of spacers analyzed	No. of spacers with homologs in	
		Phages[a]	Plasmids
Chlorobium tepidum TLS	62		1
Clostridium tetani Massachusetts E88	62	1	
Corynebacterium efficiens YS-314T	22		1
Escherichia coli ECOR42	14		1
Escherichia coli ECOR44	10	1	
Escherichia coli ECOR47	17	1	
Escherichia coli ECOR49	11		1
Listeria innocua Clip11262	9	3	
Listeria monocytogenes EGD-e	4	1	
Methanothermobacter thermoautotrophicum ΔH	169	9	
Mycoplasma gallisepticum R	71		
Neisseria meningitidis Z2491 (serogroup A)	16		
Photorhabdus luminescens laumondii TT01	65	7	
Porphyromonas gingivalis W83	44		
Pyrobaculum aerophilum IM2	129		
Salmonella typhimurium LT2 SGSC1412	57	1	
Shigella sonnei 53G	3		
Streptococcus agalactiae NEM316	13	1	
Streptococcus agalactiae 2603V/R	25	1	1
Streptococcus pyogenes MI GAS SF370	9	8	
Sulfolobus solfataricus P2	424	6	3
Sulfolobus tokodaii 7	471	2	2
Thermoanaerobacter tengcongensis MB4T	306		
Yersinia pestis CO-92 (Biovar Orientalis)	16	4	
Yersinia pestis KIM5P12 (Biovar Mediaevalis)	10	1	

Table 3. Features of the sequences most similar to CRISPR spacers from the genus *Sulfolobus*

Strain	ORF	Replicon	Activity	Alignment[a]
S. solfataricus P2	ORF406	pNOB8	Transposase	tgaatagcaacatcgtgtaacctcatcctcagccttc taaaaggcaacatcgtgcaacctcatcctcat-cttc
	ORF1025	pNOB8	NTPase	ttgtctgtcggtgaagcagtagtatttctaagaggccgtcc ctatctgtcggtgaagccgtagcatttctaagaggccgtcc
	ORF315	pNOB8	Resolvase	cctaatatcctcgggtacttatagaaccctccttctggtc cctaatattctaggatacttatagaaccctccttctggtc
	ORF121	SIRV1	Resolvase	aaagcggttgttttccagttccagaaactggaattcttat gaagtgcctgttttccagttccagaaactggaattcttaa
	ORF510	SIRV1	Unknown	atgttctttttccagaactgtaactataattttgttgatat atgttctttttccagatgtgtaactataattttgttgatat
	ORF134	SIRV1	Structural	tggtaaatagctctgttaggcccagttattccatattctg tgatatatagctctgttaggcccagttattccatattgtg
	ORF356	SIRV1	Glycosyl transferase	atcatttatgcacatttcaactccatttccaatatgaat ttttcctatacacatttgaactccatttccaatatgaat

超嗜热古菌

CRISPR: biological function



Milestone Discovery

www.sciencemag.org SCIENCE VOL 315 23 MARCH 2007

CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes

Rodolphe Barrangou,[1] Christophe Fremaux,[2] Hélène Deveau,[3] Melissa Richards,[1] Patrick Boyaval,[2] Sylvain Moineau,[3] Dennis A. Romero,[1] Philippe Horvath[2]*

Clustered regularly interspaced short palindromic repeats (CRISPR) are a distinctive feature of the genomes of most Bacteria and Archaea and are thought to be involved in resistance to bacteriophages. We found that, after viral challenge, bacteria integrated new spacers derived from phage genomic sequences. Removal or addition of particular spacers modified the phage-resistance phenotype of the cell. Thus, CRISPR, together with associated *cas* genes, provided resistance against phages, and resistance specificity is determined by spacer-phage sequence similarity.

嗜热链球菌-*S. thermophilus*

Abstract

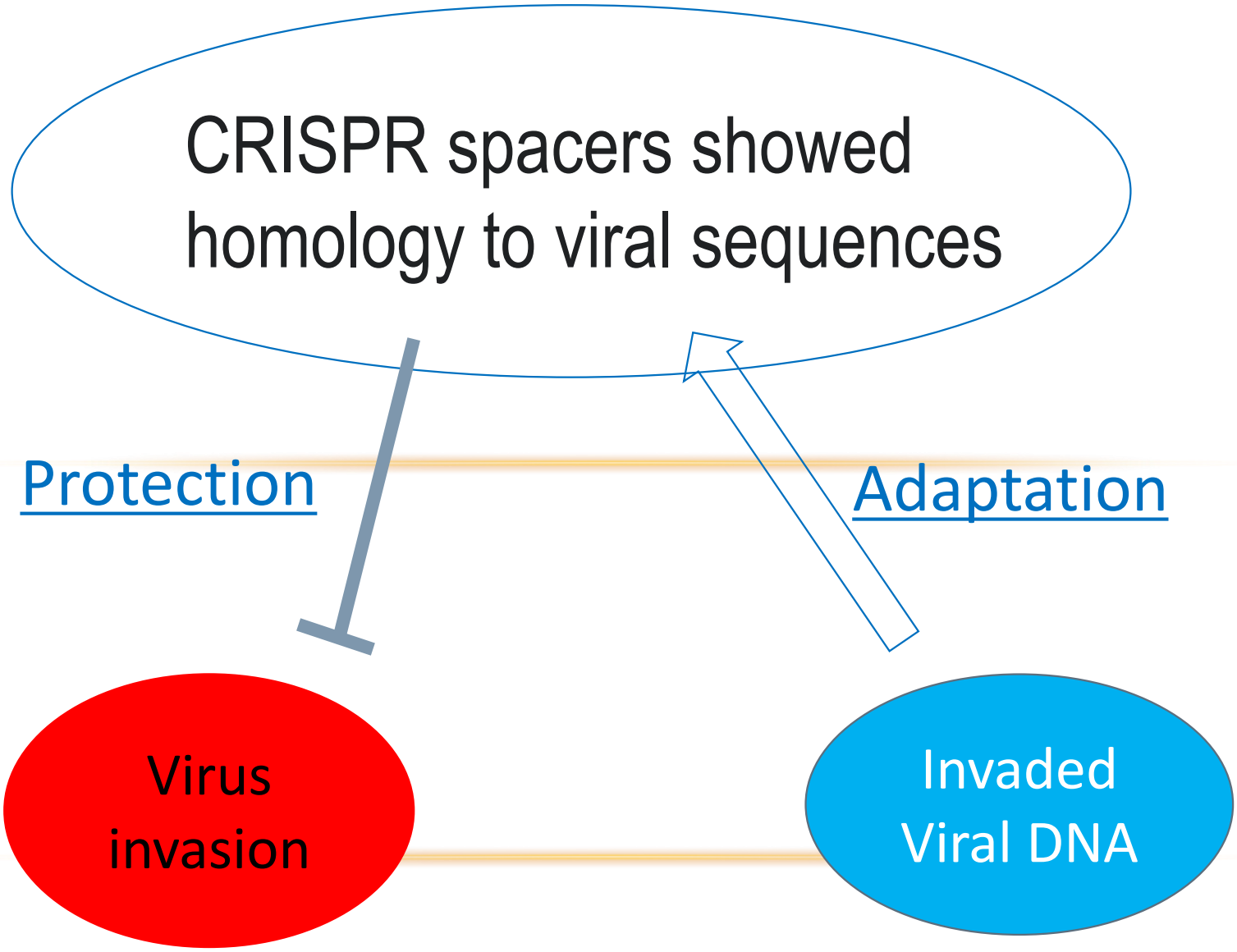
…….

We found that, after viral challenge, bacteria integrated new spacers derived from phage genomic sequences.

Removal or addition of particular spacers modified the phage-resistance phenotype of the cell.

Thus, CRISPR, together with associated cas genes, provided resistance against phages,
and resistance specificity is determined by spacer-phage sequence similarity.

CRISPR: biological function



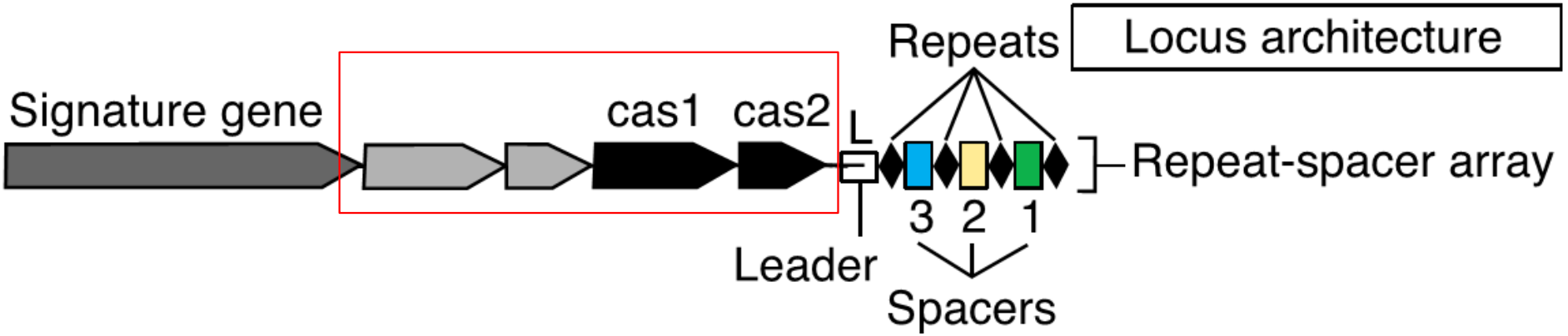
CRISPR: Molecular mechanism

1. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP et al.. **Small CRISPR RNAs guide antiviral defense in prokaryotes**. *Science*. 2008; **321**:960-4.

2. Marraffini LA, Sontheimer EJ. **CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA**. *Science*. 2008; **322**:1843-5.葡萄状球菌

3. Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L et al.. **RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex**. *Cell*. 2009; **139**:945-56.

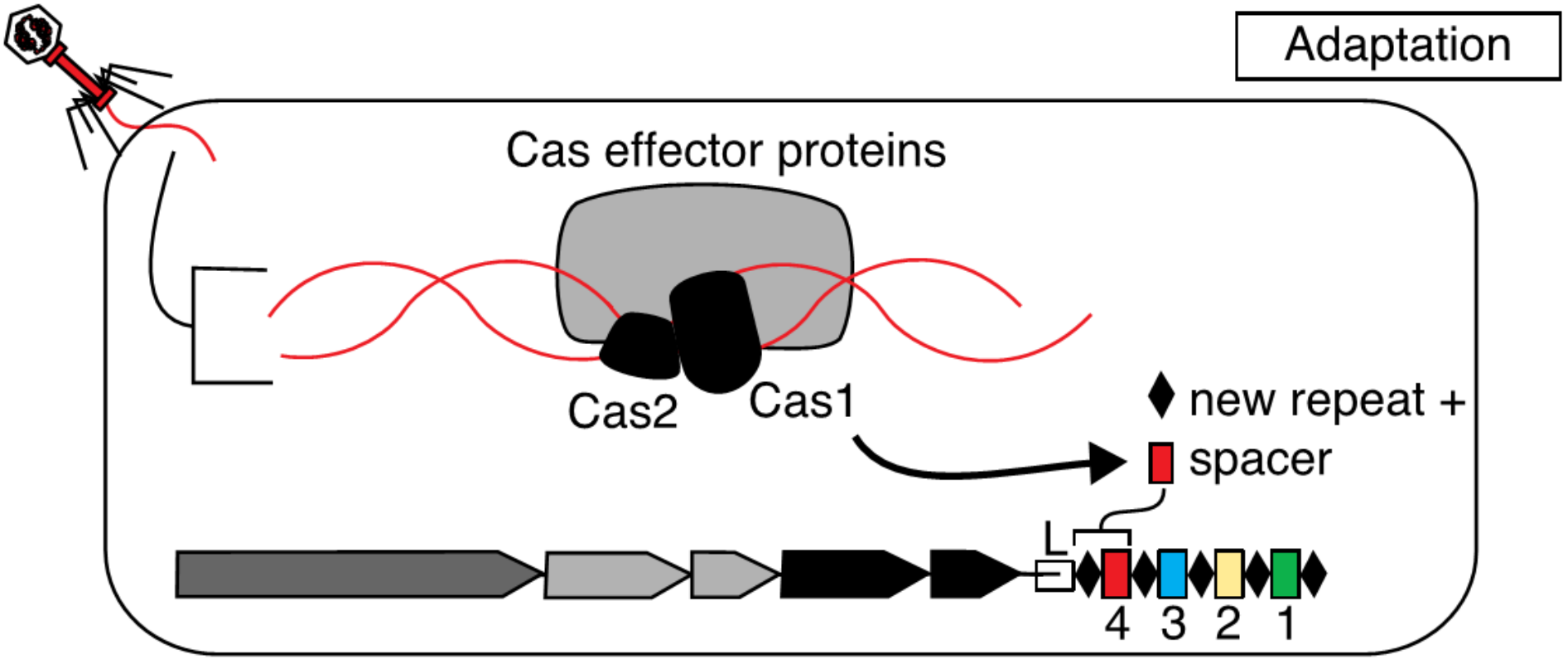
CRISPR associated (Cas) protein



Cas encodes proteins involved in the three stages of CRISPR-encoded immunity, namely adaptation, expression and interference

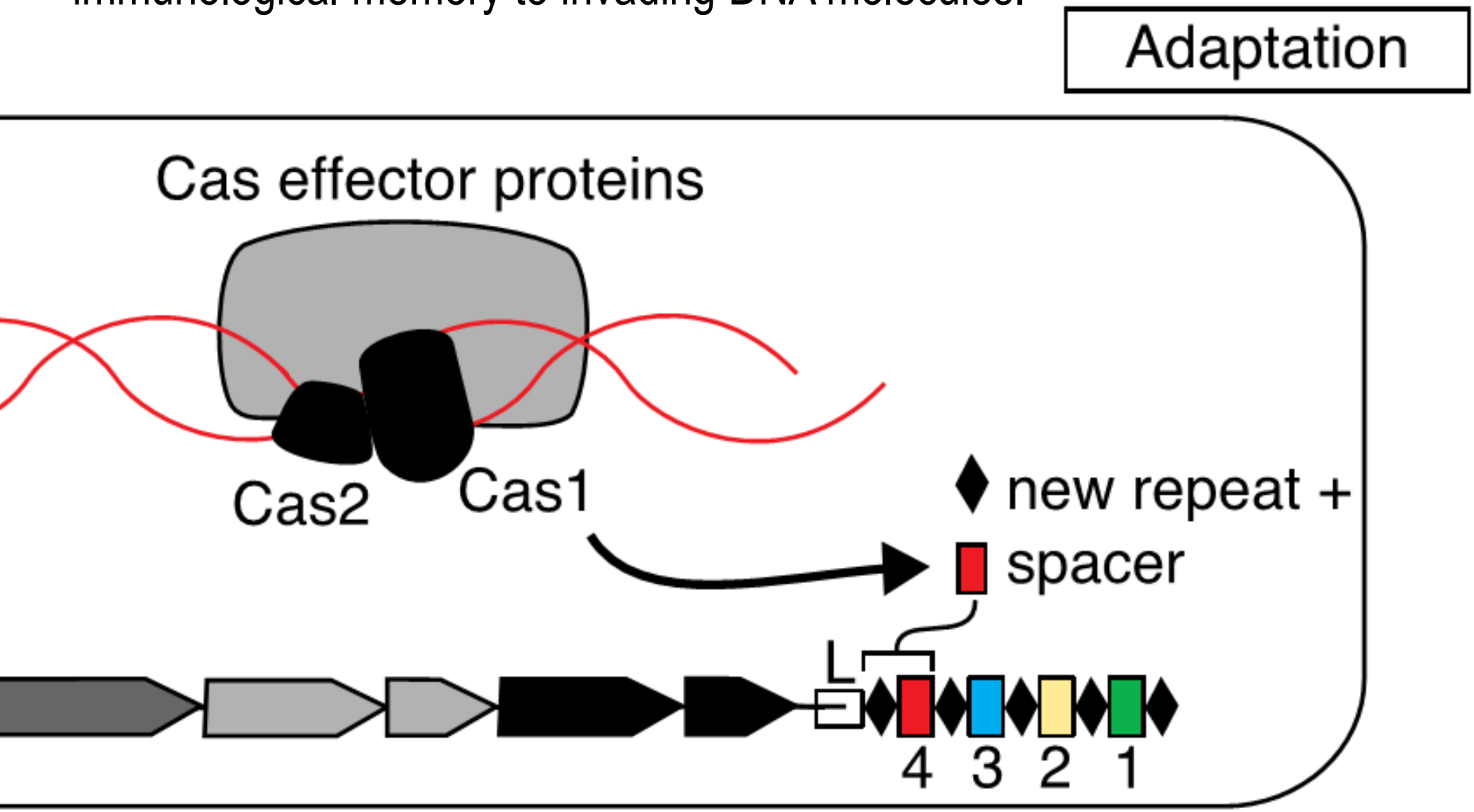
CRISPR-Cas systems and adaptive immunity

1. Adaptation

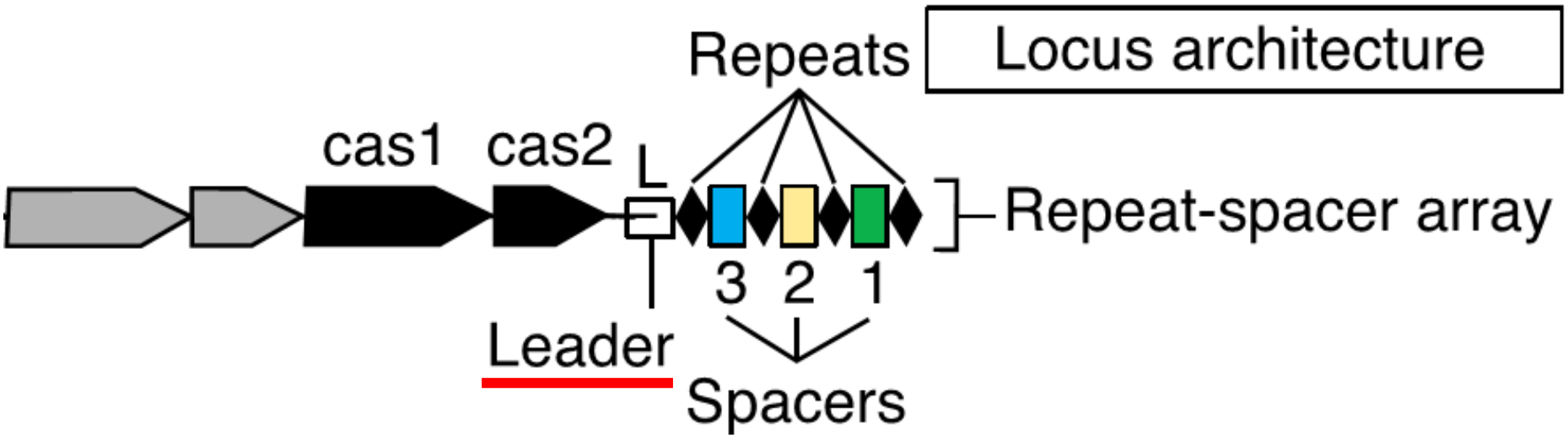


1. Adaptation

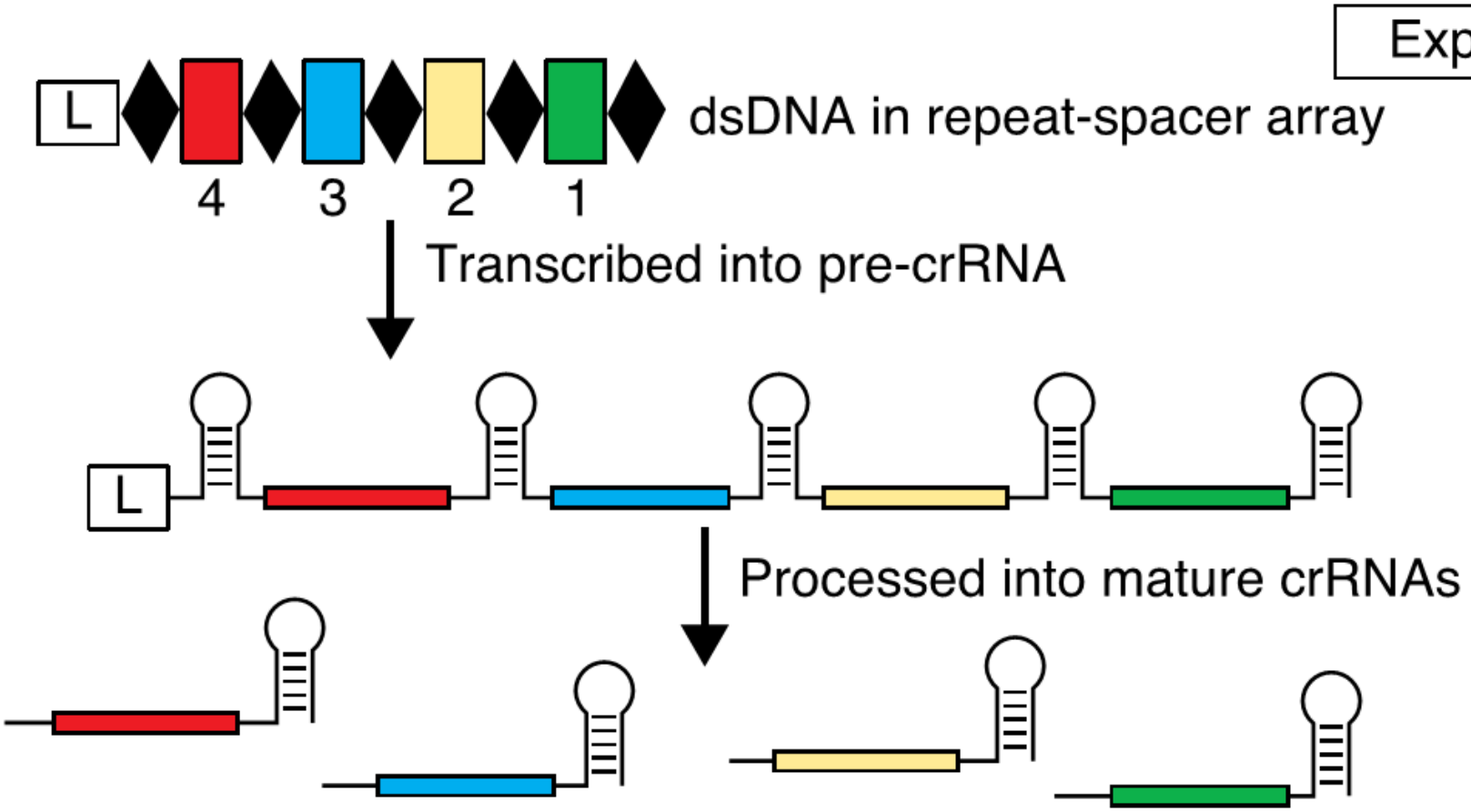
CRISPR-Cas protein machinery involved process-identification and integrating the short segments of foreign invading DNA (termed spacers) into the CRISPR array locus in host cell genome, forming an immunological memory to invading DNA molecules.



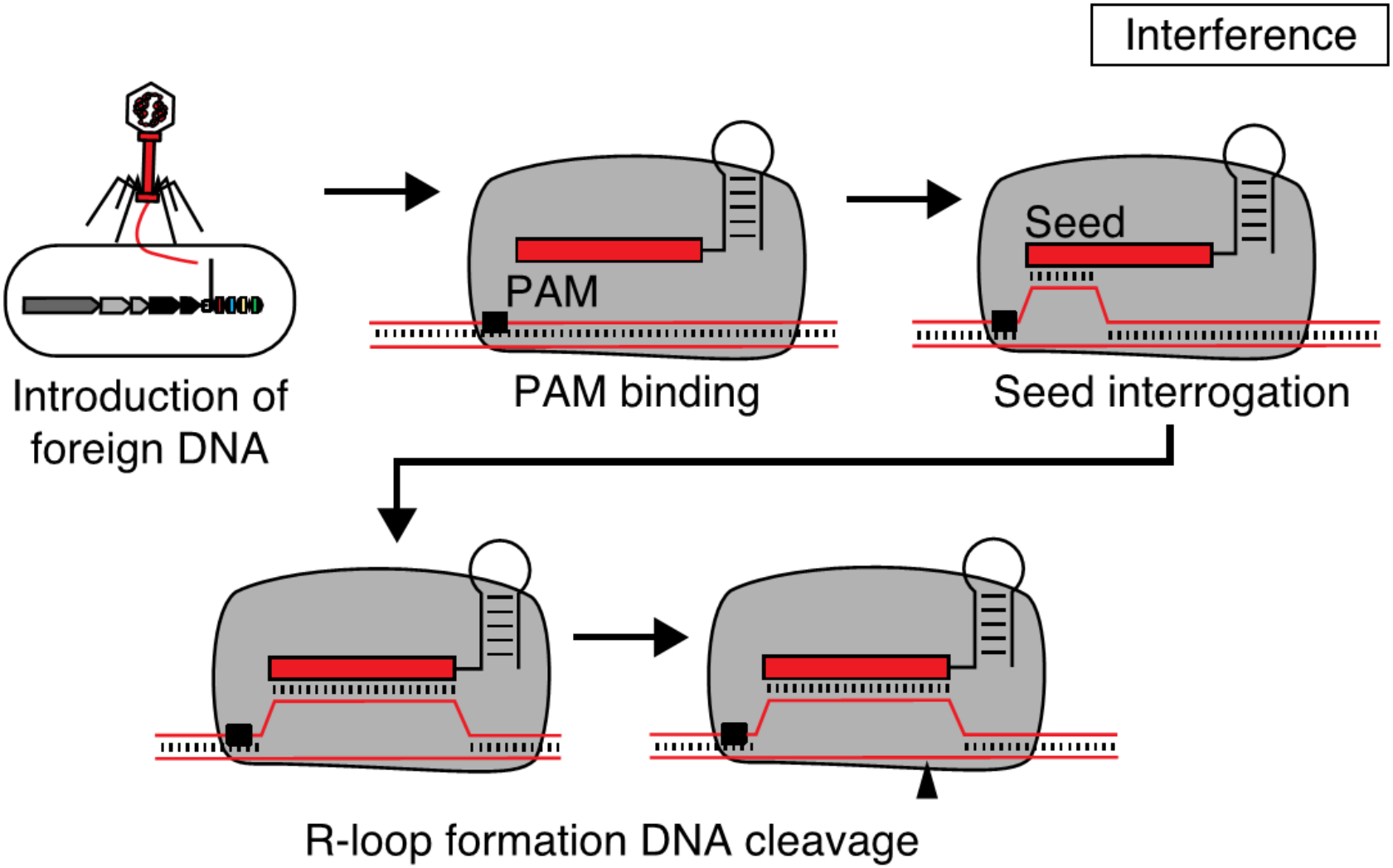
2. Expression



2. Expression



3. Interference



Key

CRISPR-Cas systems and adaptive immunity. CRISPR repeats, together with CRISPR spacers, constitute repeat-spacer arrays that define clustered regularly interspaced short palindromic repeats (CRISPRs). These CRISPR arrays are typically flanked by CRISPR associated sequences (cas) that encode Cas proteins involved in the three stages of CRISPR-encoded immunity, namely adaptation, expression and interference.

During adaptation, Cas proteins sample invasive DNA, leading to the genesis of a new repeat-spacer unit that is inserted in a polarized manner in the CRISPR array.

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CRISPR/Cas *in vivo*: Bacterial Adaptive Immunity

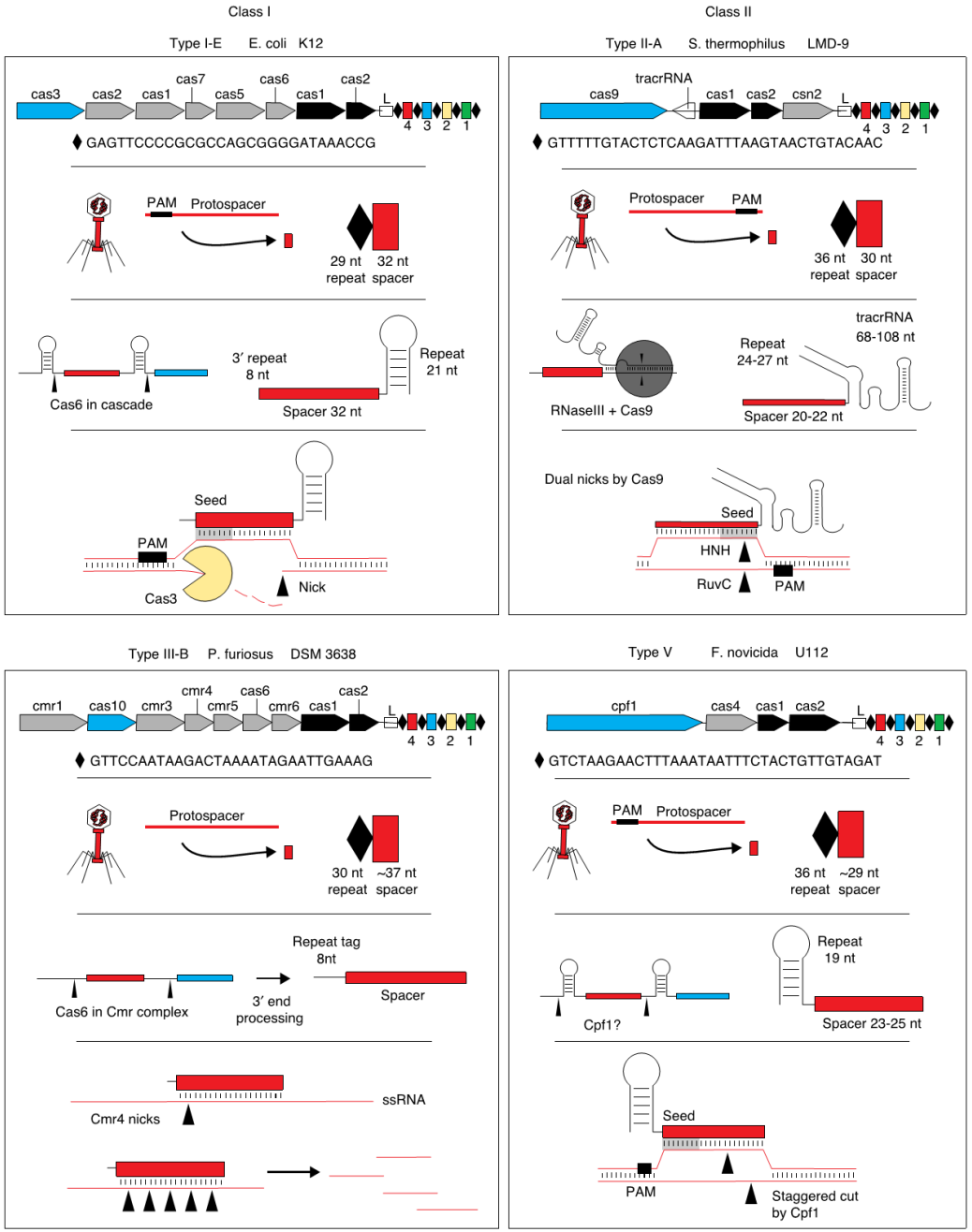
Evolutionary significance

Found in 90% of archaea and 40% of bacteria tested so far

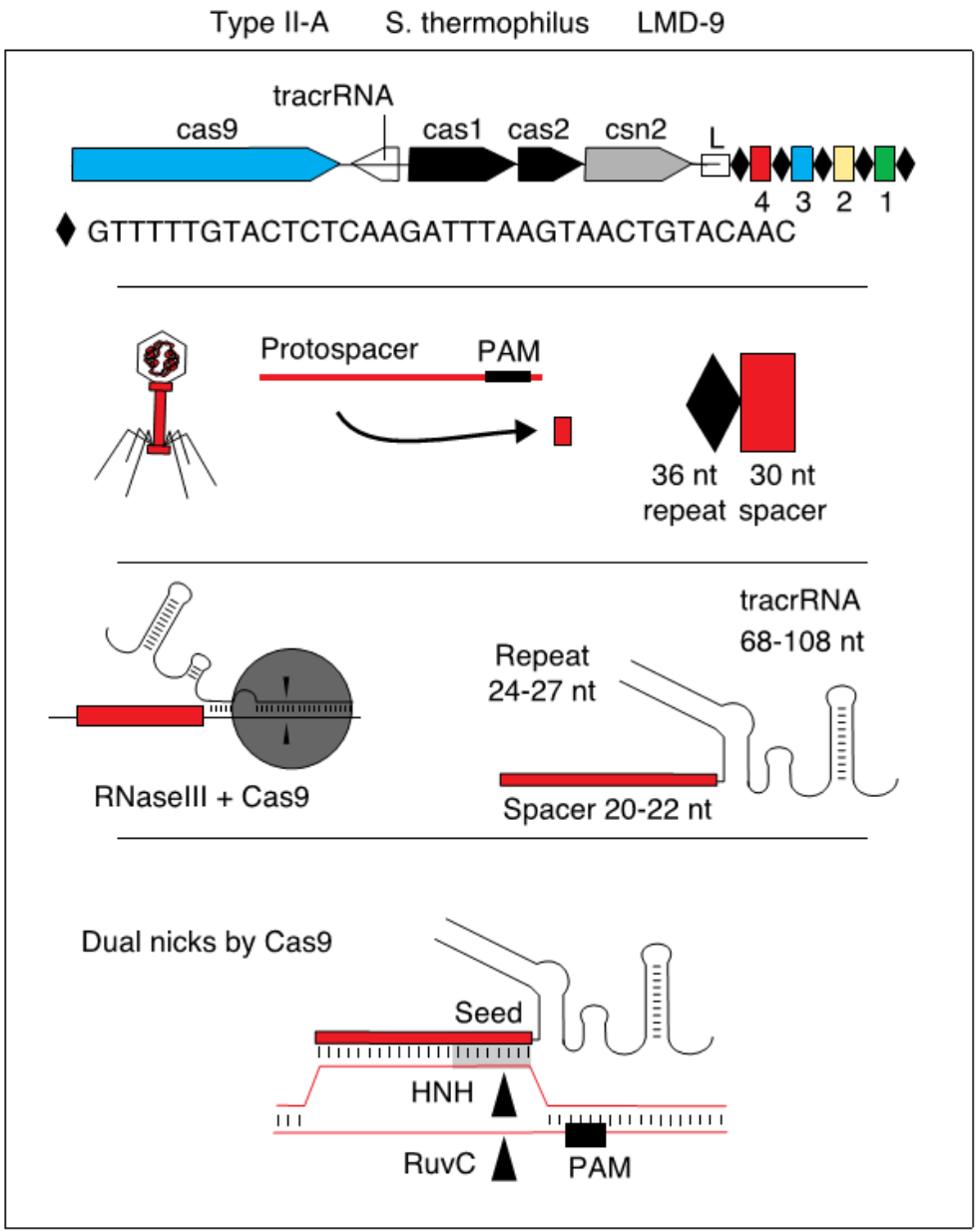
CRISPR/Cas

Developed as Molecular Biology Tools used for genome editing in Eukaryotic

Diversity of CRISPR-Cas Machines in Prokaryotes



Class II type Cas system



Engineering Type II CRISPR/Cas system

Three-component System

- Cas9
- trans-activating CRISPR RNA (tracrRNA)
- crRNA

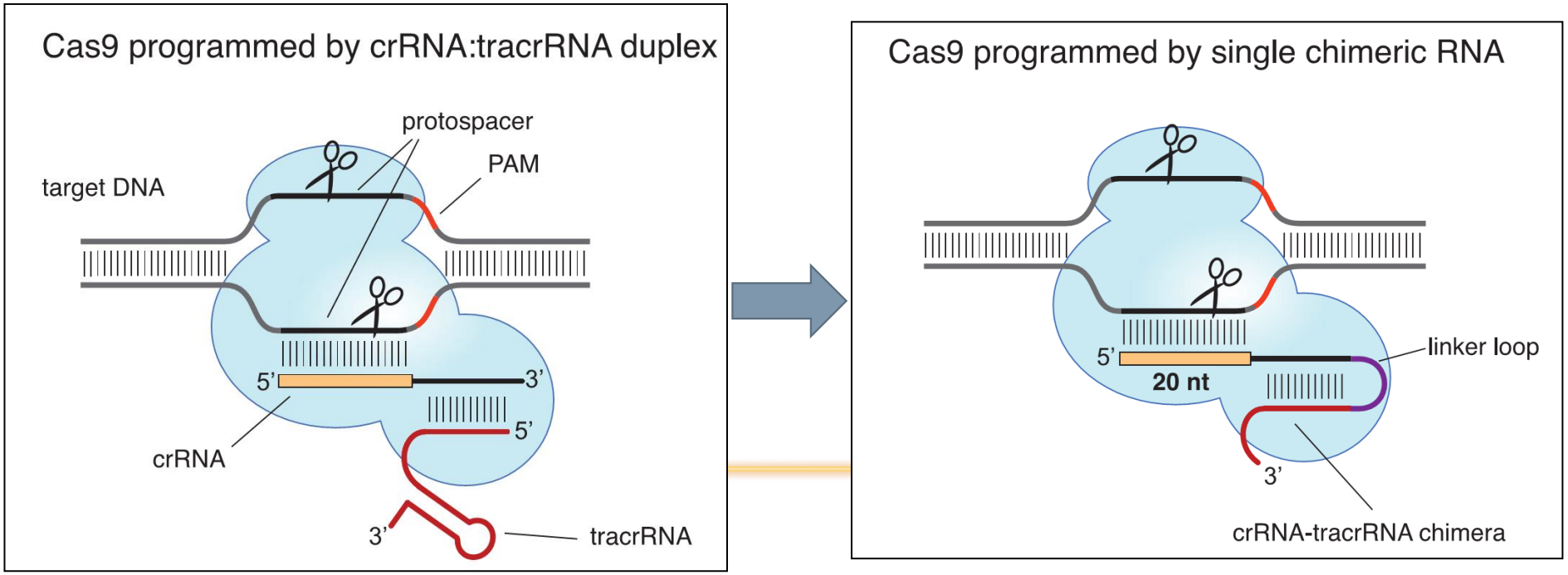
17 AUGUST 2012 VOL 337 SCIENCE

RESEARCH ARTICLE

A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity

Martin Jinek,[1,2]* Krzysztof Chylinski,[3,4]* Ines Fonfara,[4] Michael Hauer,[2]†
Jennifer A. Doudna,[1,2,5,6]‡ Emmanuelle Charpentier[4]‡

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. We show here that in a subset of these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA structure that directs the CRISPR-associated protein Cas9 to introduce double-stranded (ds) breaks in target DNA. At sites complementary to the crRNA-guide sequence, the Cas9 HNH nuclease domain cleaves the complementary strand, whereas the Cas9 RuvC-like domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.



single guide RNA (sgRNA)

Streptococcus pyogenes
酿脓链球菌

Engineering Type II CRISPR/Cas system

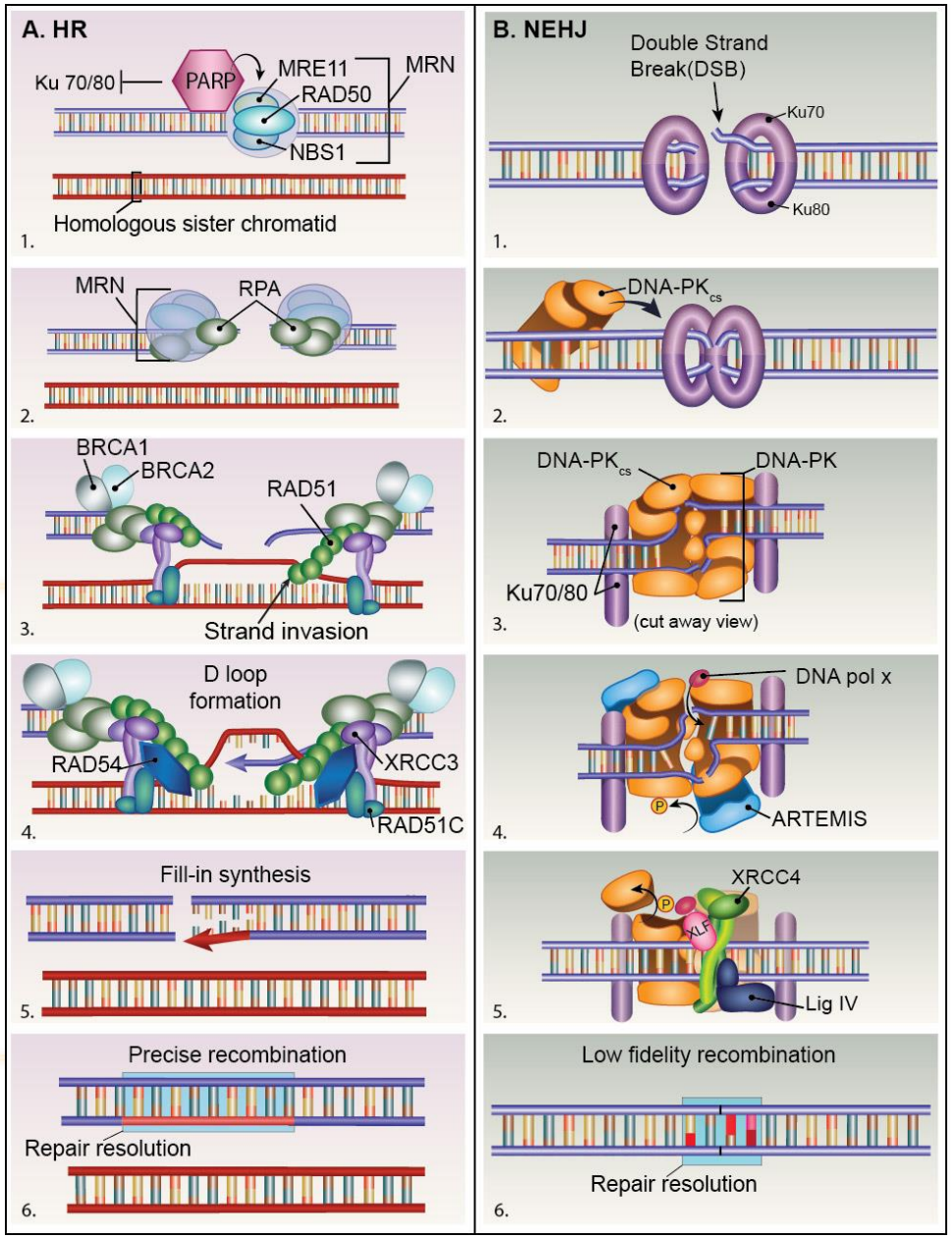
Three-component System

- Cas9
- trans-activating CRISPR RNA (tracrRNA)
- crRNA

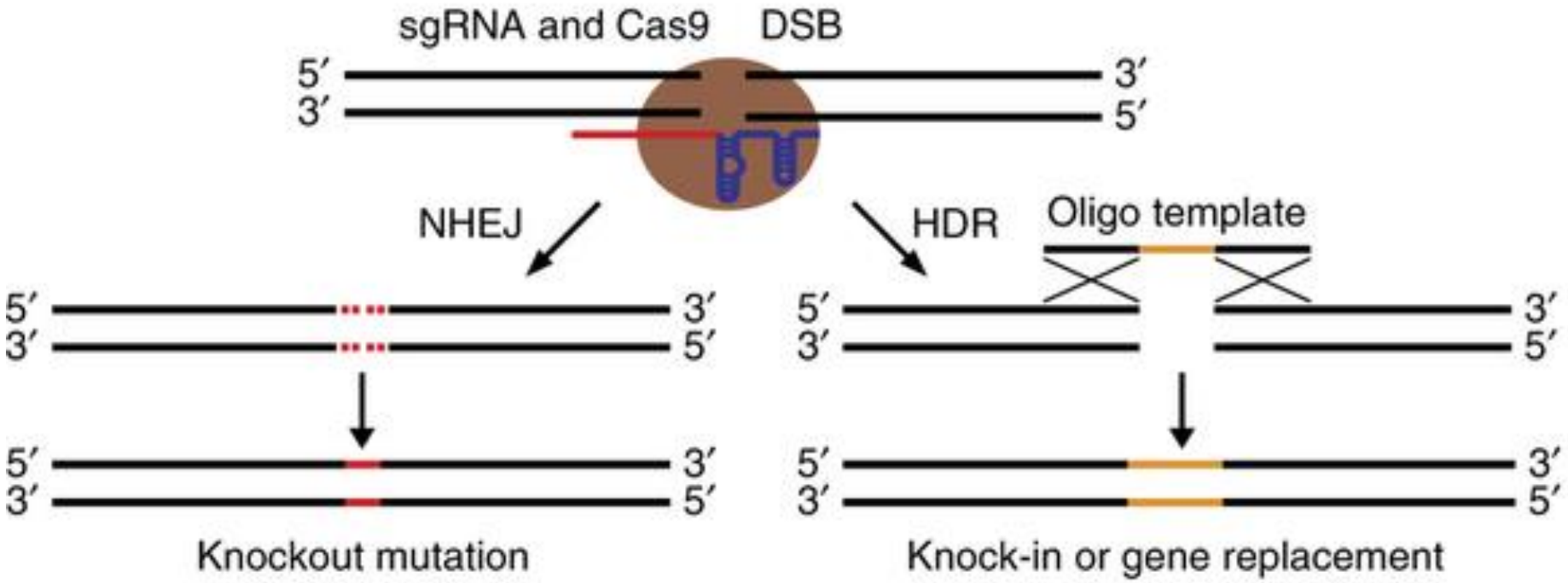
Two-component System

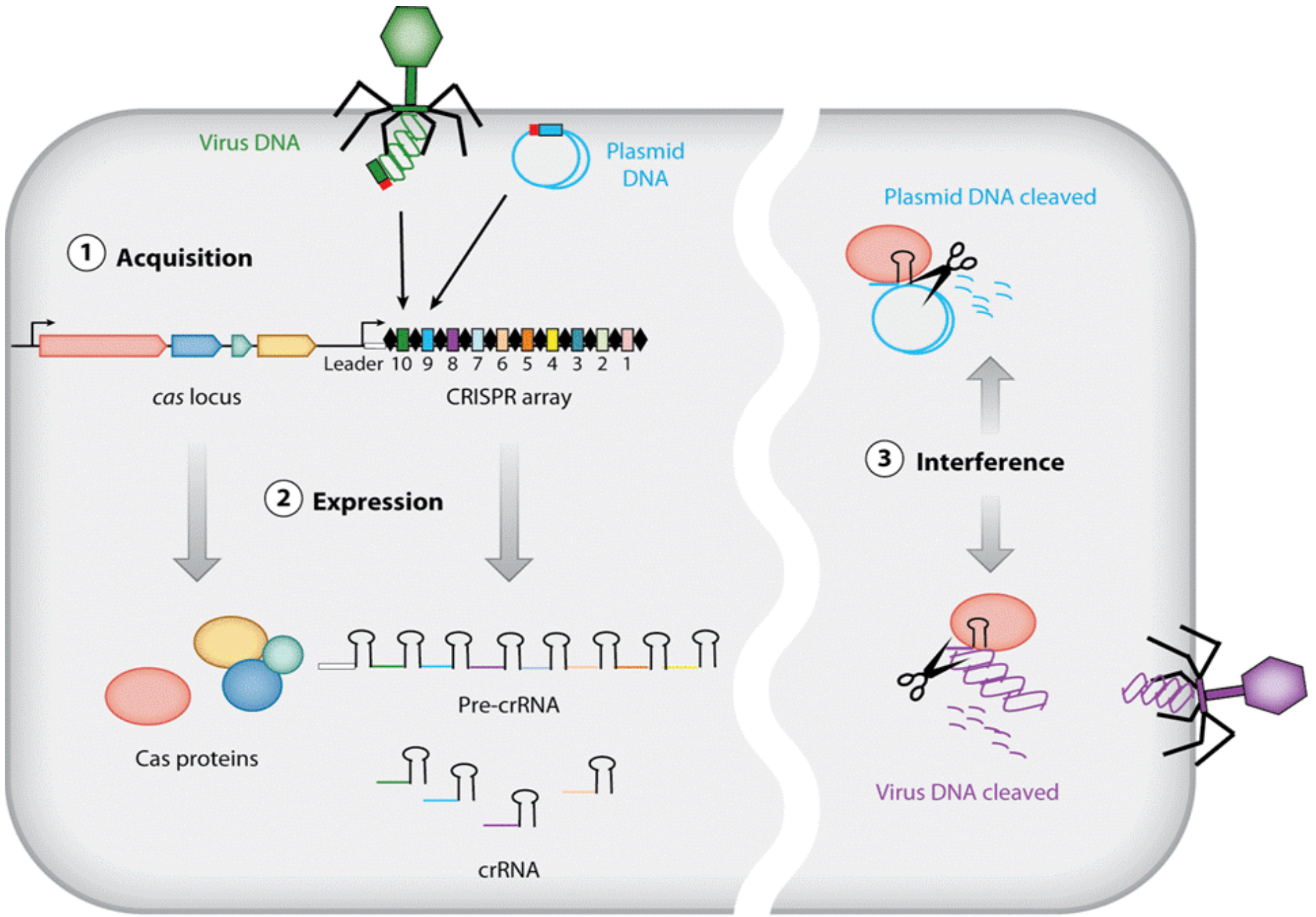
- Cas9
- sgRNA

DNA repairing machine in eukaryote



Mutation introduced in DNA repair




Virus DNA
Plasmid DNA
1 Acquisition
Leader 10 9 8 7 6 5 4 3 2 1
cas locus
CRISPR array
2 Expression
Cas proteins
Pre-crRNA
crRNA
Plasmid DNA cleaved
3 Interference
Virus DNA cleaved

Key

CRISPR-Cas systems and adaptive immunity. CRISPR repeats, together with CRISPR spacers, constitute repeat-spacer arrays that define clustered regularly interspaced short palindromic repeats (CRISPRs). These CRISPR arrays are typically flanked by CRISPR associated sequences (cas) that encode Cas proteins involved in the three stages of CRISPR-encoded immunity, namely adaptation, expression and interference.

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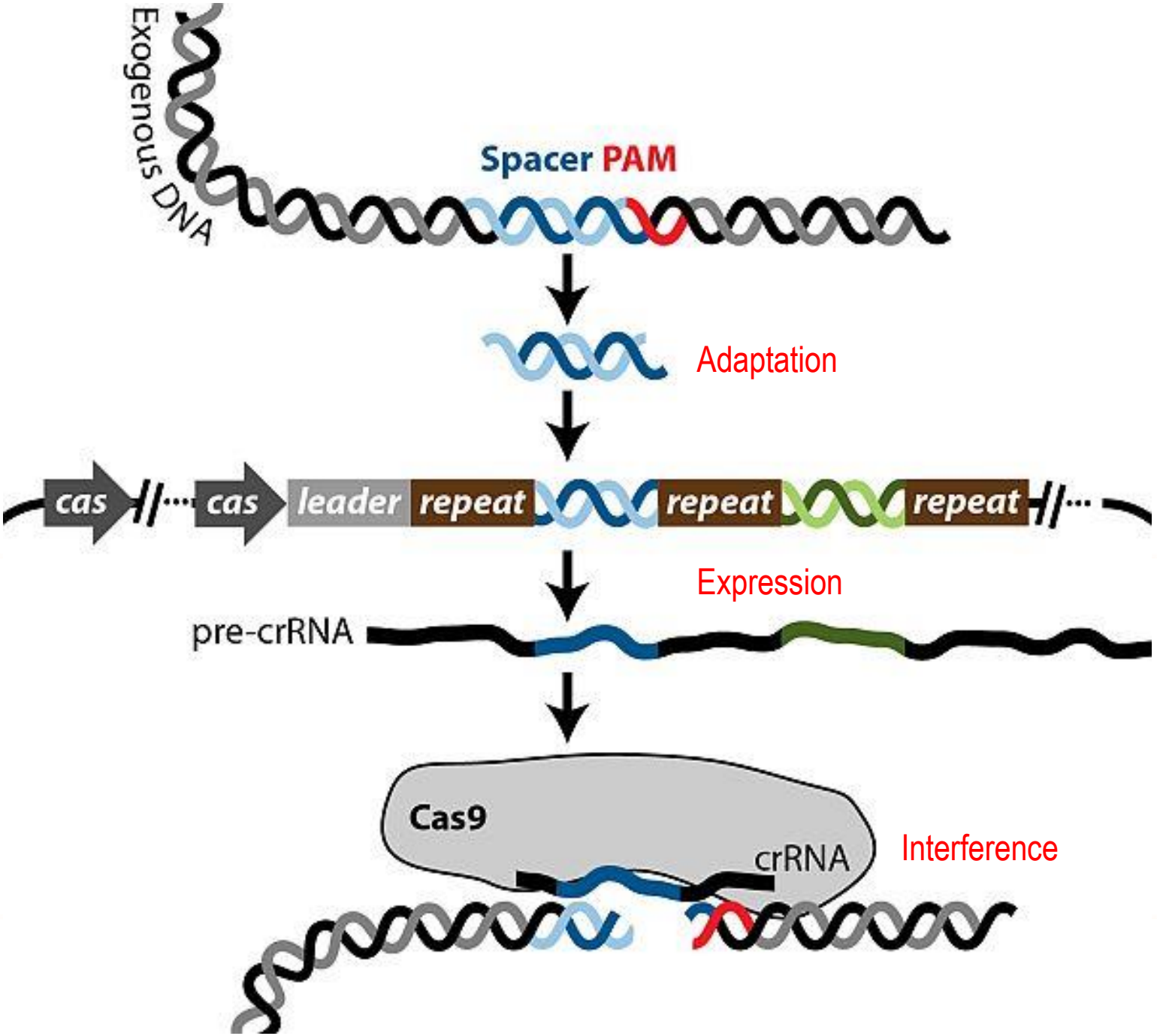
Key

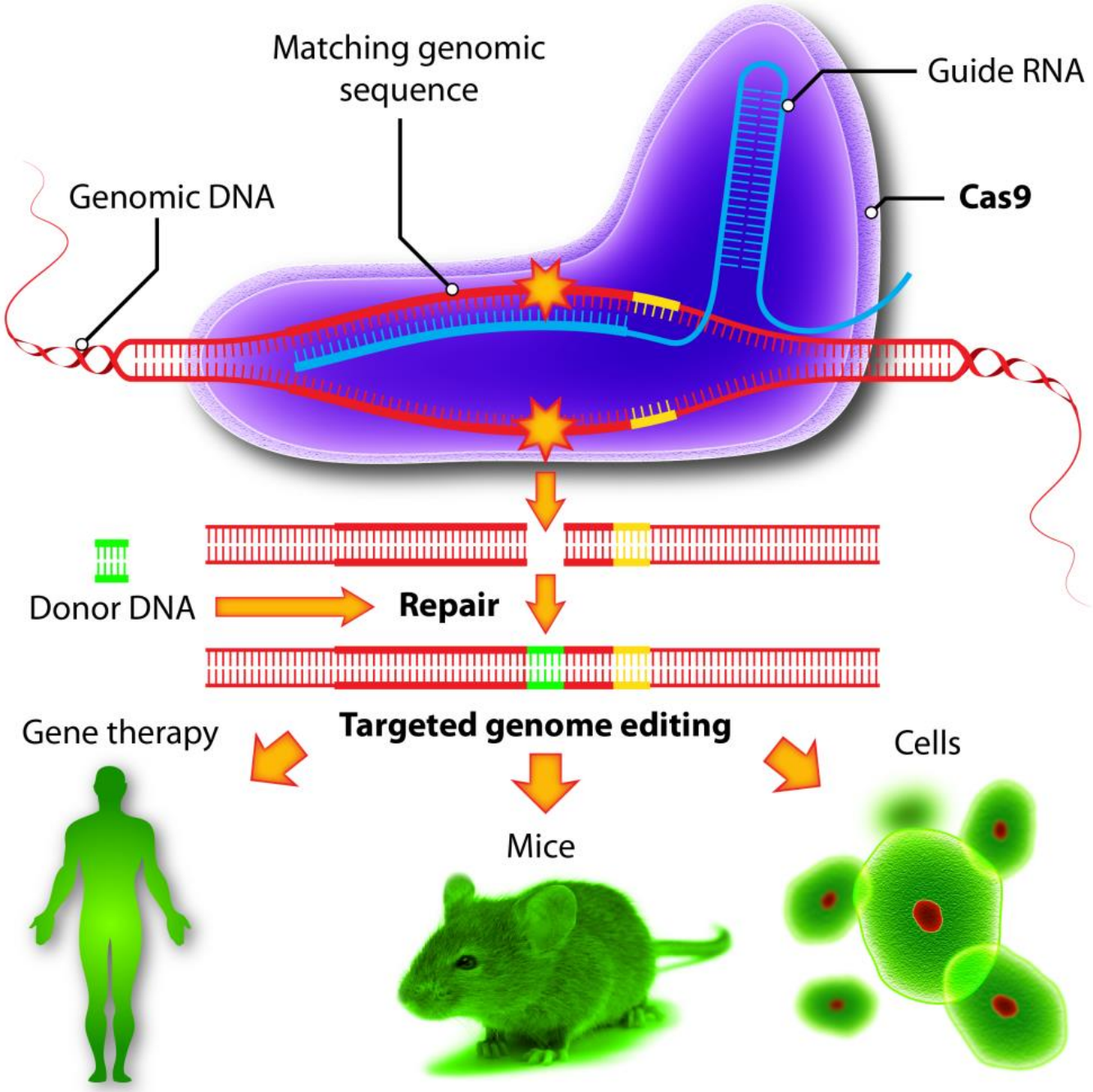
In the acquisition/adaptation phase, foreign DNA is incorporated into the bacterial genome at the CRISPR loci.

CRISPR loci is then transcribed and processed into crRNA during crRNA biogenesis. Expression

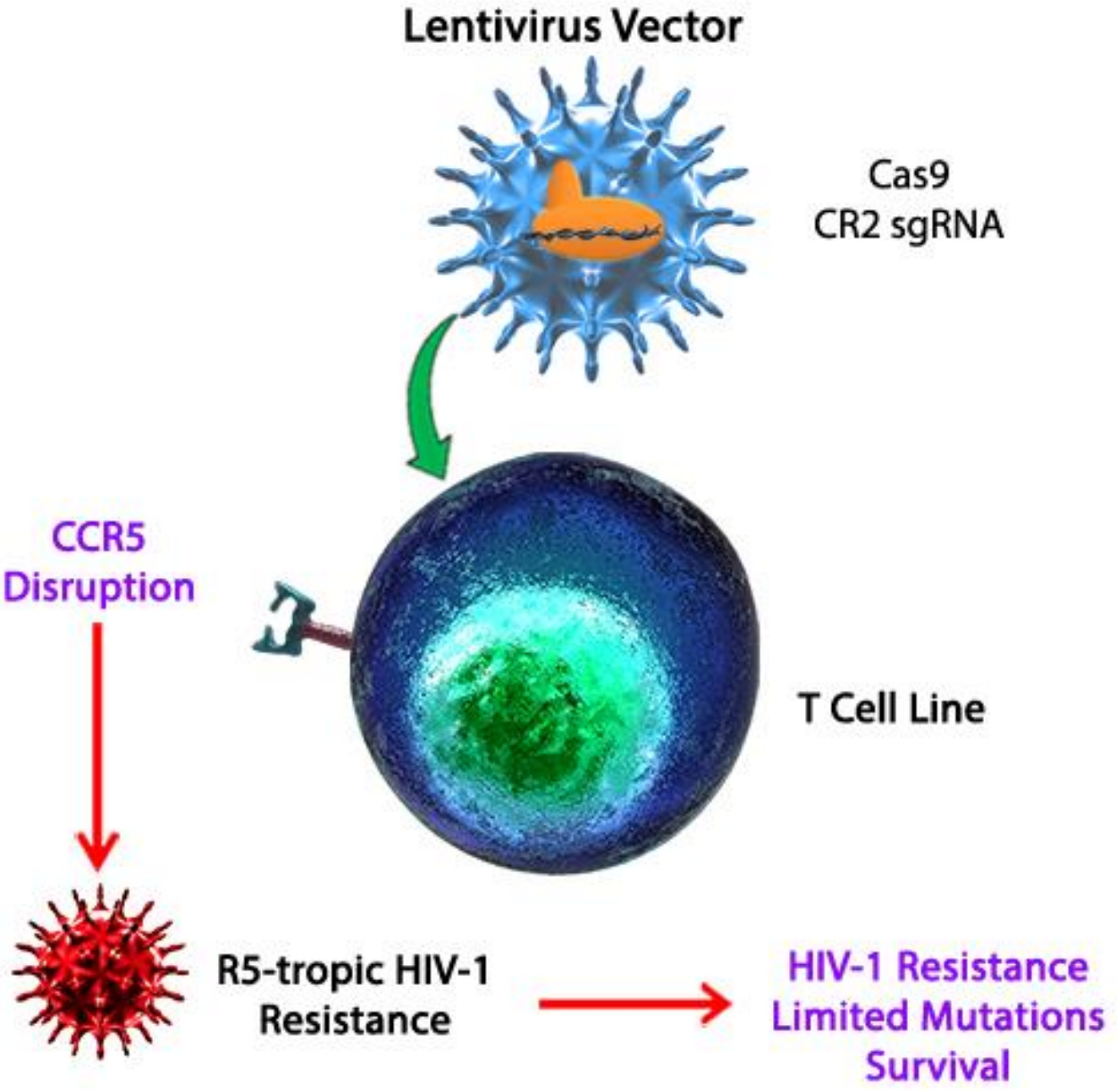
During interference, Cas9 endonuclease complexed with a crRNA and separate tracrRNA cleaves foreign DNA containing a 20-nucleotide crRNA complementary sequence adjacent to the PAM sequence.

Key

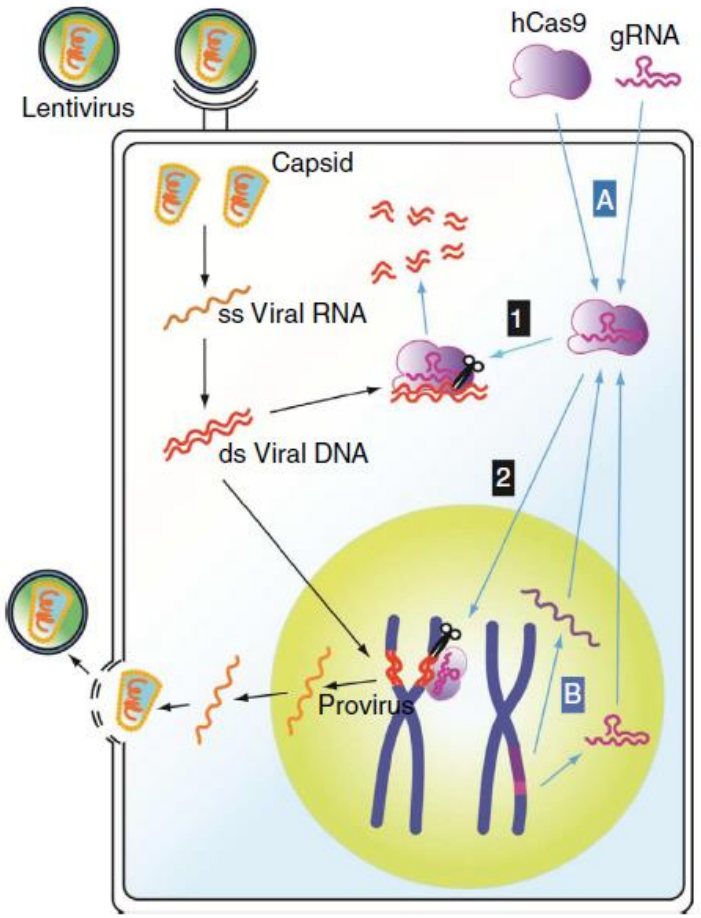

Exogenous DNA
Spacer PAM
Adaptation
cas
cas
leader
repeat
repeat
repeat
Expression
pre-crRNA
Cas9
crRNA
Interference


Matching genomic sequence
Guide RNA
Genomic DNA
Cas9
Donor DNA
Repair
Gene therapy
Targeted genome editing
Cells
Mice

Modified cell resistant to HIV



Genome Editing Cuts Out HIV



Researchers use the CRISPR/Cas9 method to remove the virus from the host genome in human cell lines.

W. Hu et al., "RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection," *PNAS*, doi:10.1073/pnas.1405186111, 2014.

CRISPR Takes the PERV Out of Pig Organs

GENOME EDITING

Genome-wide inactivation of porcine endogenous retroviruses (PERVs)

Luhan Yang,[1,2,3]*† Marc Güell,[1,2,3]† Dong Niu,[1,4]† Haydy George,[1]† Emal Lesha,[1] Dennis Grishin,[1] John Aach,[1] Ellen Shrock,[1] Weihong Xu,[6] Jürgen Poci,[1] Rebeca Cortazio,[1] Robert A. Wilkinson,[5] Jay A. Fishman,[5] George Church[1,2,3]*

The shortage of organs for transplantation is a major barrier to the treatment of organ failure. Although porcine organs are considered promising, their use has been checked by concerns about the transmission of porcine endogenous retroviruses (PERVs) to humans. Here we describe the eradication of all PERVs in a porcine kidney epithelial cell line (PK15). We first determined the PK15 PERV copy number to be 62. Using CRISPR-Cas9, we disrupted all copies of the PERV *pol* gene and demonstrated a >1000-fold reduction in PERV transmission to human cells, using our engineered cells. Our study shows that CRISPR-Cas9 multiplexability can be as high as 62 and demonstrates the possibility that PERVs can be inactivated for clinical application of porcine-to-human xenotransplantation.

Science 27 November 2015: Vol. 350

“一段重复序列的故事”