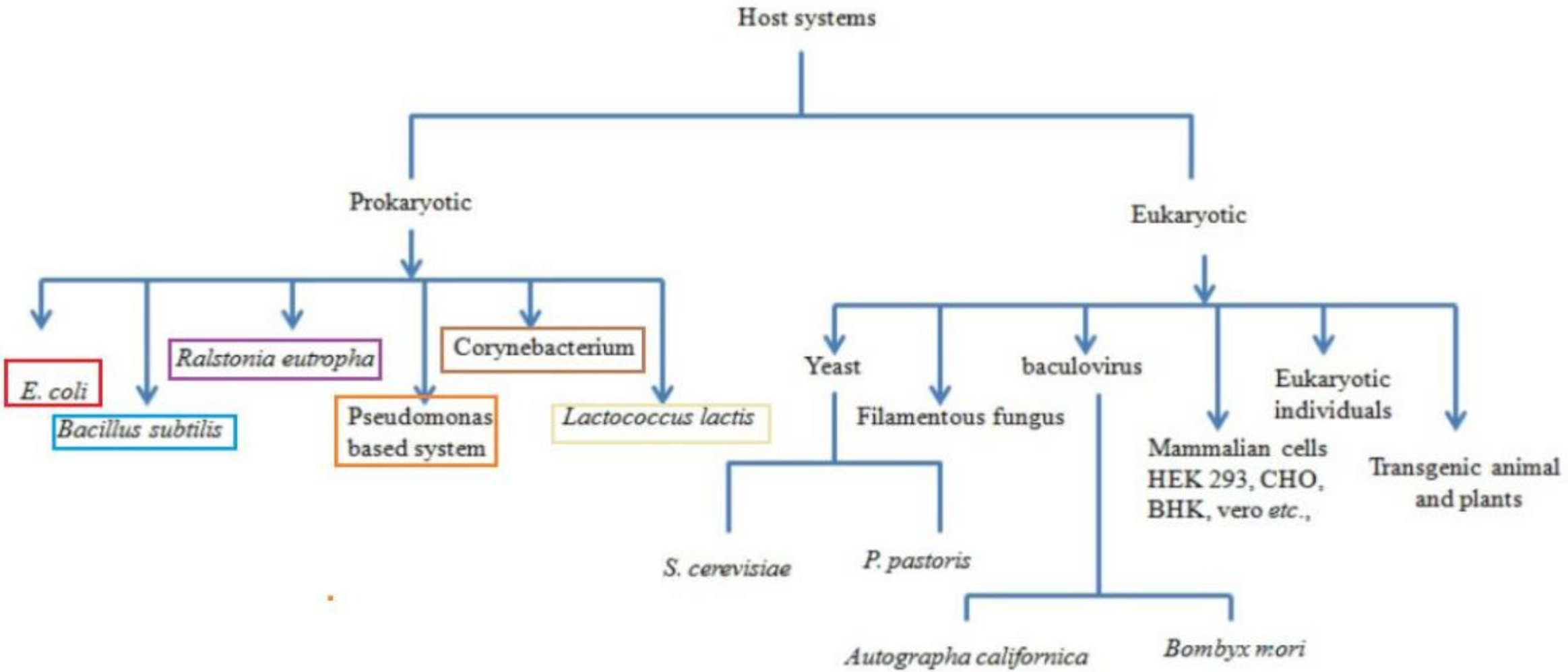
Heterologous and Homologous Expression of Proteins



Different host systems available for the production of recombinant Proteins

علت تنوع میزبان های پروکاریوتی:

۱- non-pathogenic و non-toxigenic

۲- sec-system

۳- growth on a wide variety of substrates

۴- competence cell

Table I: Comparison of cell-based protein expression systems

Expression System	Ease of Handling and Scale-Up*	Protein Expression Level	Cytotoxic Mammalian Proteins	Percent Yield (Based on Dry Weight)	PTMs†	Applications
Bacterial	****	Up to 10–30 g/L	Yes	1–5%	+	Functional assays Structural analysis Antibody generation Protein interactions
Yeast	***	Up to 30 g/L	Yes	1%	++	Functional assays Structural analysis Antibody generation Protein interactions
Insect	**	Up to 500 mg/L	Yes	30%	+++	Functional assays Structural analysis Antibody generation
Mammalian	*	Under 10 mg/L		<1%	++++	Functional assays Protein interactions Antibody generation

*Most difficult handling: ****; easiest handling: *.
†Very minimal PTM: +; PTM the closest to that in naturally occurred proteins: ++++.

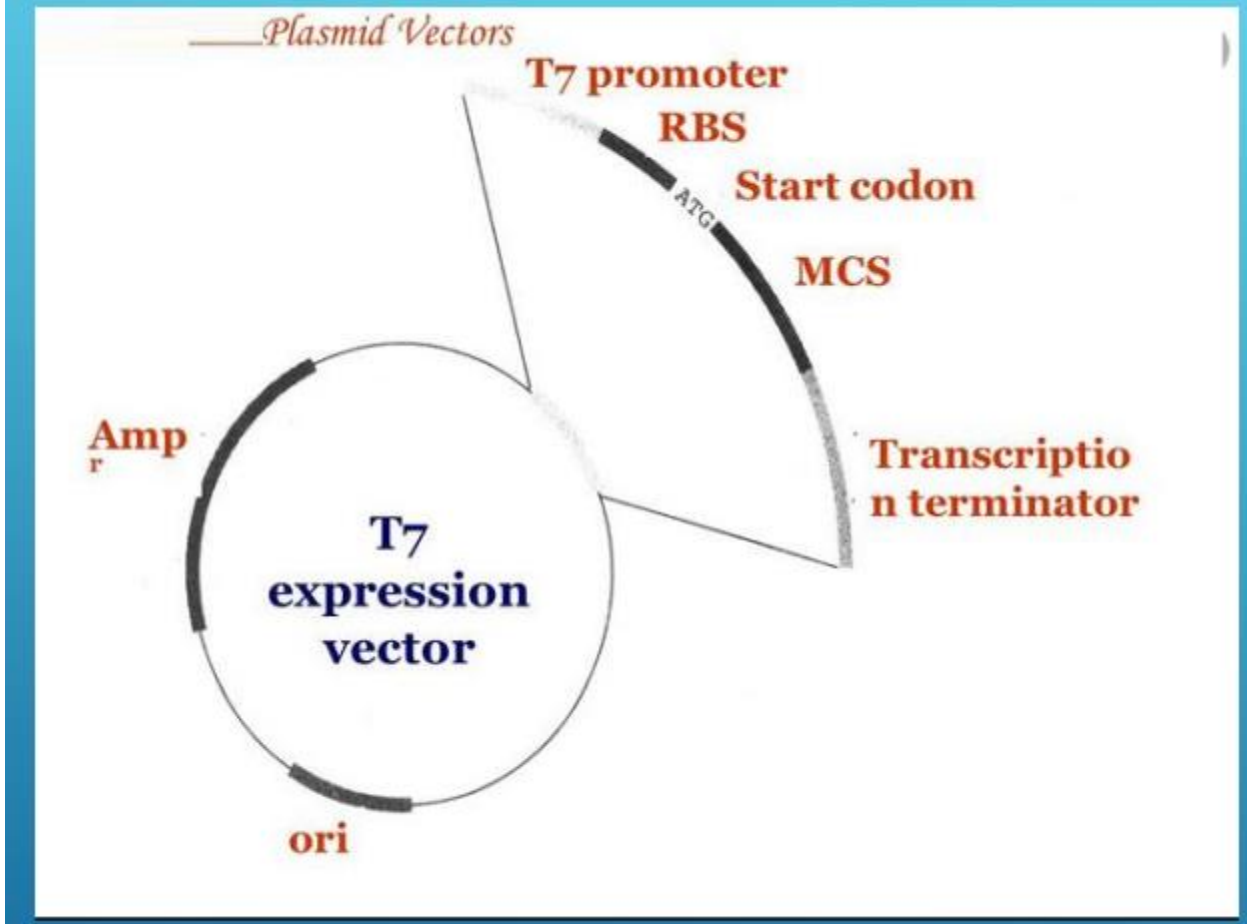
معایب اصلی میزبان های پروکاریوتی بعنوان سیستم بیان پروتئین های هترولوگوس

۱- PTM=post-translational modifications

۲- Folding (نیازمند آنزیم دی سولفید ایزومراز و سیستم چاپرونی GRP78/calnexin/calreticulin)

۳- Splicing

۴- Cutting

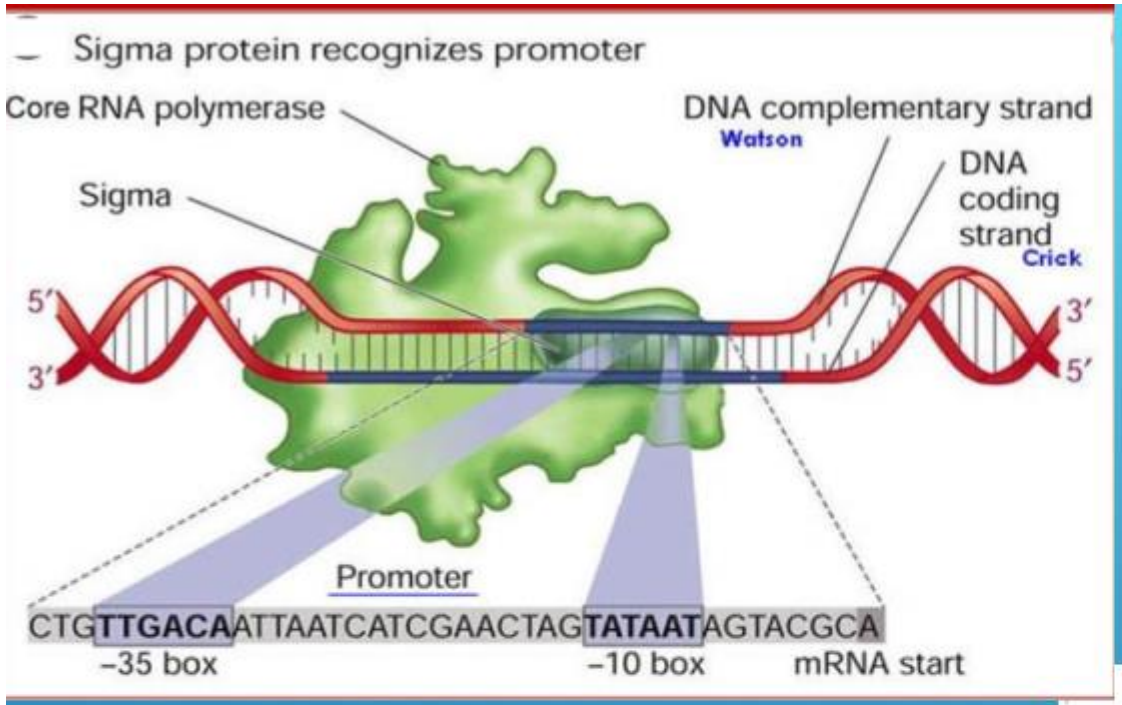


بیان ژن های بیگانه در E. coli

۱- Promoter

۲- Terminator

۳- RBS



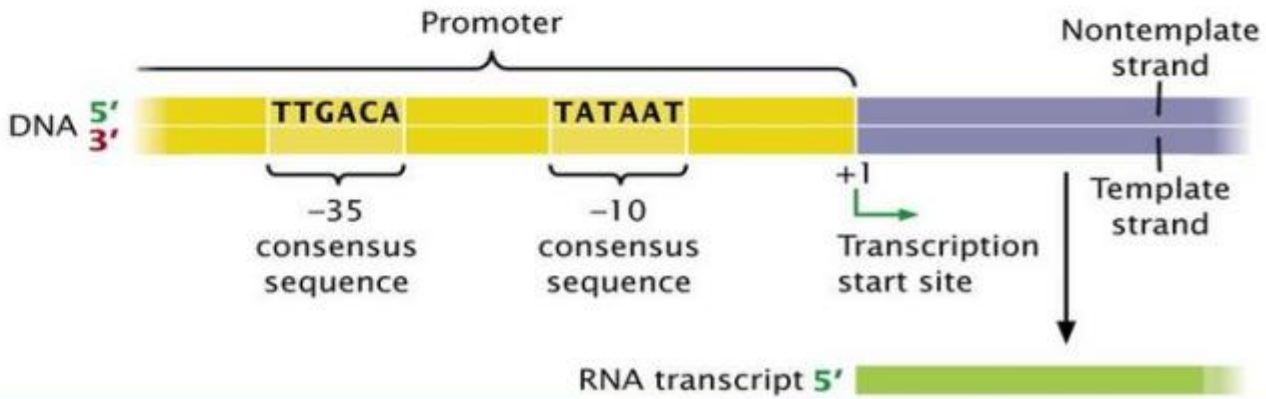
قدرت یک پروموتور:

۱- Affinity binding site

۲- Regulatory sites

۳- سرعت RNA elongation پلیمراز

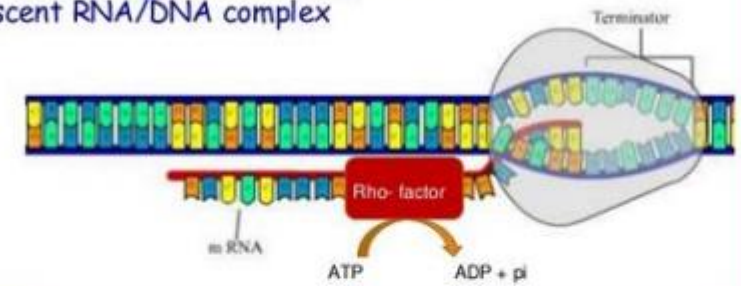
Upstream consensus sequences in bacterial promoters

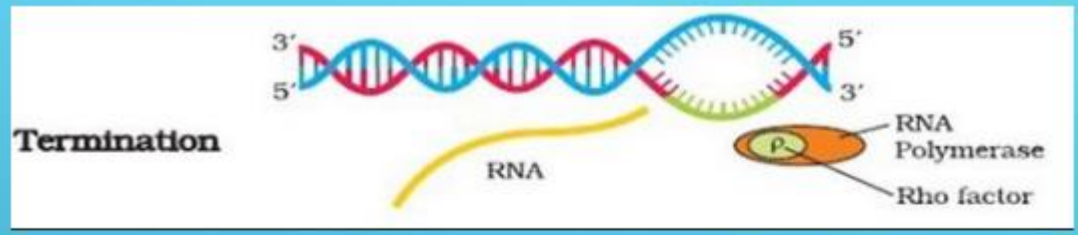


Termination

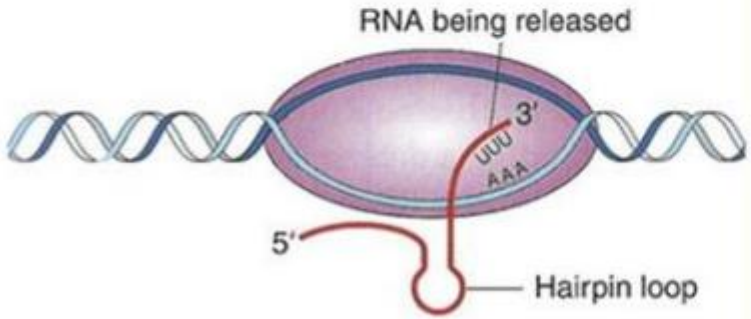
ho factor is an ATP dependent RNA-DNA helicases

ecognizes and bind to the termination signals and
isrupts the nascent RNA/DNA complex





Bacterial termination: intrinsic mechanism

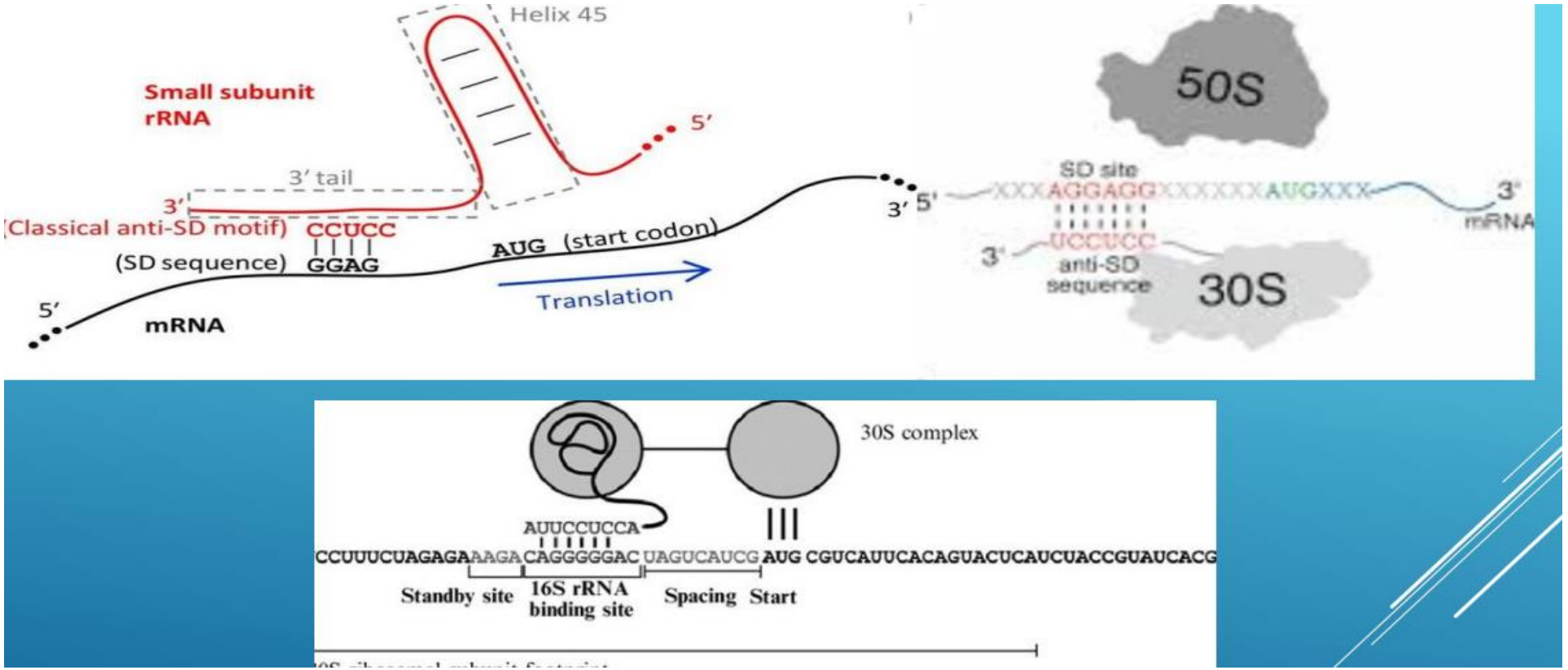


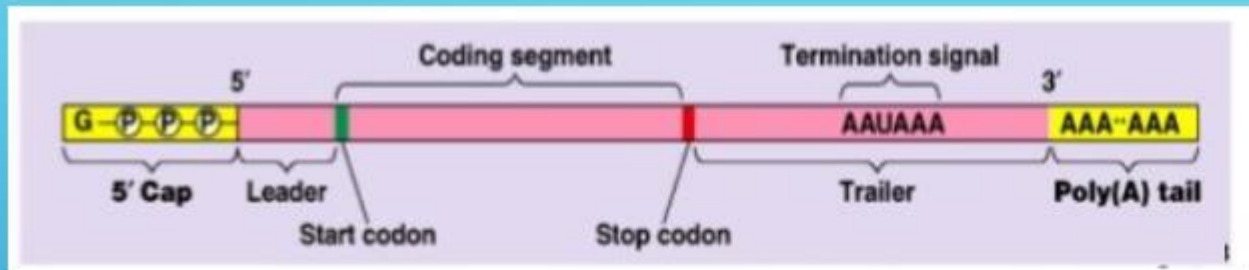
Prokaryotes:

- mRNA transcript has a Shine-Dalgarno sequence
- rRNA on ribosome small subunit has a complementary section: anti Shine-Dalgarno sequence

Eukaryotes

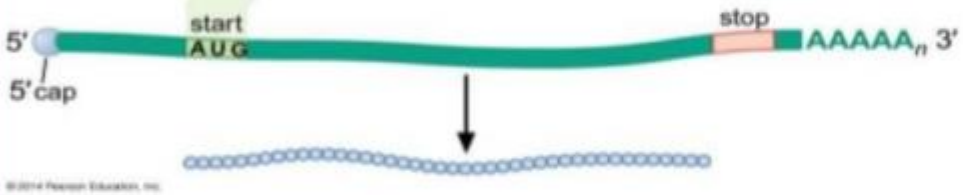
- Ribosome small subunit recognizes and bind to mRNA at 5′ cap





Kozak sequence

5′ NN $\frac{G}{A}$ NN A U G G N N 3′

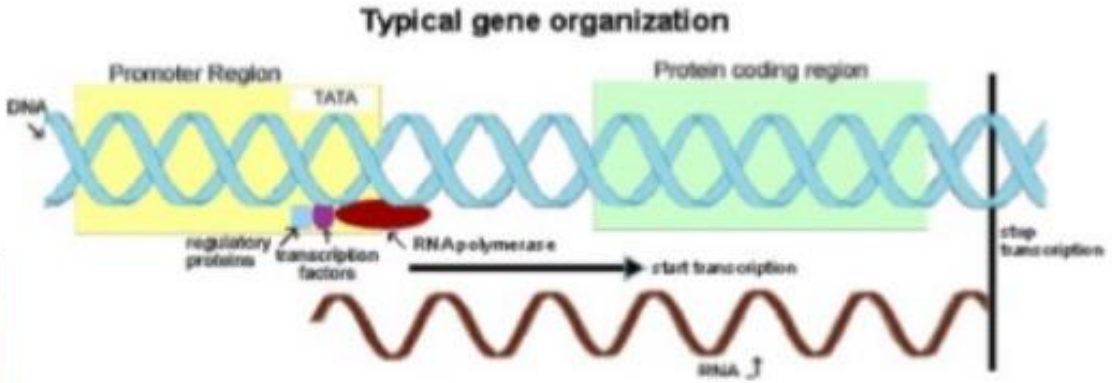


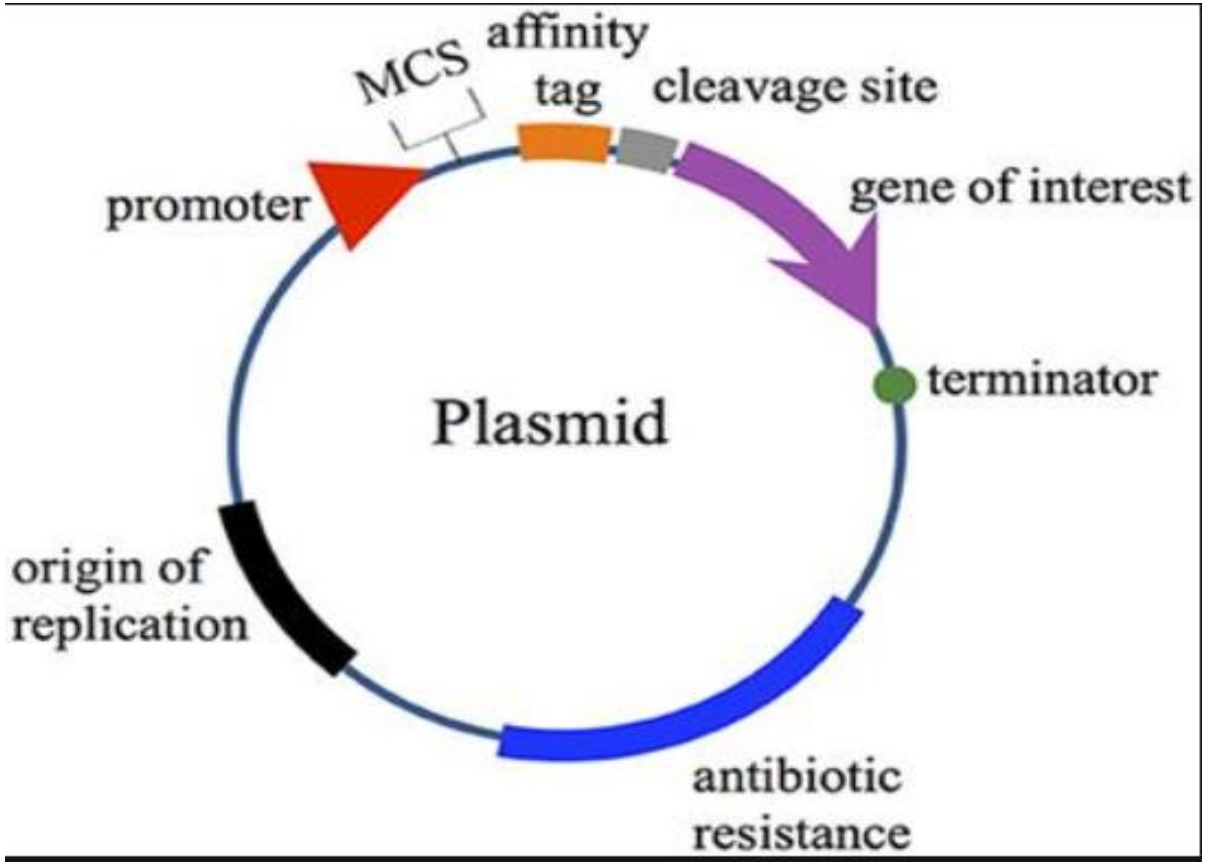
Organism(s)	Consensus Sequence*
Vertebrates	GCCRCCATGG
Terrestrial plants	AACAATGGC
Drosophila melanogaster (fruit fly)	CAAAATG
Saccharomyces cerevisiae (baker's yeast)	AAAAAAATGTCT
Dictyostelium discoideum (slime mold)	AAAAAAATGRNA
Plasmodium spp. (malarial protozoa)	TAAAAAATGAAN

*R = purine; N = any base.

Function of promoter

- RNA polymerase binding site
- Initiation of transcription
- control by regulatory sequences => control the expression of genes





Constitutive promoters: level up (low, middle): در همه شرایط بیان شده و تحت تیمار خاصی قرار نمی گیرند.

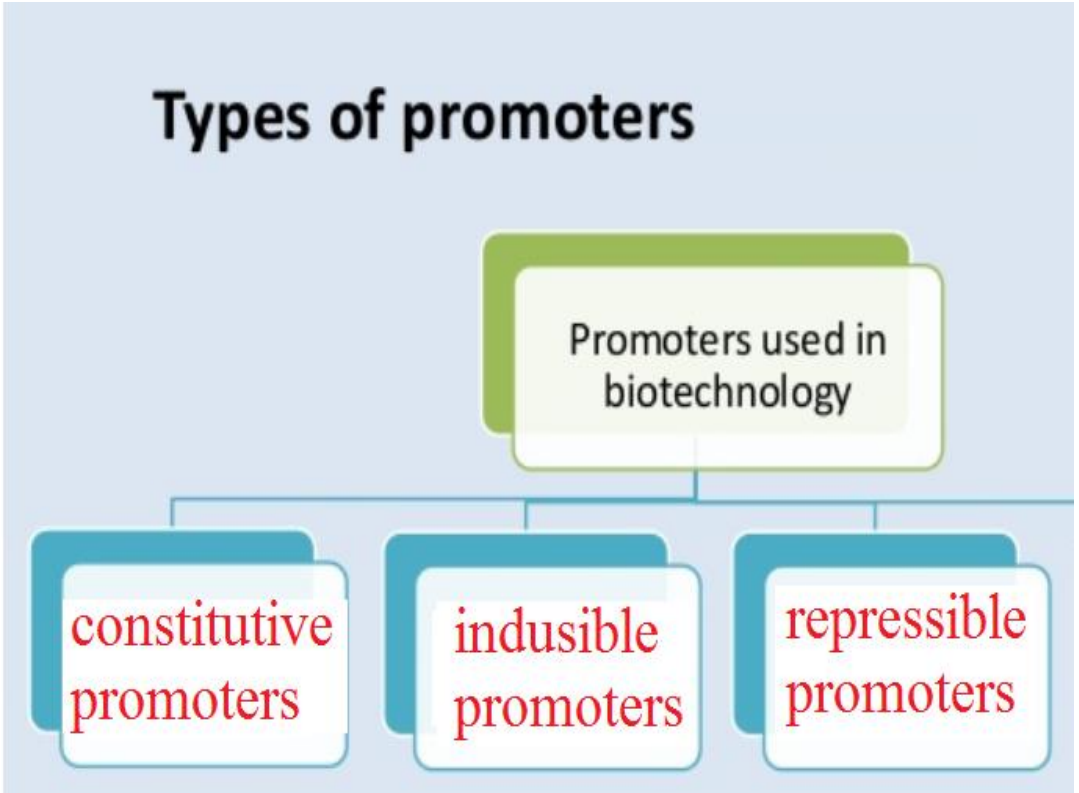
Inducible: normal (turn/shut off): در حضور ماده تنظیم کننده القاء کننده: gene on

Repressible: normal (turn on): در حضور ماده تنظیمی مهار کننده: gene off

چرا بایستی توالی های تنظیمی (القاء کننده یا مهار کننده شیمیایی) برای کنترل ژن نوترکیب کلون شده استفاده نمود:

۱-اثرات سایتوتوکسیک پروتئین هترولوگوس

۲-بیان هر نوع پروتئین هترولوگوس همراه است با رشد کندتر میزبان و بتدریج حذف خواهد شد.



inducible expression system

s.no.	Host vector system	Inducible expression /promoter
1.	*E .coli*	a.lac Promoter
		b.tac Promoter
		c. *λPL Promoter*
		d.T7 Expression System
2.	**Yeast** *Saccharomyces cerevisiae,* *Pichia pastoris and* *Schizosaccharomyces pombe.*	 *a.GAL System* *b. CUP1 System* a. Alcohol oxidase (*AOX1*) *a.nmt1*

Promoters

- *E.coli* natives
  - *lac, trp, tac, trc, ara*
- Viral, but recognised by *E.coli*
  - λ_L, λ_R, T5
- T7, T7*lac*
  - requires its own RNA polymerase

promoter	-35 region	spacer	-10 region
P_{lac}	TTtACA	18 bp	TATgtT
P_{lacUV5}	TTtACA	18 bp	TATAAT
P_{trp}	TTGACA	17 bp	TtaAcT
P_{tac}	TTGACA	17 bp	TATAAT
λP_L	TTGACA	17 bp	gATAcT
λP_R	TTGACt	17 bp	gATAAT
Consensus	**TTGACA**	**17 bp**	**TATAAT**

lac & *trp*

lac

- Promoter of the *lac* operon
- Repressed by *lacI* gene, which binds downstream of the promoter
- Regulated by galatose or its analogues, in expression work non-hydrolysable IPTG used.

trp

- Promoter of tryptophane biosynthetic enzymes
- Repressed by Trp, so induction done by causing a Trp deficiency with indole-2-acrylic acid

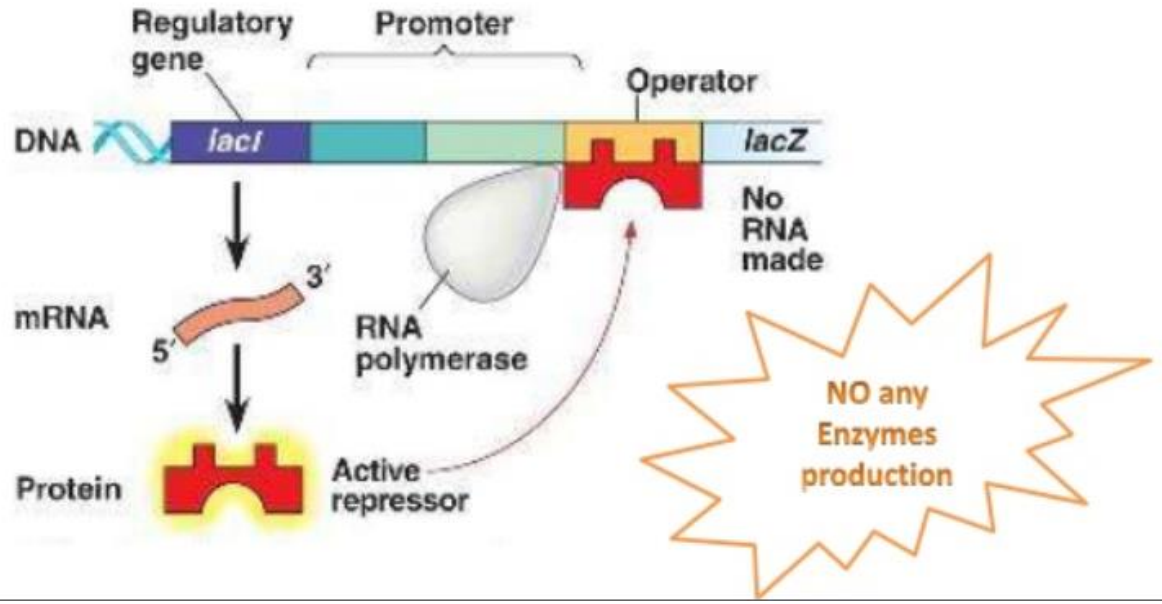
E.coli's own promoters are the first ones ever used to drive overexpression of proteins in bacteria. These are strong promoters, and can be induced with relatively inexpensive chemicals,

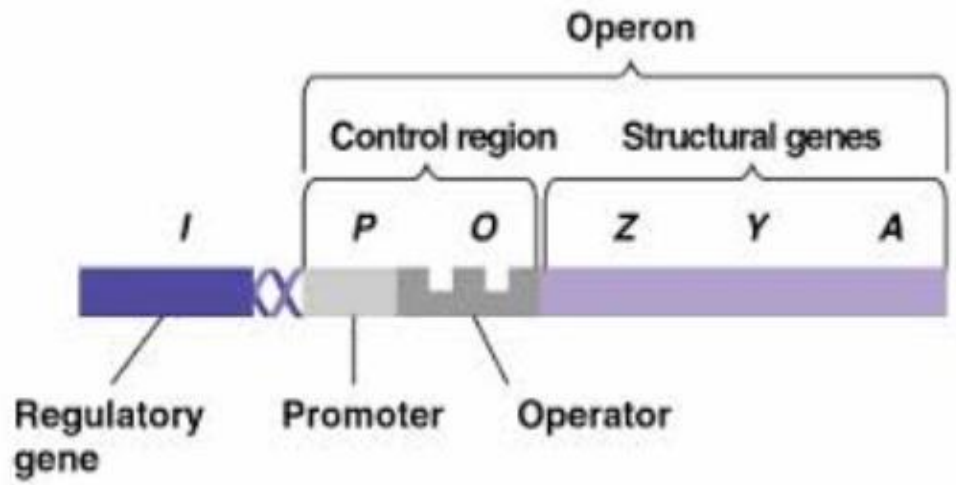
lacI promoter

lacI^Q promoter

Lac-operon function

- **when only glucose is present**

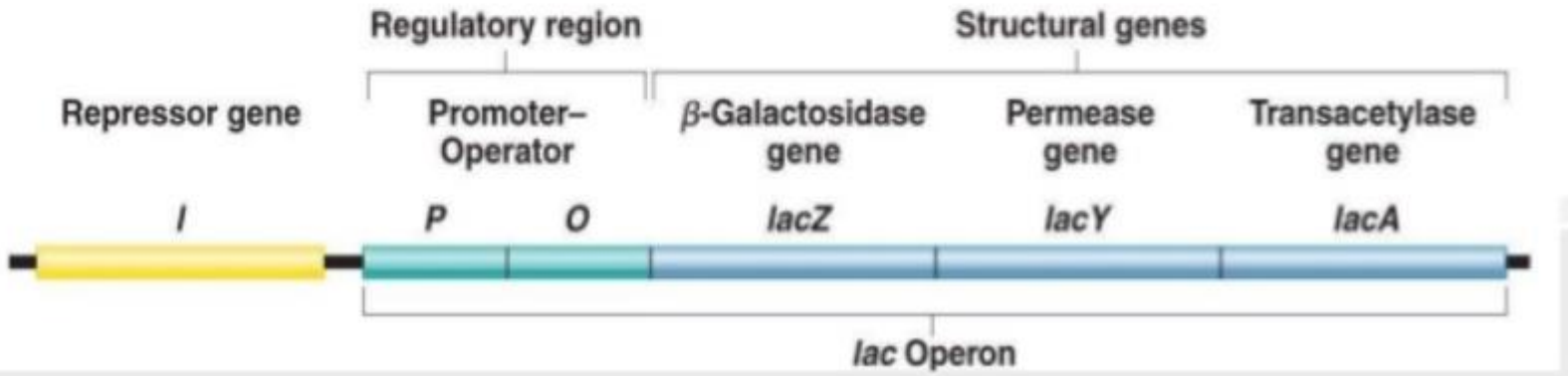




Lac Operon In Ecoli

- **Promoter (P)** - aids in RNA polymerase binding
- **Operator (O)** - "on/off" switch - binding site for the repressor protein
- **Repressor (lacI) gene**

Repressor gene (lacI) - produces repressor protein with two binding sites, one for the operator and one for lactose



tac & trc

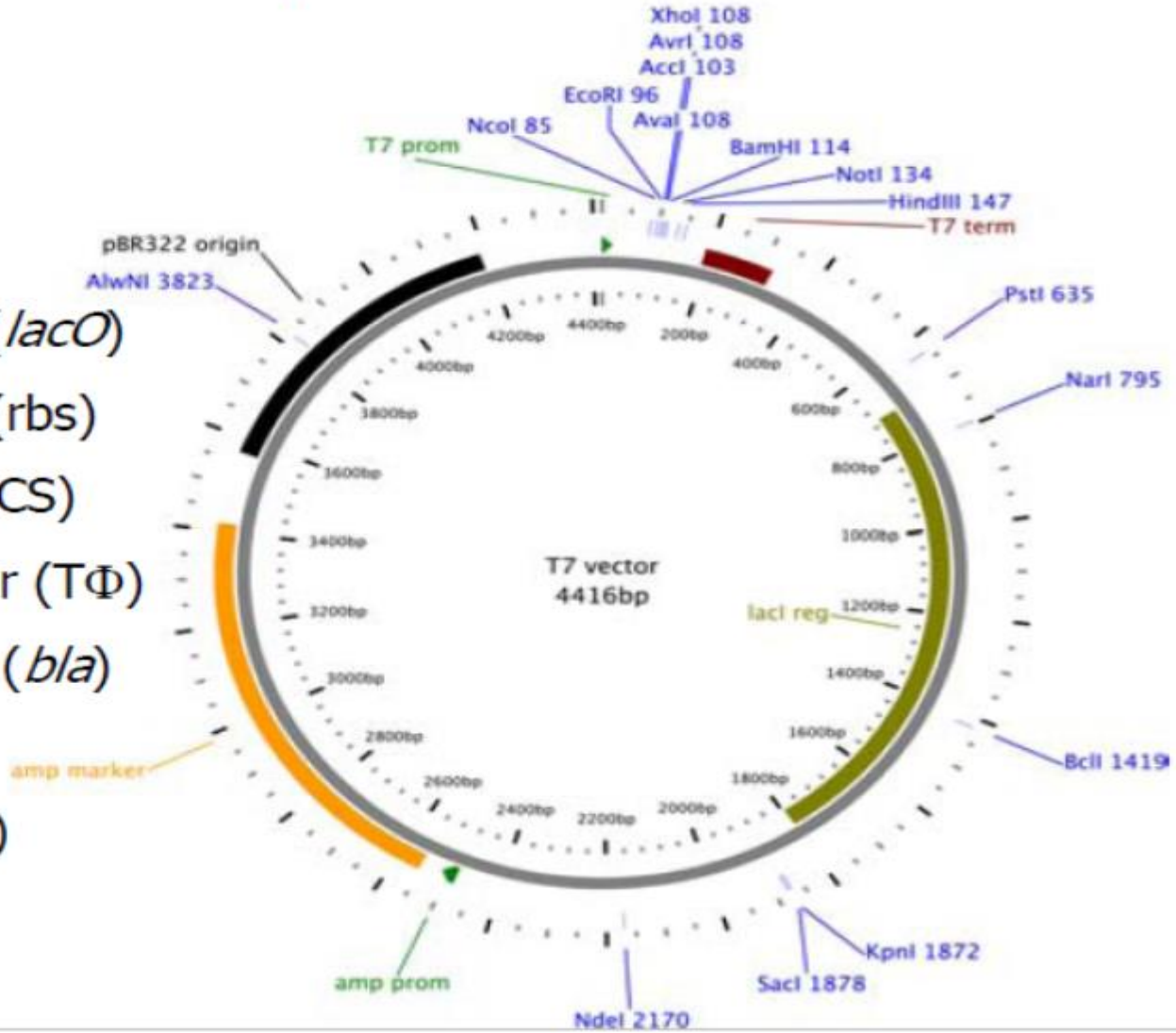
Synthetic promoters created by fusion of *trp* and *lac* promoters

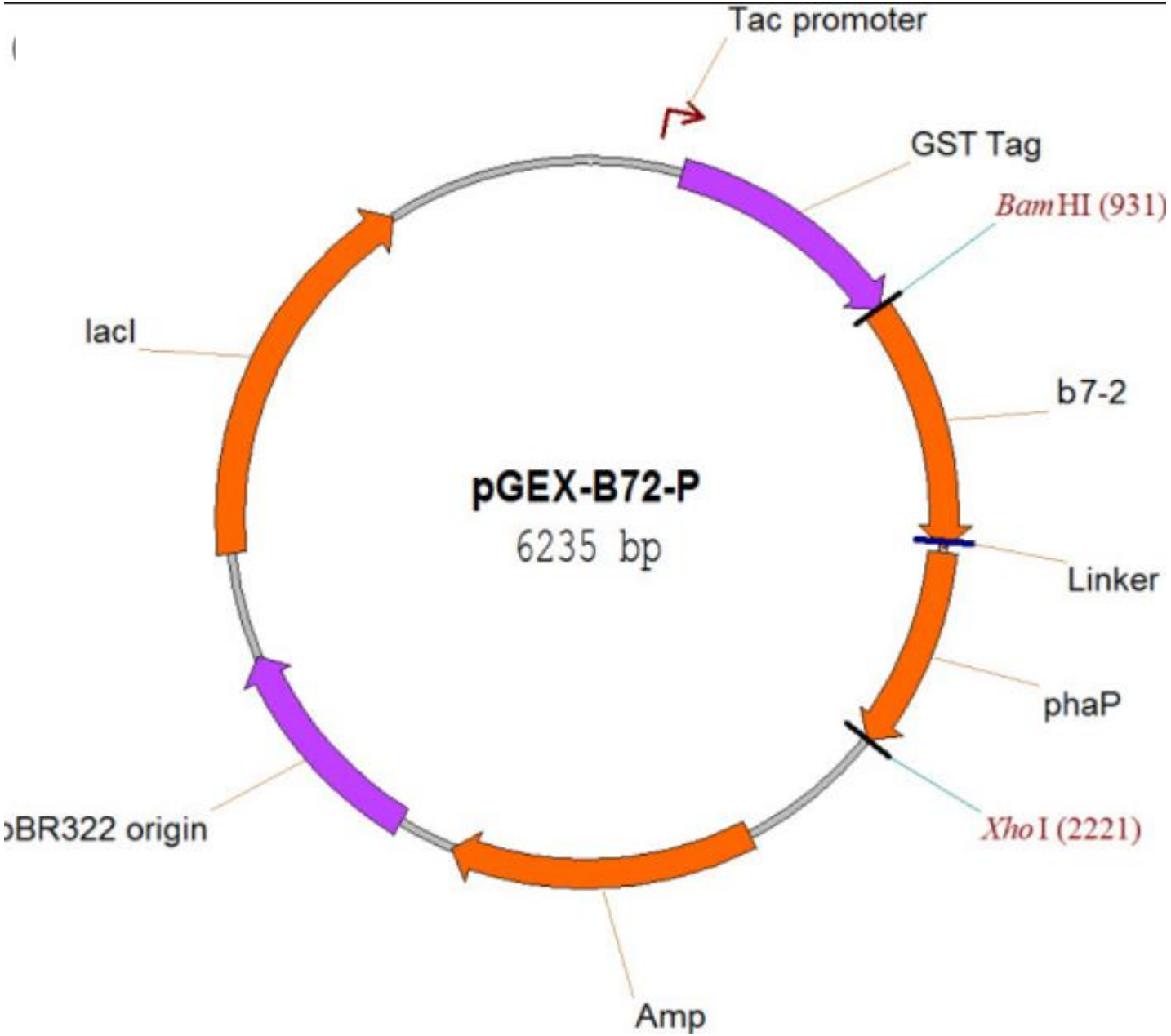
- -35 part from *trp*, -10 from *lac*
- Regulation from *lac* system, *ie.* induced by IPTG
- Originally shown to be much stronger than either of the parent promoters
- Now found in pGEX and pMAL vectors

Although not naturally found in *E.coli* the synthetic tac and trc promoters can be classified as *E.coli* promoters, as they are created by fusing different elements of the *lac* and *trp* promoters making them more powerful than either of the parental promoters alone. Several commercial vector systems still use these, inclusing pMAL and pGEx series, and pTRC series from Invitrogen,

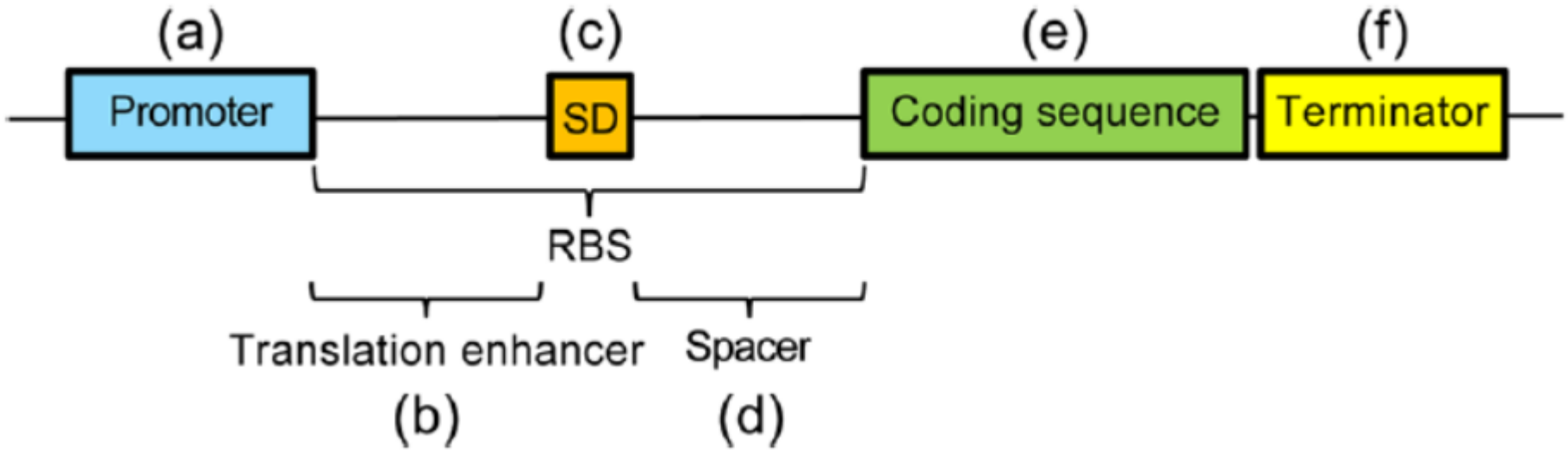
Elements of an expression vector

- Expression cassette
  - Promoter (T7)
  - Regulator binding site (*lacO*)
  - Ribosome binding site (rbs)
  - Multiple cloning site (MCS)
  - Transcription terminator (TΦ)
- Antibiotic resistance gene (*bla*)
- Origin of replication (ori)
- Other control genes (*lacI*)




Tac promoter
GST Tag
BamHI (931)
lacI
b7-2
pGEX-B72-P
6235 bp
Linker
phaP
XhoI (2221)
pBR322 origin
Amp

Gene cassette



مشکلات مرتبط با تولید پروتئین نوترکیب در اشریشیاکلي و راه حل ؟

1. عدم سیستم Splicing
2. تغییرات پس از ترجمه
3. امکان هضم پروتئین نوترکیب
4. خالص سازی
5. امکان خاتمه پیش از موعد

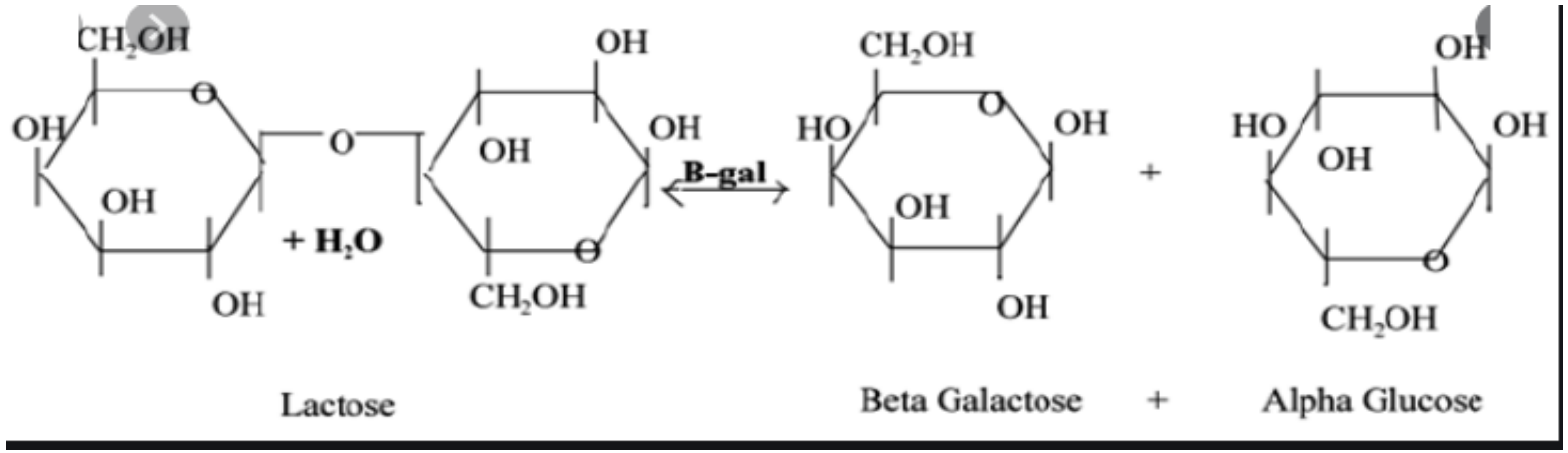
Blue-White Screening & Protocols for Colony Selection

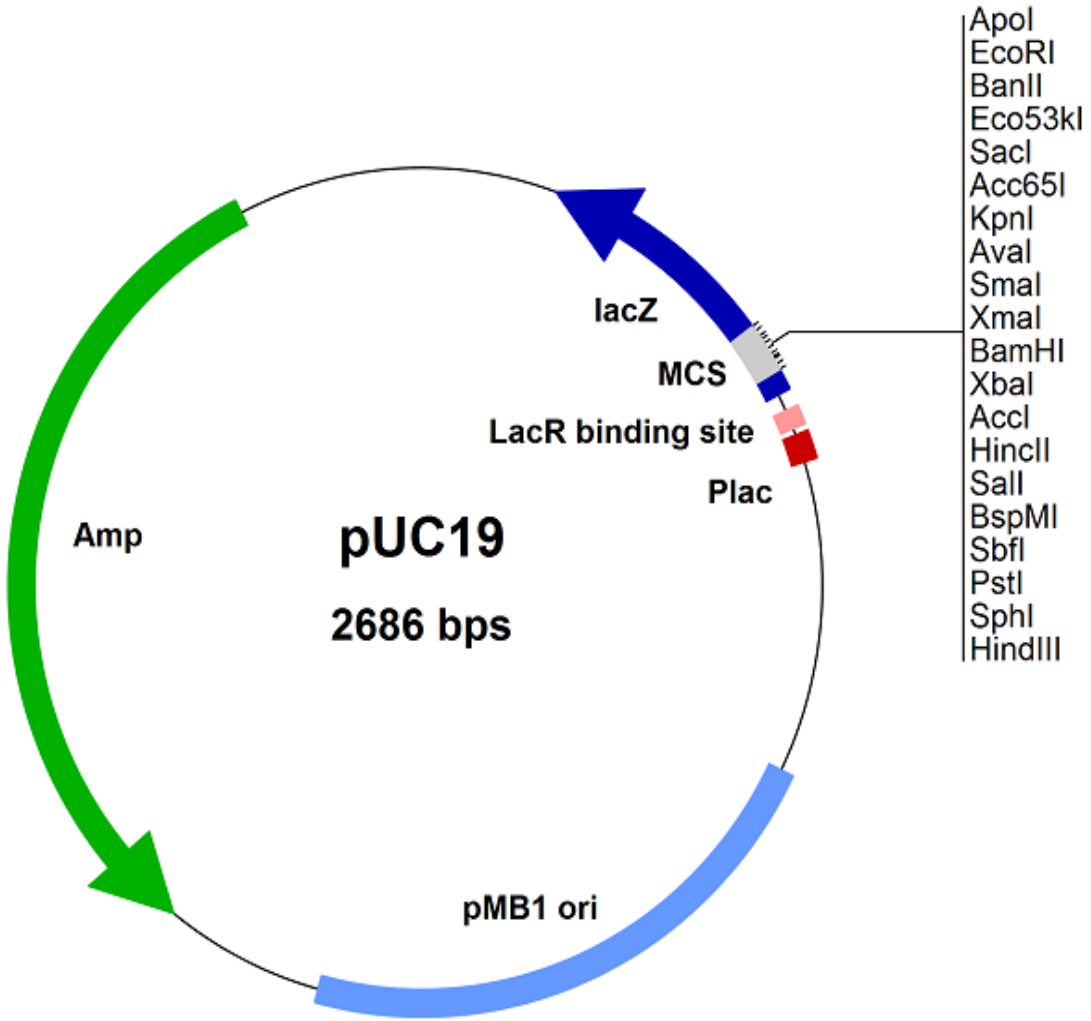
Alpha-complementation

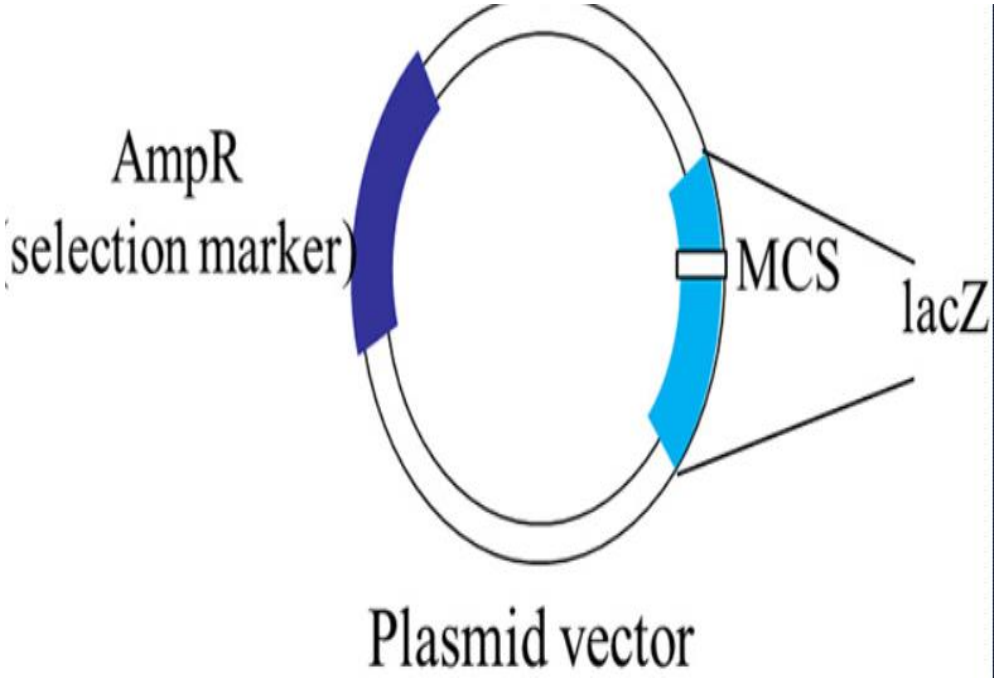
آنزیم بتاگالاکتوزیداز بصورت تترامر است که هر مونومرش از دو بخش lacz-alpha و lacz-omega ساخته شده است. محققان پی برده اند اگر قطعه آلفا حذف شود، آنزیم غیرفعال خواهد بود، با این وجود قطعه آلفا می تواند مجددا بازیابی گردد از طریق پلاسمید حاوی قطعه آلفا

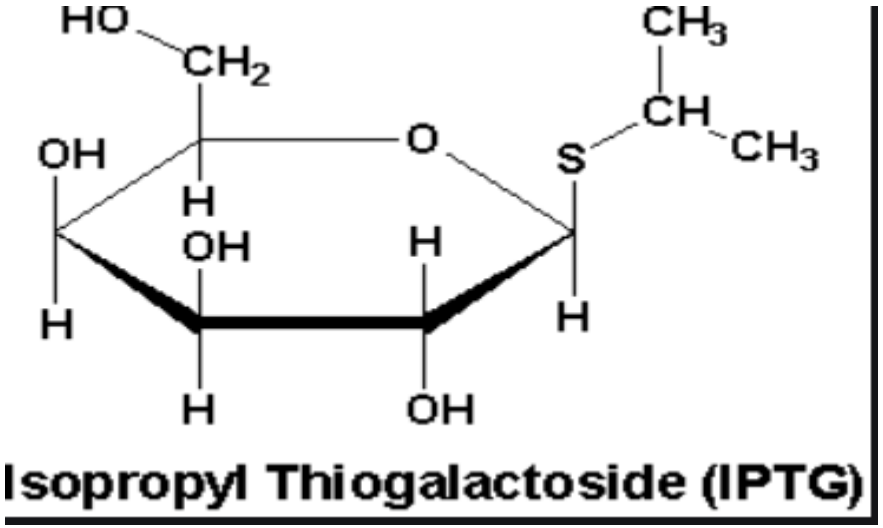
Vectors contains lacz-alpha (pUC19 and pBlueScript and their derivatives)

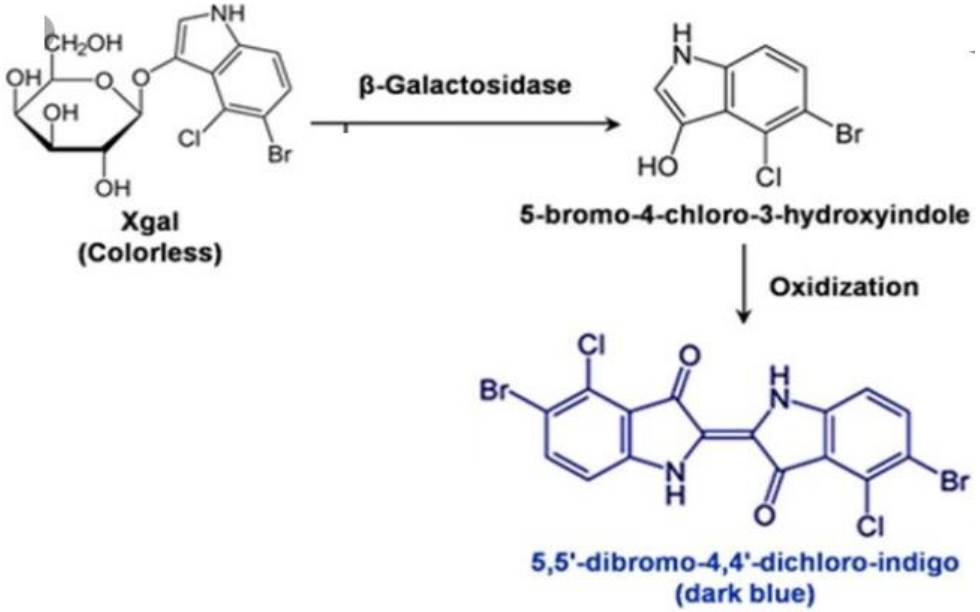
E. coli contains lacz-alpha deletion mutant (JM109, DH5-alpha, XL1-Blue)

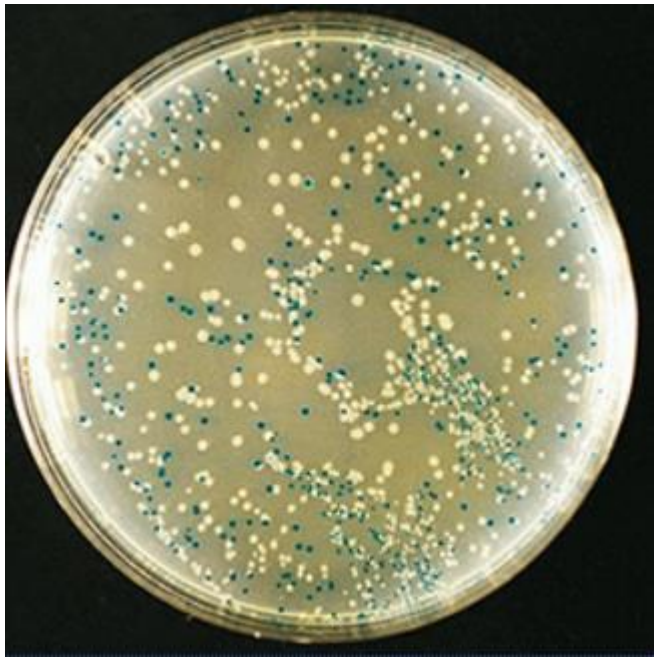


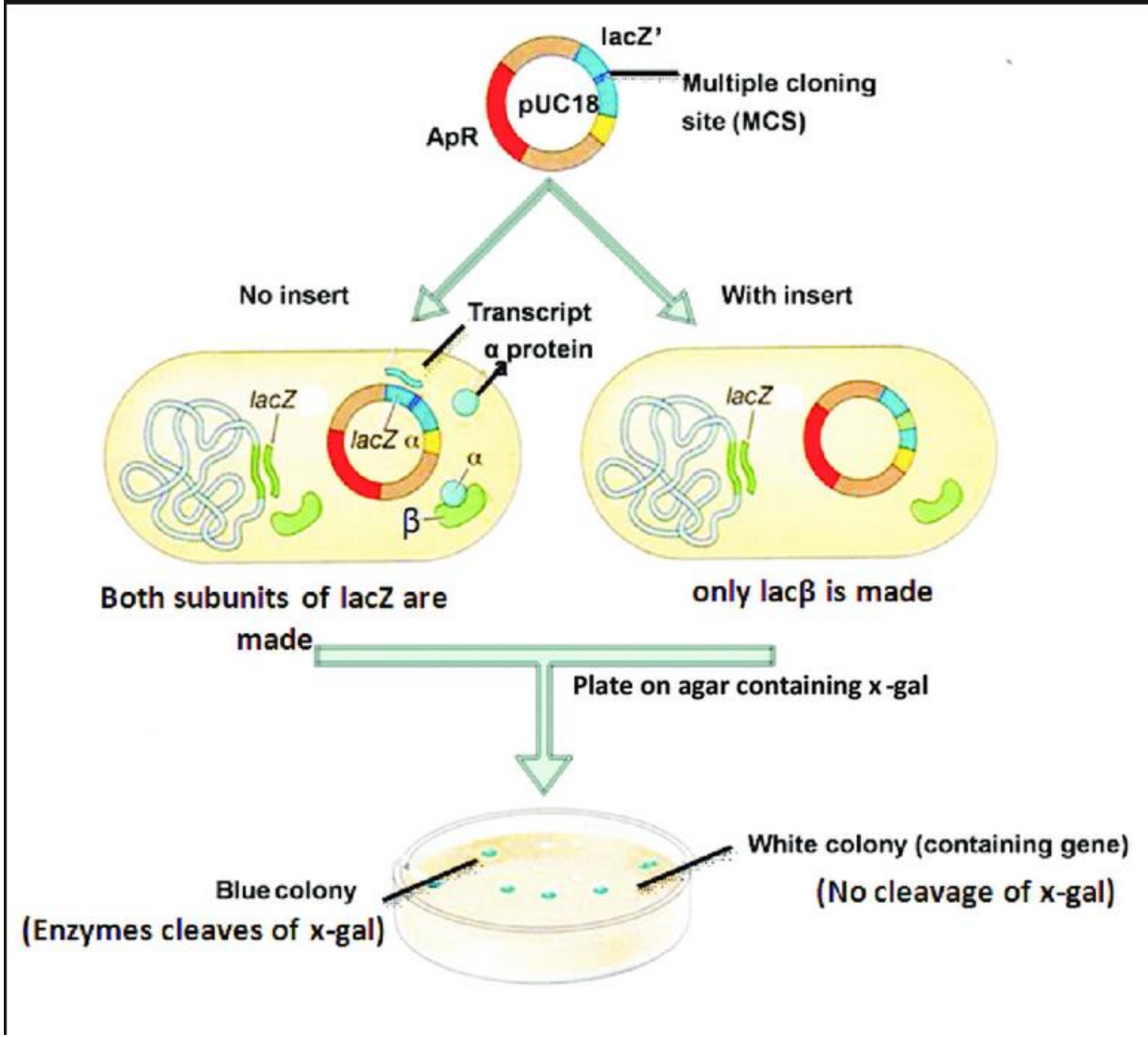

lacZ
MCS
LacR binding site
Plac
Amp
pUC19
2686 bps
pMB1 ori
ApoI
EcoRI
BanII
Eco53kI
SacI
Acc65I
KpnI
AvaI
SmaI
XmaI
BamHI
XbaI
AccI
HincII
SalI
BspMI
SbfI
PstI
SphI
HindIII


AmpR
(selection marker)
MCS
lacZ
Plasmid vector


HO
CH2
OH
H
OH
H
H
O
S
CH3
CH
CH3
H
H
OH
H
OH
Isopropyl Thiogalactoside (IPTG)


β-Galactosidase
Xgal
(Colorless)
5-bromo-4-chloro-3-hydroxyindole
Oxidization
5,5'-dibromo-4,4'-dichloro-indigo
(dark blue)




lacZ'
pUC18
ApR
Multiple cloning site (MCS)
No insert
With insert
Transcript
α protein
lacZ
lacZ α
α
β
Both subunits of lacZ are made
only lacβ is made
Plate on agar containing x-gal
Blue colony
(Enzymes cleaves of x-gal)
White colony (containing gene)
(No cleavage of x-gal)

کاربرد آنزيم هاي مهم در مهندسي ژنتيک

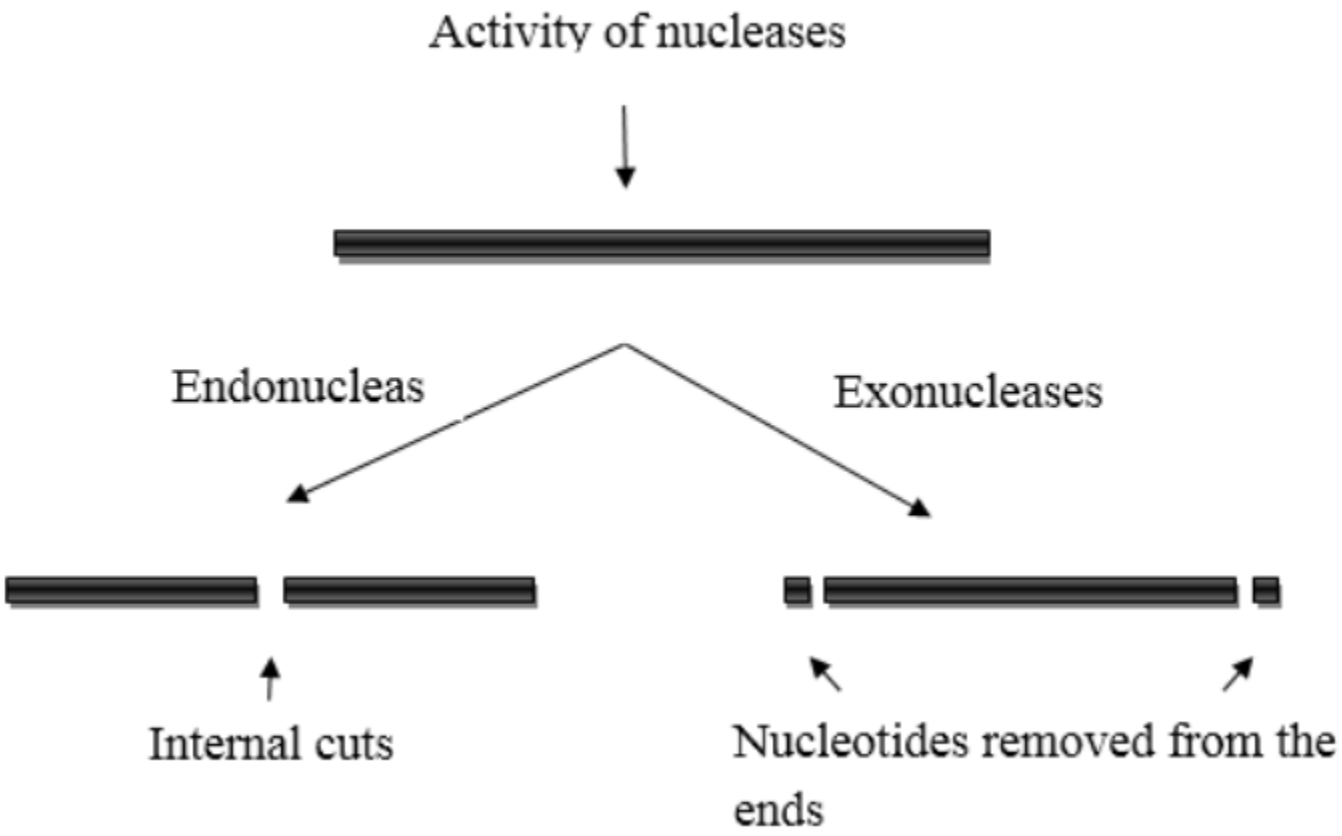
نوکلئازها

لیگازها

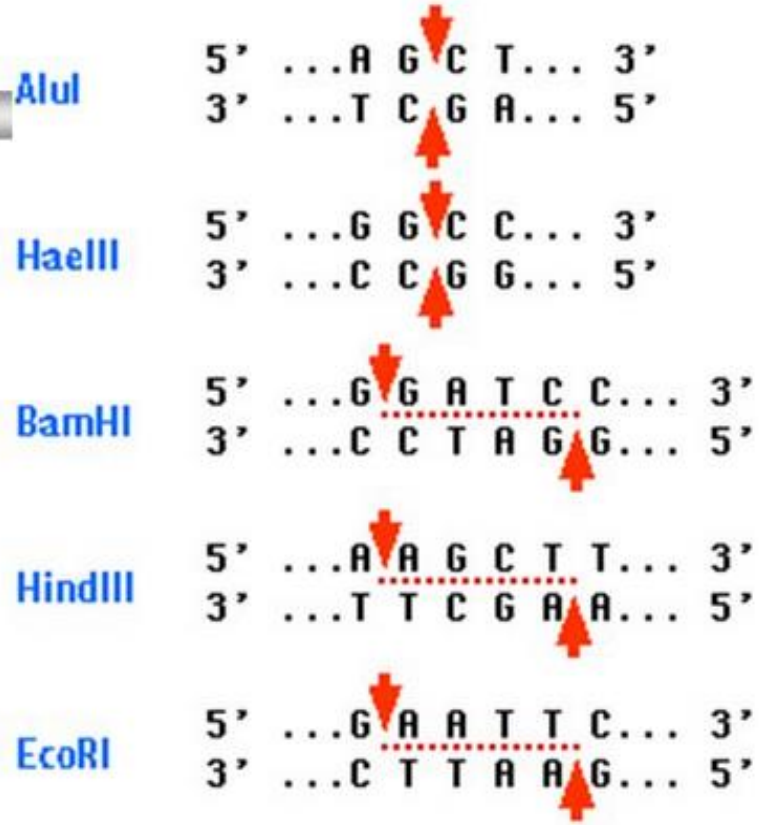
پلیمرازها

تغییر دهنده گروههاي شیمیایي

توپوازومرازها

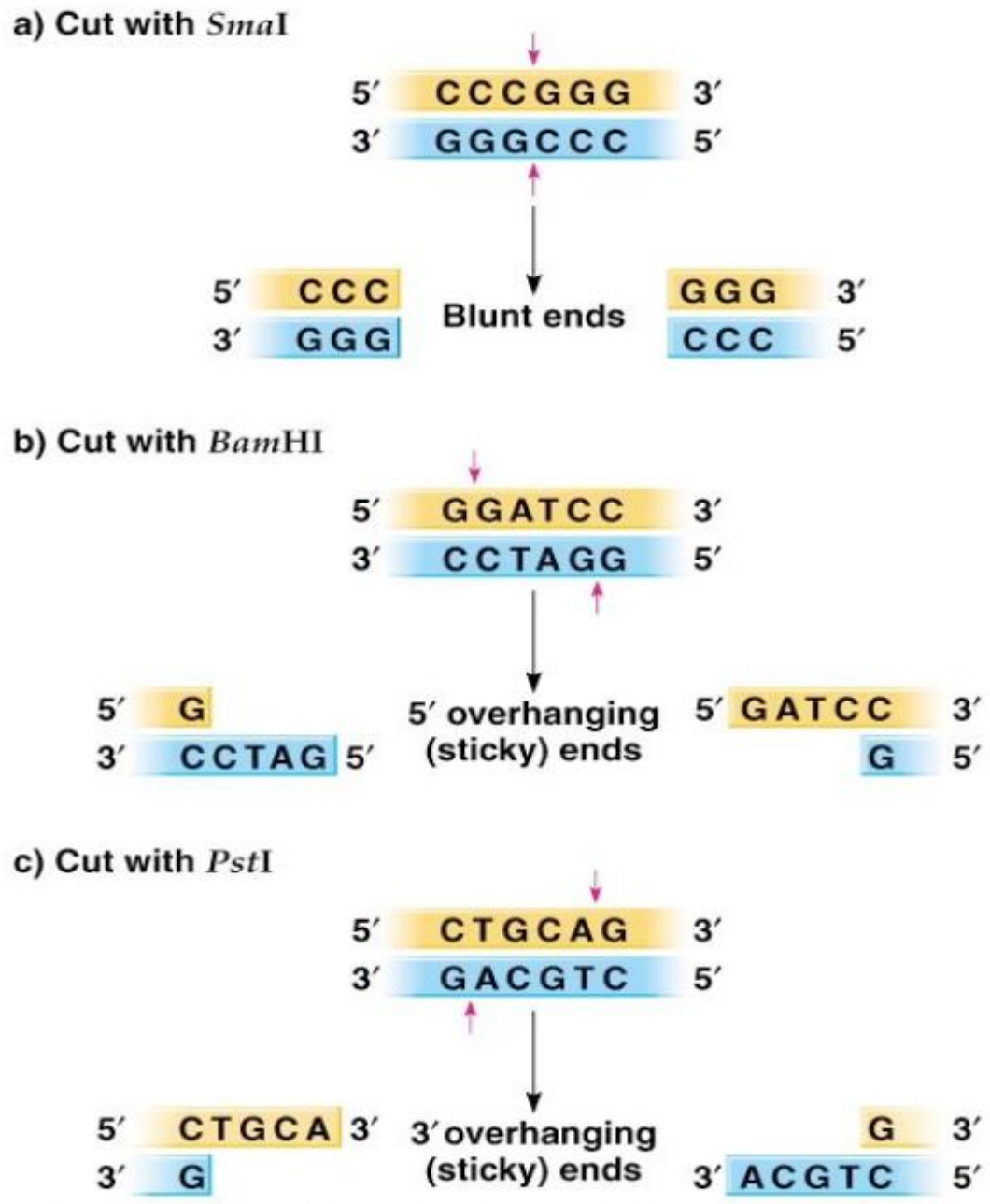


Blunt & Sticky ends



AluI and HaeIII produce blunt ends

BamHI HindIII and EcoRI produce "sticky" ends



- Apart from restriction enzymes, there are four useful nucleases that are often used in genetic engineering.
- These are
  - **Bal 31** and
  - **Exonuclease III** (exonucleases), and
  - **Deoxyribonuclease I** (**DNase I**) and
  - **S1-nuclease** (**endonucleases**).

Exonuclease III (*E.coli*)

A double-strand specific, nonprocessive 3´→5´ exodeoxyribonuclease activity; however, 3´-overhangs of ≥4 bases are protected from Exo III activity (1).

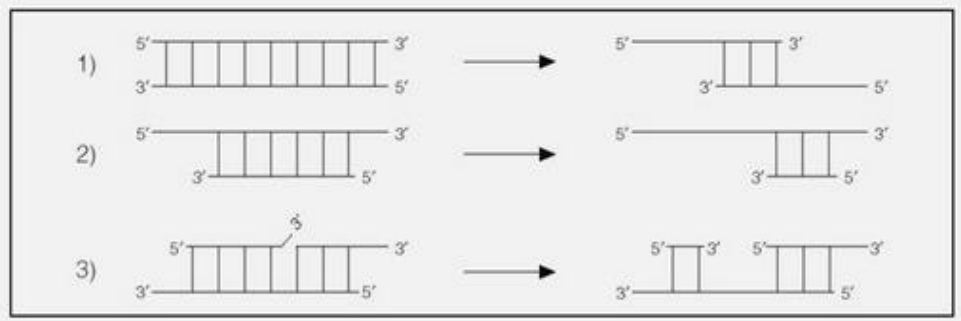


Figure 1. Exonuclease III catalyzes the stepwise removal of mononucleotides starting from a 3´-OH at: 1) blunt ends, 2) recessed ends and 3) nicks. Exonuclease III will also act on 3´-overhangs of less than 4 bases (not shown). Note that the 3´-overhangs shown in 3) are ≥4 bases and therefore not susceptible to Exonuclease III activity.

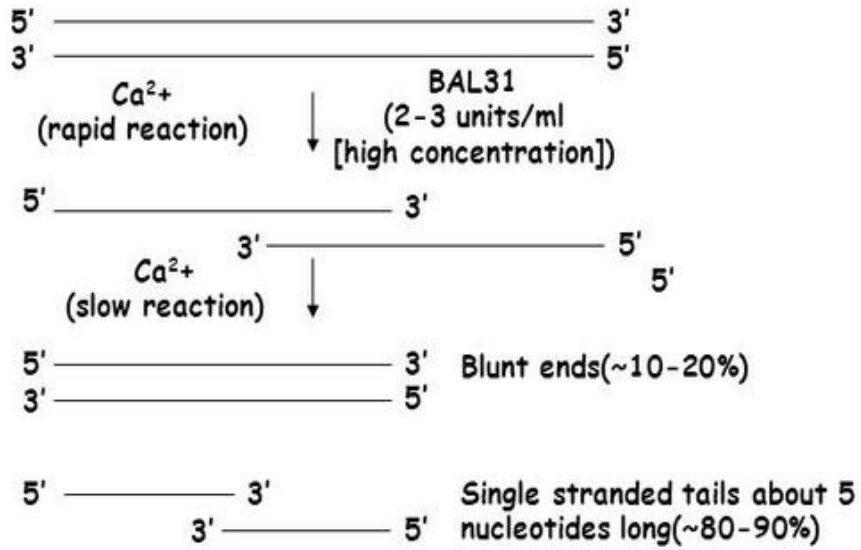
*Bal*31 NUCLEASE

SOURCE

Alteromonas espejiana

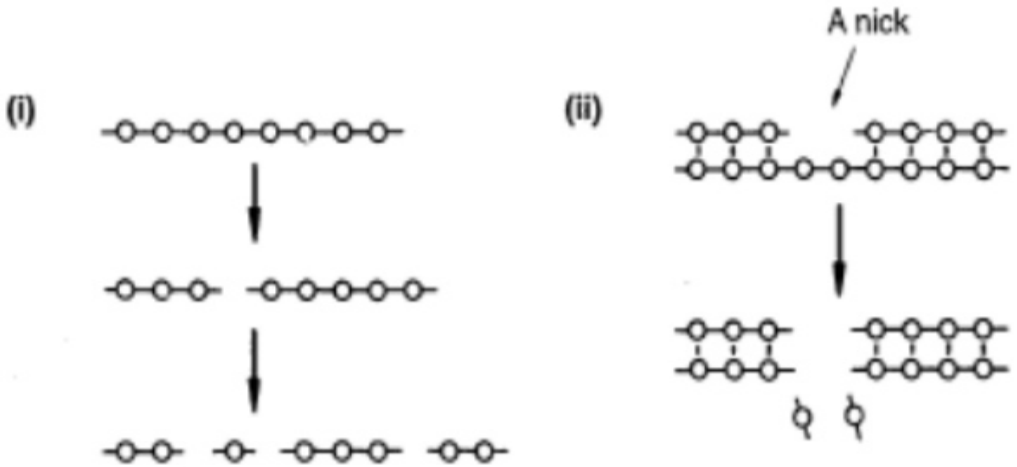
FUNCTION

- 3′→5′ exonuclease activity that eliminates mononucleotides from dsDNA
- 5′→3′ exonuclease activity that works efficiently on ssDNA
- Endonuclease activity that degrades ssDNA slowly and cleaves supercoiled dsDNA as well as mutagenically altered dsDNA



Aspergillus oryzae

S1 nuclease



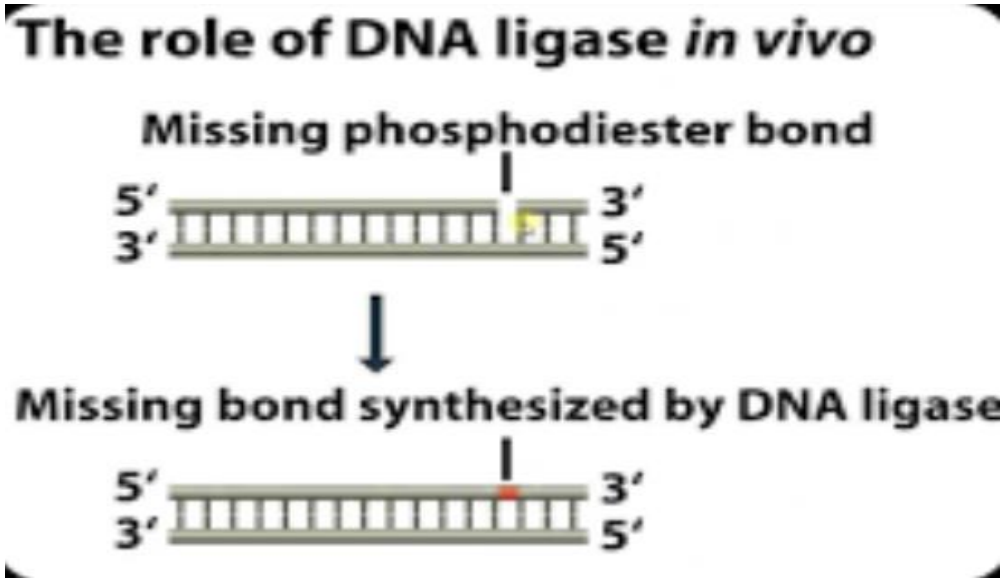
MUNG BEAN ENDONULEASE

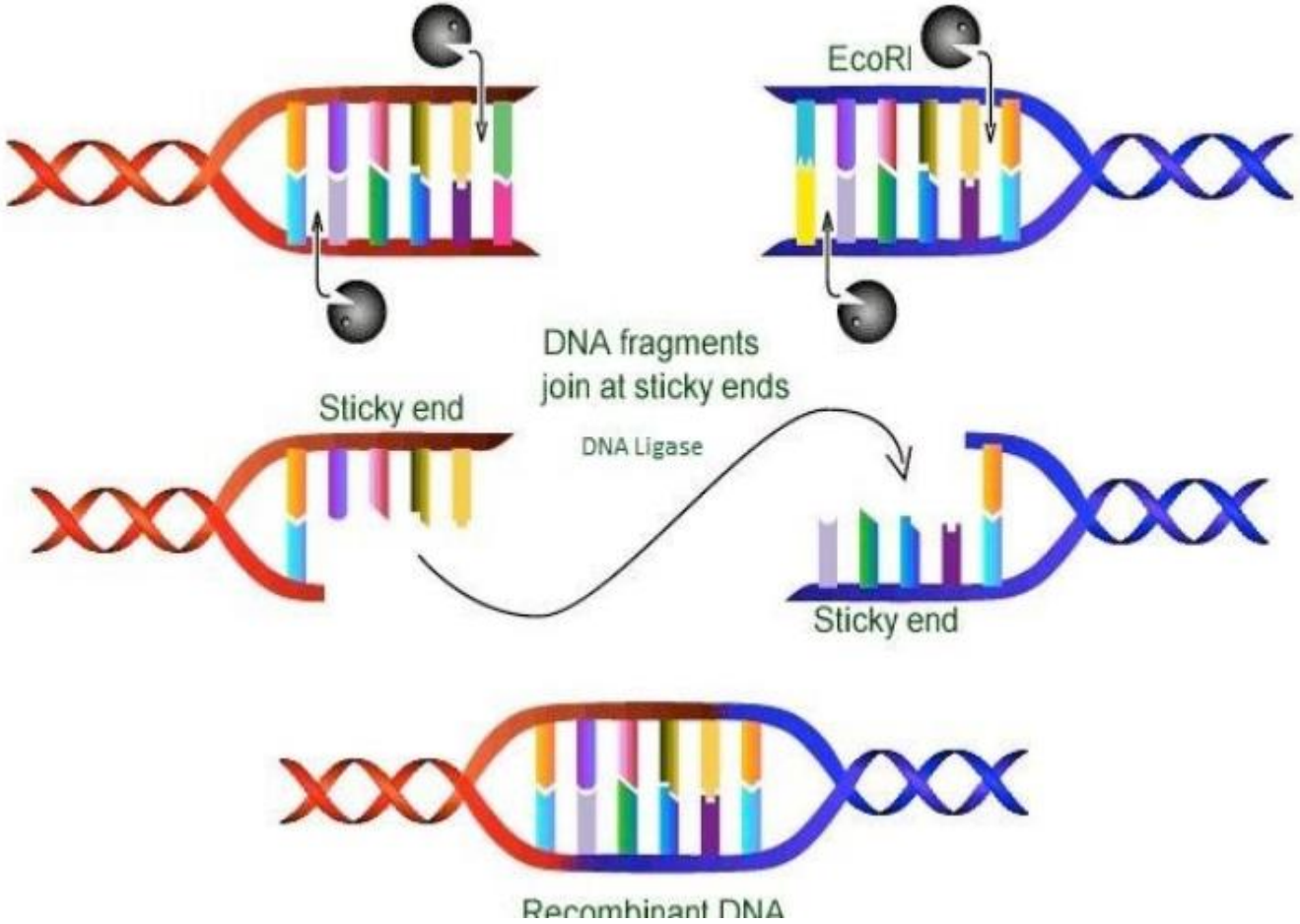
SOURCE

Mung bean sprouts

FUNCTION

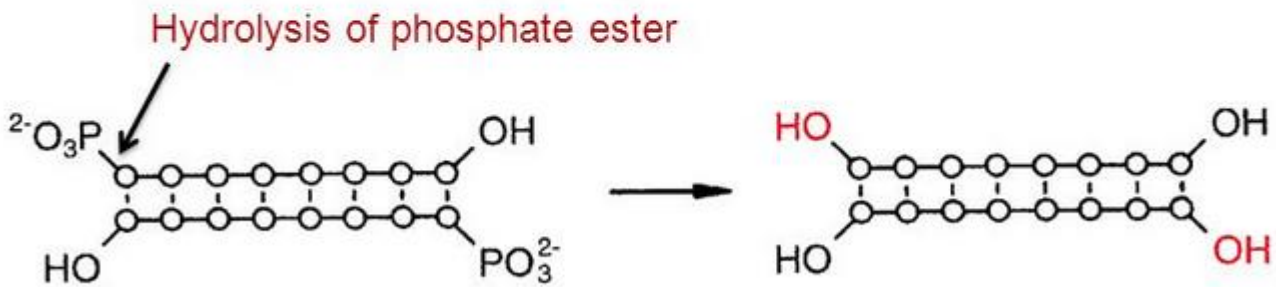
- Single strand specific nuclease that degrades DNA and RNA to 5'-P mononucleotides
- ds DNA, dsRNA and RNA:DNA hybrid are resistant to this enzyme
- Works on nick after it has been enlarged to a gap of many nucleotides


The role of DNA ligase *in vivo*
Missing phosphodiester bond
5′
3′
3′
5′
Missing bond synthesized by DNA ligase
5′
3′
3′
5′


EcoRI
DNA fragments
join at sticky ends
Sticky end
DNA Ligase
Sticky end
Recombinant DNA

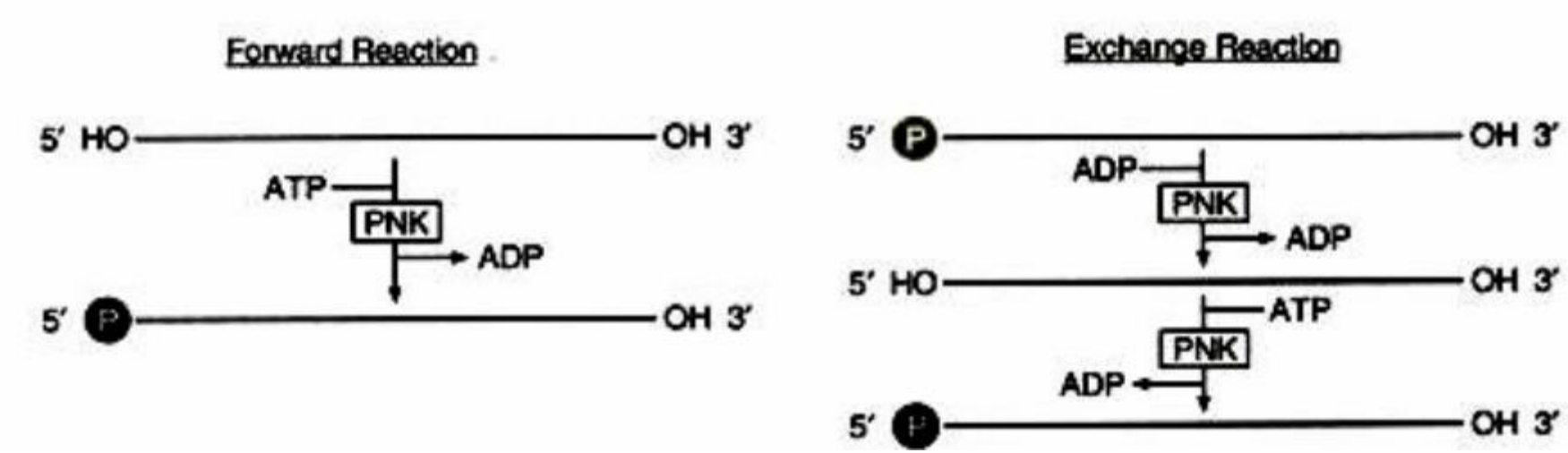
DNA modification enzymes

- **Alkaline phosphatase**:
  - removes the 5' phosphate groups from DNA, normally the vector DNA



Polynucleotide Kinase:

Polynucleotide kinase (PNK) is an enzyme that catalyzes the transfer of a phosphate from ATP to the 5′ end of either DNA or RNA. It is a product of the T4 bacteriophage, and commercial preparations are usually products of the cloned phage gene expressed in E. coli.



Terminal Transferase:

Terminal transferase catalyzes the addition of nucleotides to the 3′ terminus of DNA. Interestingly, it works on single-stranded DNA, including 3′ overhangs of double-stranded DNA, and is thus an example of a DNA polymerase that does not require a primer. It can also add homo-polymers of ribo-nucleotides to the 3′ end of DNA. The much preferred substrate for this enzyme is protruding 3′ ends, but it will also, less efficiently, add nucleotides to blunt and 3′-recessed ends of DNA fragments. Cobalt is a necessary cofactor for