Advanced tools for molecular microbiology

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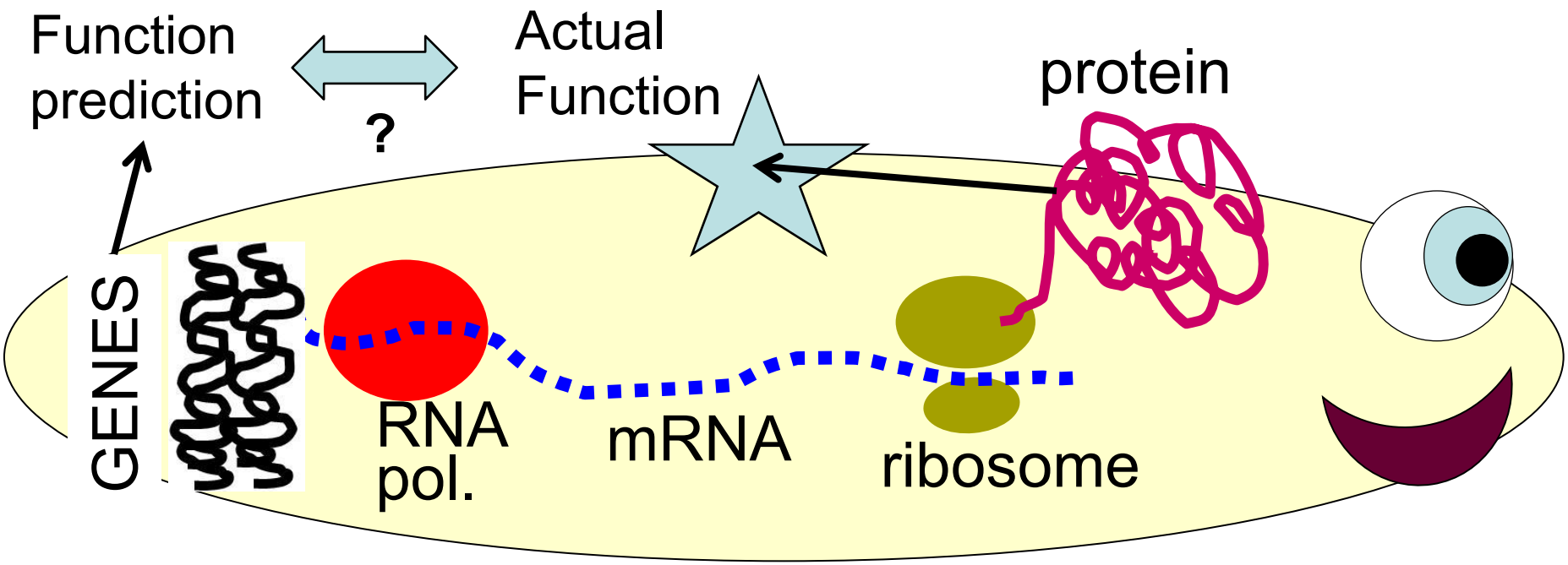
Advanced tools for molecular microbiology

Learning outcomes. Be able to…

- **Explain why we need molecular tools to make knockouts, and to enable heterologous expression**
- **Describe, compare, and contrast 3 different methods for making gene knockouts**
- **Describe, compare, and contrast 2 different strategies for heterologous expression of genes**

Why do we need these molecular tools?

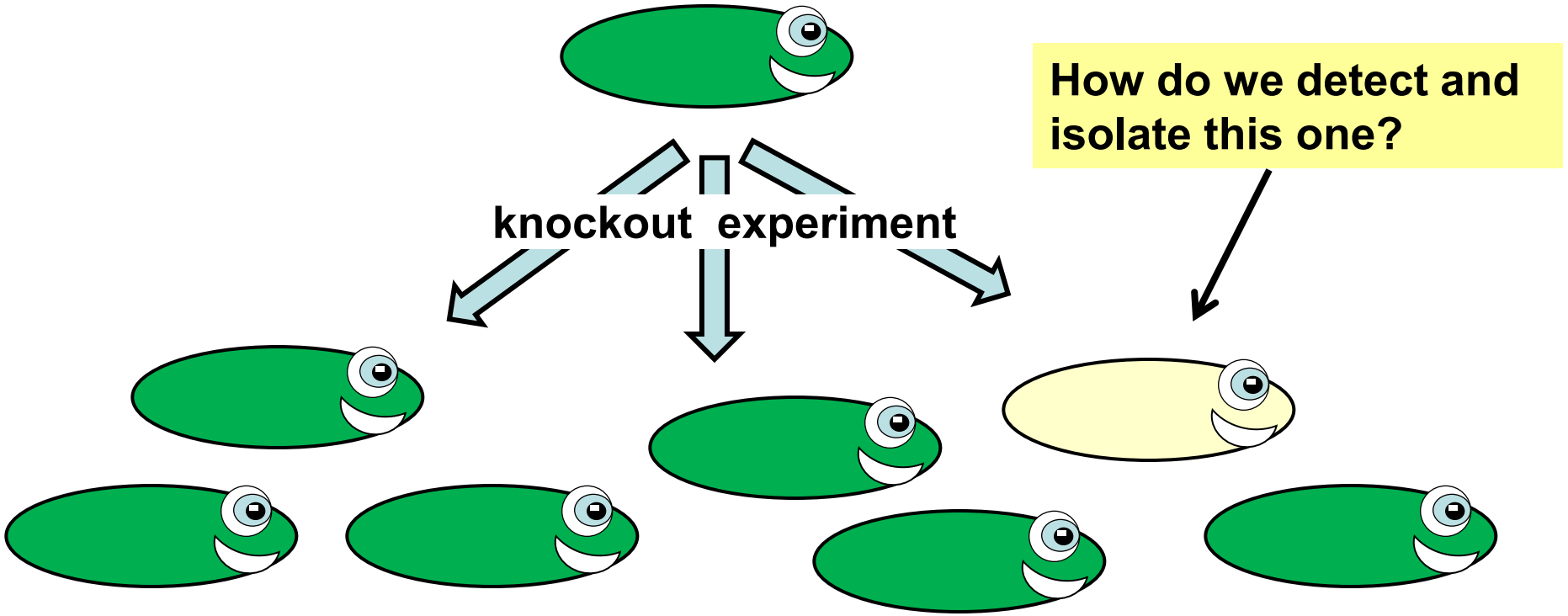
- **Genome sequencing of microbes has yielded very large amounts of data… we can *predict* many functions, but…**
- **Rigorous link of genes → functions lacking in many cases**
- **Gene knockout and heterologous expression methods can test the linkage between genes and functions**



Targeted approaches for studying gene function

- **Knockouts and heterologous expression are both targeted approaches to studying gene function**

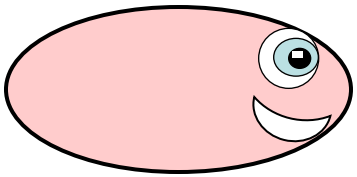
- **With these methods, we need a specific hypothesis and a specific screening method to detect phenotype changes.**



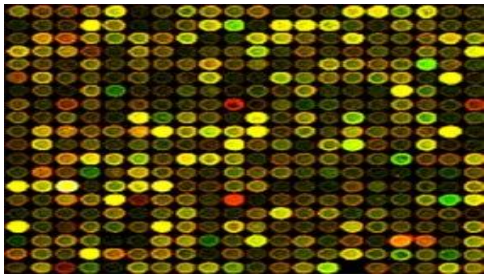
…some untargeted approaches for comparison…

- **Not all methods of linking genes with functions are 'targeted' in the same way as knockouts / expression**

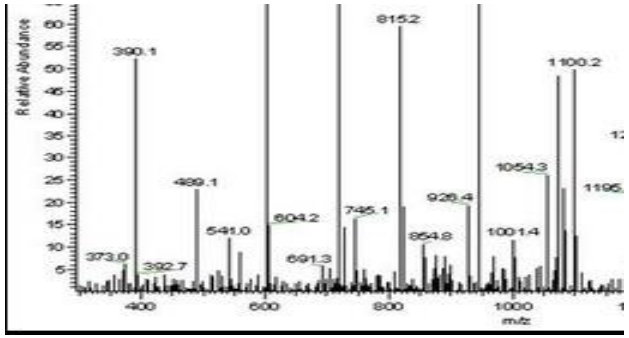
- **Untargeted methods have some advantages**
  - they give a holistic view of gene expression
  - they can reveal unexpected patterns and correlations



Transcriptomics



Metabolomics



Proteomics

Random mutagenesis (eg. with Tn5) would also be an <u>UNtargeted</u> approach to linking gene & function

Part 1. Methods for making gene knockouts

- **Double crossover**
- **Recombineering**
- **Cre/loxP**

Strategies for making gene knockouts in microbes

- All methods aim to specifically delete one or more genes ie. not random mutagenesis like chemical or transposon

- A segment of the normal DNA is replaced with a piece of foreign DNA – this contains selectable marker genes

- Minimal requirements:
  - A selectable marker gene that works in that host
  - A method for introducing DNA into the cells
  - An active homologous recombination system

Strategies for making knockouts

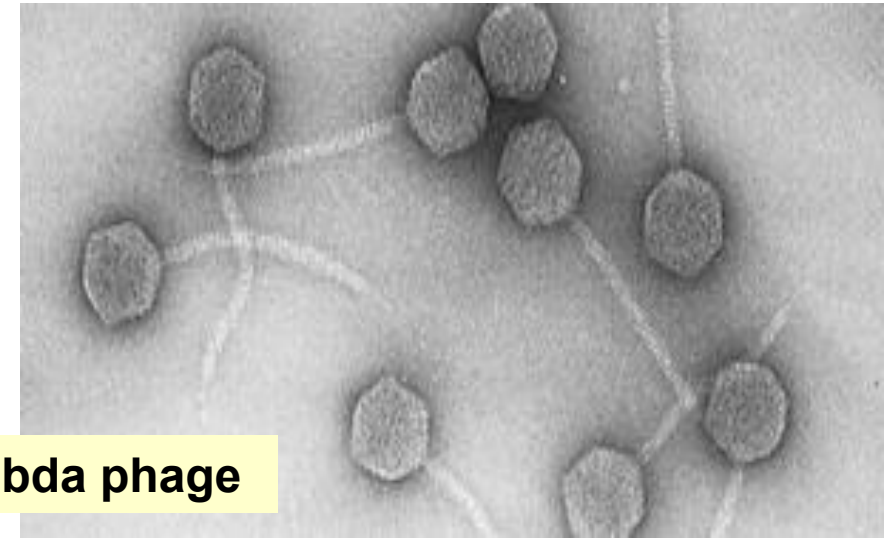
- Different mechanisms of DNA recombination can be used to make gene knockouts. Each involves distinct types of DNA-handling enzymes.

- Homologous recombination host enzymes eg. RecA

- Recombineering Enhanced homologous recombination. Phage enzymes eg. λ Exo/Beta/Gam

- Site-specific recombination

  Phage enzymes eg. Cre



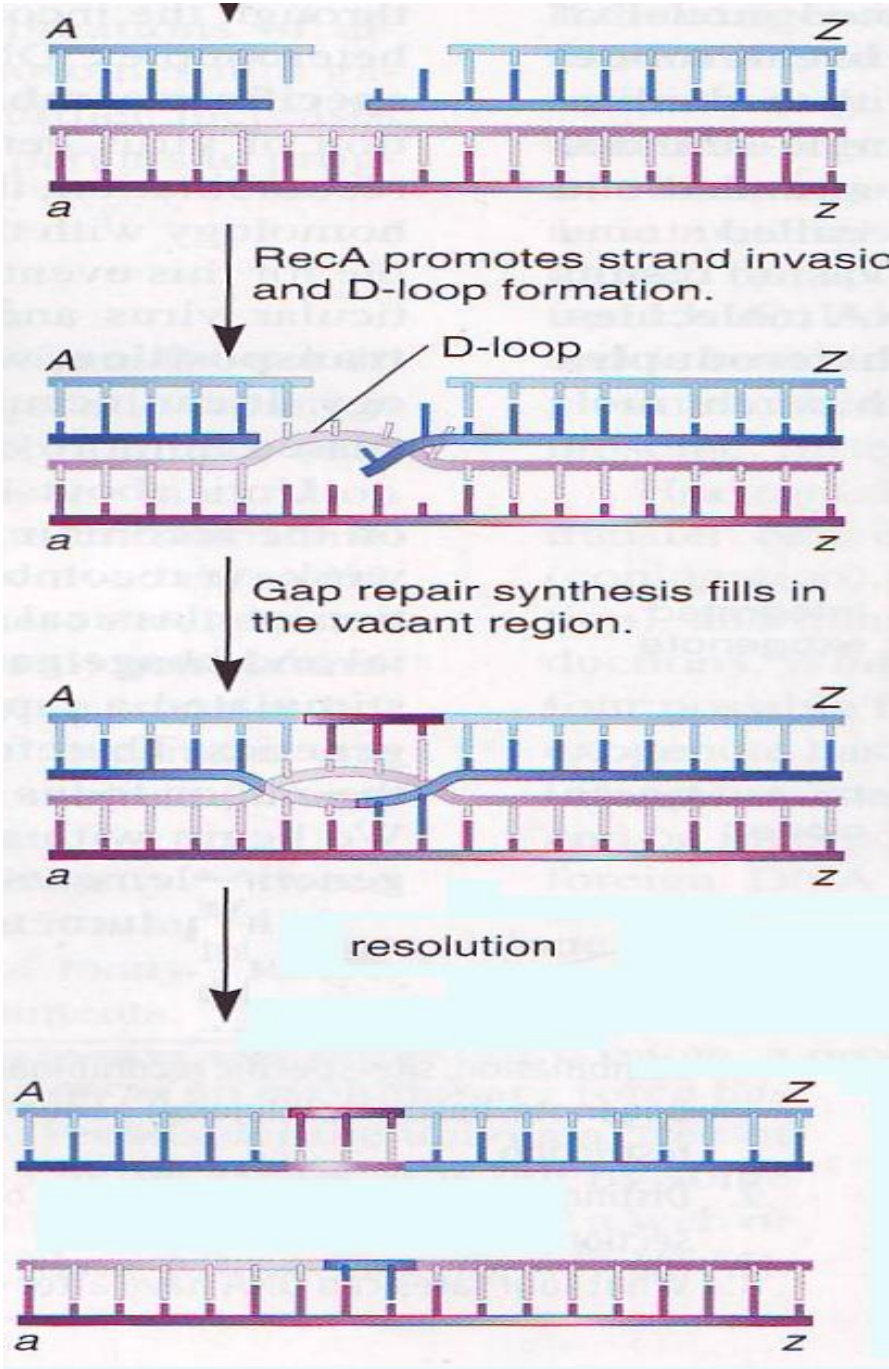
Lambda phage

Strategies for making knockouts

Homologous recombination

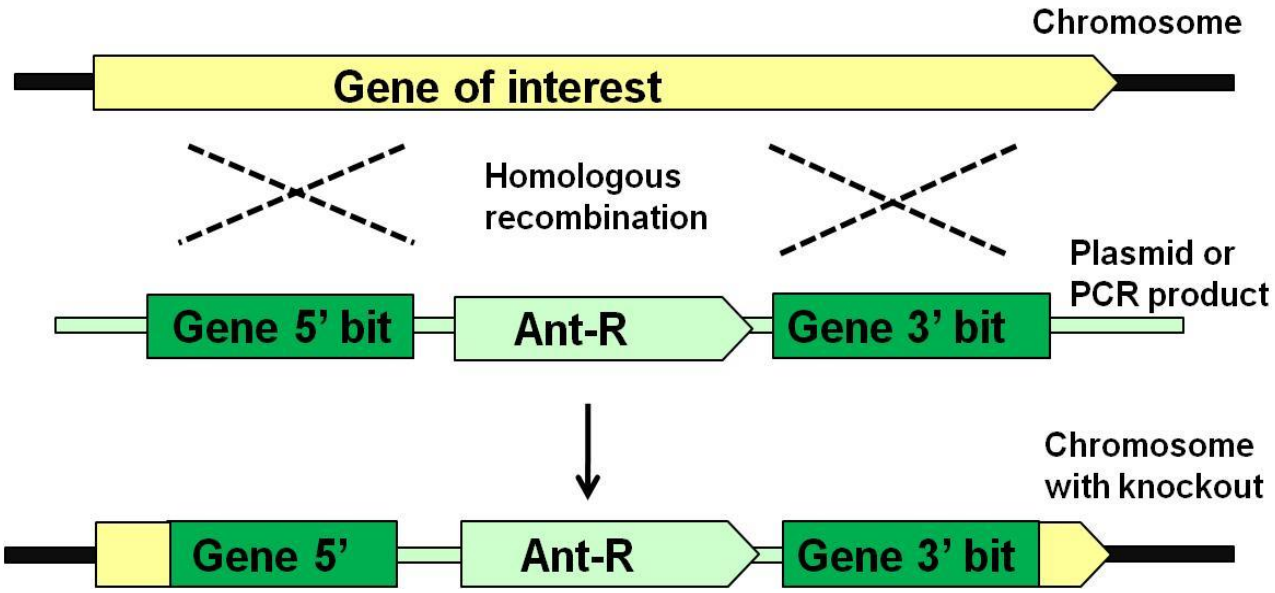
- Requires host enzymes eg. RecA, RecBCD
- RecA has homologs in every organism. Crucial role in DNA repair in all types of cells.

E.coli strains used for cloning are often recA mutants; this improves stability of recombinant plasmids



Homologous recombination : Double Crossover

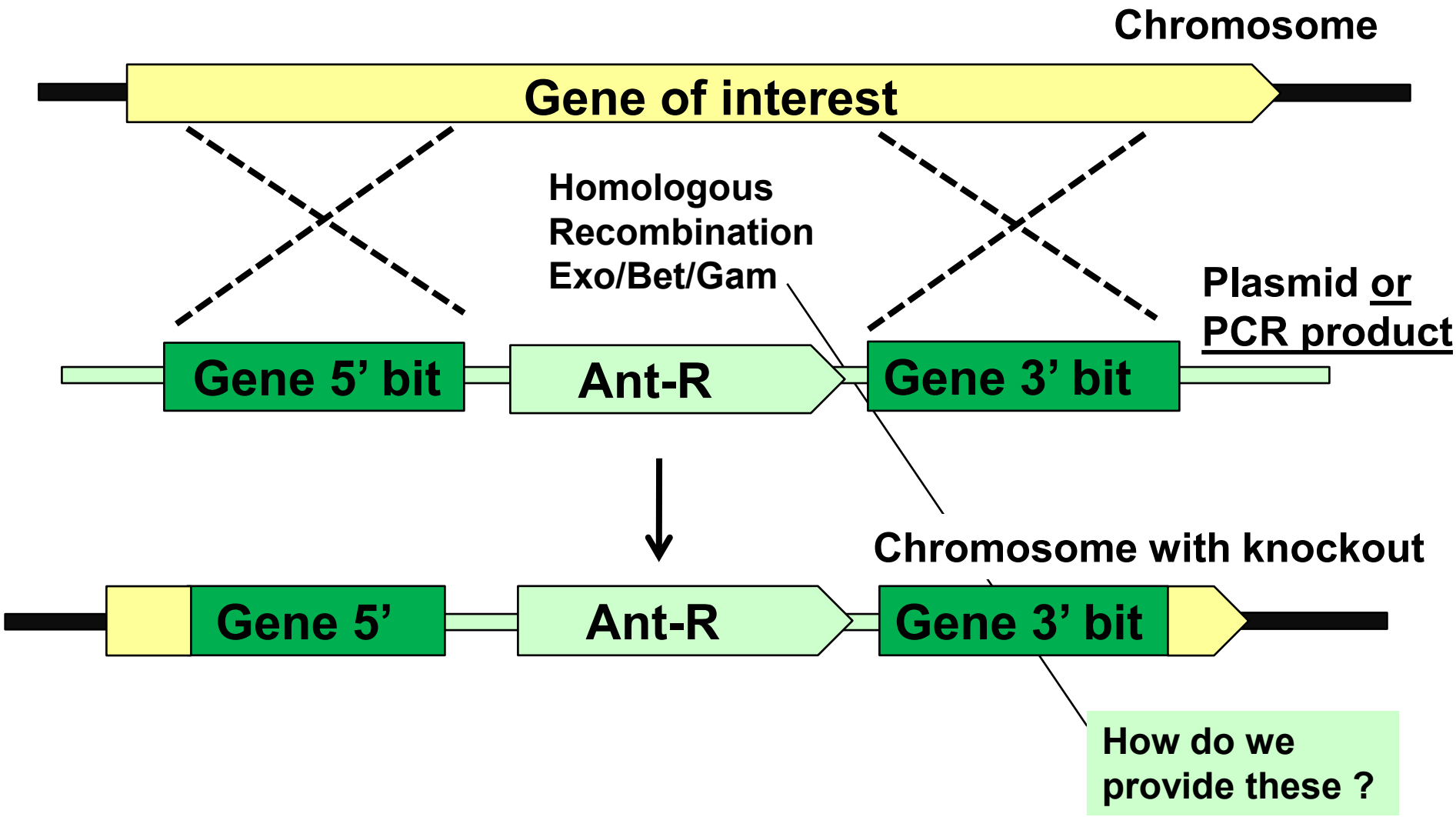
- Can fully remove target gene
- Not a ‘clean’ deletion, replacement by resistance gene
- Simple setup, just need to add one DNA to cells
- Dependent on host enzymes for recombination



Recombineering

- Use of phage lambda Exo / Bet / Gam enzymes to increase the frequency of homologous recombination
- Higher efficiency than plain homol. recomb.
- Can fully remove target gene
- Not a ‘clean’ deletion, replacement by resistance gene
- Complex setup, need to add multiple DNAs to cells
- Dependent on both host enzymes and introduced enzymes to enable recombination

Recombineering



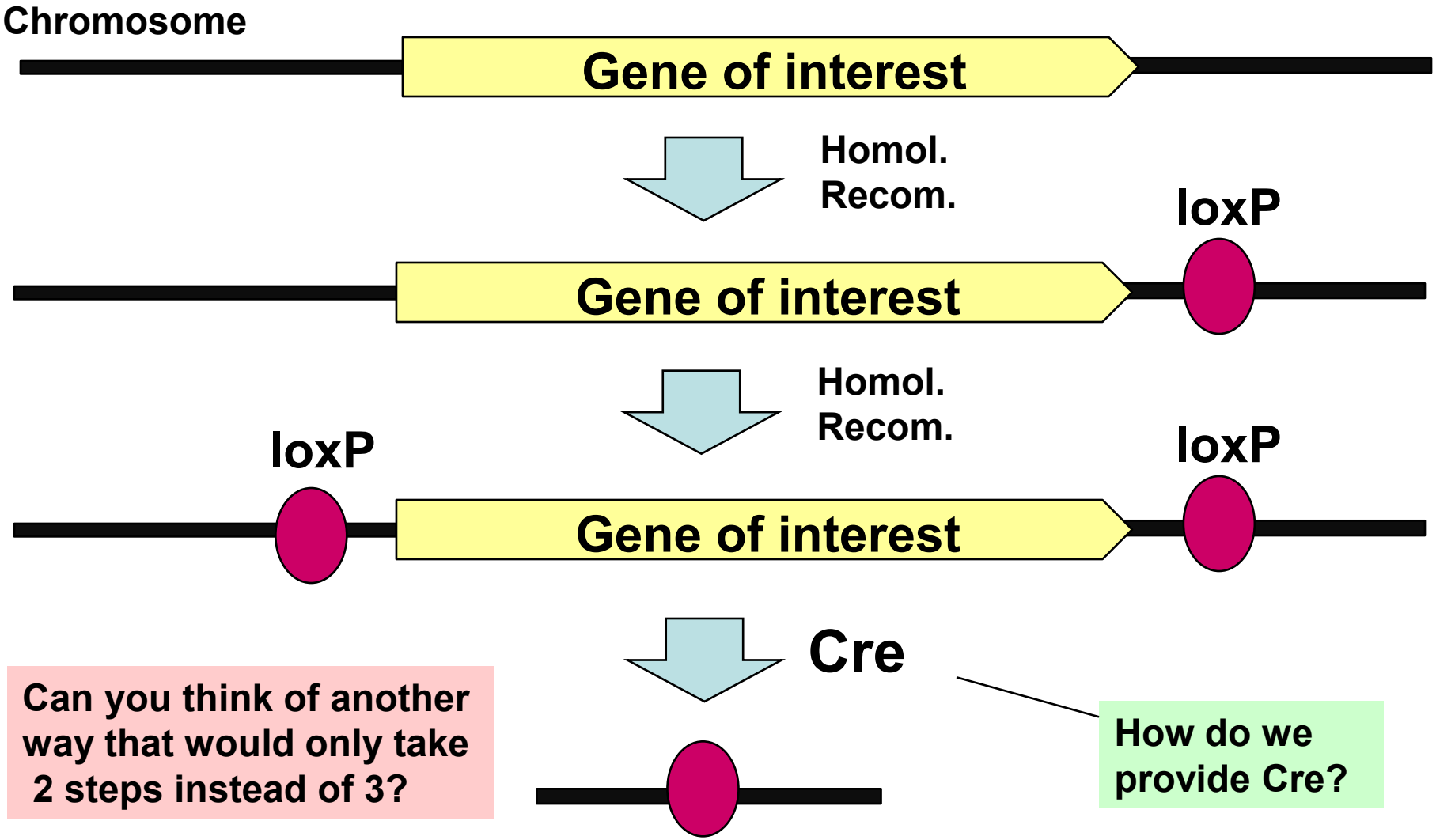
Site specific recombination : Cre-*loxP*

- **Initial constructs are complex to make, initial homologous recombination or cloning needed to prepare target**
- **Can fully remove target gene**
- **Gene removed cleanly, replaced by a few nucleotides**
- **NOT dependent on host enzymes for recombination**
- **Can be used in combination with other methods eg. for removing resistance genes in double X-over strains**

The FLP - FRT system is similar to Cre - loxP

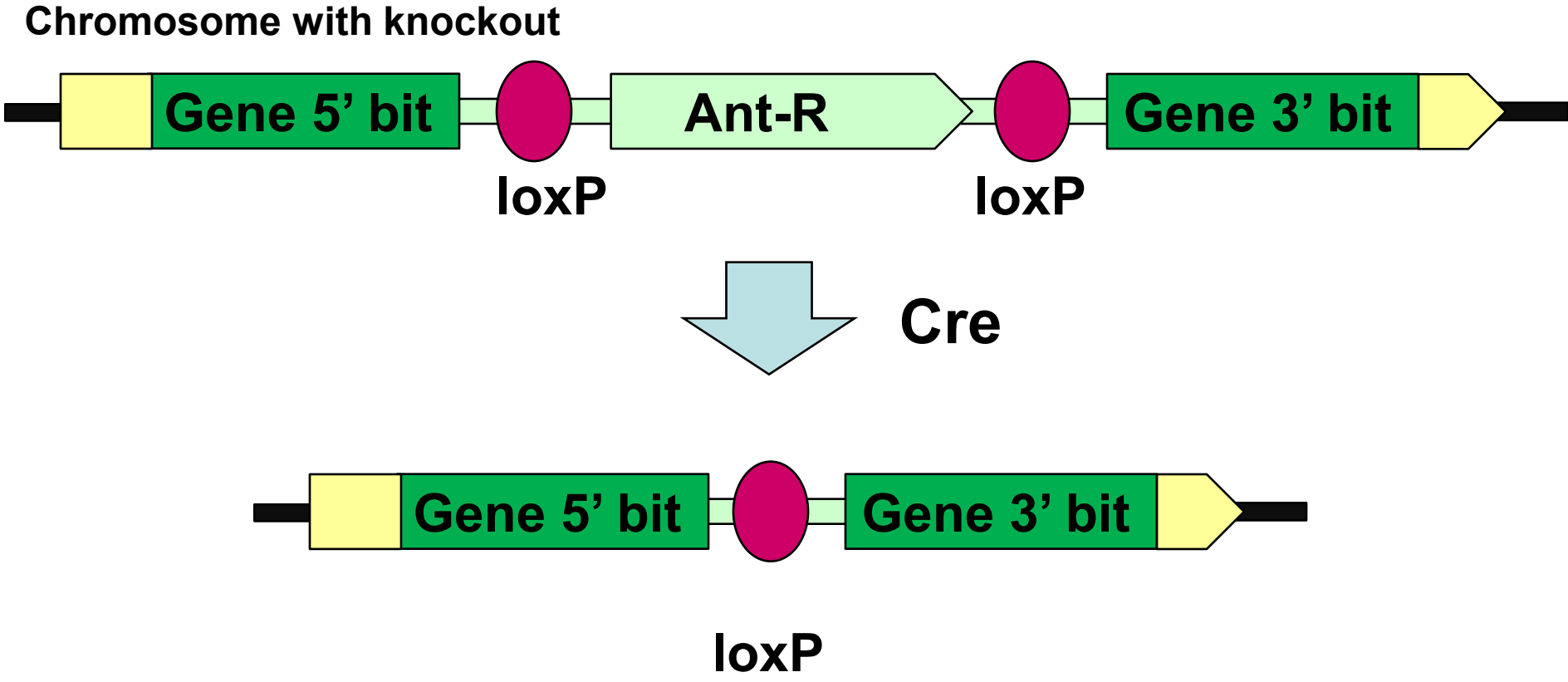
Site specific recombination : Cre-*loxP*

- Cre/*loxP* can be used as the primary gene knockout system. Note req'ment for homol.recom. to set it up



Unmarking resistance genes using Cre-*loxP*

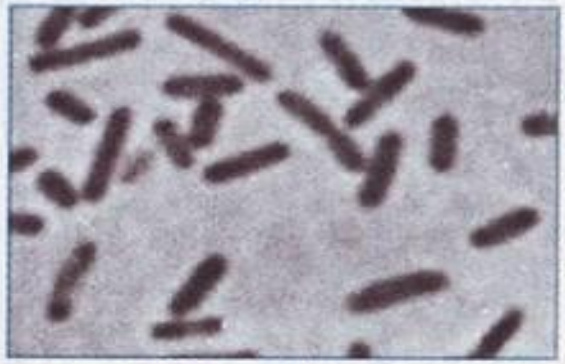
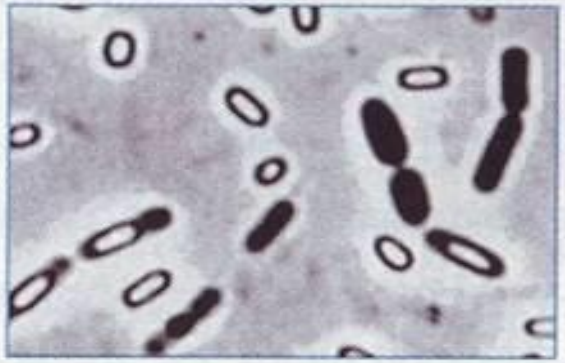
- Cre/*loxP* can be used as a secondary mechanism in the knockout process to remove resistance genes
- Knockout construct needs to provide 2 x *loxP* sites



Part 2. Advanced cloning + expression vectors

- **Yeast integrative plasmid pMIRY**
- **Fosmid vector in E.coli EPI300**

Prokaryotic vs. eukaryotic microbial hosts

Prokaryotes		Eukaryote
Escherichia coli	*Bacillus subtilis*	*Saccharomyces cerevisiae*
		
Well-developed genetics Many strains available Best-known prokaryote	Easily transformed Nonpathogenic Naturally secretes proteins Endospore formation simplifies culture	Well-developed genetics Nonpathogenic Can process mRNA and proteins Easy to grow
Potentially pathogenic Periplasm traps proteins	Genetically unstable Genetics less developed than in *E. coli*	Plasmids unstable Will not replicate most prokaryotic plasmids

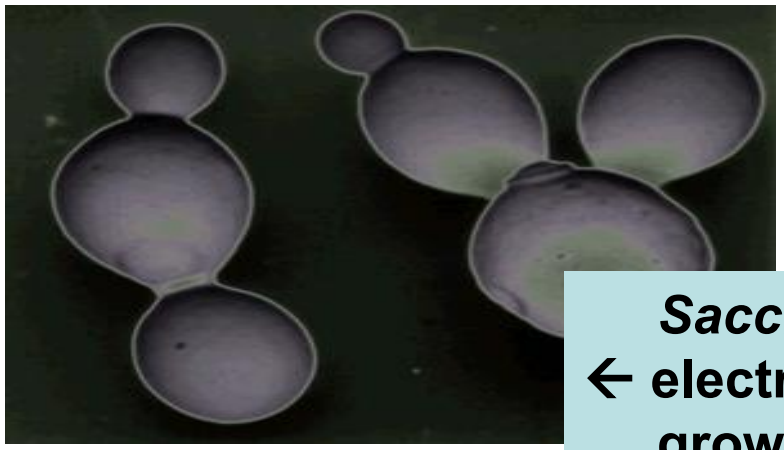
Advantages Disadvantages

Brock

Heterologous expression systems:

pMIRY plasmid in *Saccharomyces*

- An amplifiable yeast integrative plasmid (YIp)
  → Why is this useful?
- Yeasts provide eukaryotic protein processing activities (eg. folding, stabilisation, organelle targeting)
- Integration into chromosome gives high stability of recombinant clones; more stable than plasmid
- Amplifiable vector: more expression due to increased gene dosage, compensates for chromosomal location

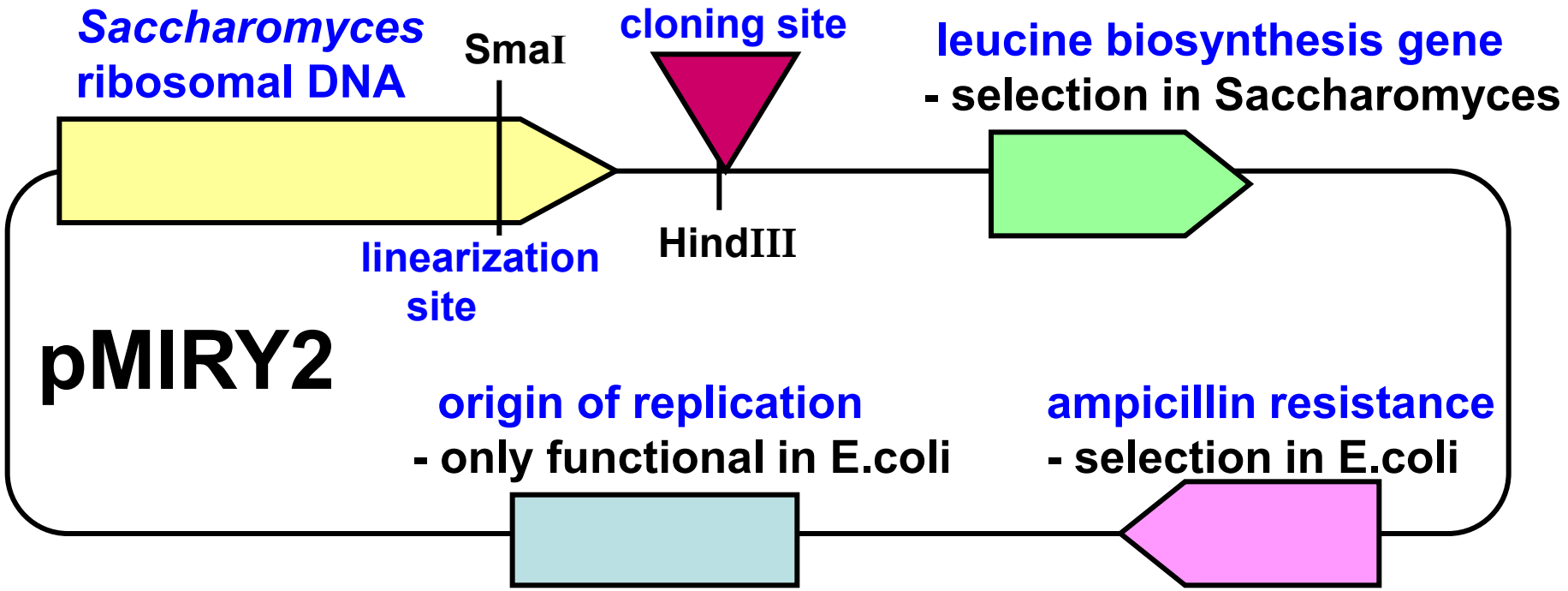


Saccharomyces
← electron micrograph
growing on agar →

Heterologous expression systems:

pMIRY2 plasmid in *Saccharomyces*

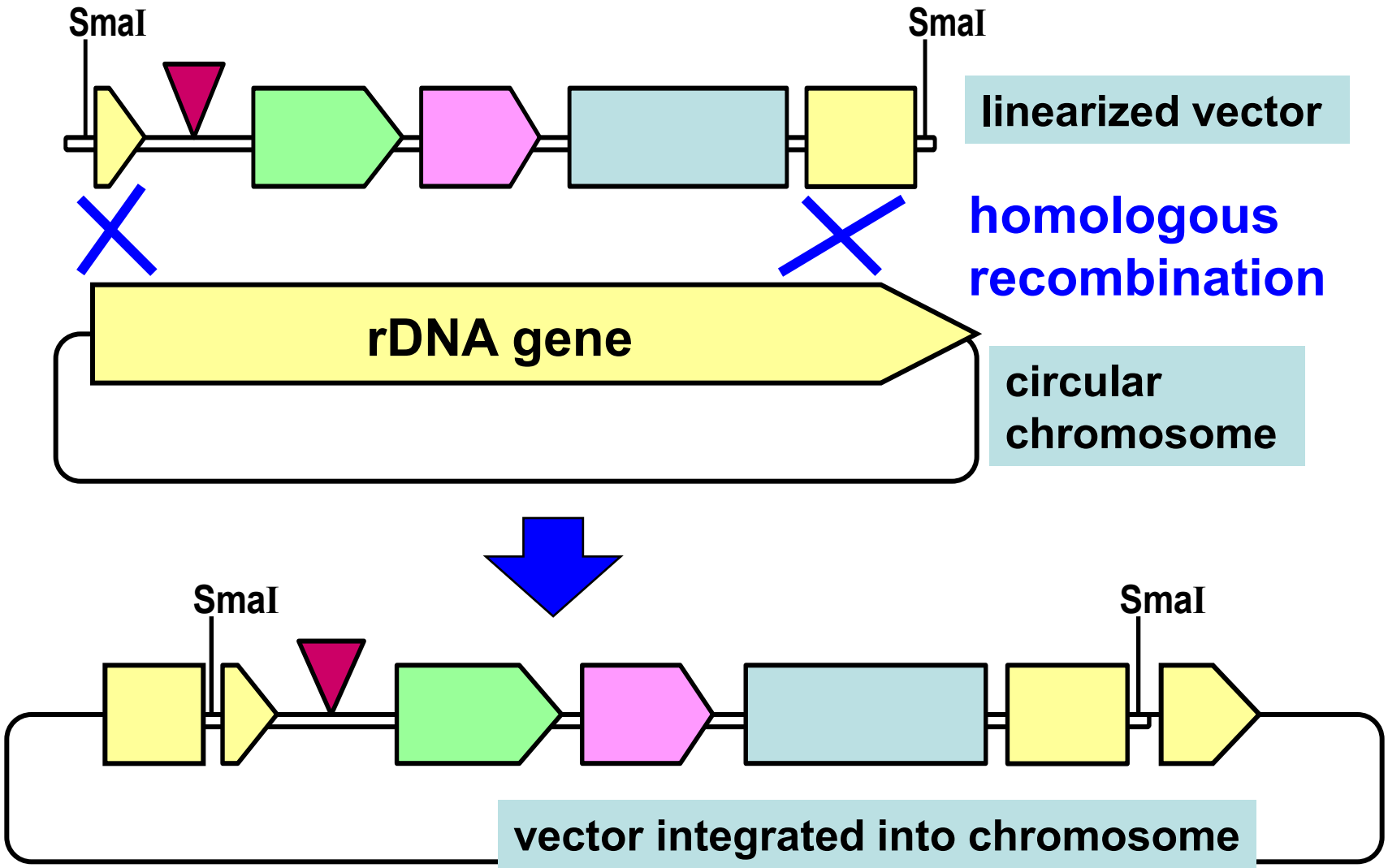
- Replicates as a plasmid in *E.coli*, use ampicillin to select
- Cut with SmaI → linearizes vector → transform into yeast
- Linear vector integrates into ribosomal DNA by homologous recombination



Heterologous expression systems:

pMIRY2 plasmid in *Saccharomyces*

How does integration occur?



Heterologous expression systems:

pMIRY2 plasmid in *Saccharomyces*

How does amplification work?

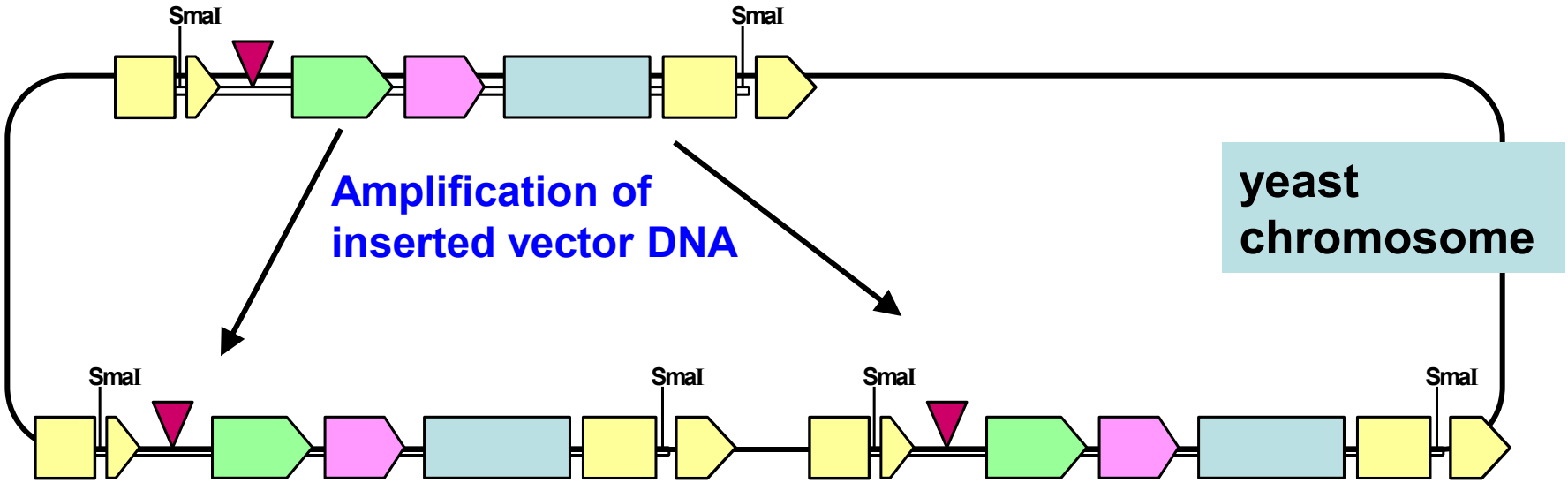
- Leucine biosynthesis gene intentionally functions poorly

  → duplication of gene region gives better biosynthesis

  → selective advantage to mutants with multiple copies

  → amplified mutants take over culture

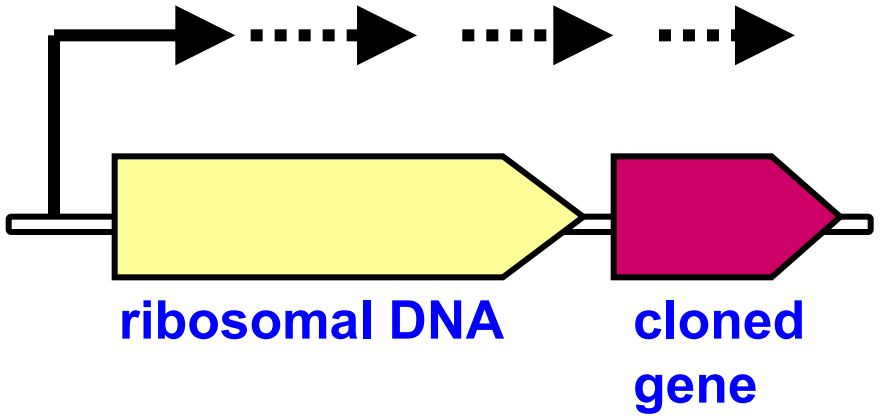
- Mechanism of amplification: homol. recomb.?



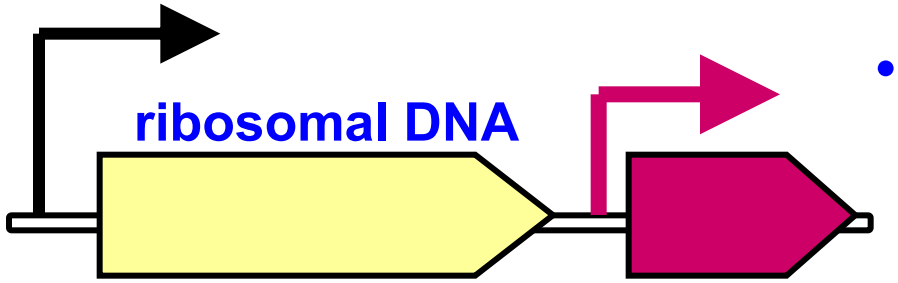
Heterologous expression systems:

pMIRY2 plasmid in *Saccharomyces*

What about a promoter for expression of cloned gene?



- **Option 1.** Rely on read-through expression from 'upstream' rDNA promoter



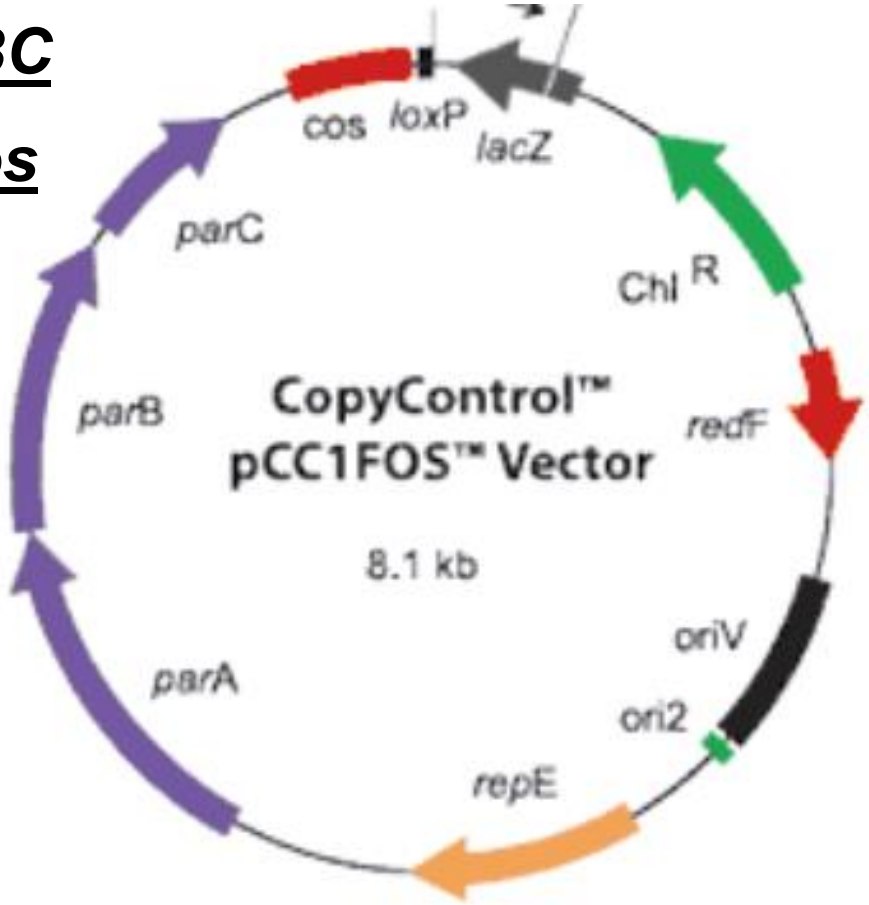
- **Option 2.** Add a promoter
  Promoter must be recognised by yeast RNA polymerase!

commonly used yeast promoters:

- **ADH** (alcohol dehydrogenase): constitutive promoter
- **GAL** (β-galactosidase): inducible promoter

Cloning systems: pCC1FOS in E.coli EPI300

- **A fosmid vector is a plasmid that allows stable cloning of large DNA segments. It has several important features:**
  - **Controllable copy number (~1-50) *repE / ori2 /oriV***
  - **Partitioning system *parABC***
  - **Lambda packaging site *cos***



Fosmid allows cloning of 40 kb DNA segments, and tolerates 'difficult' seqences eg. repeats

Need proper *E.coli* strain (EPI300) to get copy control system to work – this is partly host-encoded

Cloning systems: pCC1FOS in E.coli EPI300

- **A clone library made in a fosmid is called a fosmid library. Fosmid libraries are very useful for :**

  - retrieving whole genes from a genome starting with a DNA fragment (via PCR or hybridisation)

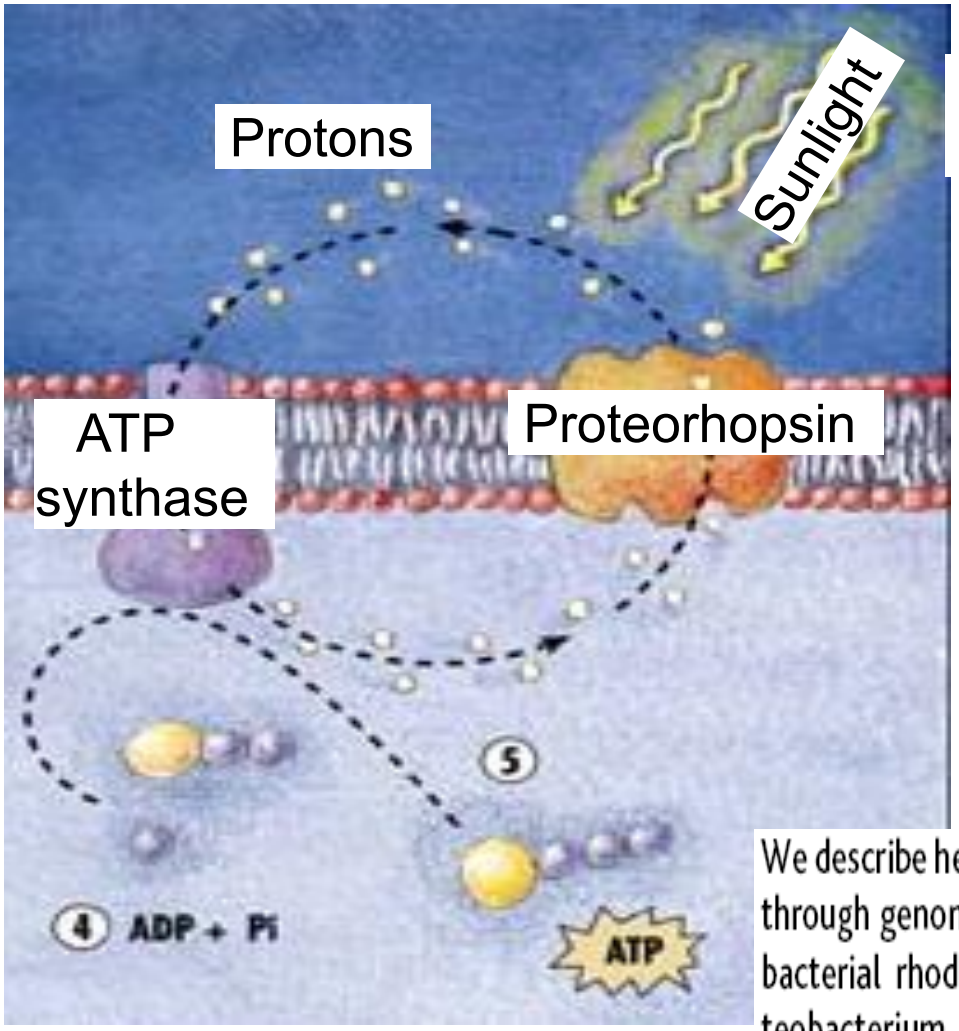
  - retrieving whole genes from a genome via functional assay for the encoded functions

  - closing gaps in raw genome sequences

  - screening metagenomic DNA for enzyme activities or the presence of specific sequences

These applications depend on 2 key features of the fosmid: large insert DNA size, and stable propagation of this DNA.

Breakthroughs enabled by fosmid vectors



15 SEPTEMBER 2000 VOL 289 SCIENCE

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]*

We describe here a type of rhodopsin derived from bacteria that was discovered through genomic analyses of naturally occuring marine bacterioplankton. The bacterial rhodopsin was encoded in the genome of an uncultivated γ-proteobacterium

archaeal-like rhodopsins are broadly distributed among different taxa, including members of the domain *Bacteria*. Our data also indicate that a previously unsuspected mode of bacterially mediated light-driven energy generation may commonly occur in oceanic surface waters worldwide.

Picture credits

http://www.versailles.inra.fr/urgv/microarray.htm

http://textbookofbacteriology.net/phage_2.html

http://www.epibio.com/item.asp?id=385

http://www.whoi.edu/science/B/people/mhahn/Beja_2000.pdf

http://www.mbari.org/twenty/proteorhodopsin.htm