Chapter 6

Manipulation of Gene Expression in Prokaryotes

6.1. Gene Expression from Strong and Regulatable Promoters

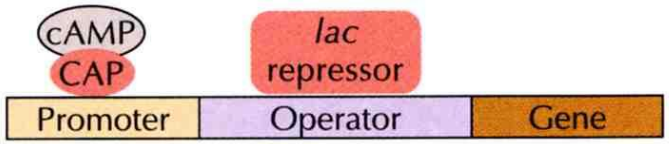
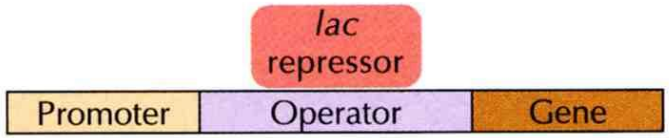
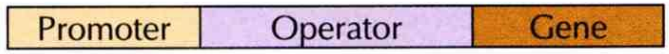
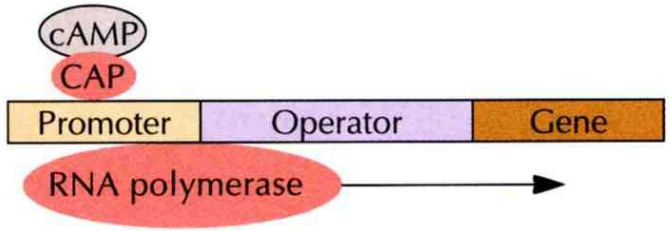
Gene Expression from Strong and Regulatable Promoters

- Constitutive expression of foreign protein
  - Can be detrimental to the host because of the energy drain
  - Can cause plasmid instability
- Regulatable promoters
  - *E. coli lac*, *trp*, *tac* (-10 *lac* + -35 *trp*) promoter
  - Bacteriophage λ *p*L promoter
  - Bacteriophage T7 gene10 promoter

Lac Operon

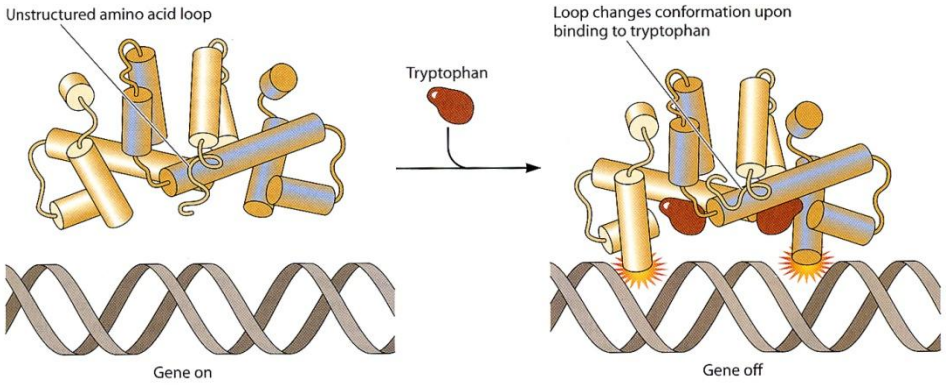
- Genes: *LacZ*, *LacY*, *LacA* for lactose utilization
- Repressor: *LacI*
  - Represses gene expression by binding to lac operator
  - DNA binding activity is inhibited by binding to lactose or isoproply-β-D-thiogalactopyranoside (IPTG)
- Activator: CAP (catabolite activator protein)
  - Activates gene expression by binding to the promoter
  - DNA binding activity is increased by cAMP binding under low glucose conditions

Lac Promoter

Glucose concentration	Lactose concentration	cAMP concentration	*lac* promoter-operator region	Level of transcription
Low	Low	High		Low
High	Low	Low		Low
High	High	Low		Low
Low	High	High		High

Trp Promoter

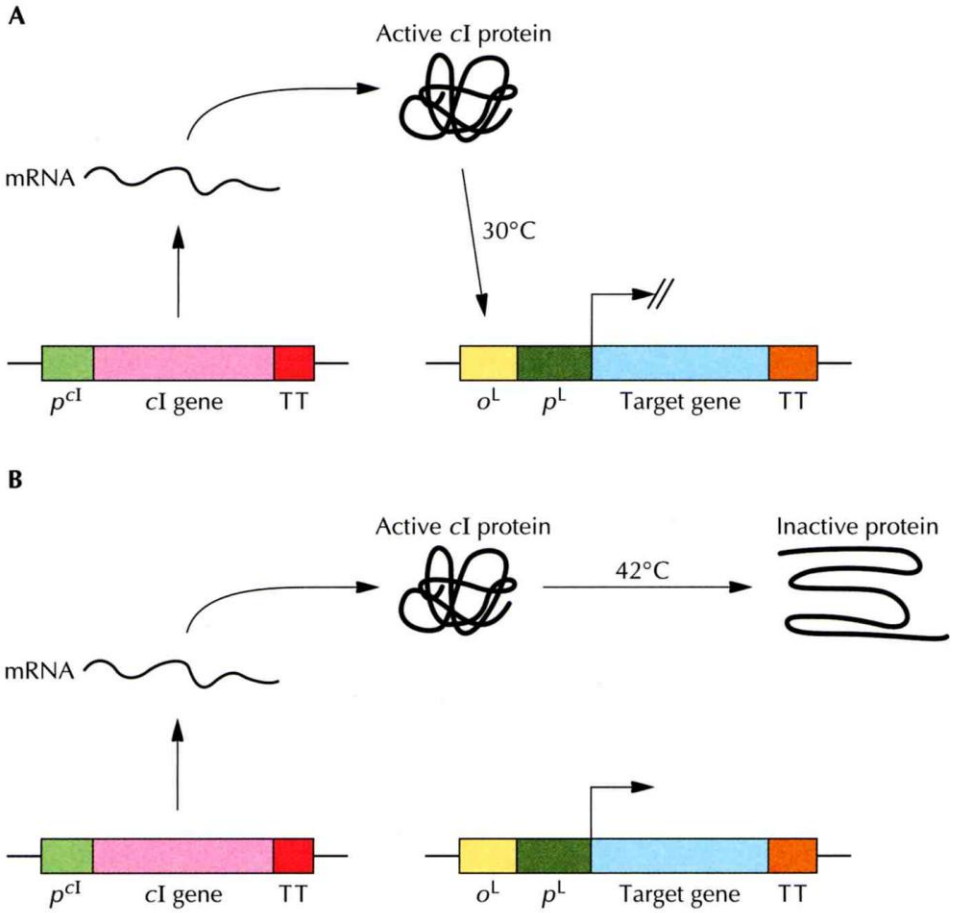
- Genes: genes for Trp biosynthesis
- Trp Repressor
  - Binding to the operator in the presence of Trp
  - Leaky expression



- *tac*, *trc* promoter
  - -35 trp promoter --- 16 (tac) or 17 (trc) bp --- -10 lac promoter
  - IPTG inducible
  - Stronger and more effective than the original promoters

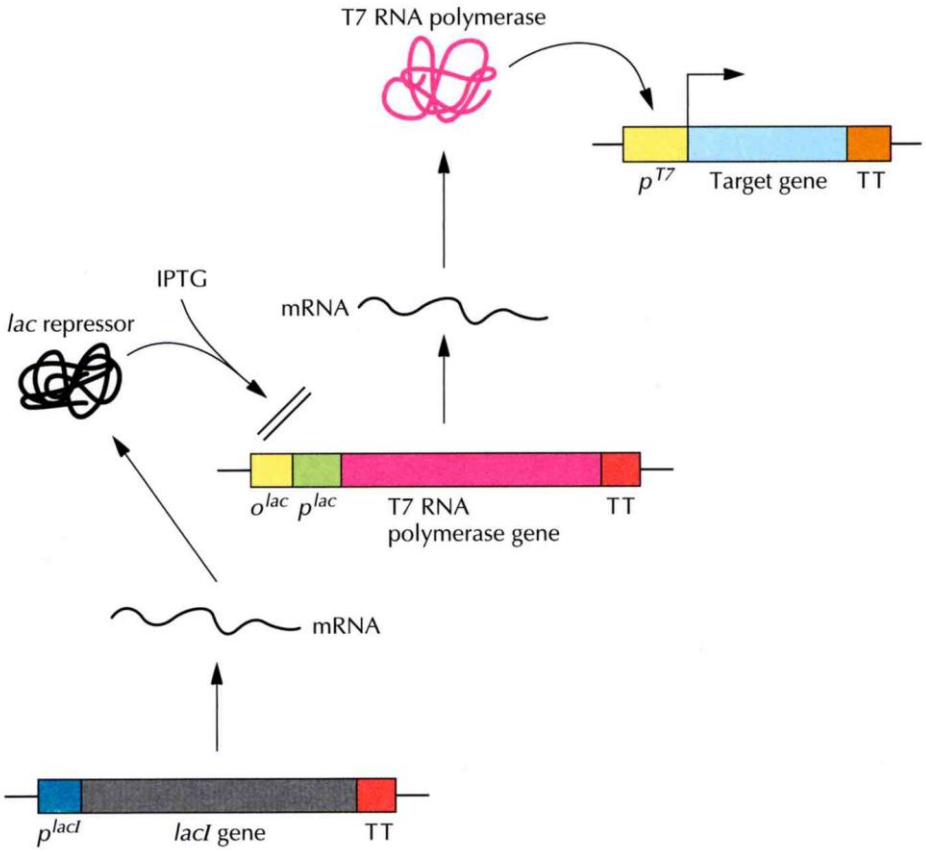
Bacteriophage λ P^L Promoter

- Repressor : *c*I
  - cI857: temperature-sensitive *c*I
    - Active at low temperature (28~30°C)
    - Inactive at high temperature (42°C)

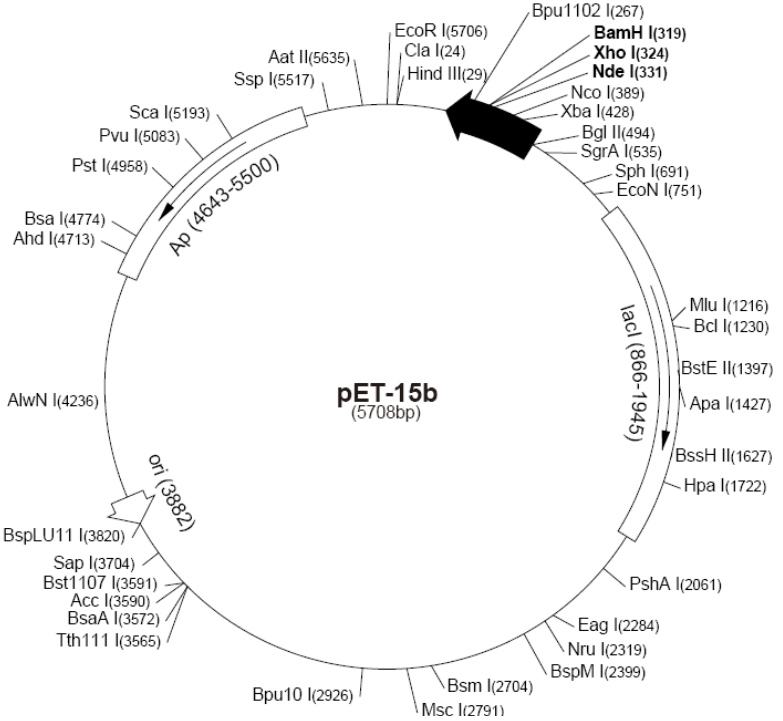


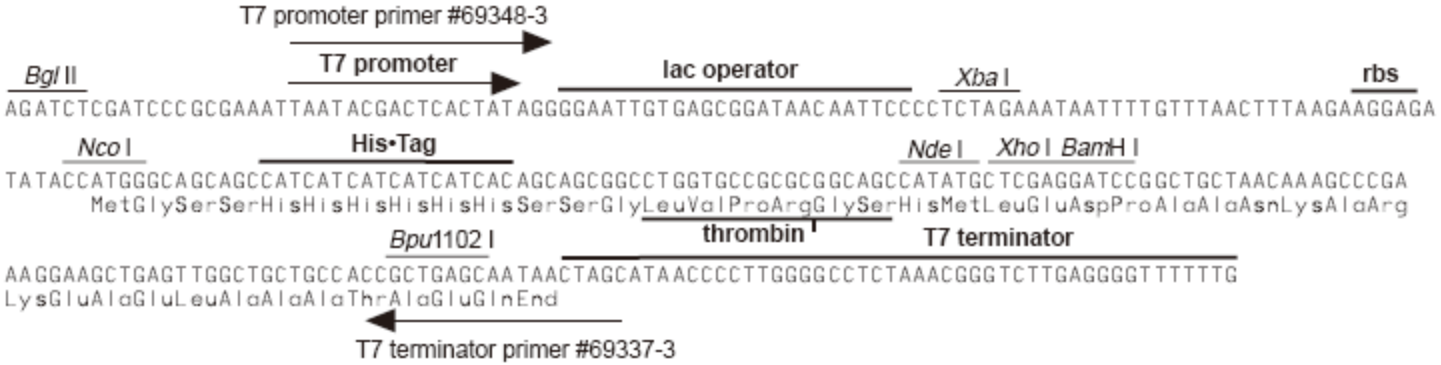
T7 Promoter

- T7 promoter
  - Transcription by T7 polymerase
- T7 polymerase under the control of the *lac* promoter
  - Induction of T7 promoter-regulated genes by IPTG

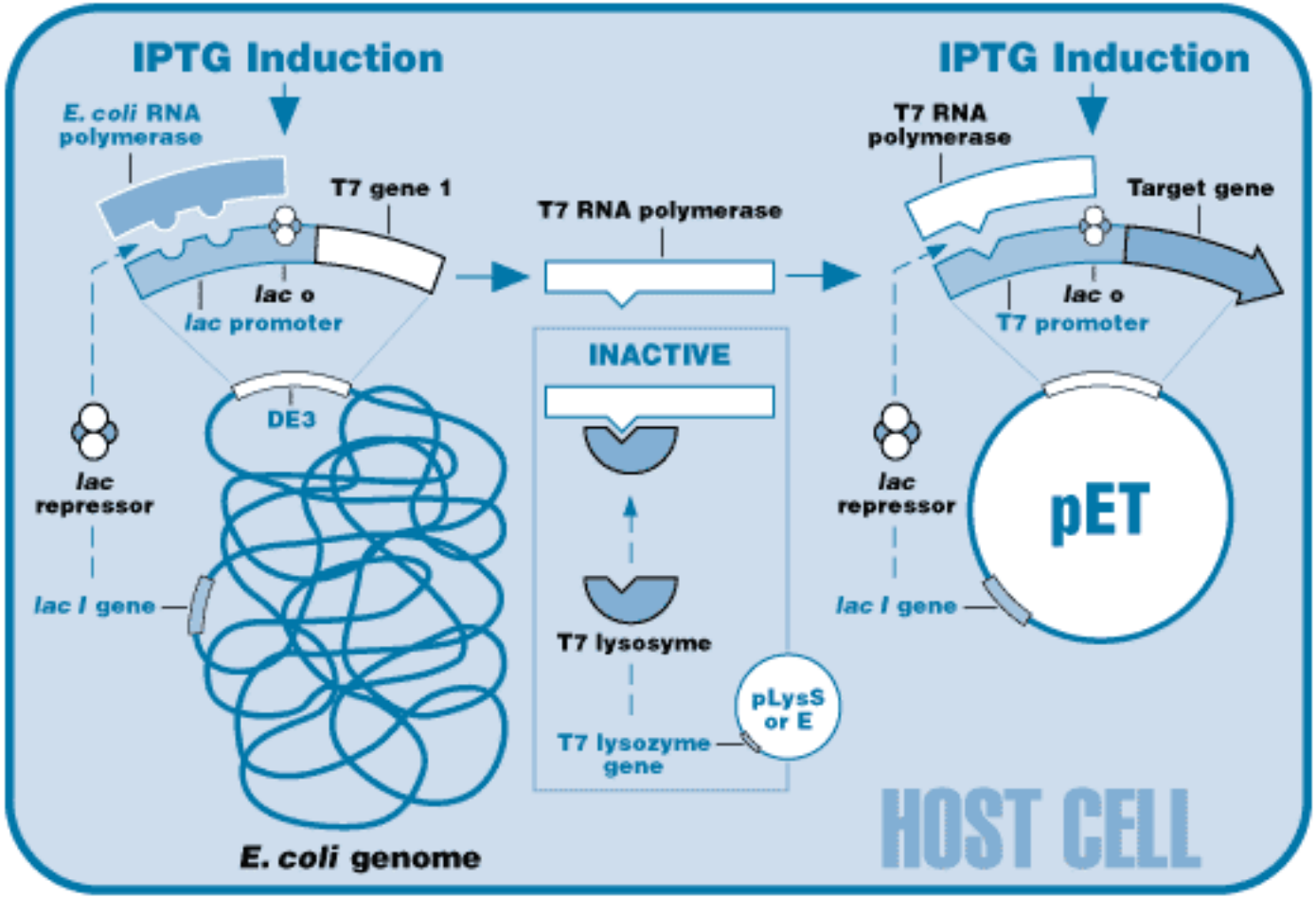


pET Expression Vector





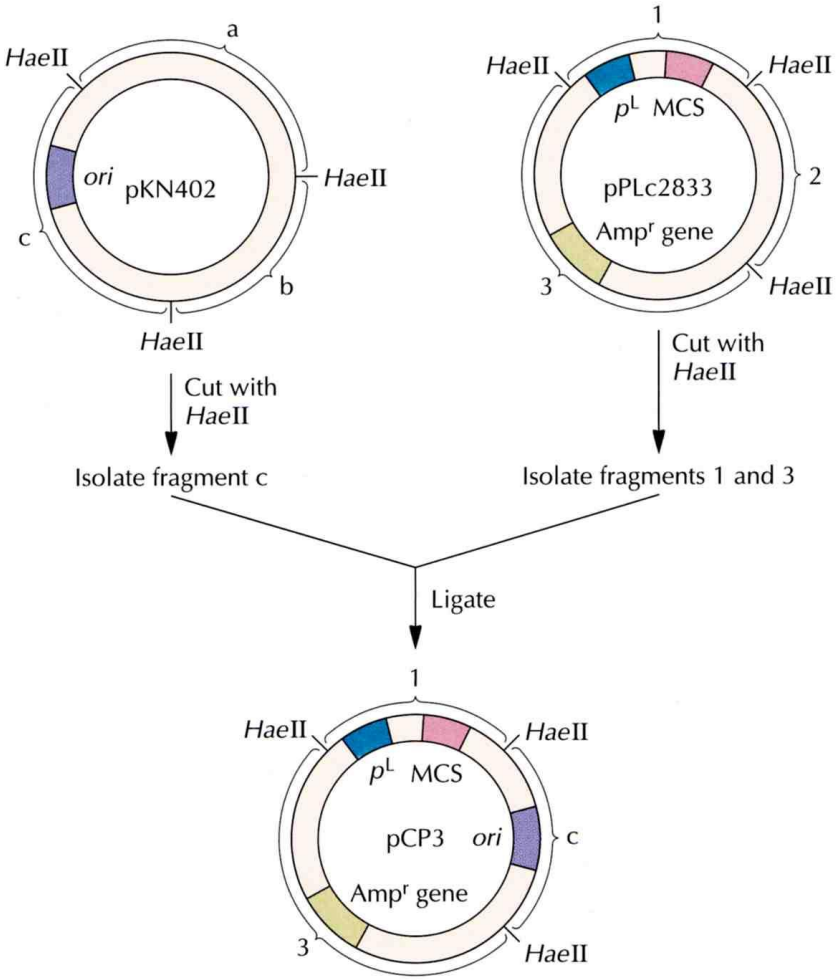
PET Expression System



E.coli DE3 (pLysS)

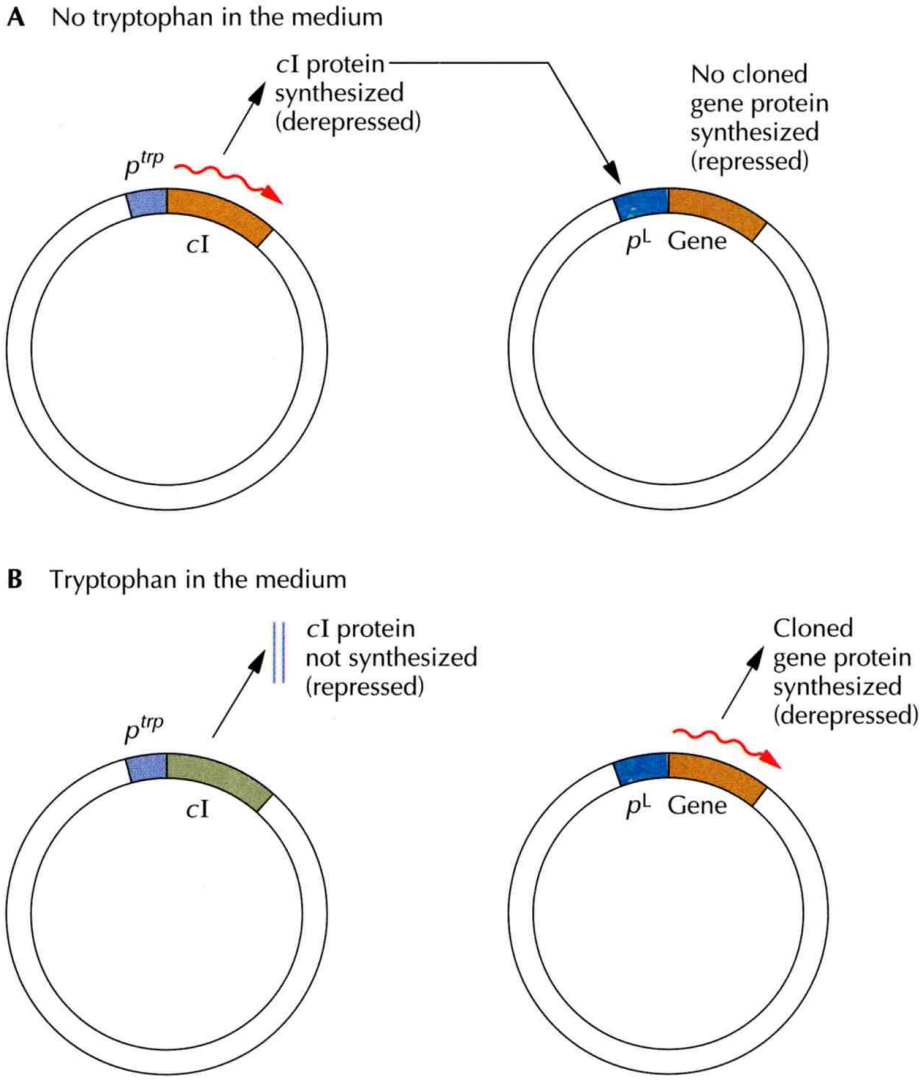
Temperature-Dependent Regulation of Plasmid Copy number

- pKN402
  - Increase in copy number at high temperature
  - 28°C: 82 → 42°C: 521
- pCP3
  - Contains pKN402 replication origin
  - High level protein expression at 42°C



Dual Plasmid System

- Two plasmids
  - *c*I under the control of *trp* promoter
  - Protein expression under the control of p^L promoter
- Control gene expression
  - Without Trp
    - Repression of protein expression
  - With Trp
    - Induction of protein expression
- Inexpensive system for large scale protein production



Expression in Other Microorganisms

- Universal gram (-) bacterial expression vector
  - Tn5 promoter in a broad-host-range plasmid pRK290
  - Efficient gene expression in different bacterial hosts
- Modification of promoter strength
  - *Lactococcus lactis* constitutive promoter
  - Screening strong promoters from spacer region (between -35 and -10) library

TTGACANNNNNNNNNNNNNNNTGRTATAAT

6.2. Fusion Proteins

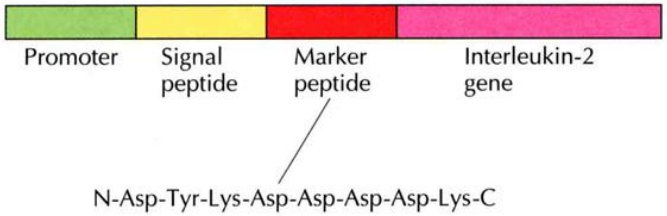
Fusion Proteins

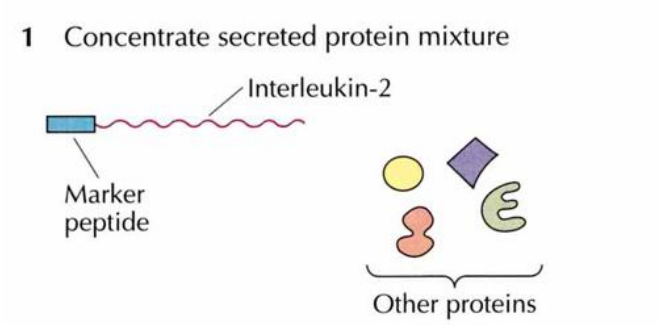
- Fusion protein
  - In frame fusion of a target protein with a stable host protein
  - Resistance to proteolysis
- Cleavage of fusion proteins
  - Linkage of two proteins with nonbacterial protease recognition sequence
  - Ile-Glu-Gly-Arg : C-terminal end cleavage by factor X_a

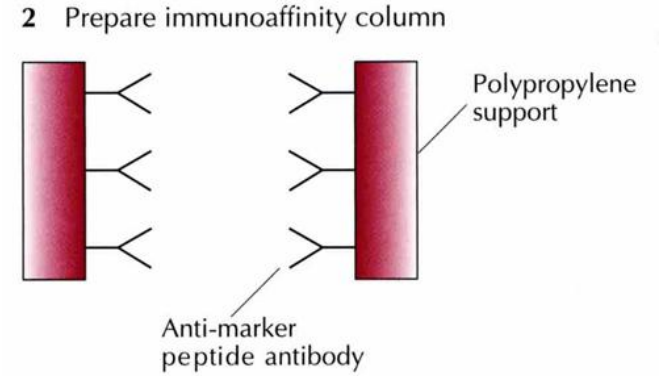
Uses of Fusion Proteins

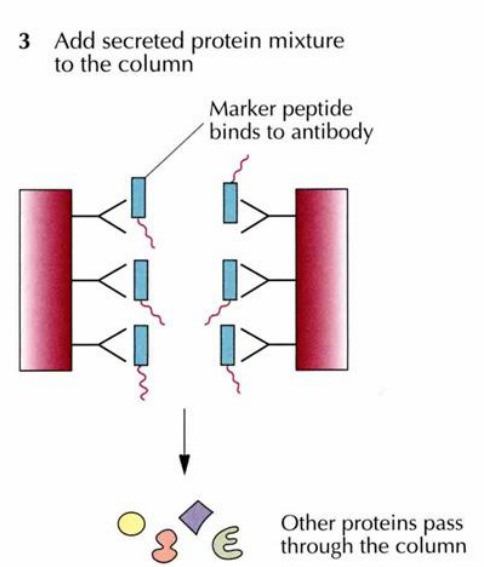
- Raising antibody
- Simplifying purification
  - His tail (6-10 aa): Ni^{2+} binding, elution by imidazole
  - Strep-tag (10 aa):Streptavidin binding, elution by iminobiotin
  - MBP (40 kDa): Amylose binding, elution by maltose
  - GST (25 kDa): Glutathione binding, elution by reducing agent
  - Flag (8 aa): Flag Ab binding, elution by low calcium
  - ZZ (14 kDa): IgG binding, elution by low pH

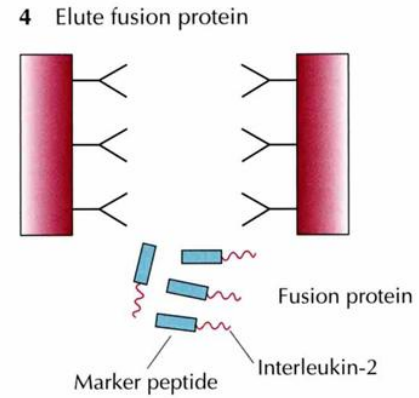
Purification of Fusion Proteins by Immunoaffinity Chromatographic Purification





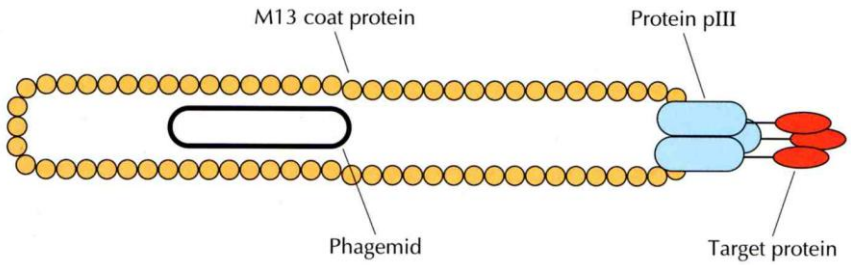


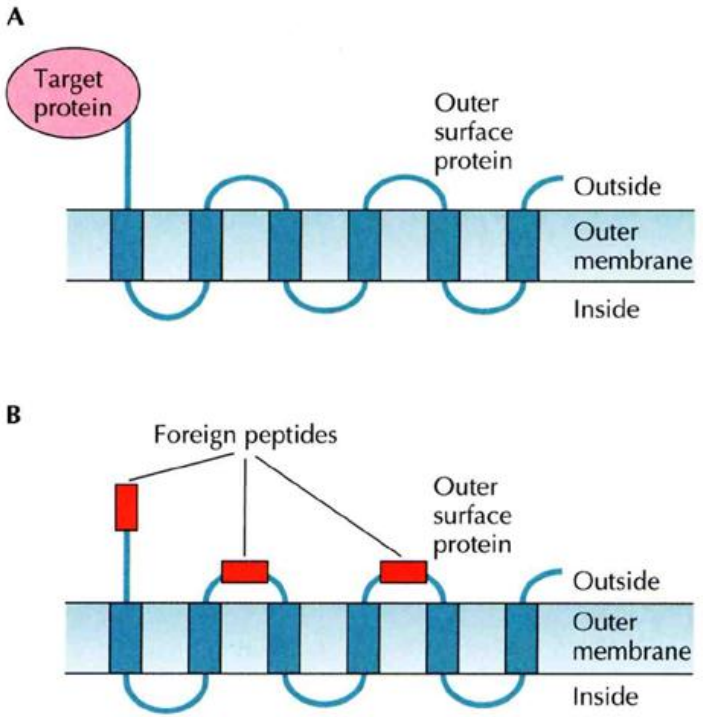




Surface Display

- Display proteins as fusion proteins with a surface proteins (filament, pilus protein)
  - Phage display
    - Protein pIII of M13 phage
  - Bacterial display
    - *E. coli* OmpA, OmpF : outer membrane protein
    - *E. coli* PAL: peptidoglycan-associated lipoprotein
    - Target protein in N or C terminus, or in the middle



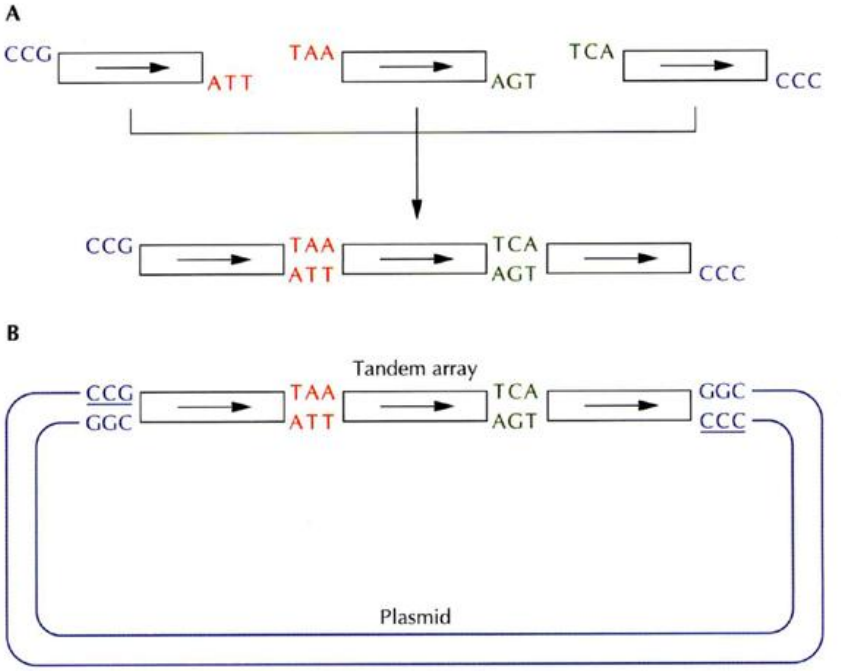


Usage of Surface Display

- Screening of cDNA libraries
- Overexpression of peptides or proteins
  - Expression of antigenic determinant of the parasite *Plasmodium falciparum* (causing malaria) by inserting into surface-exposed loops of the major outer membrane protein from *P. aeruginosa* (OprF)
  - Possible usage as vacccines

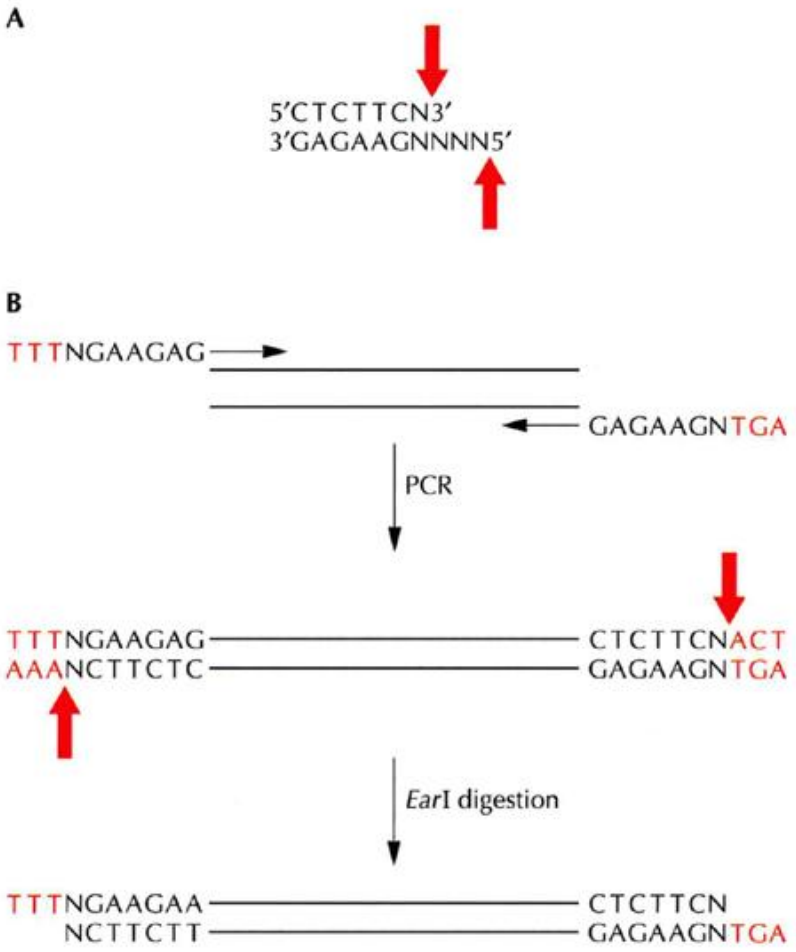
Unidirectional Tandem Gene Array

- Protein expression levels
  - Multiple copies of genes in a low-copy-number plasmids > Single copy of gene in a high-copy-number plasmid
  - Generation of tandem gene array
    - Using complementary 3-nucleotide extensions



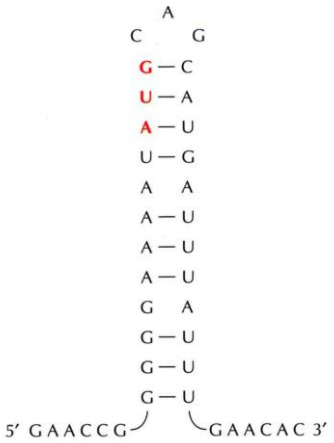
Unidirectional Tandem Gene Array

- TypeIIS restriction enzyme
  - Cut random sequence outside of the recognition site
- Use pfu polymerase for PCR



6.3. Translation Expression Vectors

Translation Expression Vectors

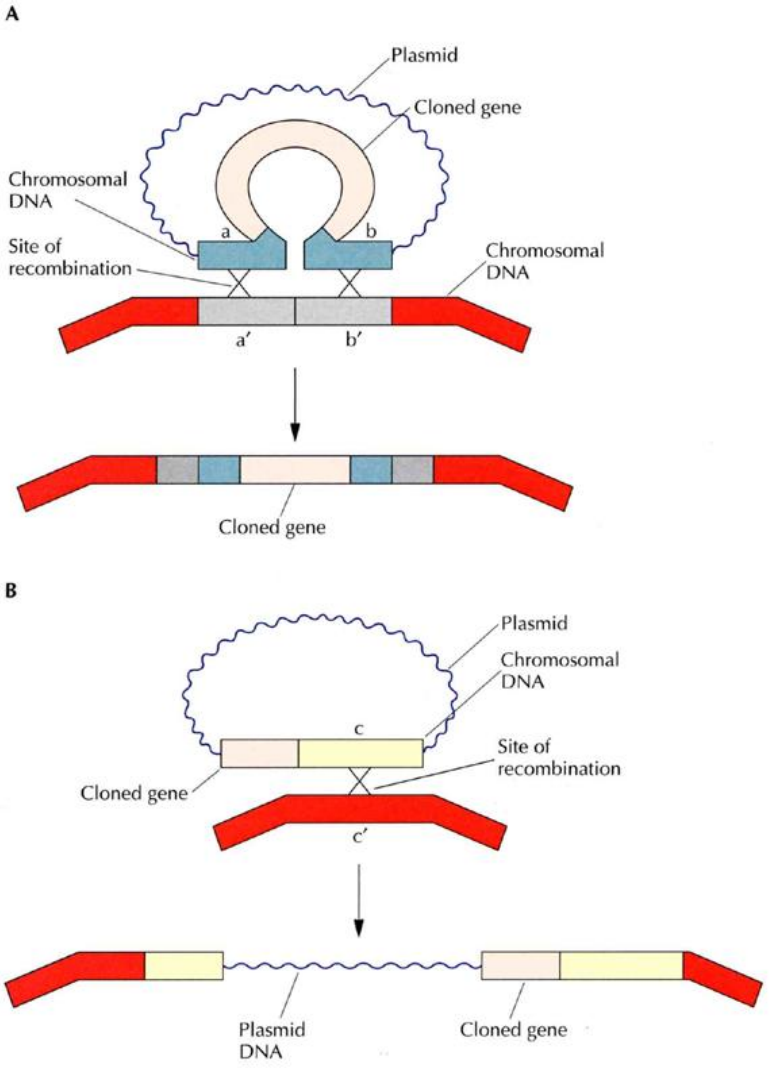


- Translation efficiency
  - Ribosome binding site
    - UAAGGAGG
    - Precise distance from the starting codon
    - No secondary structure formation preventing ribosome binding
  - Codon usage
    - Rarely used codons in *E. coli* (AGG, AGA, AUA, CUC, CGA)
    - Solutions
      - Expression in eukaryotes
      - Change codons
      - Provide tRNAs for rare codons
        - Strain is commercially available

6.4. DNA Integration into the Host Chromosome

DNA Integration into the Host Chromosome

- DNA integration into chromosome
  - Stable expression
  - Integration into non-essential gene
  - Homologous recombination through chromosomal sequence
    - Double cross over or
    - Single cross over
  - Use nonreplicating plasmid for integration



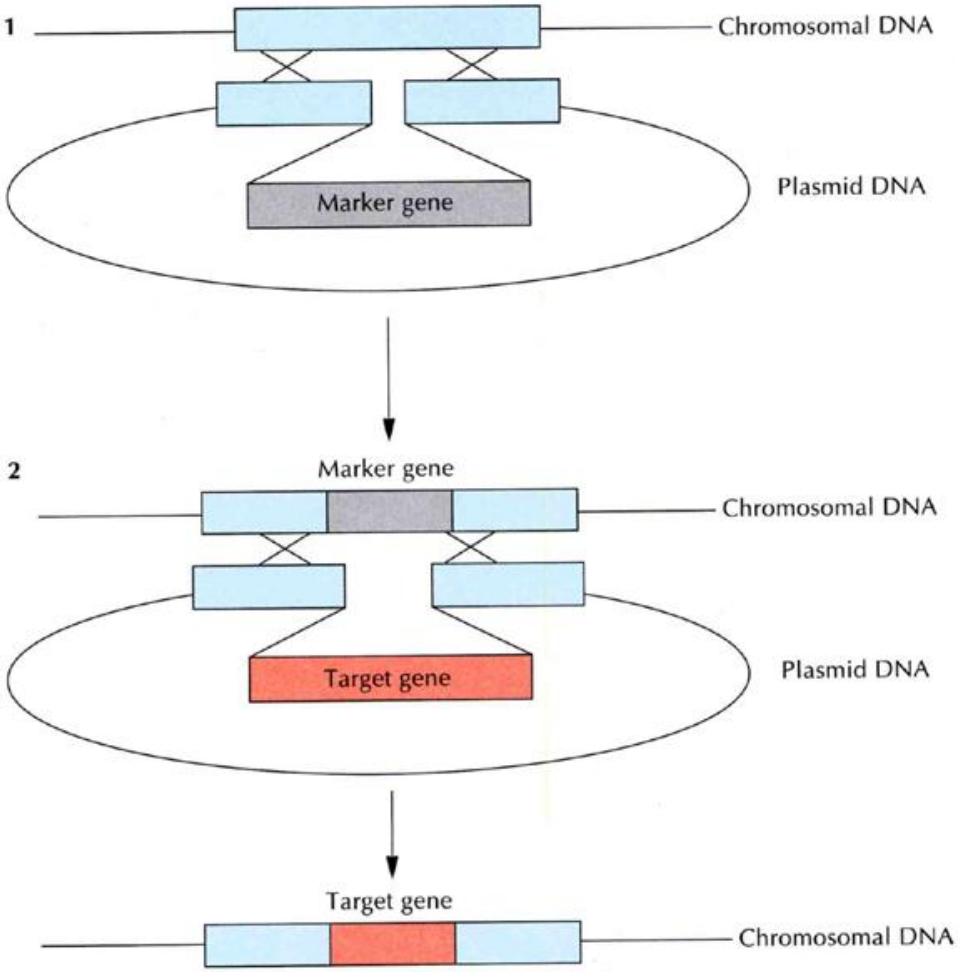
Multiple Integration

- Expression of α-amylase in *B. subtilis*
  - Integration of plasmid with α-amylase gene and chloramphenicol resistance marker on *B. subtilis* chromosome
  - Isolation of clones with multiple integration by selection under high chloramphenicol

Copies/genome	α-amylase activity (U/ml)
2	500
5	2,300
7	3,100
9	4,400
Multicopy plasmid	700

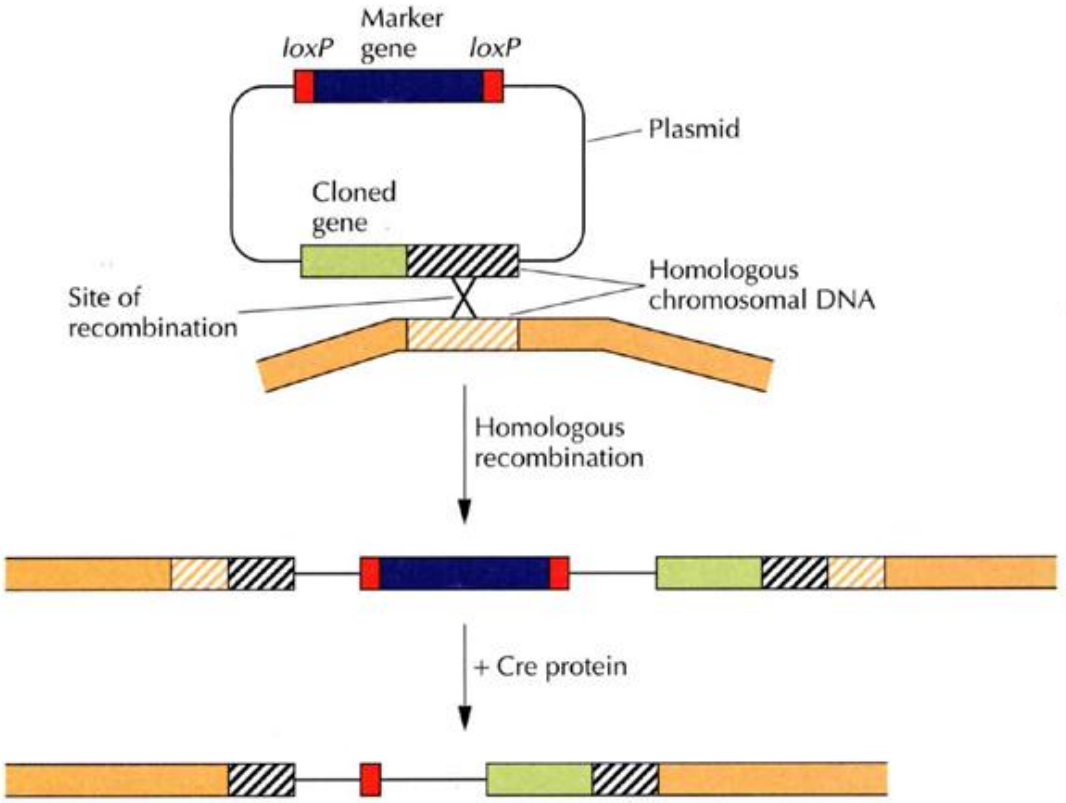
Multiple integration at specific sites

- Integration of a marker
- Replacement of the marker with a target gene



Removing Selectable Marker Genes

- Cre-LoxP system
  - Cre recombinase
  - LoxP site: 34-bp recombination sites
  - Removal of a marker flanked by loxP site by expression of Cre enzyme (under the control of lac promoter)



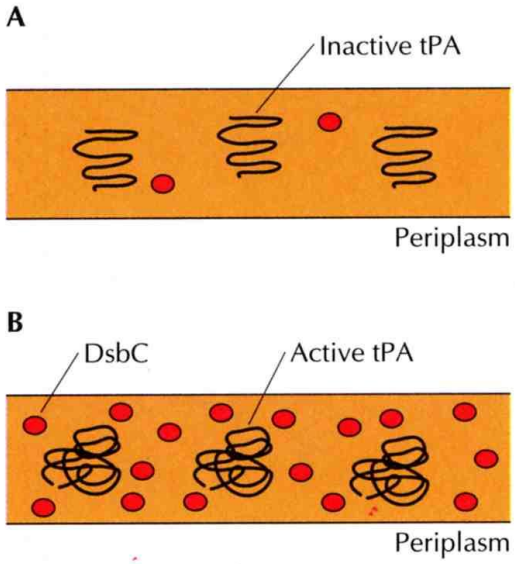
6.5. Protein Stability, Folding, and Secretion

Increasing Protein Stability

- Protein half life
  - A few minutes to hours
- Factors affecting protein stability
  - N terminal amino acid
    - M, S, A , T, V, G at N terminus of β-galactosidase: > 20 h
    - R: ~ 2 min
  - Internal PEST sequence
    - Facilitate degradation

Protein Folding

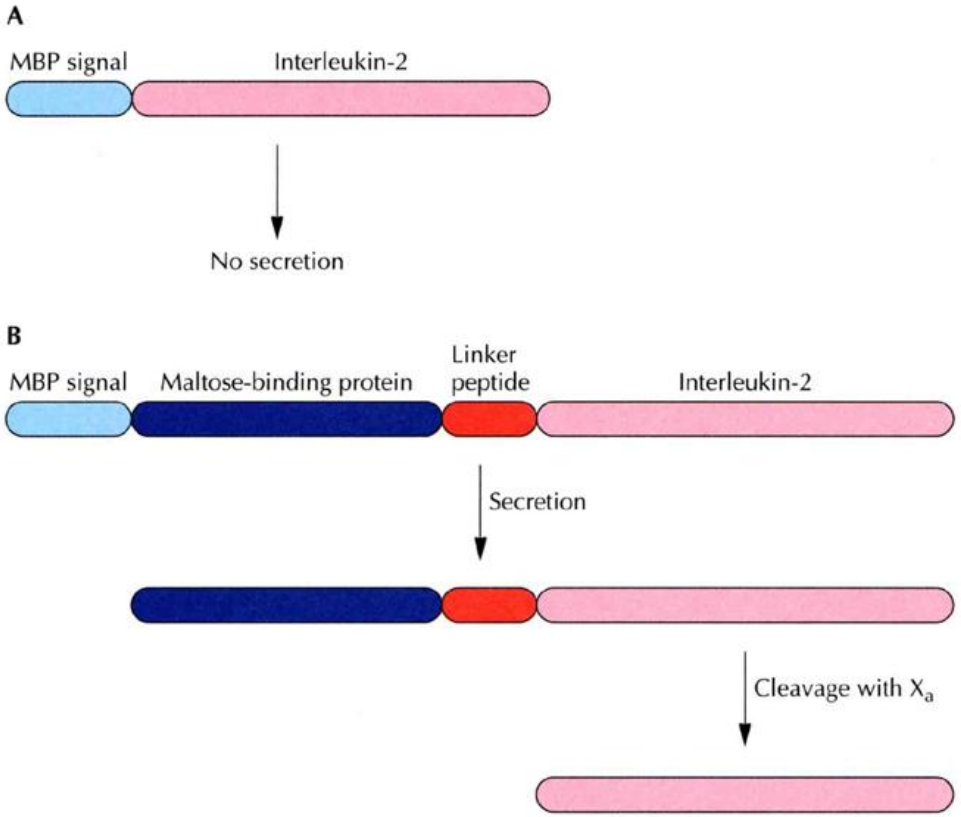
- Inclusion body
  - Insoluble aggregates of expressed protein
- Strategies to prevent inclusion body formation
  - Tagging with other proteins
    - Thioredoxin, GST
  - Proteins with multiple disulfide bonds
    - Expression with signal peptide for secretion into periplasmic space
    - Coexpression of high levels of periplasmic DsbC enzyme involved in disulfide bond formation



tPA: tissue plasmogen : 17 S-S bonds

Increasing Secretion

- Advantages of secretion of a target protein
  - Protein stability
    - Proinsulin: more stable in periplasm
  - Easy to purify
- Protein secretion
  - Addition of signal peptide
  - Fusion with a secretory protein



Permeabilization of *E. coli*

- Inducible expression of bacteriocin release protein
  - Activation of phospholipaseA
  - Permeabilization of inner and outer membrane
- Translational overload
  - Inhibition of protein secretion
  - Expression of limiting components protein secretion pathway

Bacteria with Increased Permeability

- L-form bacteria
  - Bacterial lacking cell wall
  - Generation of L-form bacteria
    - Spontaneous mutations
    - Treatment with penicillin
      - Inhibition of the final step in cell wall biosynthesis
    - Treatment with lysozyme
      - Hydrolysis of saccharide linkage
  - High yield of protein secretion
- Spheroplasts, protoplasts
  - Complete loss of cell wall

6.6. Production of Recombinant Proteins

Overcoming Oxygen Limitation

- Oxygen limitation
  - Slow growth
  - Enter stationary phase
  - Protease production
- Use of protease-deficient host strains
  - Proteases are also required for cell growth
  - rpoH (heat shock sigma factor) and degP (protease for high temperature growth) mutants
    - Decrease in protein degradation of secretory proteins
- Bacterial hemoglobin
  - Hemoglobin-like molecule in Vitreoscilla bacterium
  - Expression in *E. coli* to increase protein synthesis

Metabolic Load

- Impairment of normal cellular function of host cells by expression of foreign DNA
  - Replication and maintenance of high copy number plasmid
  - Limitation of dissolved oxygen
  - Depletion of certain aminoacyl-tRNAs and/or drain energy
  - Prevent proper localization of host proteins by foreign secretory proteins
  - Interference of host cell function by foreign proteins

Effects of a Metabolic Load

- Decrease in cell growth rate
  - Loss of plasmid or a portion of plasmid DNA
- Decrease in energy-intensive metabolic processes
  - Nitrogen fixation
  - Protein synthesis
- Changes in cell size and shape
- Increase in extracellular polysaccharide production
- Increase in translational errors

Prevention of Metabolic Load

- Prevention of metabolic load
  - Low copy number plasmid for expression
  - Integration of DNA into host chromosome
  - Inducible promoter
- Optimization for the maximum yield
  - Protein expression levels
  - Cell density

Increasing Cell Density

- Elimination of growth inhibitory waste products
  - e.g. prevention of acetate formation
    - Use glucose analogue α-glucoside to reduce glucose uptake
    - Use ptsG (enzyme II in the glucose phosphotransferase system) mutant
    - Expression of acetolatase synthase