Practical and technical applications of thermostable enzymes

- **higher reaction rate at higher T**
- **lower resk of microbial contaminations**

Bioconversion reactions and applications of thermostable enzymes

Enzyme	Temperature range (°C)	Bioconversions	Applications
α-Amylase (bacterial)	90–100	Starch → dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-Amylase (fungal)	50–60	Starch → dextrose syrups	Production of maltose
Pullulanase	50–60	Starch → dextrose syrups	Production of glucose syrups
Xylanase	45–65, 105[a]	Craft pulp → xylan + lignin	Pulp and paper industry
Chitinase	65–75[b]	Chitin → chitobiose	Food, cosmetics, pharmaceuticals, agrochemicals
		Chitin → *N*-acetyl glucosamine (chitibiase)	
		N-acetyl glucosamine → glucosamine (deacetylation)	
		Chitin → chitosan (deacetylase)	
Cellulase	45–55, 95[c]	Cellulose → glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65–85	Protein → amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30–70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry
DNA polymerase	90–95	DNA amplification	Genetic engineering/PCR

DNA polymerase from *Thermus aquaticus*: Taq polymerase

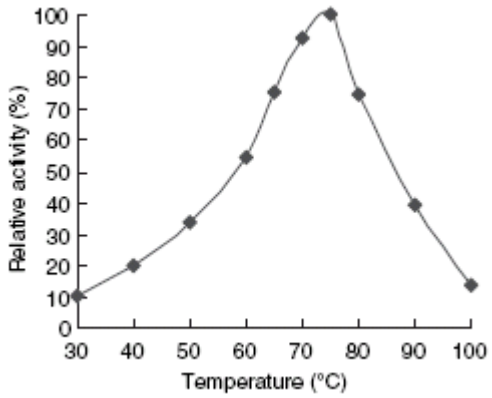
XYLANASE

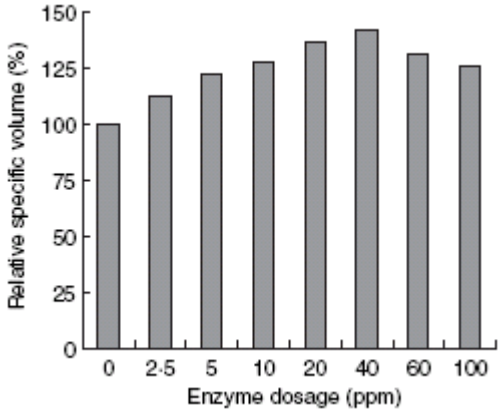
Pulp and paper industry

Bakery

Higher quality of bread

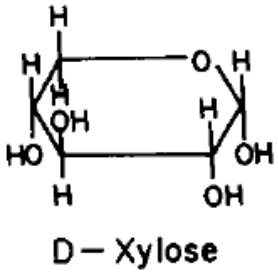
Enzyme active at 60-75°C , also during cooking

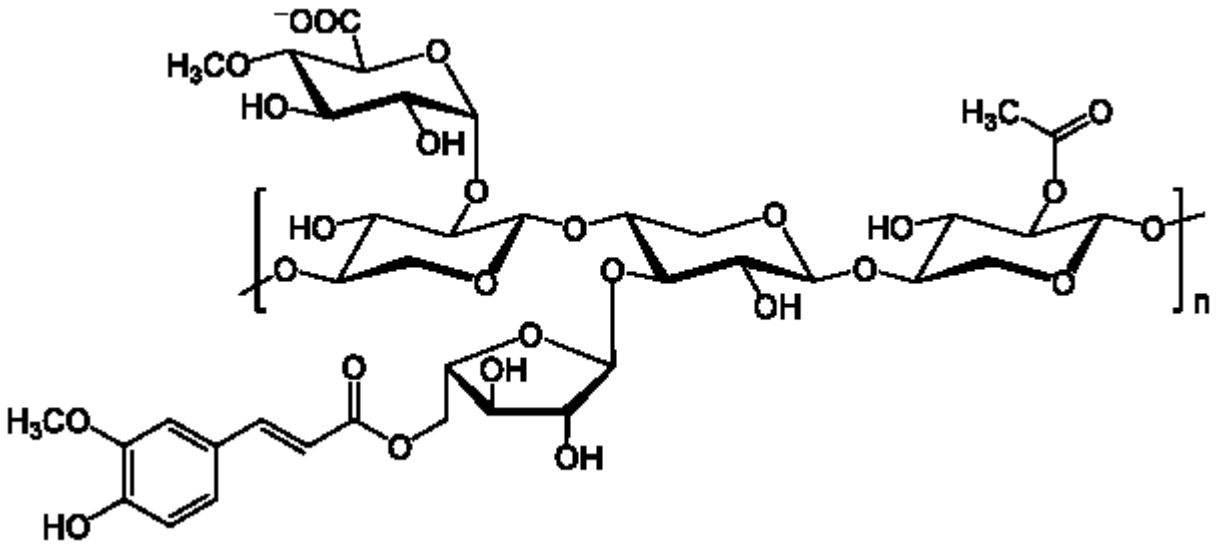




Xylan: a group of hemicelluloses present in plant cell walls

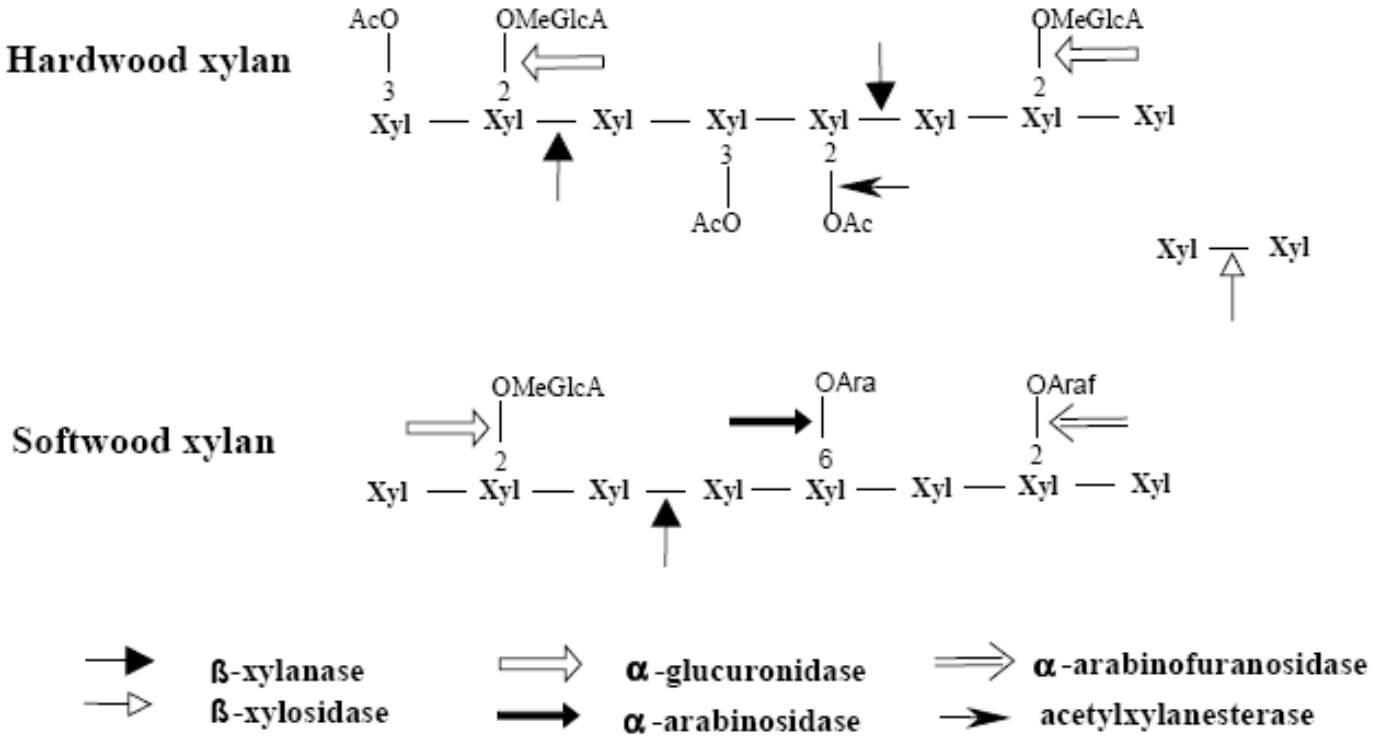
Ubiquitous as cellulose in plant cell walls and contain predominantly β-D-xylose units.
Typically the content of xylans in hardwoods are 10 - 35 % of the hemicelluloses and in softwood are 10 - 15 % of the hemicelluloses.





Example of xylane structure (ester of ferulate)

Enzymes degrading hemicellulose



Endo-ß-1,4-xylanases catalyse the random hydrolysis of ß-1,4-glycosidic bonds in xylans.

Proteases in detergents

Source microorganisms and properties of thermostable proteolytic enzymes

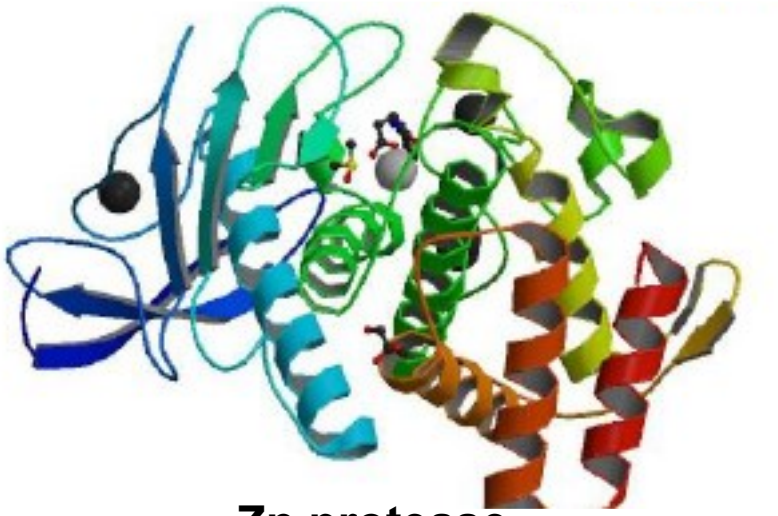
Organism	Enzyme properties	
	Optimal temperature (°C)	Optimal pH
Bacillus brevis	60	10.5
Bacillus licheniformis	70	9.0
Bacillus stearothermophilus	60	–
Bacillus stearothermophilus	85	–
Bacillus sp. JB-99	80	6–12
Bacillus stearothermophilus TP26	75	–
Bacillus sp. no. AH-101	80	12.0–13.0
Bacillus thermoruber	45	9
Pyrococcus sp. KODI	100	7
Staphylothermus marinus	–	9
Thermoacidophiles (archeal and bacterial origin)	60–70	7.0–8.5
Thermococcus aggreganes	90	7.0
Thermococcus celer	95	7.5
Thermococcus litoralis	85	8.5
Thermotoga maritema	95	9.5

Proteases in detergents

Bacillus thermoproteolyticus neutral proteinase

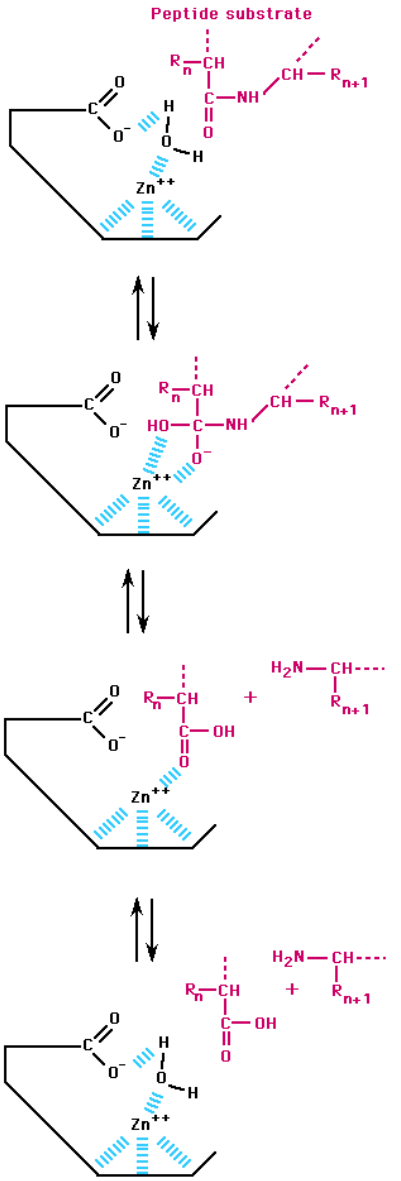
Thermolysine

Stable at 50-60°C



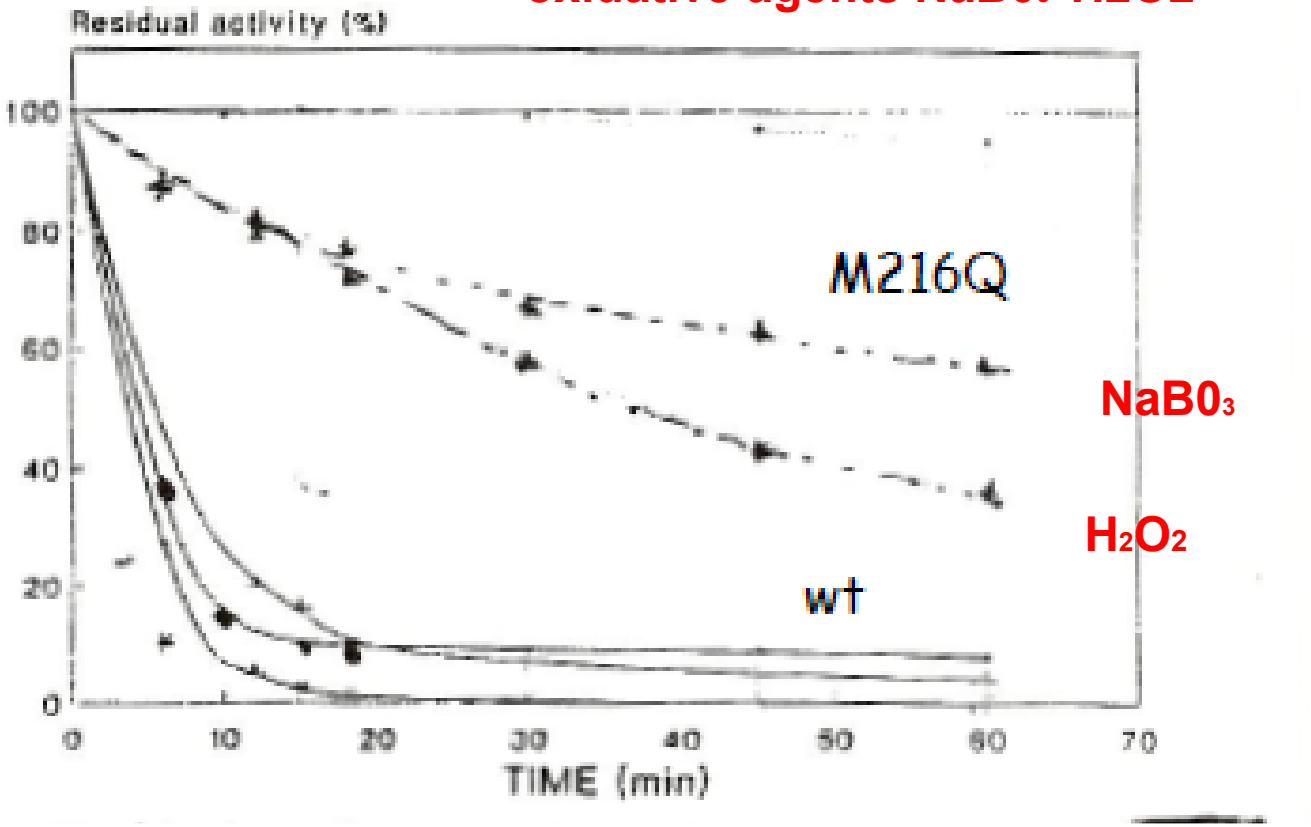
Zn protease

Catalytic mechanism of metallo proteinases



Stabilization of a protease towards oxidatve agents (peracids and H2O2): Genetic engineering

Mutation of Metionine into Glutamine: mutant displays higher stability towards oxidative agents $NaBO_3$ H_2O_2



«natural» methods for increasing enzyme stability

Glycosylation of proteins is a natural tool for increasing protein stability

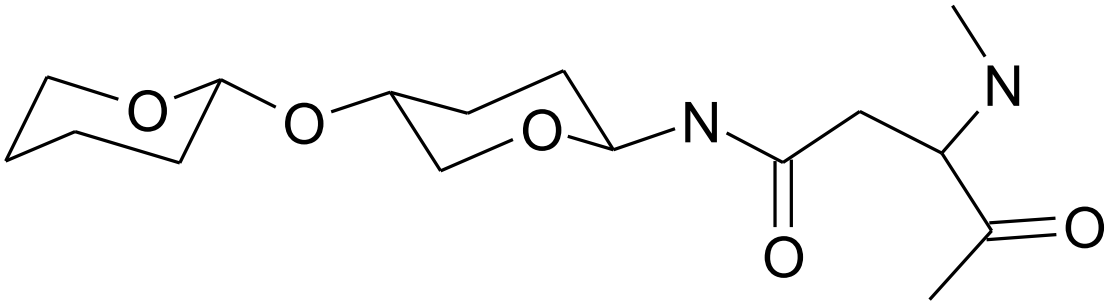
Several roles have been suggested for the carbohydrate moieties of glycoproteins among which

- **stabilization of protein conformation,**
- **protection from proteolysis**
- **Prevention of aggregation**

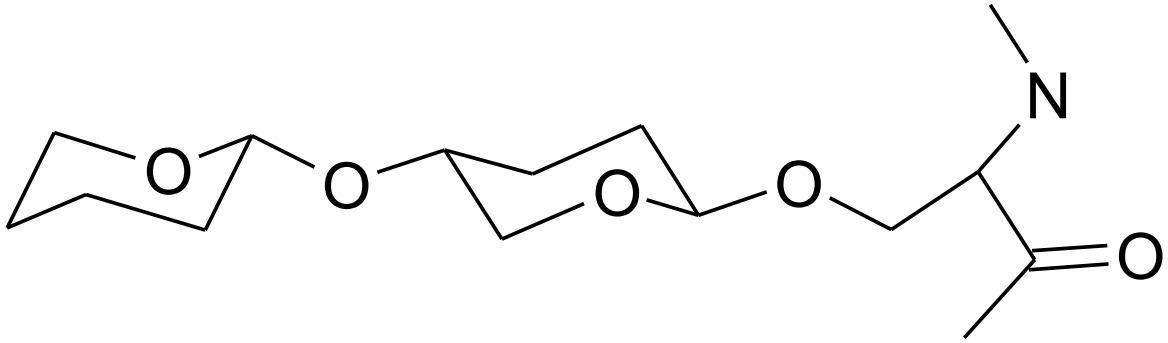
There are two main type of protein glycosylation:

- **N-glycosylation, in which the glycan (10-35 mannose residues) is attached to an Asn residue present in the tripeptide consensus sequon Asn-XSer/Thr (where X can be any amino acid except Pro),**

- **O-glycosylation, in which the glycan is attached to a Ser or Thr residue.**

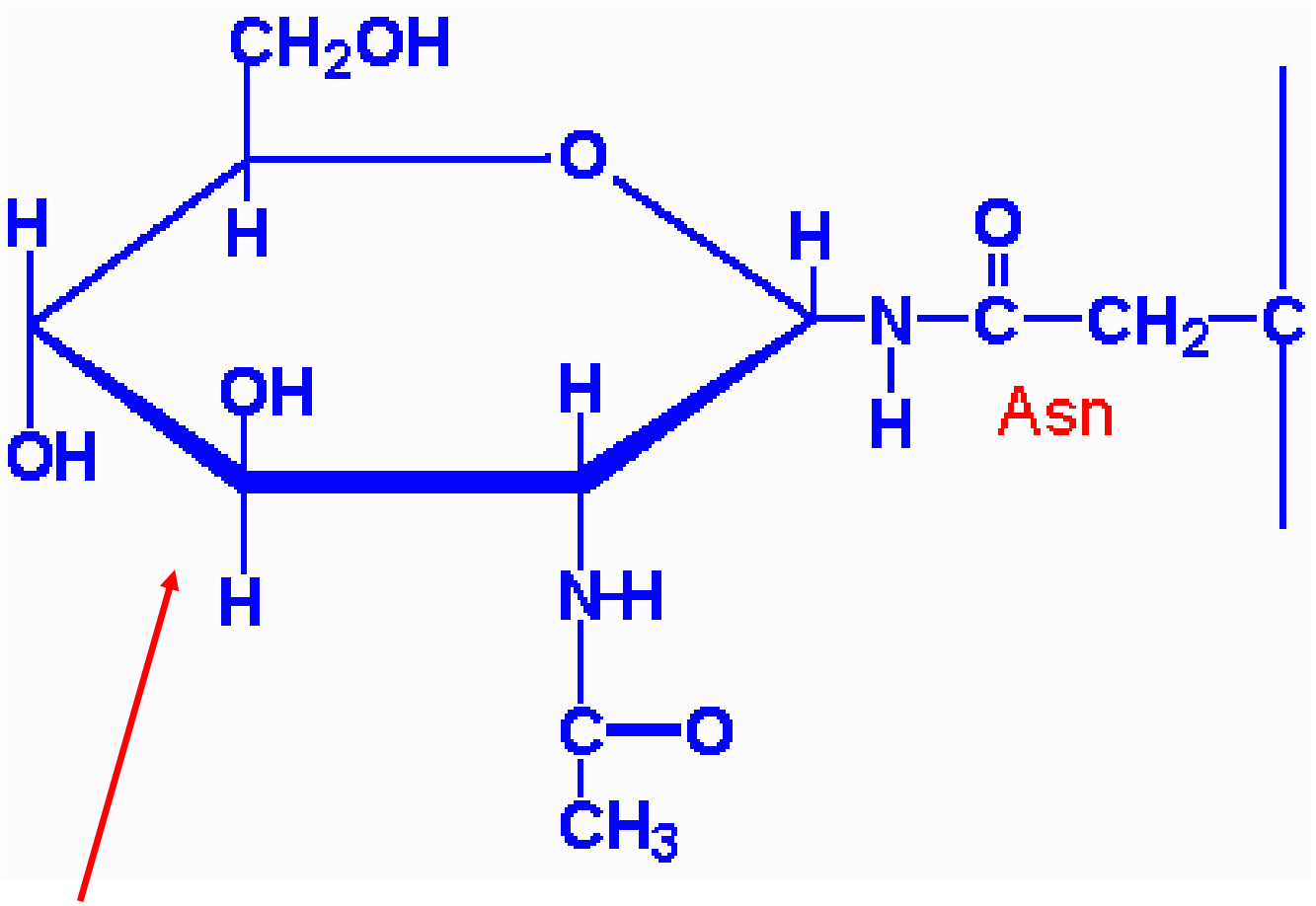


Asn



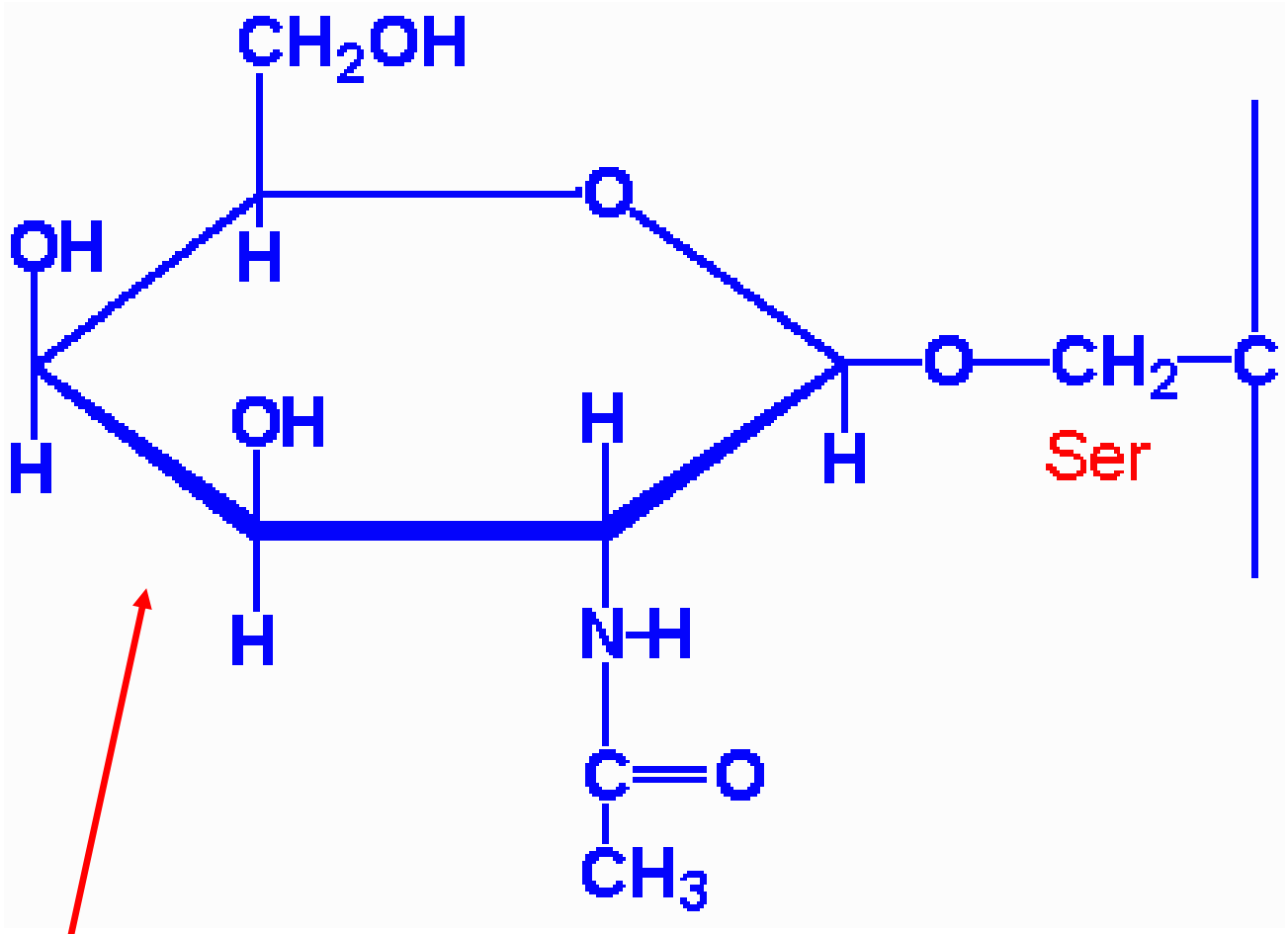
Ser

N-glycosidic bond



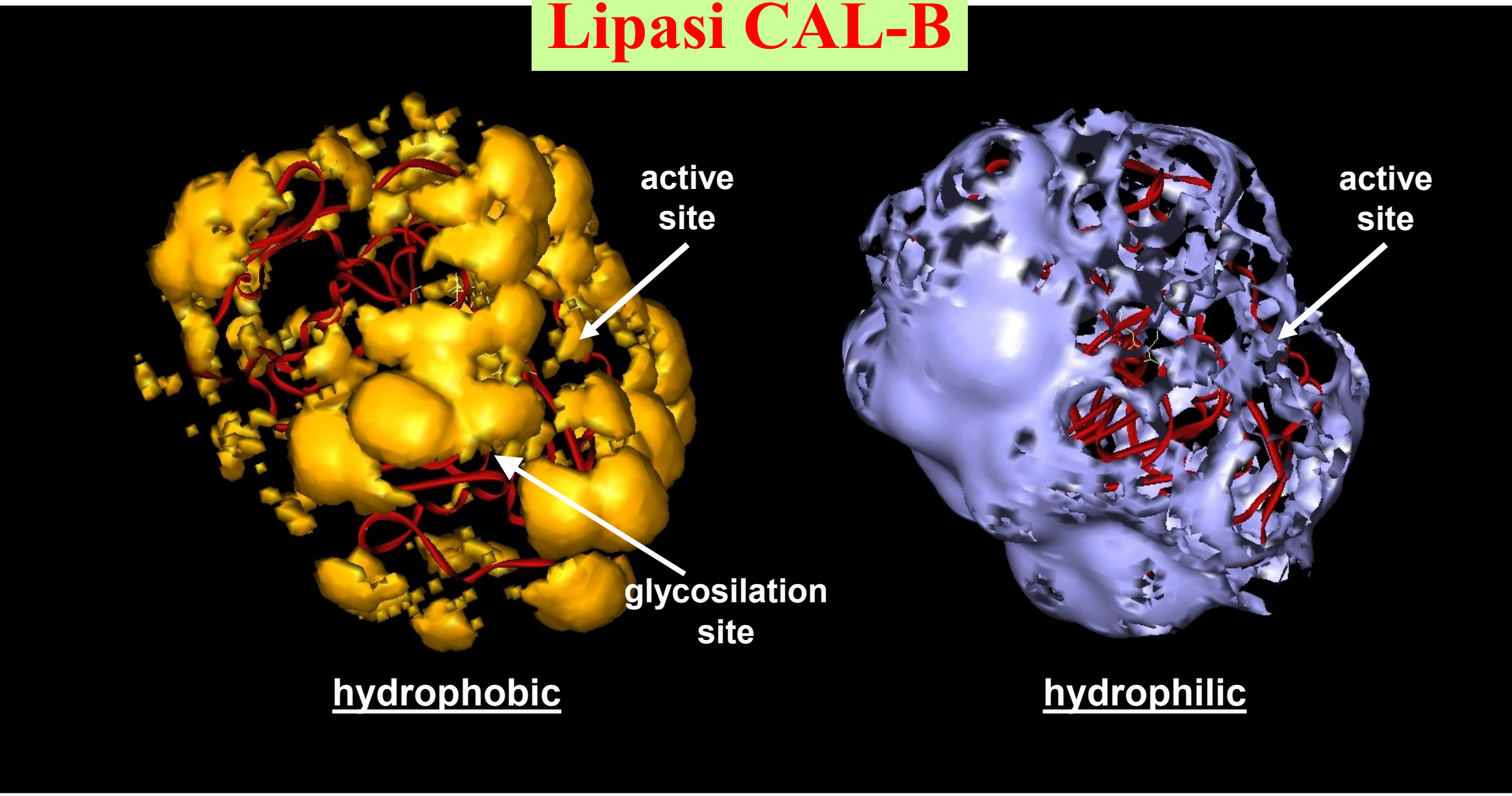
2-N-Acetylglucosammine

O-glycosidic bond



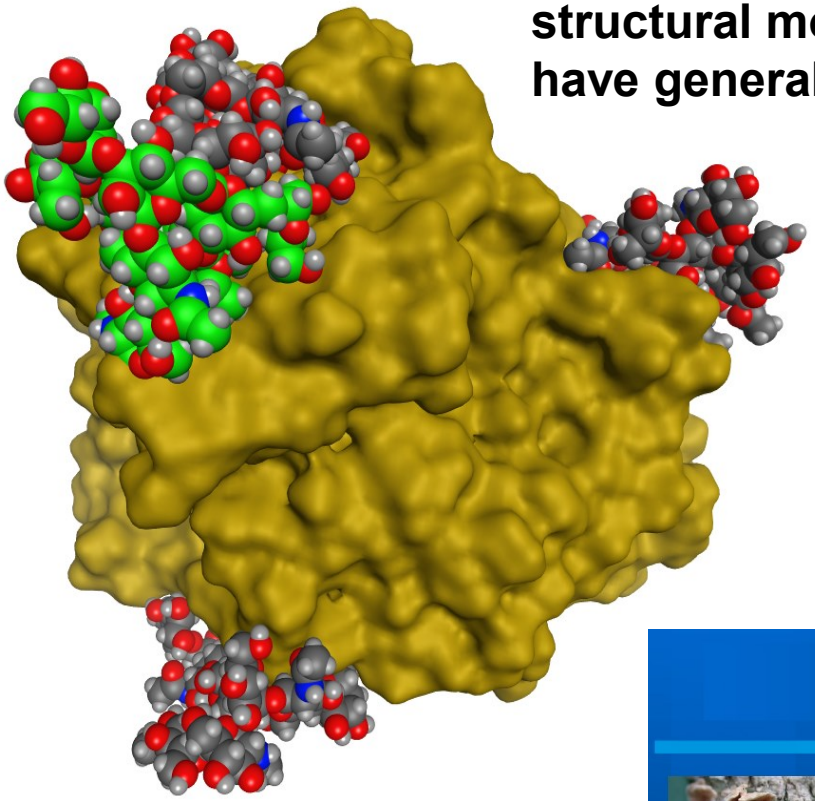
2-N-Acetylglucosammine

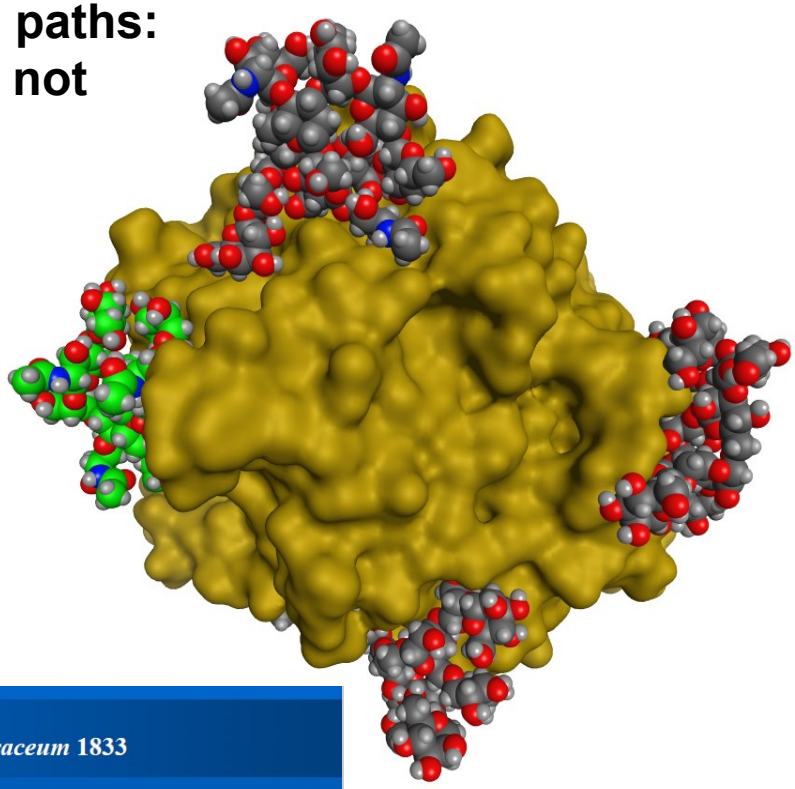
Lipasi CAL-B



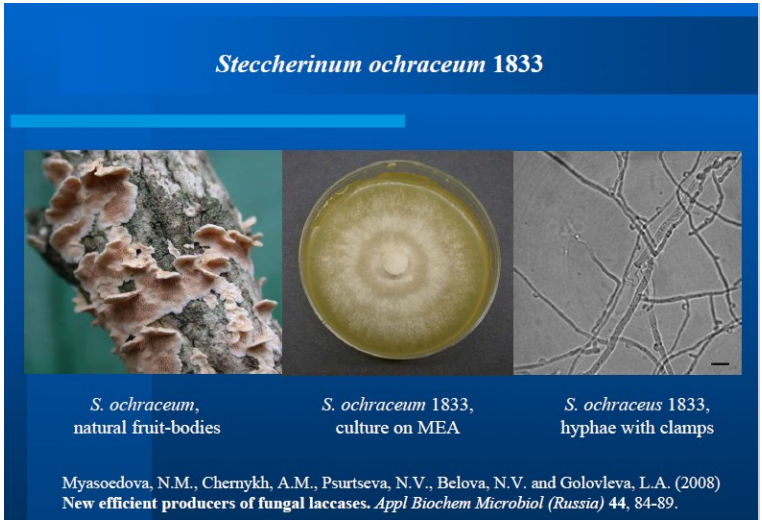
Glycans are generally insertedin the proximity of hydrophobic regions on the surface for stabilizing against hydration

Glycosylation occurs with broadly heterogeneous paths: structural models does not have general validity





Laccase from fungi: degrades lignin



Glycosylation improves thermal stability

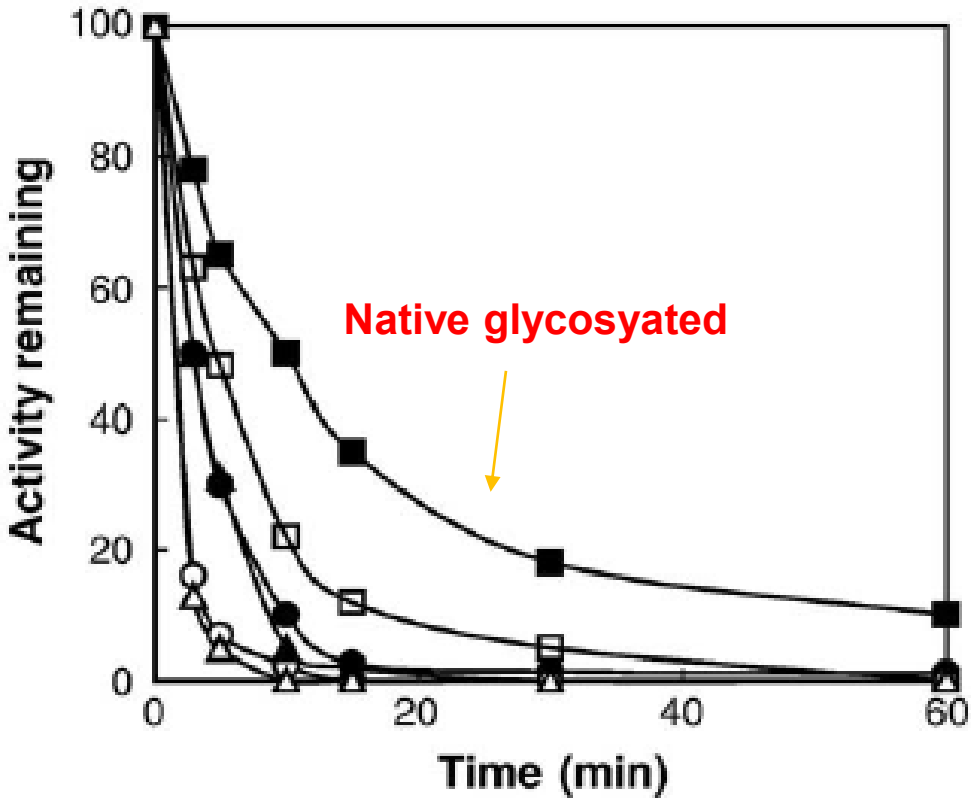


Fig. 4. Irreversible thermoinactivation of native and deglycosylated forms of glucoamylase at 70°C: Native (●), deglycosylated (○) at pH 3; native (■), deglycosylated (□) at pH 4.8; native (▲), deglycosylated (△) at pH 8. Further details are described under Materials and methods.

Glycosylation prevents aggregation

- improves the solubility of unfolded or partially folded proteins

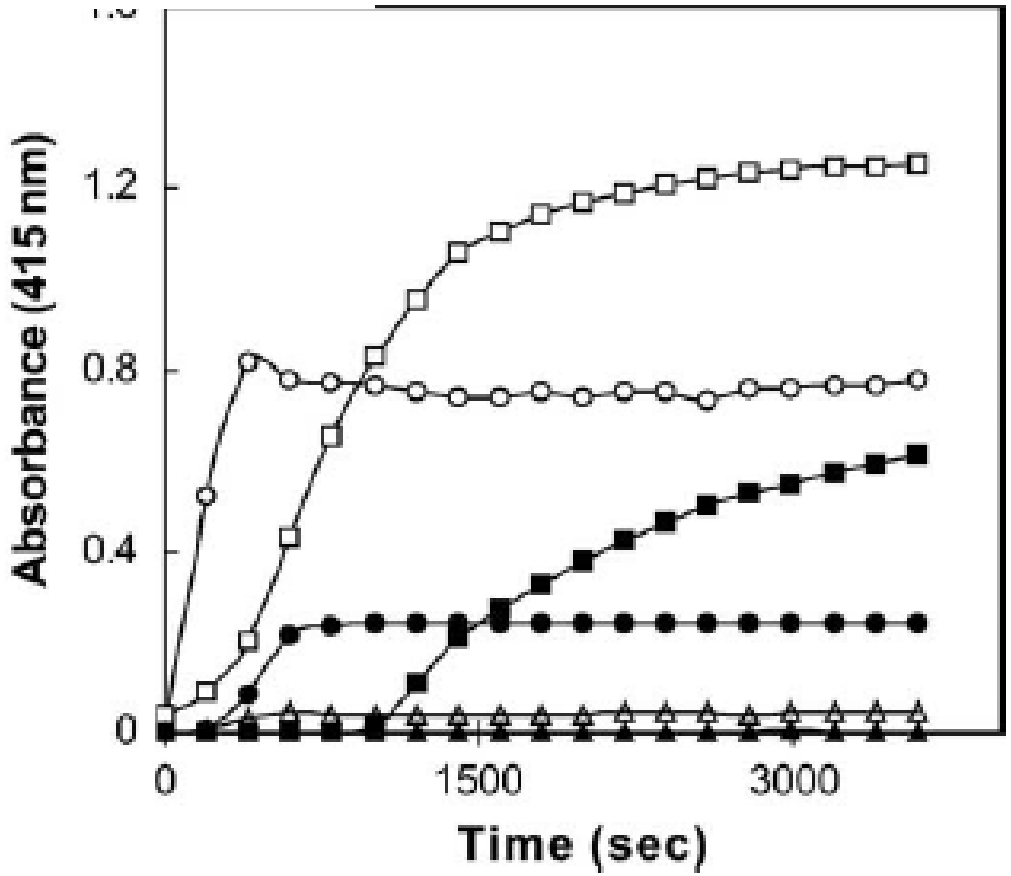
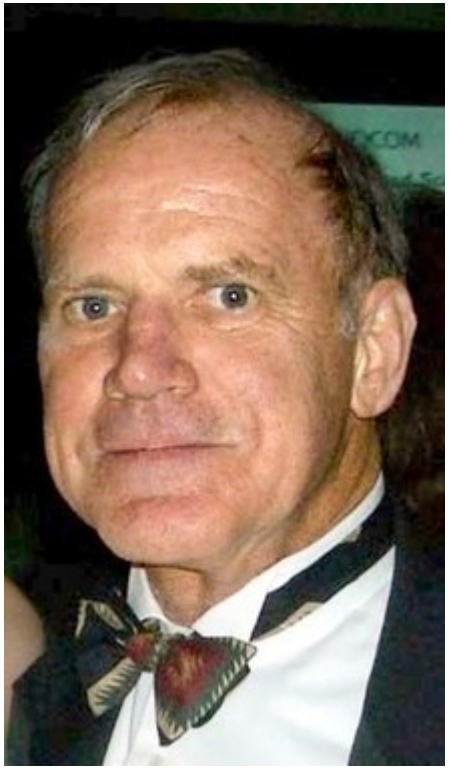


Fig. 5. Aggregation of native and deglycosylated forms of glucoamylase at 70°C: Native (●), deglycosylated (○) at pH 3; native (■), deglycosylated (□) at pH 4.8; native (▲), deglycosylated (△) at pH 8. For further details, please see Materials and methods.

Thermostable DNA polymerase in molecular biology



Kary Banks Mullis (Lenoir, 28 dicembre 1944)
biochimico statunitense,
vincitore del Premio Nobel per la Chimica nel 1993 assieme a Michael Smith.

Indice
•1

Ha ottenuto il dottorato di ricerca all'Università della California a Berkeley nel 1973.
Mullis ha ricevuto il premio Nobel per lo sviluppo della tecnica della reazione a catena della polimerasi (*Polymerase Chain Reaction* o PCR), un processo già descritto da Kjell Kleppe e da Har Gobind Khorana, Nobel nel 1968. La tecnica consente l'amplificazione in vitro di frammenti di DNA. I miglioramenti apportati da Mullis hanno reso la PCR una tecnica fondamentale in biochimica e nella biologia molecolare, con innumerevoli applicazioni in campo medico, agricolo, e investigativo.
Mullis è stato insignito nel 1993 anche del Japan Prize.
Surfista e contestatore nella Berkeley degli anni sessanta, Mullis è considerato un personaggio alquanto originale e discusso, che spesso si è scontrato con le posizioni "ortodosse" della comunità scientifica. È noto per essere fortemente critico nei confronti della teoria diffusamente accettata del legame HIV - AIDS, per lo scetticismo rispetto alle cause del riscaldamento globale e del buco nell'ozono. In una famosa intervista si domandò provocatoriamente se avrebbe mai scoperto la PCR se non avesse assunto LSD, concludendo che ne dubitava seriamente, in quanto poteva letteralmente veder lavorare i singoli polimeri e ammettendo di avere imparato parecchio grazie a tale esperienza.
Nel suo libro del 1998 ("*Ballando nudi nel campo della mente. Le idee (e le avventure) del più eccentrico tra gli scienziati moderni*") Mullis parla della sua visione del mondo e narra di episodi curiosi ed esperienze alquanto insolite da lui esperite, parla dell'astrologia, della sua partecipazione al processo di O. J. Simpson, del suo uso di droghe, per finire addirittura con l'ipotesi di essere stato rapito dagli alieni; gli accadde una notte del 1985, in un bosco nei pressi di Mendocino County in California.
Mullis racconta come ai tempi dei suoi studi a Berkeley mandò all'autorevole rivista inglese *Nature* un articolo fortemente speculativo nel quale sosteneva che metà della materia dell'universo andrebbe all'indietro nel tempo: l'articolo fu pubblicato. Un ventennio dopo propose alla stessa rivista un articolo in cui documentava la tecnica della PCR, che gli avrebbe poi valso il Nobel, e non fu pubblicato.

DNA polymerase

Recombinant DNA

- DNA molecules formed by laboratory methods of genetic recombination to bring together genetic material from multiple sources
- Proteins resulting from the expression of recombinant DNA within living cells are termed *recombinant proteins*
- Expression of foreign proteins requires the use of specialized *expression vectors*

The first prerequisite is to obtain a sufficient DNA quantity (amplification)

DNA amplification is obtained by *molecular cloning* or by PCR (Polymer Chain Reaction)

PCR - Polymerase Chain Reaction

- PCR is a technology used mainly for amplification and/or isolation of specific DNA sequences
- The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA
- PCR employ a thermostable DNA polymerase, such as Taq polymerase (DNA polymerase from *Thermus aquaticus*)

Taq polymerase enzymatically assembles a new DNA by using single-stranded DNA as a template and DNA oligonucleotides (DNA primers), which are required for initiation of DNA synthesis

PCR - Polymerase Chain Reaction

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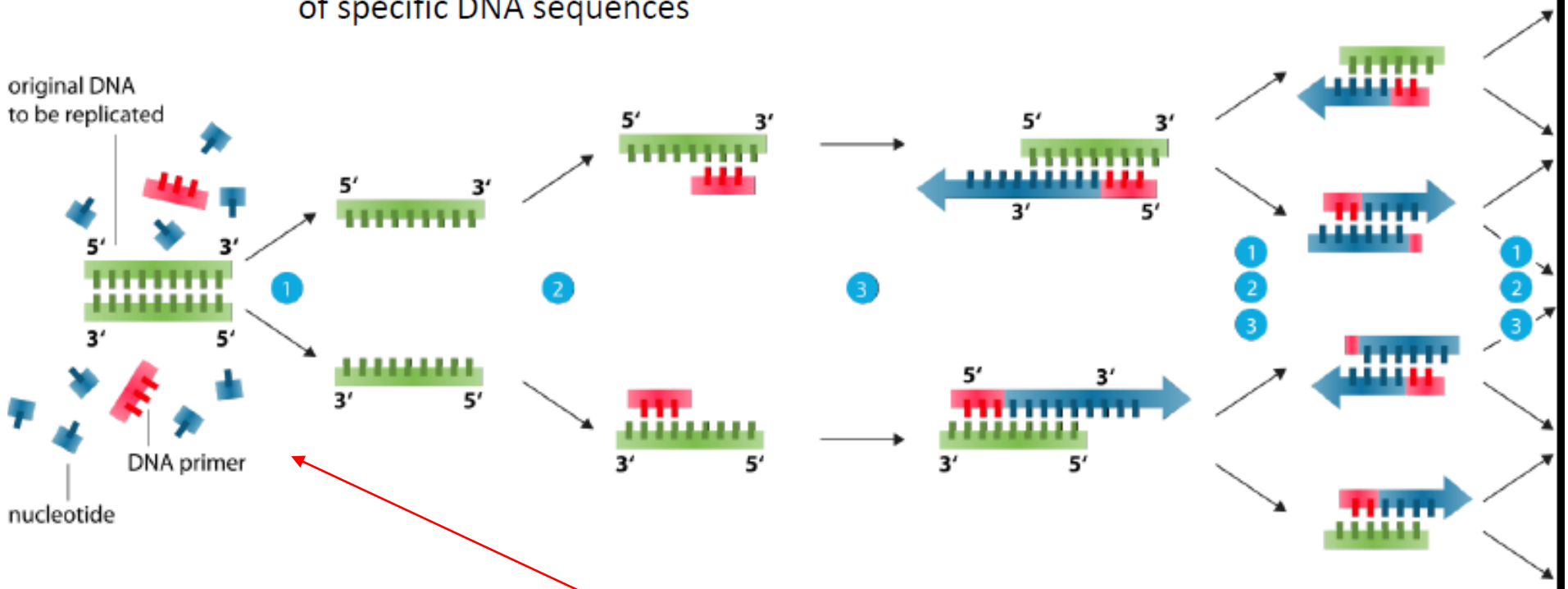

1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C

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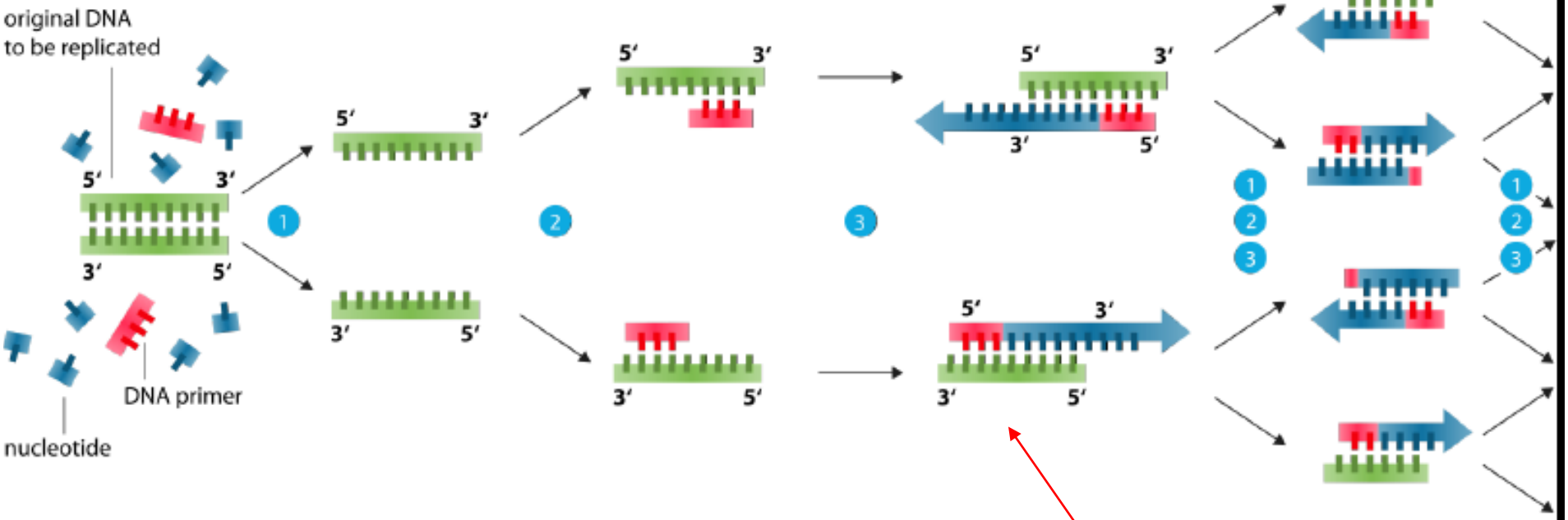
PCR - Polymerase Chain Reaction



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

Taq polymerase enzymatically assembles a new DNA by using single-stranded DNA as a template and DNA oligonucleotides (DNA primers), which are required for initiation of DNA synthesis

Molecular cloning

1. Choice of host organism and cloning vector
2. Preparation of cloning DNA vector
3. Introduction of cloning vector into host organism
4. Screening for clones with desired DNA inserts

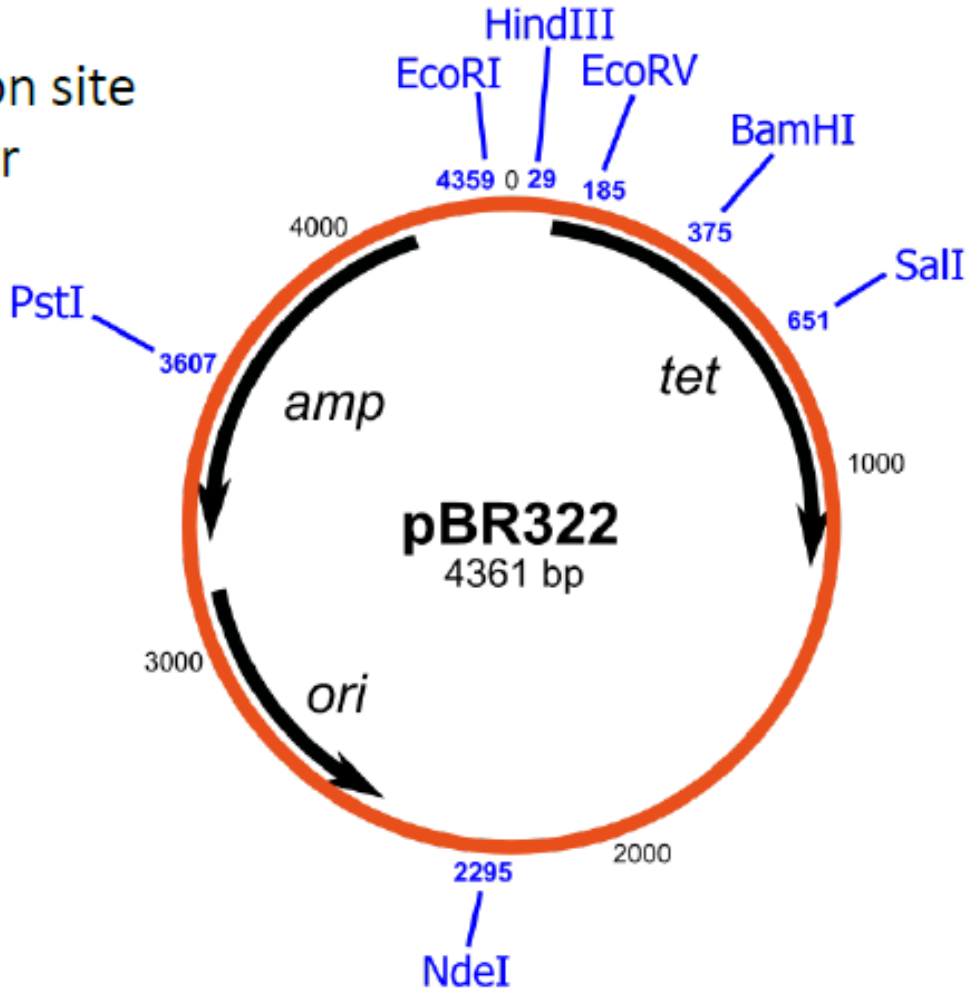
The great majority of molecular cloning experiments begin with a laboratory strain of the bacterium *Escherichia coli* and a *plasmid* cloning vector

A plasmid is a small DNA molecule physically separated from a chromosomal DNA that can replicate independently

Plasmid vector

Essential plasmid elements:

- Origin of replication
- At least one cloning/restriction site
- At least one selectable marker

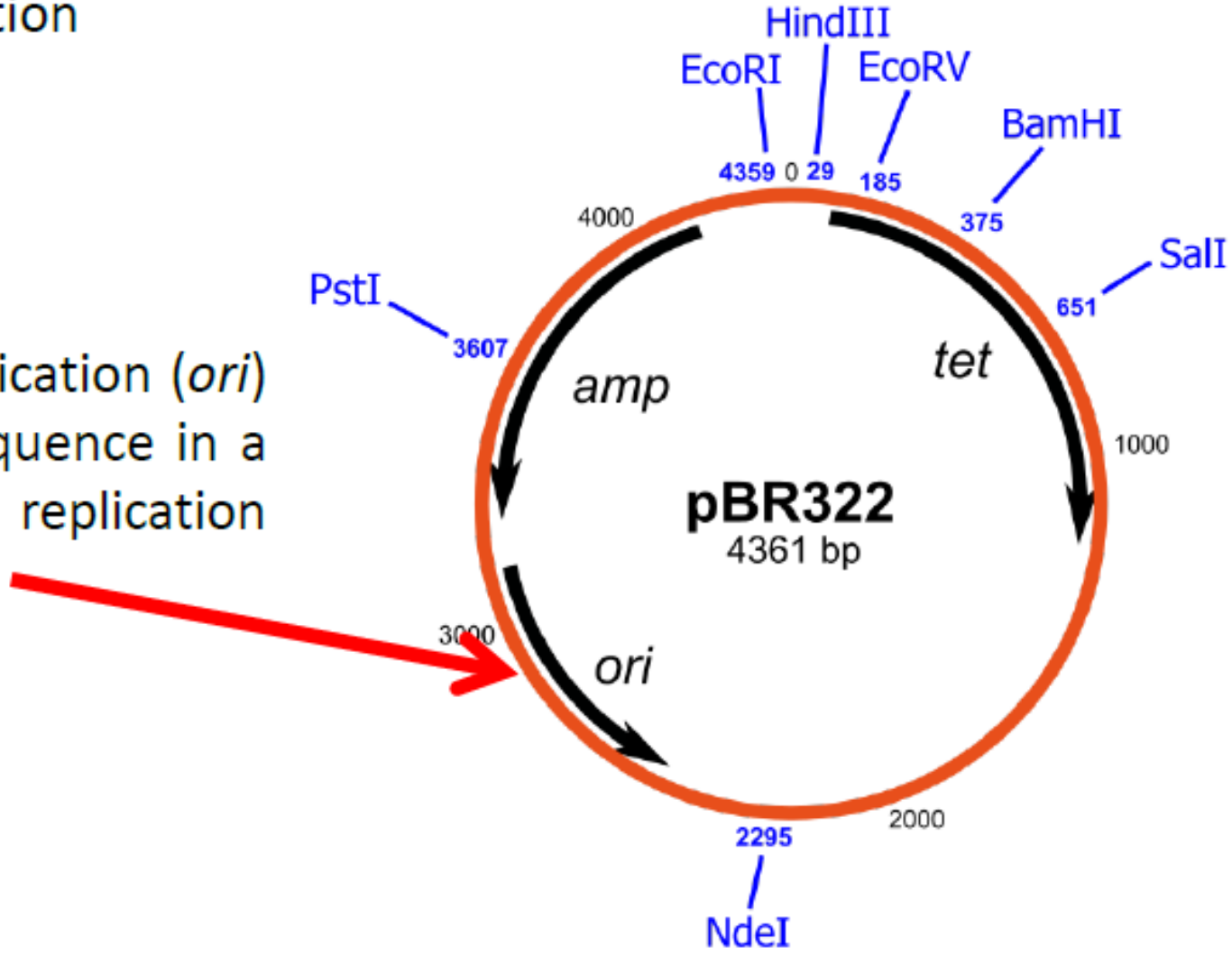


Plasmid vector

Essential plasmid elements:

- Origin of replication

The origin of replication (*ori*) is a particular sequence in a genome at which replication is initiated

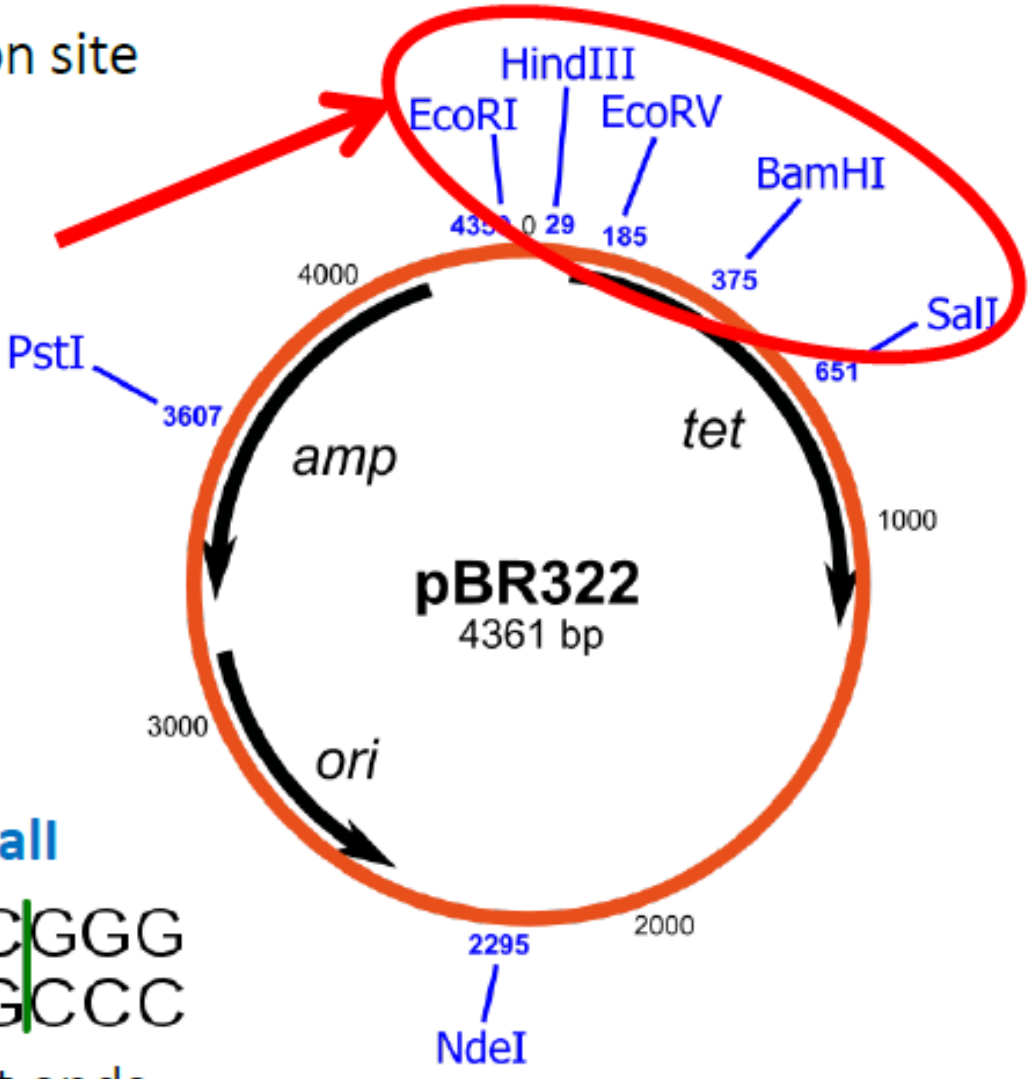


Plasmid vector

Essential plasmid elements:

- At least one cloning/restriction site

A restriction site is a nucleotide sequence specifically recognized by endonuclease enzymes (or restriction enzymes) that cuts DNA at or near the restriction sites



EcoRI

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

Sticky ends

SalI

C C C G G G
G G G C C C

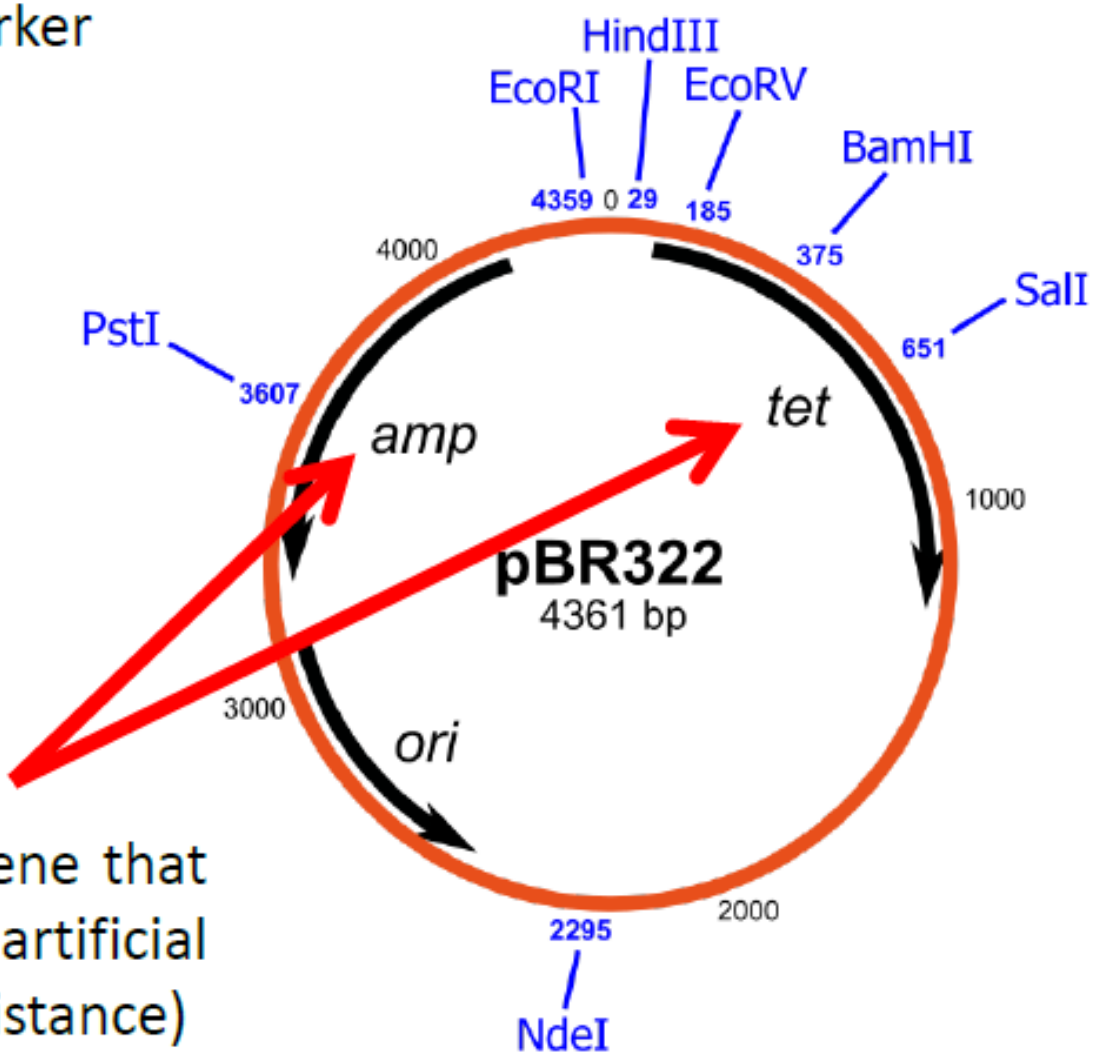
Blunt ends

Plasmid vector

Essential plasmid elements:

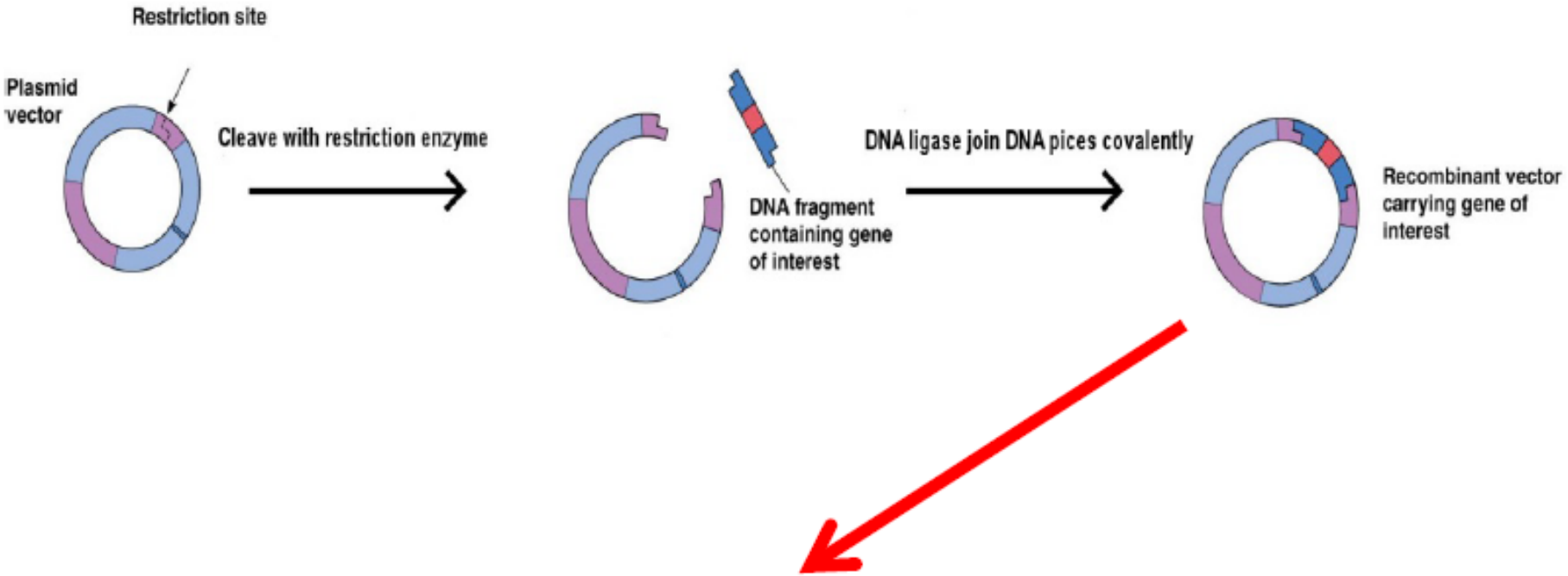
- At least one selectable marker

tet = tetracicline resistance
amp= ampicillin resistance

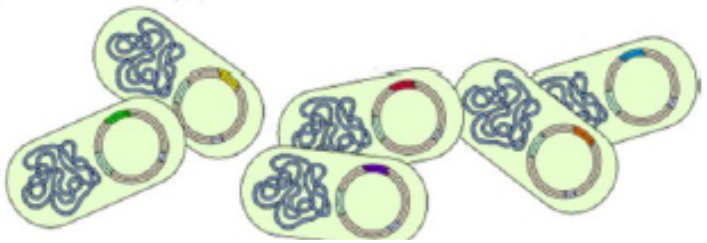


A selectable marker is a gene that confers a trait suitable for artificial selection (i.e. antibiotic resistance)

DNA insertion



Plasmid is inserted into the cloning host (i.e. *E. coli*)



Cell transformation
Insert exogenous DNA in host microorganism

Main techniques:

- Heat shock
- Divalen cations ($CaCl_2$)
- Electroporation (electric field of 10-20 kV/cm)
- Enzyme treatment

Increase cell permeation