Cloning & expressing genes in *E.coli:* the pET / BL21 system

Dr. Nick Coleman

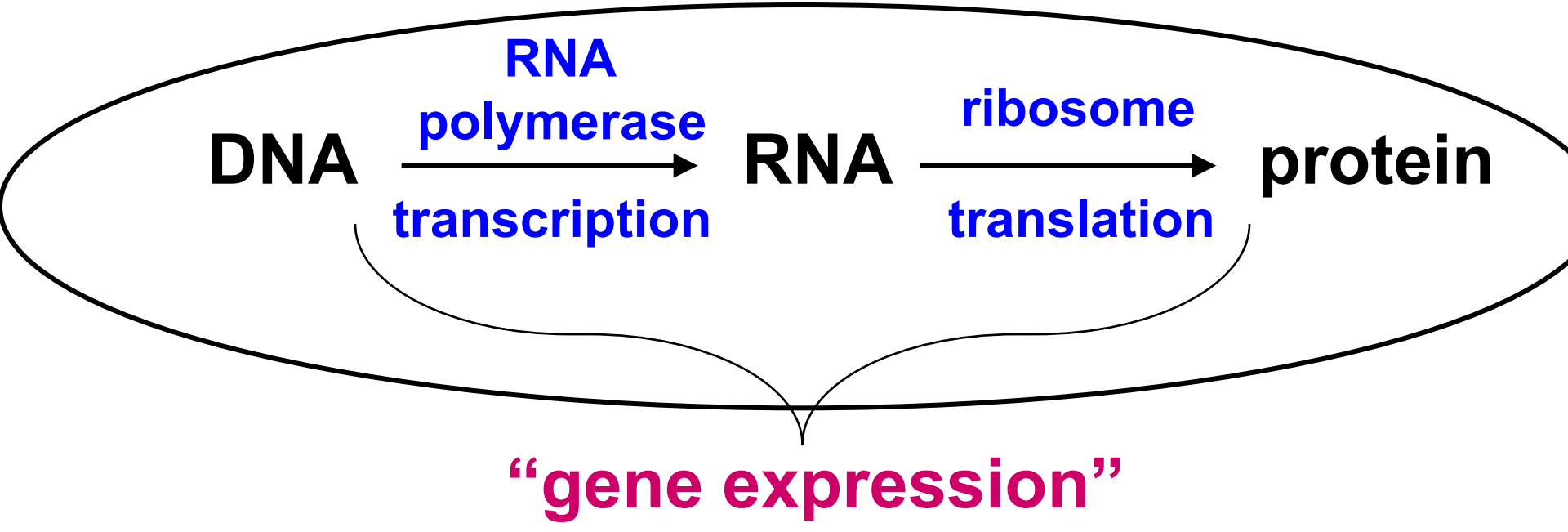
Cloning and expression in *E.coli.*

Learning outcomes. Be able to…

- **Explain the concepts and tools used for cloning genes into plasmid cloning vectors**
- **Define ‘heterologous expression’ and discuss the advantages, disadvantages, challenges of this approach**
- **Describe the details of all the separate elements of the BL21-pET cloning system, and how these elements function together to allow protein overexpression**

Functional units in recombinant DNA work

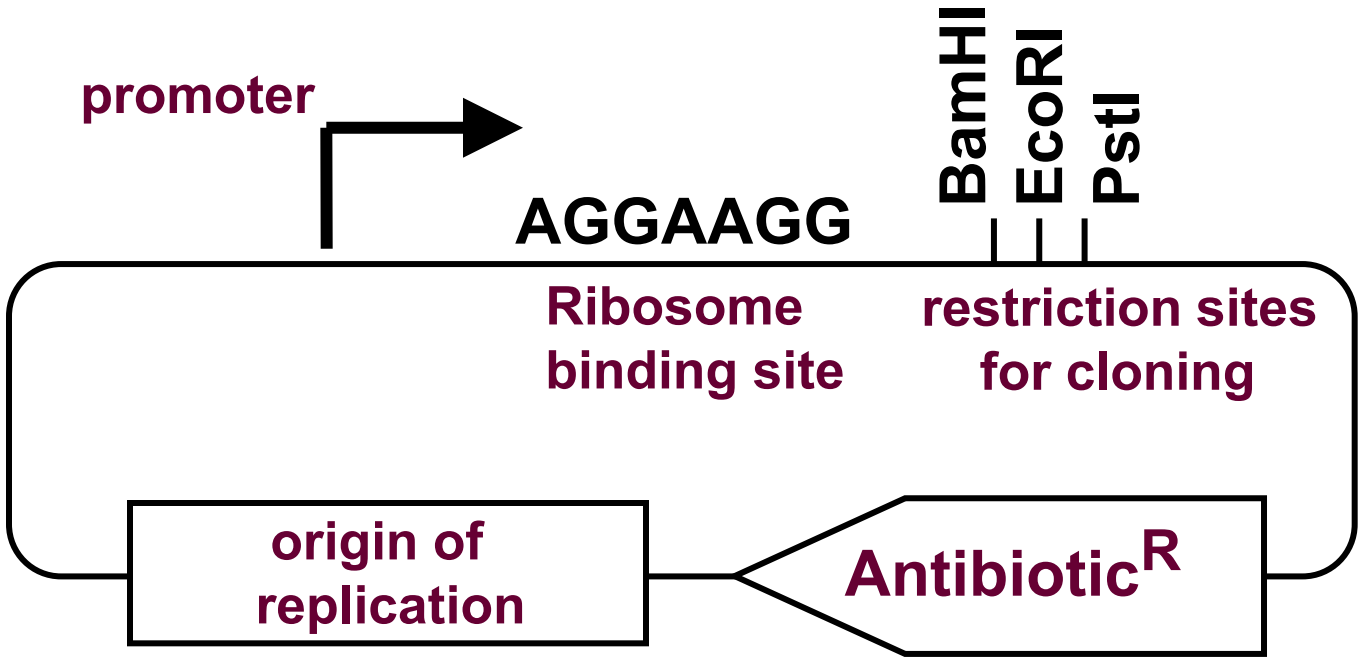
- **Gene**: DNA region encoding a protein.
- **Promoter**: regulatory DNA, activates transcription to mRNA
- **Ribosome binding site**: ensures translation
  - prokaryotes: RBS = Shine-Dalgarno seqeunce
  - eukaryotes: RBS = Kozak sequence



Functional units in recombinant DNA work

- **Vector**: DNA / RNA element that carries other genes,
  eg. plasmid, virus (incl. phage), transposon

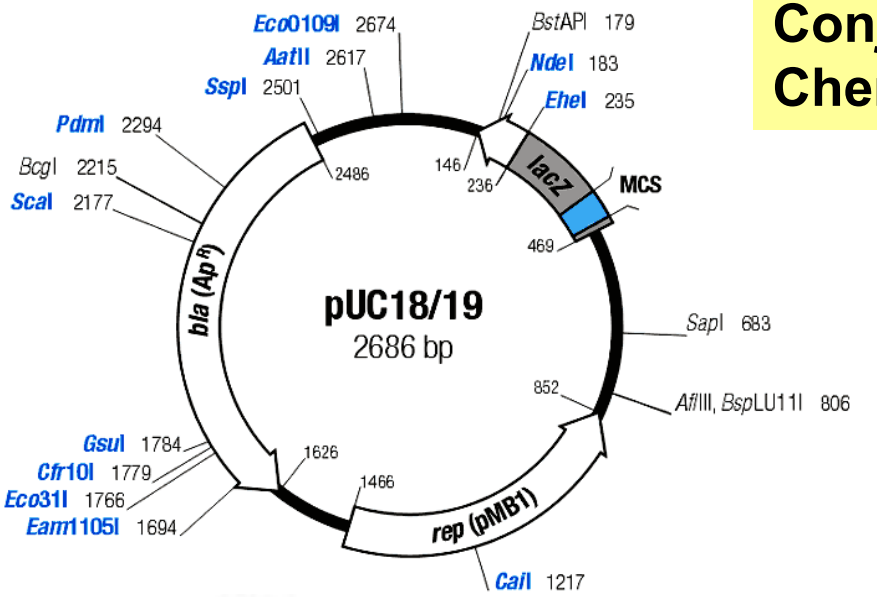
- **Marker gene**: allows <u>selection</u> or <u>detection</u>
  - **selection**: eg. resistance to antibiotics or herbicides
  - **detection**: eg. green fluorescence, bioluminescence

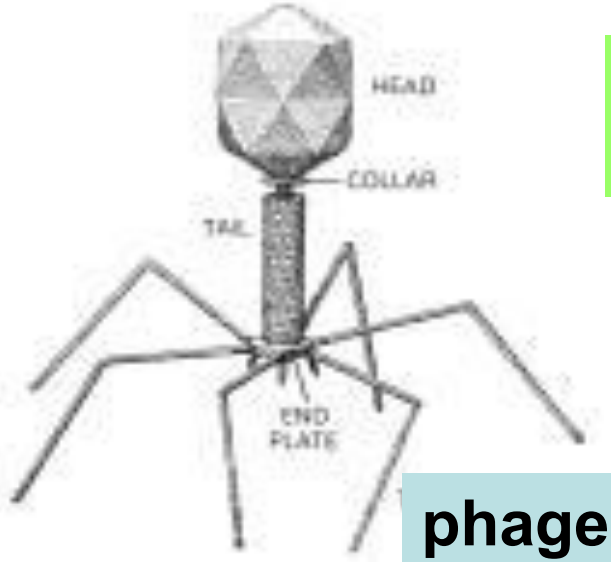


Features of a standard bacterial plasmid vector

Common vector types

plasmid:
Conjugation, electroporation, Chemical transformation



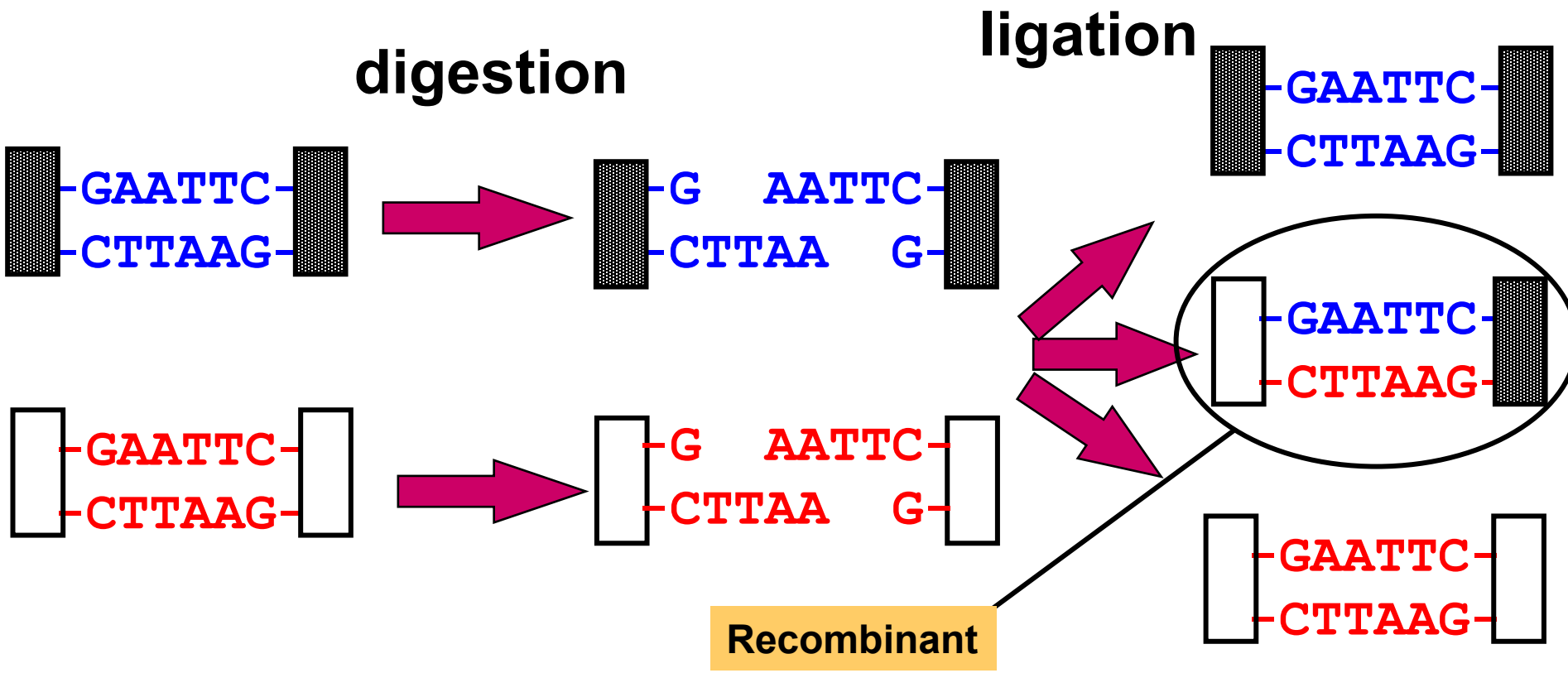


naked DNA (no vector):
natural transformation

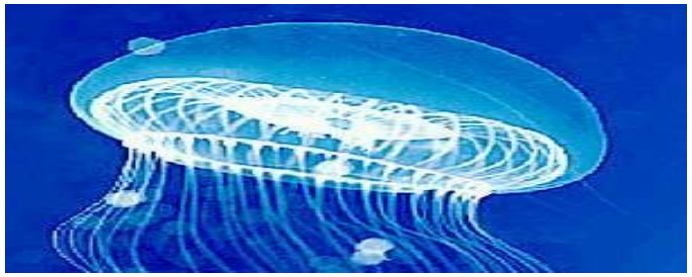
phage: transduction

Enzymes for manipulating DNA

- **Copying:** RNA or DNA polymerase.
- **Cutting**: digestion with restriction enzyme
- **Joining**: ligation using DNA ligase



Making recombinant DNA and organisms



source organism

extract DNA

PCR

gfp

gene of interest

restriction digests

ligation

gfp

recombinant plasmid

plasmid

competent bacteria

transformation

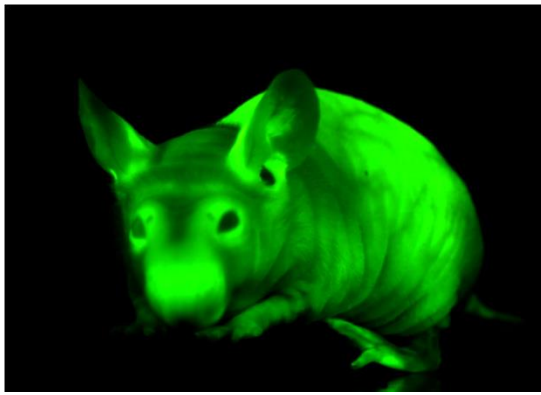
gfp

recombinant bacteria

extract DNA

transformation

conjugation

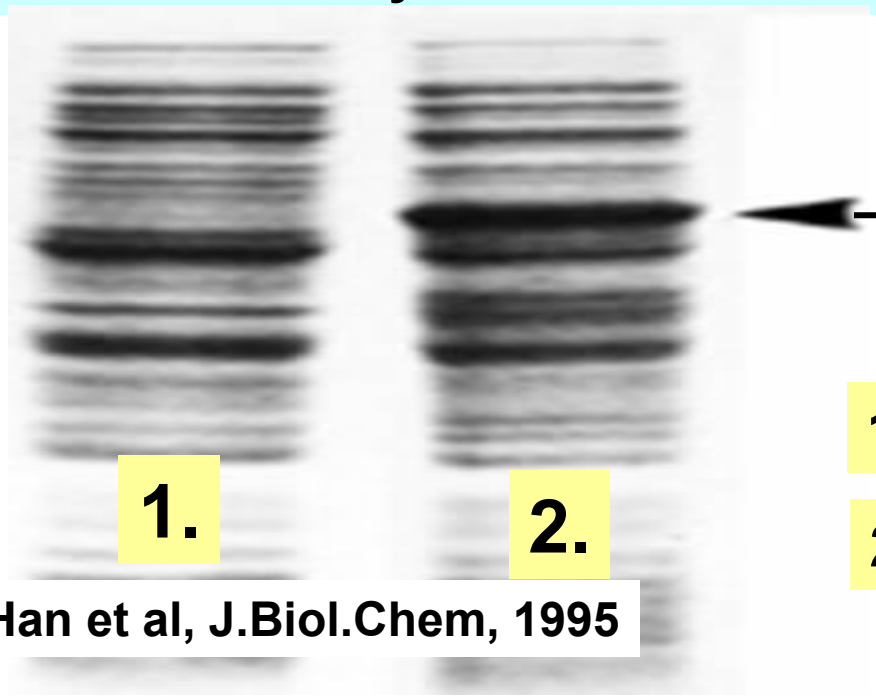


recombinant animal or plant

Heterologous expression of proteins

- Expression of a protein in 'unnatural' host
- Advantages
  - potentially high expression level of protein
  - heterologous host easier to grow / manipulate
  - can add affinity tags to aid purification

SDS-PAGE analysis of *Bacillus* cellulase expression in *E.coli*



1. Before addition of inducer

2. Three hours after inducer

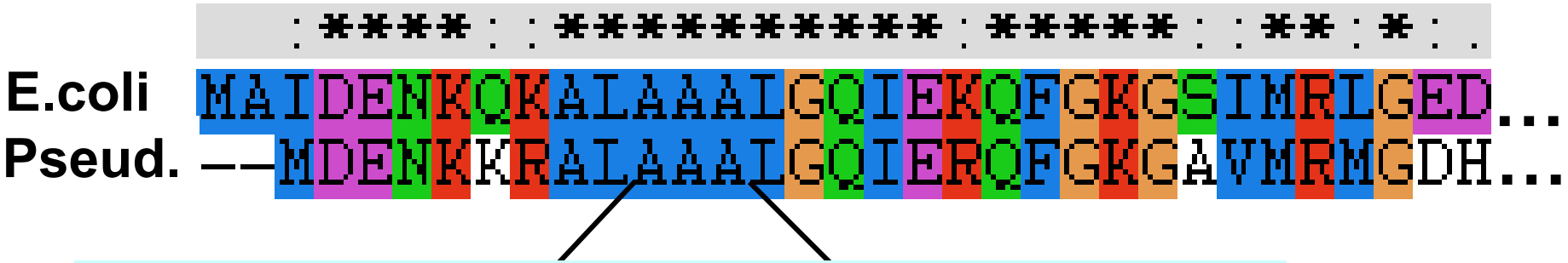
Han et al, J.Biol.Chem, 1995

Heterologous expression of proteins

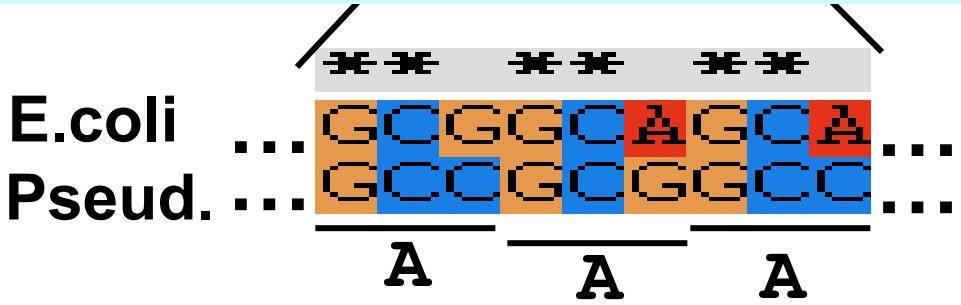
- **Disadvantages**
  - gene must be identified and cloned
  - need to provide transcription + translation signals
  - toxicity of overexpressed protein

★ codon usage / protein processing differences

eg. compare RecA proteins in *Pseudomonas* vs. *E.coli* :



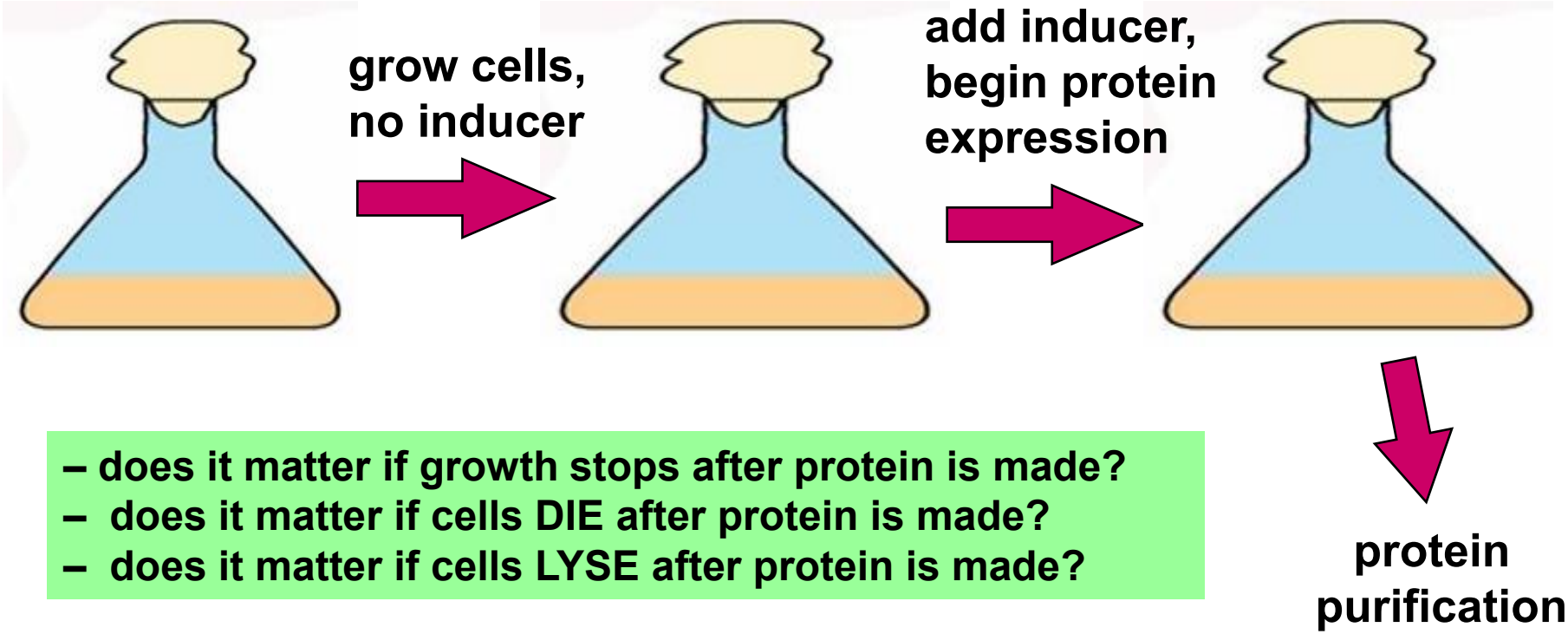
Now compare *recA* genes in *Pseudomonas* vs. *E.coli* :



pET plasmid in *E.coli* BL21(DE3)(pLysS)

- Overexpression of proteins often harms the cell
  → need to grow bacteria, then switch on expression

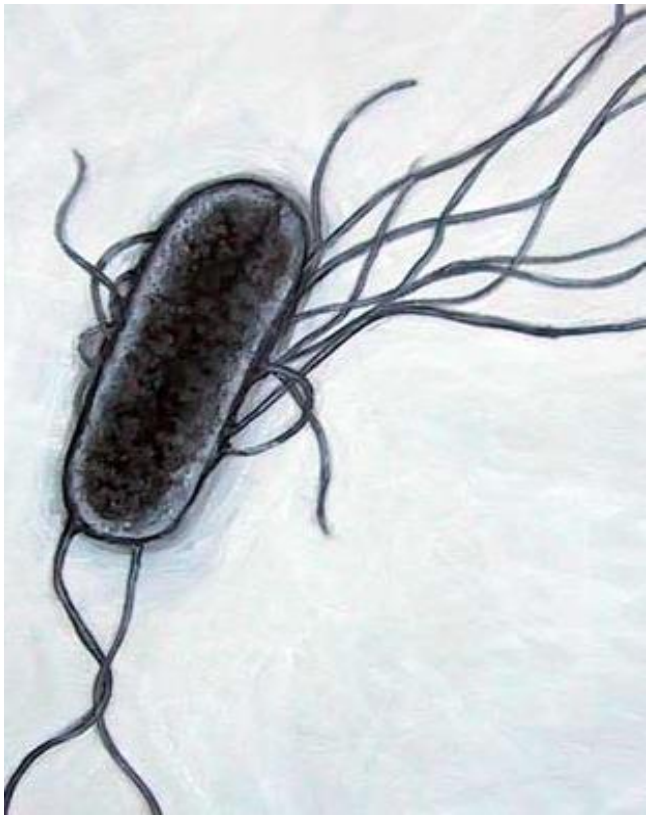
- BL21(pET) provides a way of doing this; expression is kept switched off until induced with IPTG



- does it matter if growth stops after protein is made?
- does it matter if cells DIE after protein is made?
- does it matter if cells LYSE after protein is made?

BL21(pET) expression system

***E.coli* BL21 cells provide:**

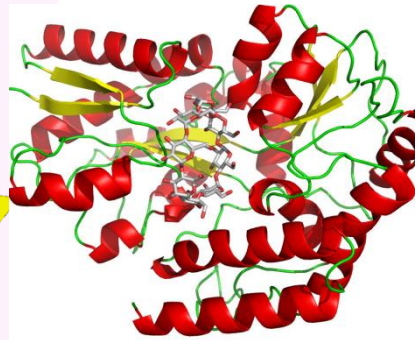


- **protein factories (ribosomes)**
- **building blocks (amino acids)**
- **transfer RNAs**
- **energy for biosynthesis**
- **post-translational modifications (or not)**
- **cofactors (NAD, FAD, iron, etc)**

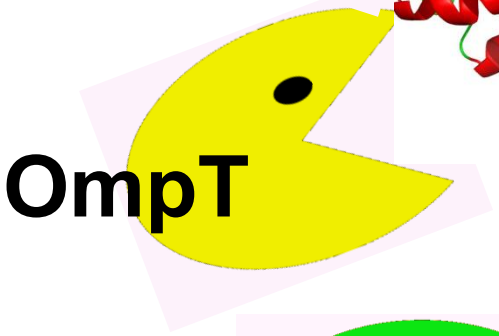
BL21(pET) expression system

E.coli BL21 cells

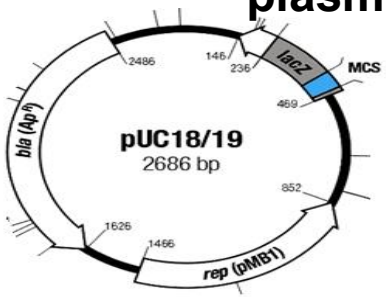
- are engineered with mutations in:
  - → protease genes (Lon, OmpT)
  - → restriction system (Hsd)

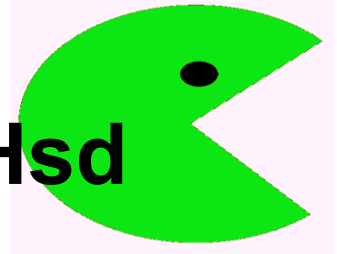


Your favourite protein

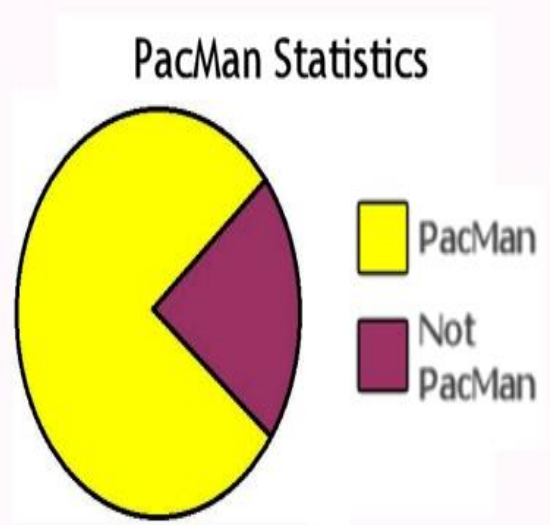


Your favourite plasmid





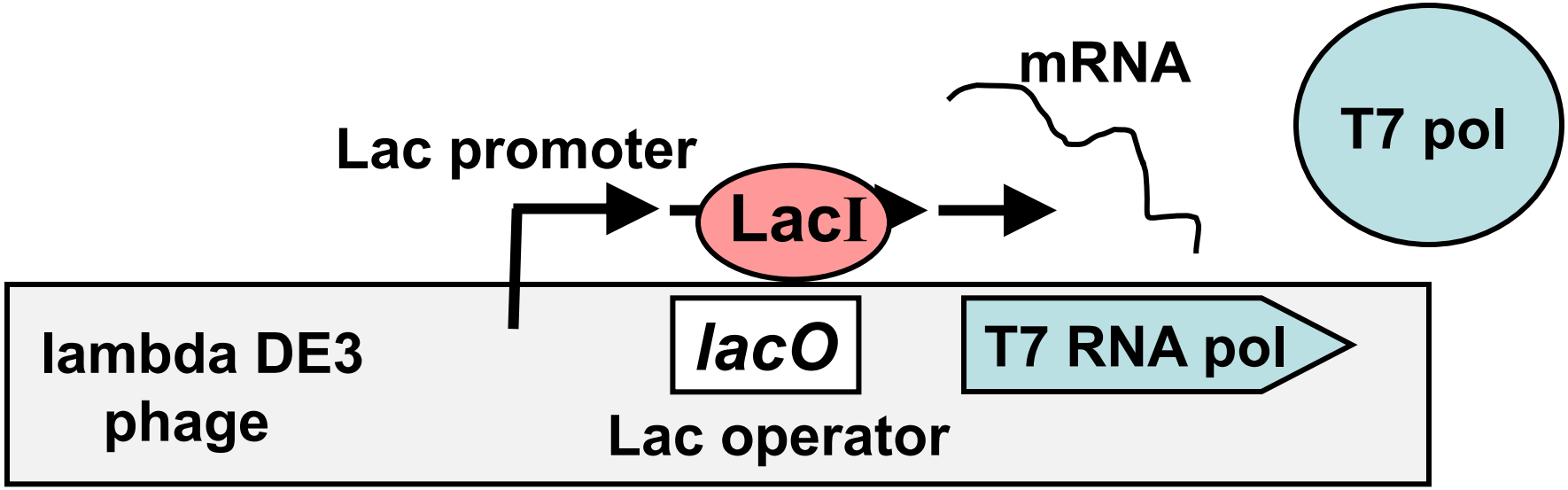
Why are these mutations helpful ?



BL21(pET) expression system

Lambda DE3 phage (integrated in *E.coli* chromosome)

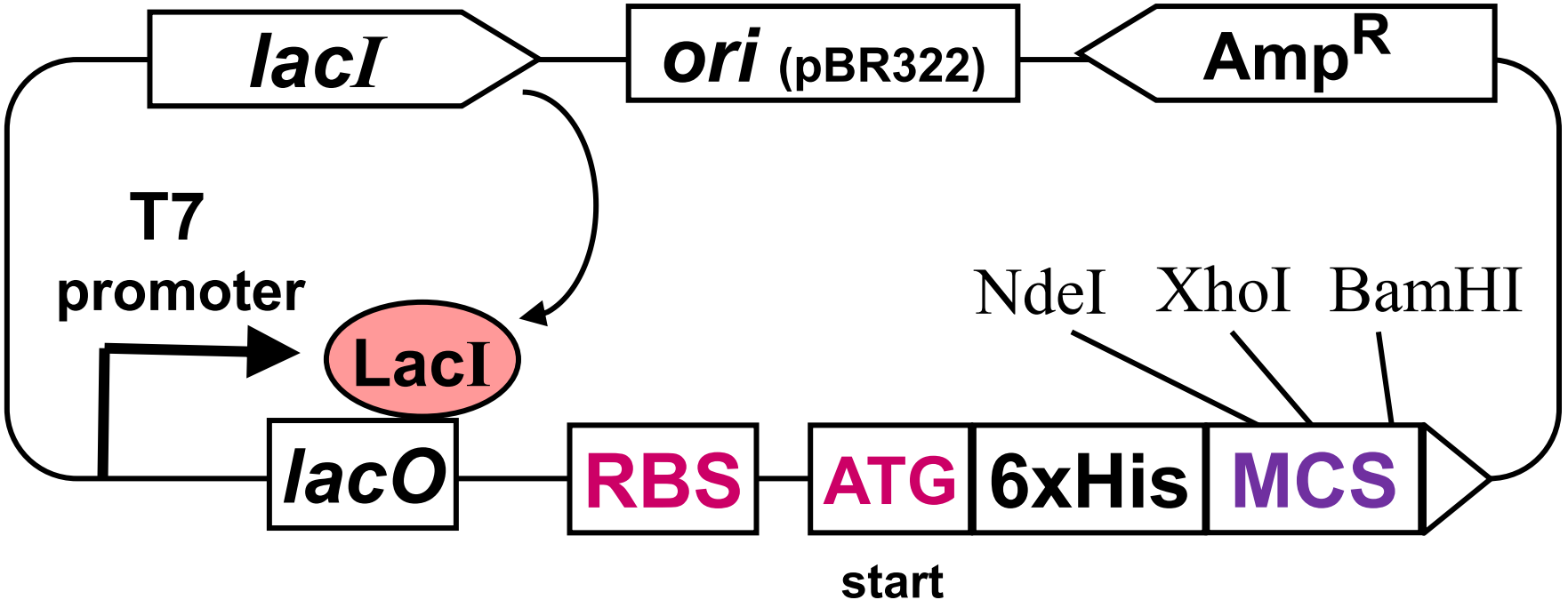
- T7 RNA polymerase gene controlled by lac promoter
- Addition of IPTG modifies LacI repressor, frees *lacO*
- *E.coli* RNA polymerase binds Lac promoter, expresses T7 RNA polymerase (this then finds the T7 promoter)



BL21(pET) expression system

pET15b plasmid – uninduced state

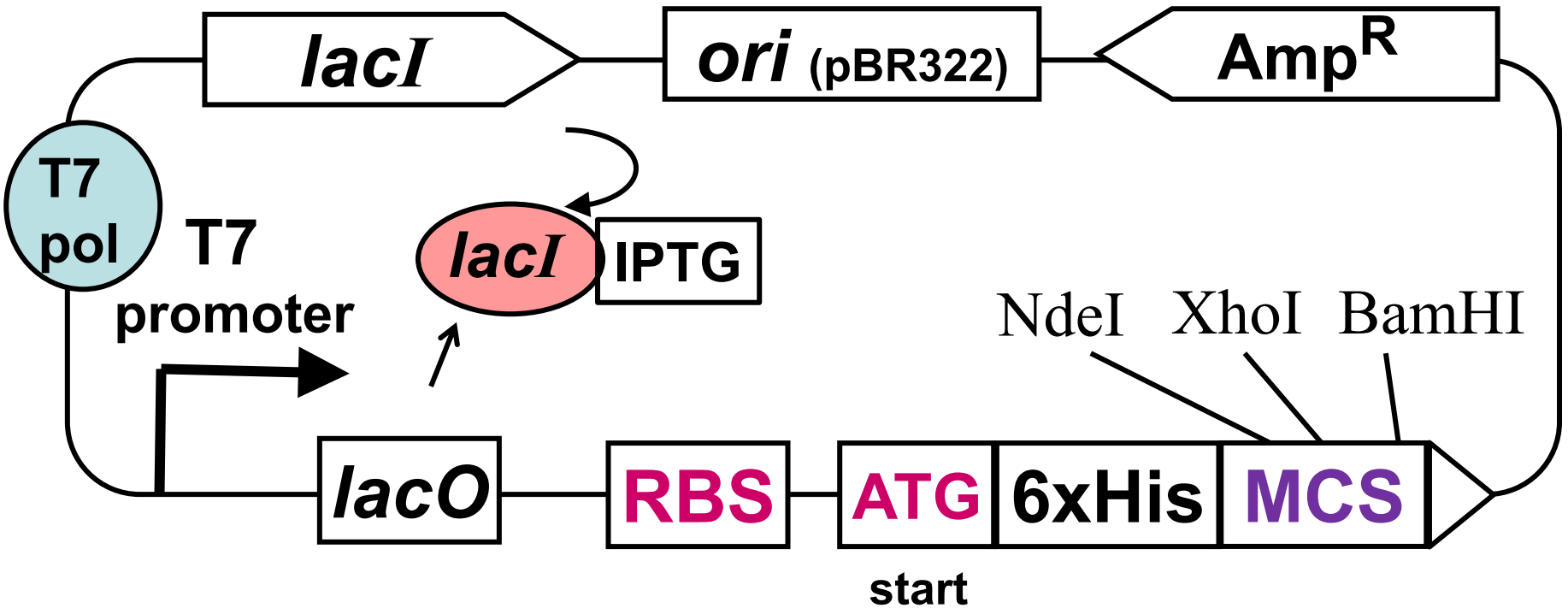
- cloned gene under control of T7 promoter / lac operator
- LacI protein binds to *lacO* DNA → expression off.
- T7 polymerase gene in DE3 not made → expression off



BL21(pET) expression system

pET15b plasmid – induction by IPTG

- IPTG modifies LacI → releases *lacO*
- T7 pol binds to T7 promoter, transcribes cloned gene
- vector provides RBS, start, and 6 x Histidine tag

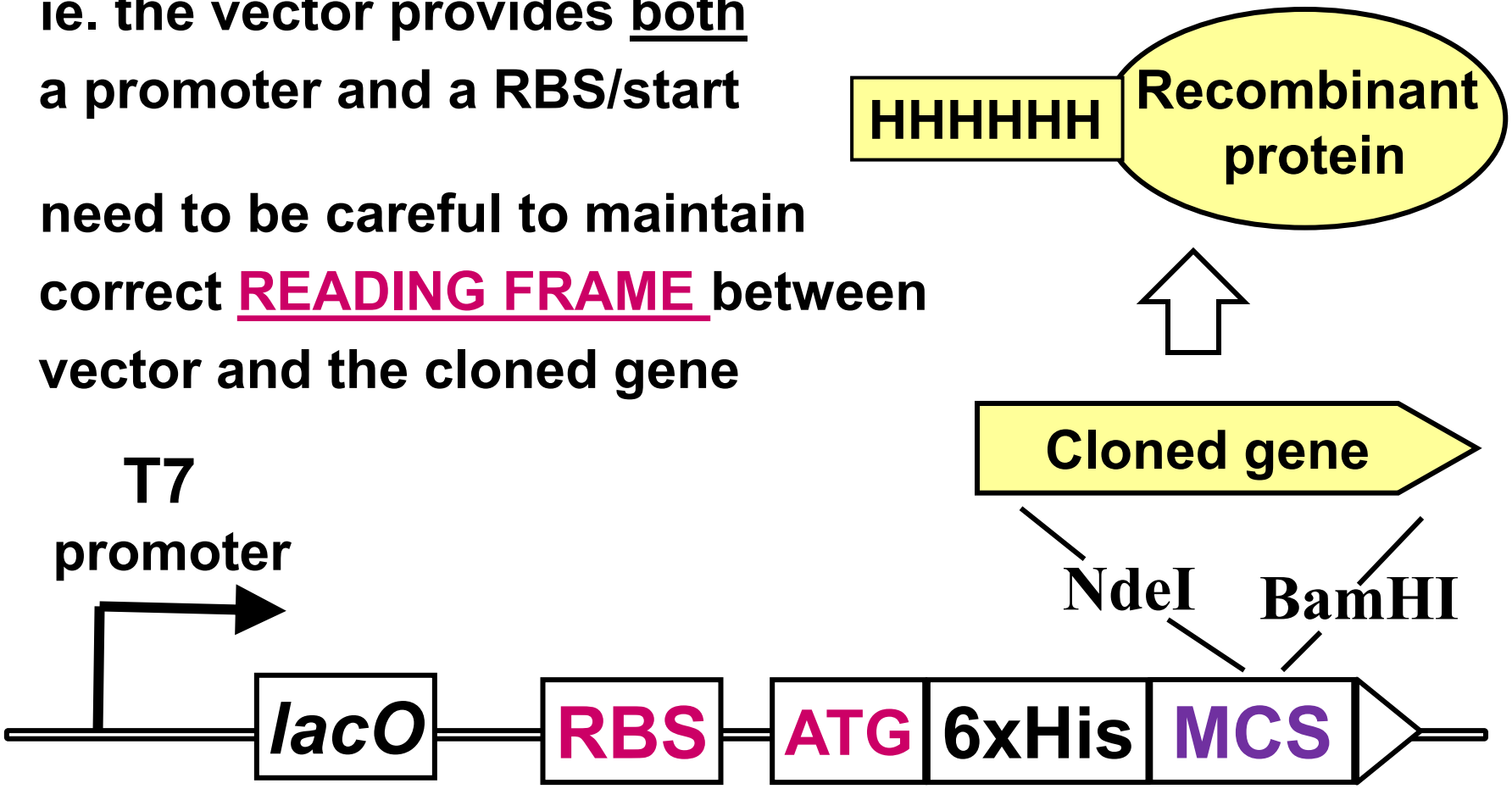


BL21(pET) expression system

- **pET15b plasmid makes a transcriptional fusion AND a translational fusion with the cloned gene**

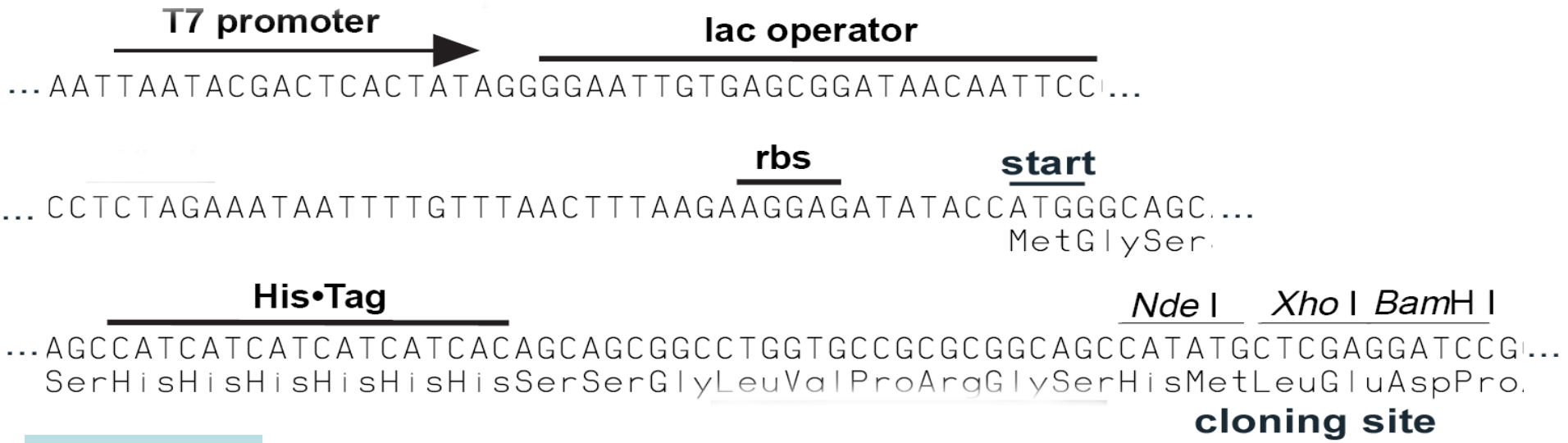
- **ie. the vector provides both a promoter and a RBS/start**

- **need to be careful to maintain correct READING FRAME between vector and the cloned gene**



BL21(pET) expression system

pET15b plasmid importance of reading frame in cloning



Example.

GGC	AGC	CAT	ATG	TAT	GAG	CCG
Gly	Ser	His	Met	Tyr	Glu	Pro

Correct clone

GGC	AGC	CAT	ATG	GTA	TGA	GCC	C
Gly	Ser	His	Met	Val	STOP		

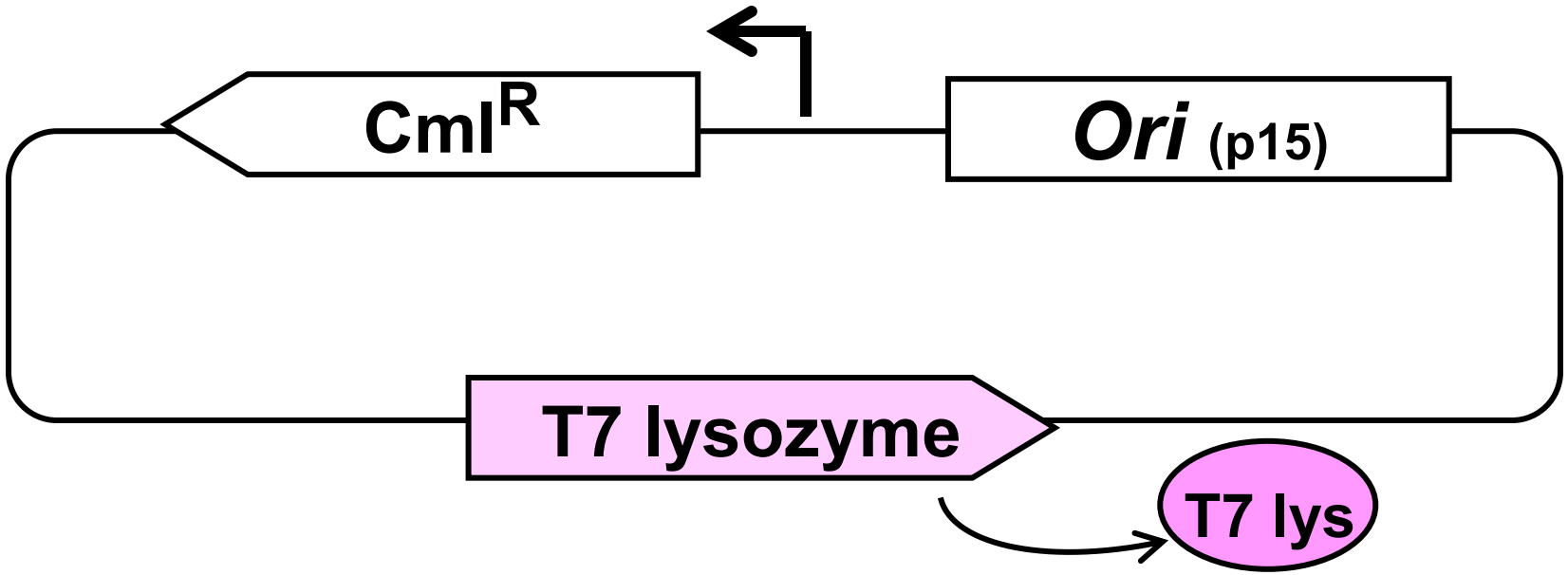
Point mutation
→ frameshift
→ Stop codon

BL21(pET) expression system

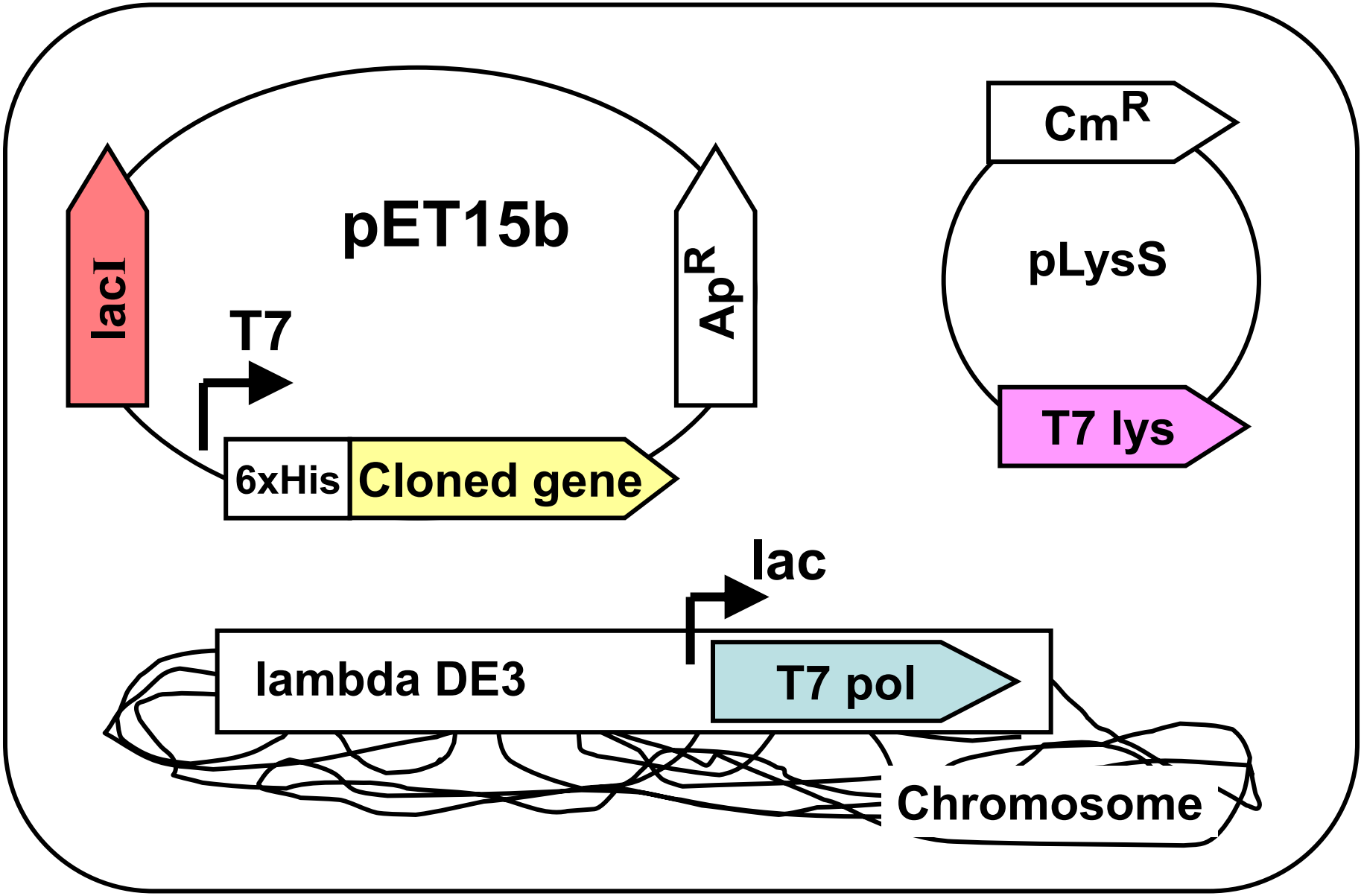
pLysS plasmid

Antibiotic^{R} type

- different replication origin AND selection to pET15b allows these two plasmids to stably co-exist in one cell
- carries and expresses T7 lysozyme gene
- T7 lysozyme is an inhibitor of T7 polymerase



Full system: *E.coli* BL21 (DE3) (pET15b) (pLysS)



BL21/pET uninduced culture:

LB + Amp + Cml medium

LacI repressor active

↓

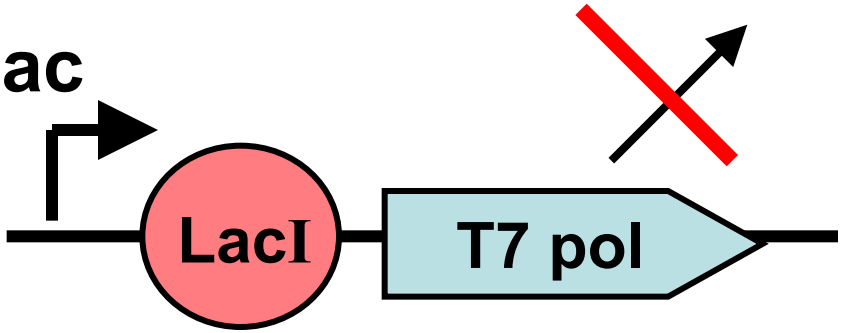
Lac promoter switched off

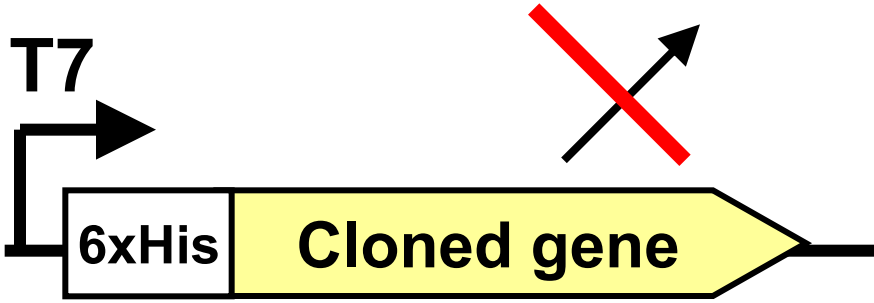
↓

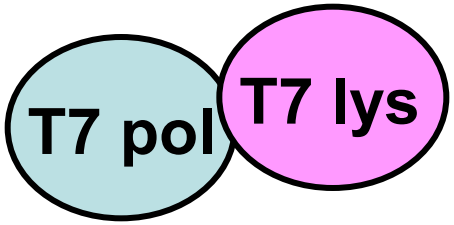
T7 polymerase not expressed

↓

Cloned gene not expressed







Traces of T7 pol protein neutralised by T7 lysozyme

BL21/pET induced culture:

LB + Amp + Cml + IPTG medium

LacI repressor inactive

↓

Lac promoter switched on

↓

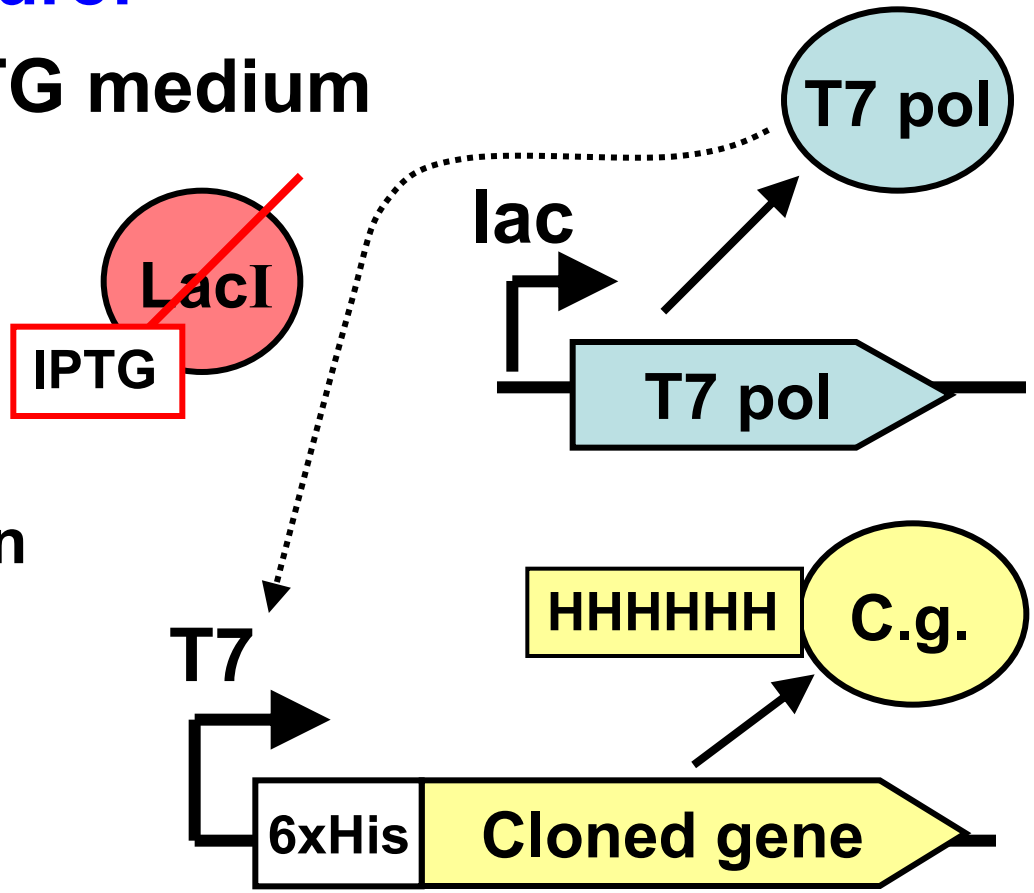
T7 polymerase expressed

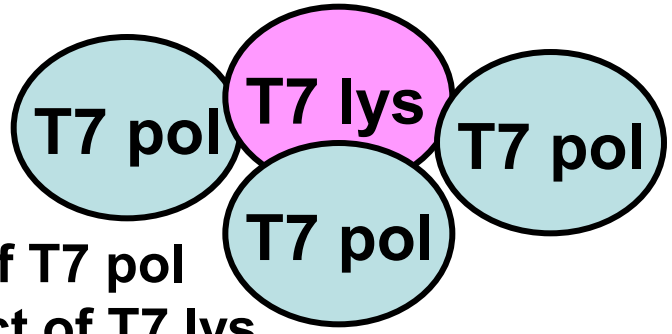
↓

Cloned gene expressed

↓

Recombinant protein





Expression of T7 pol swamps effect of T7 lys

Growing recombinant *E.coli*

Growth medium ?

- **Luria Bertani medium (LB) is the most widely used:**
  10 g tryptone, 5 g yeast extract, 5 g NaCl per litre

- **LB is widely used because it is easy to make !**
  - but not necc. best for high yield of cells, DNA, protein

- **Alternatives:**

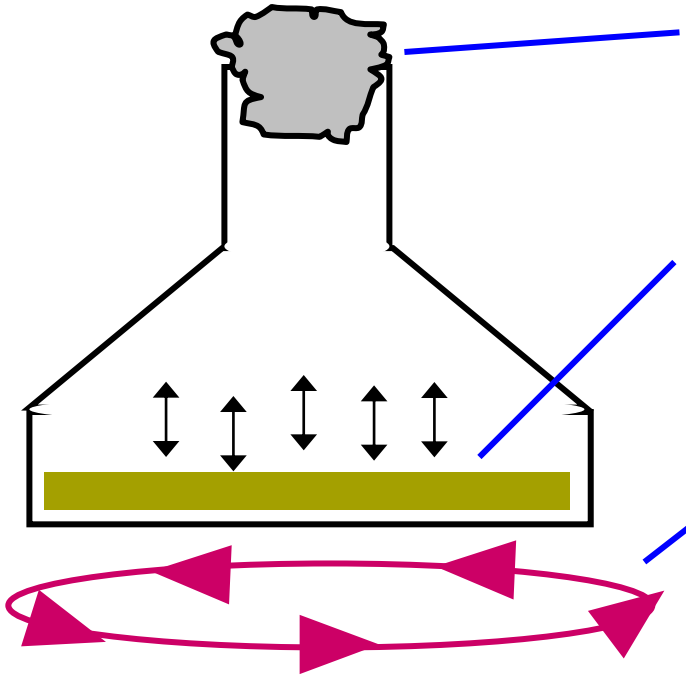
  - Terrific Broth (TB) contains higher nutrient conc's and also contains phosphate buffer for control of pH.

  - Minimal medium (eg. M9) gives better control of metabolism, eg. can choose a specific C source

Growing recombinant *E.coli*

Culture and incubation conditions ?

- ***E.coli*** **is a facultative anaerobe, it can grow aerobically (O_2 as electron acceptor) or anaerobically (fermentation)**
- **For cloning and expression experiments, we usually want AEROBIC growth of *E.coli* – this is more rapid**



Cotton wool stopper allows entry of air, not microbes

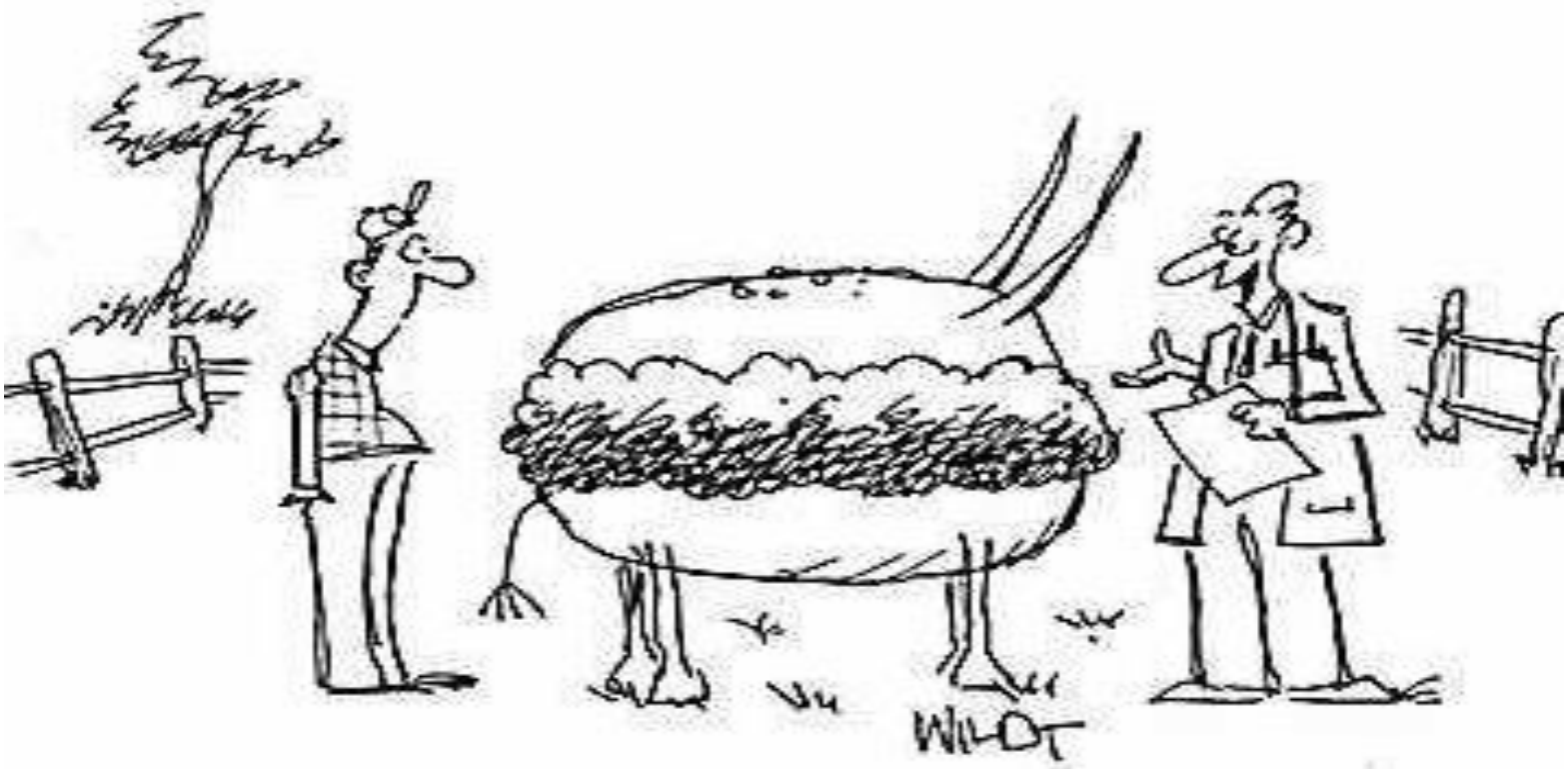
Small amount of medium in a large flask gives a high surface area / volume ratio

Rapid shaking

Rapid gas exchange

So, making recombinants

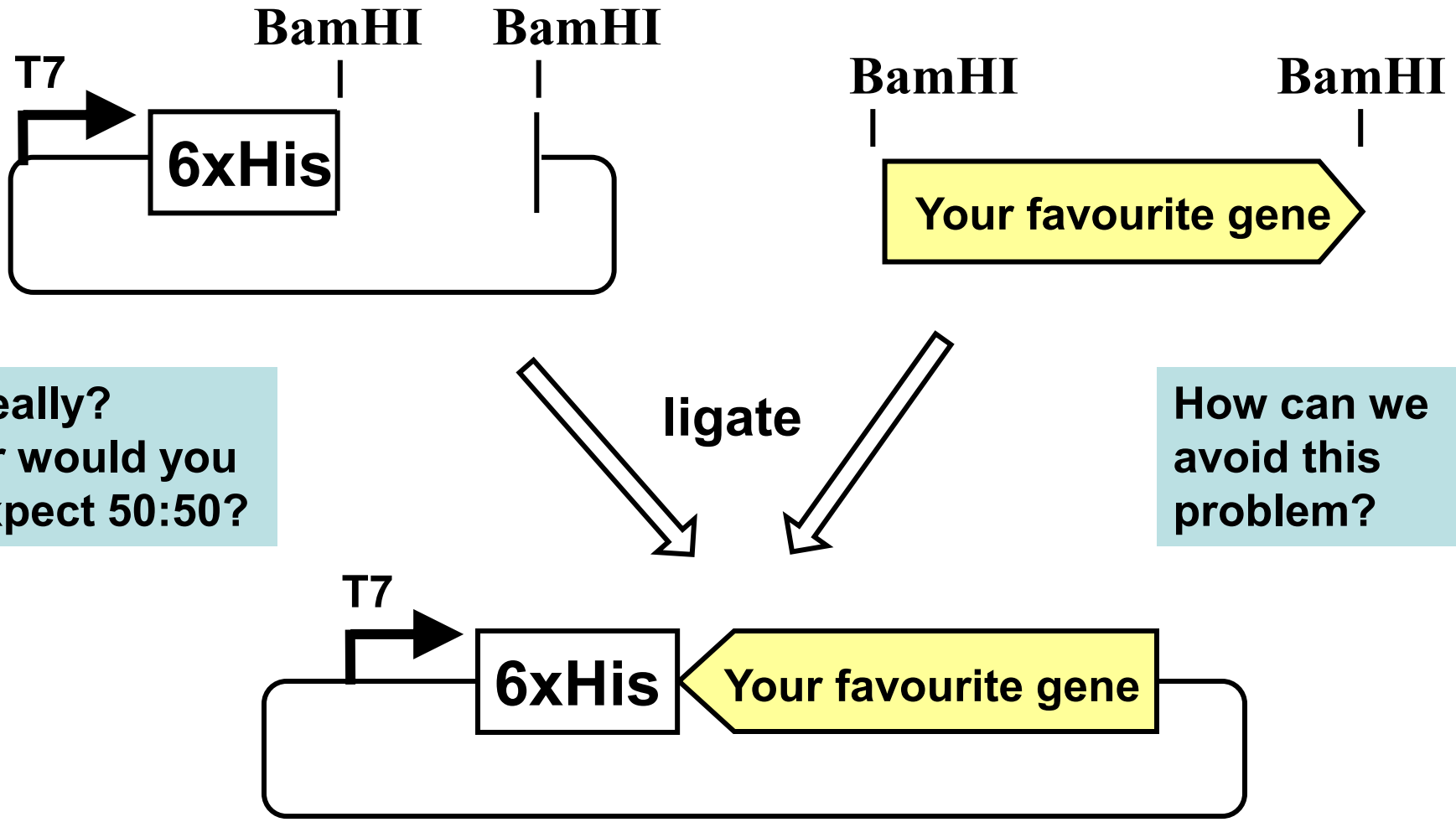
looks pretty straightforward, hey ?



"This has taken YEARS of genetic research."

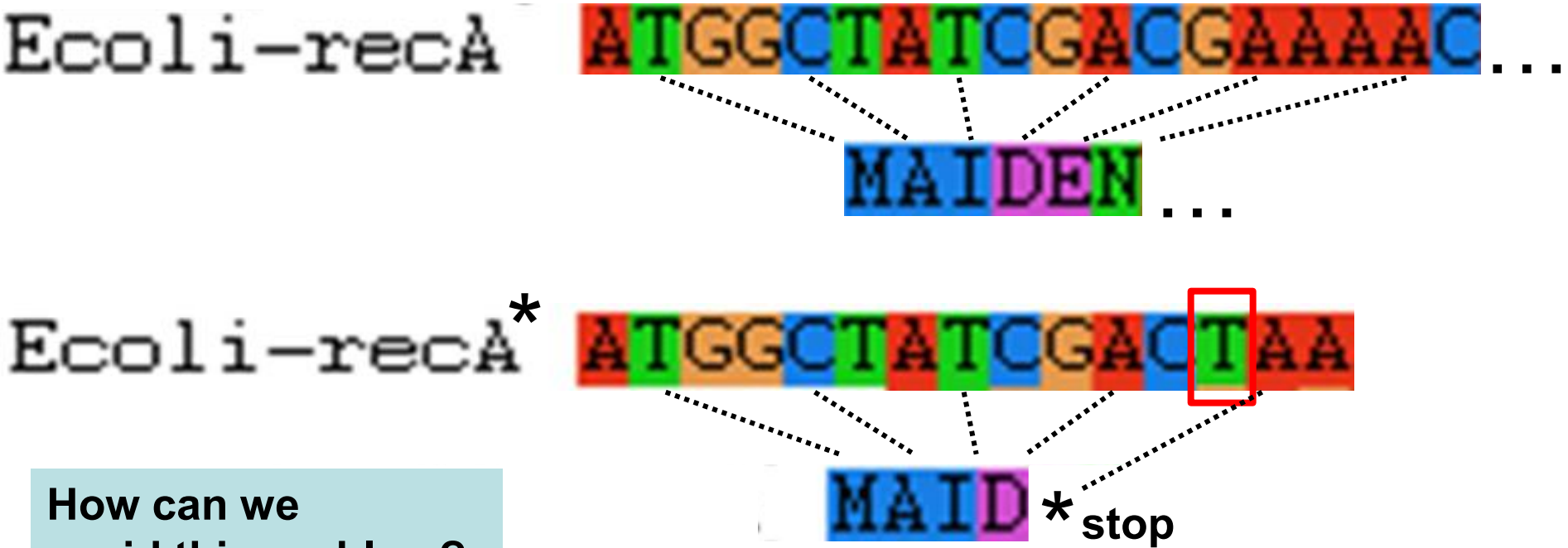
Cloning and expressing genes. Murphy's Law #1

" If you use a single restriction enzyme to clone a gene into a vector, you will get the wrong recombinants"



Cloning and expressing genes. Murphy's Law #2

" If you use PCR to amplify a gene, you will introduce a mutation at a critical point in the translated protein"

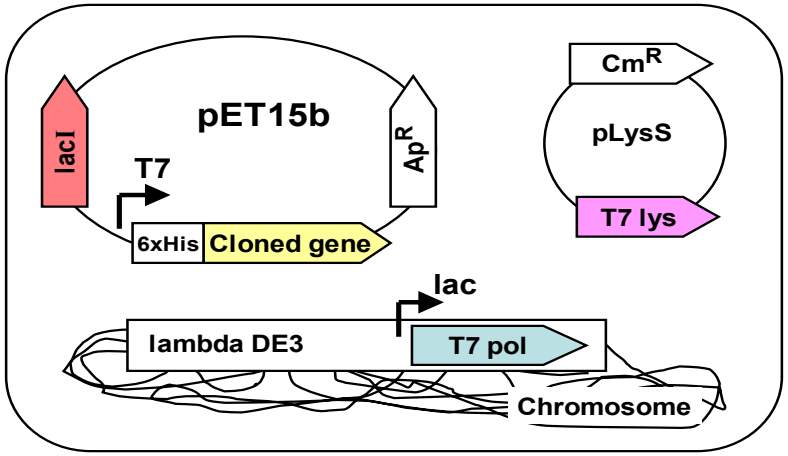


How can we avoid this problem?

Are there ways to clone genes into a vector that DON'T use PCR?

Cloning and expressing genes. Murphy's Law #3

" You will forget to add antibiotics to the medium"

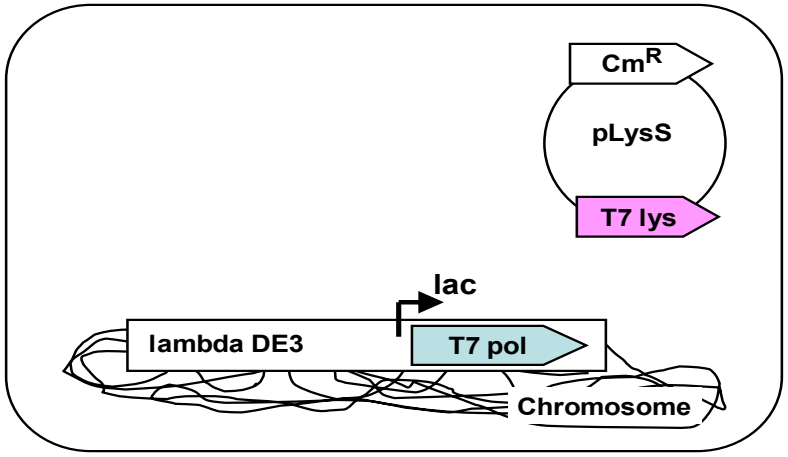


Inoculum cells:
Cml^{R} , Amp^{R}

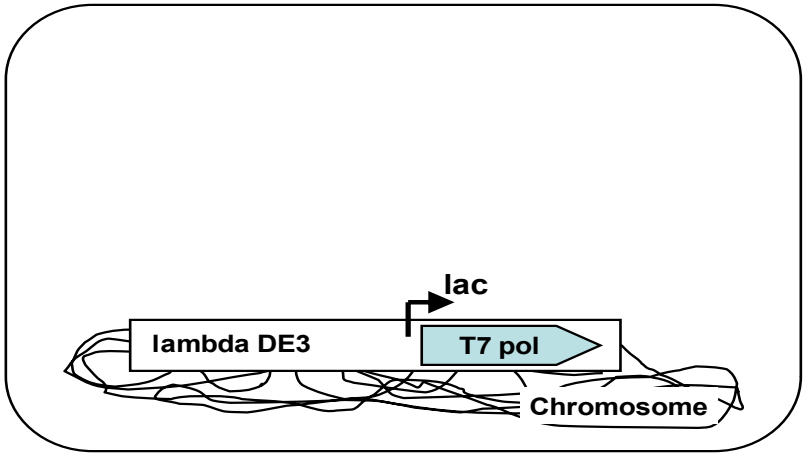
Effect of IPTG Induction ?

Growth in plain LB

After growth: Cml^{S} Amp^{S}



Growth in plain LB



Cloning and expressing genes. Murphy's Law #4

" The gene YOU want to study is always extremely toxic and/or non-functional if overexpressed in E.coli "

AND

" YOU are the one who has to figure out the above. This will take 2.5 years of your 3 year PhD project "

eg. how do you overexpress a restriction enzyme without digesting all the host bacteria's DNA?

eg. how do you overexpress membrane channel proteins that make pores in the cell envelope?

Picture credits

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