RECOMBINANT

DNA

TECHNIQUES

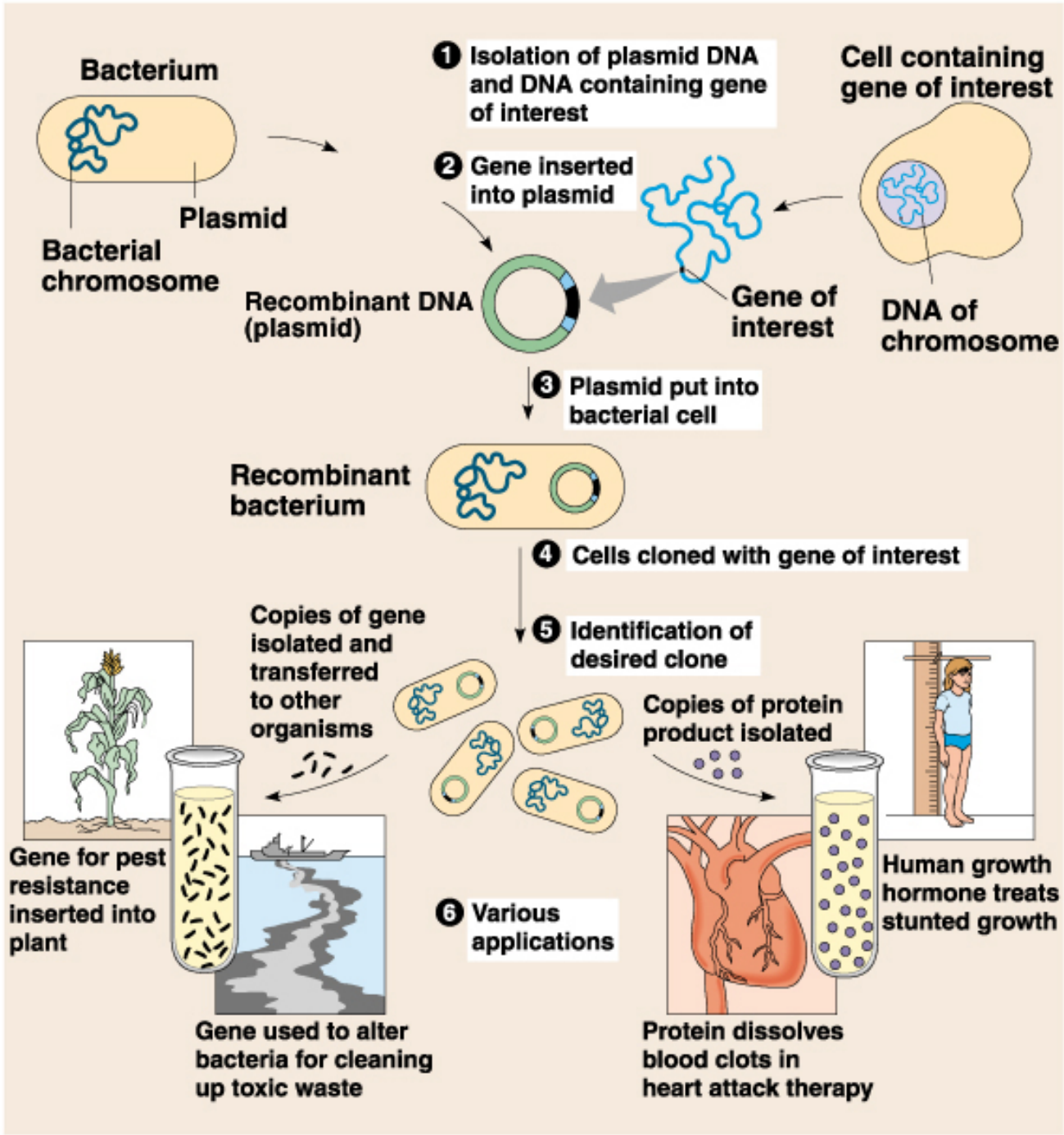
DNA RICOMBINANTE

tecnica che permette di

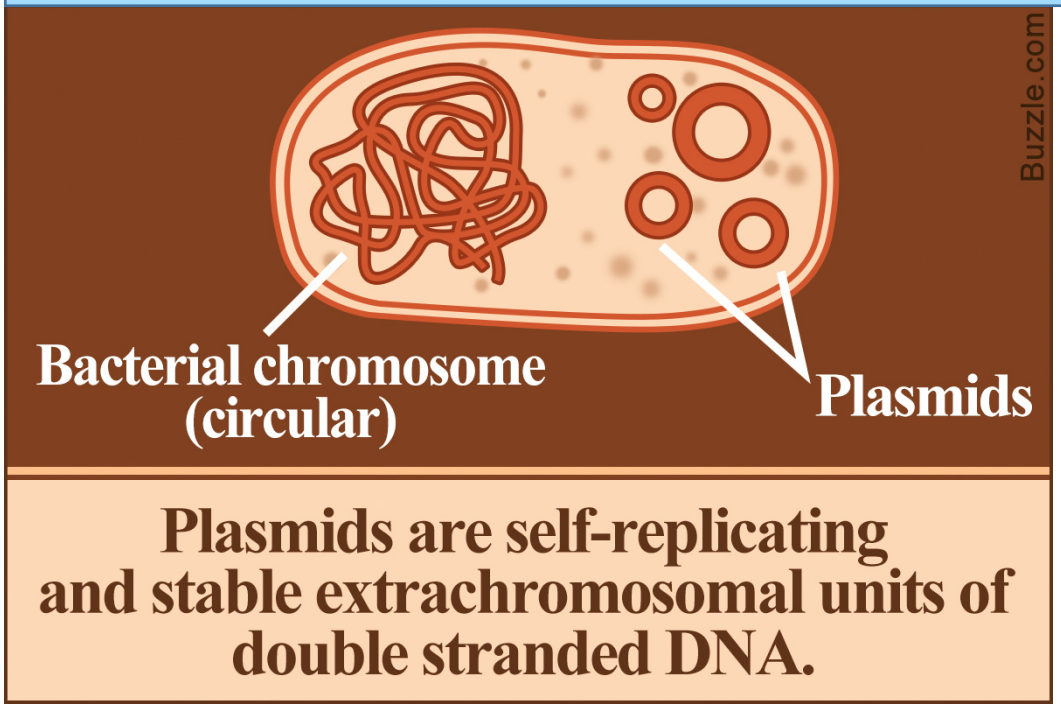
- **ottenere brevi segmenti di DNA clonati e di studiarne la sequenza nucleotidica**
- **di trasferirli nel genoma di altre cellule**
- **di controllare l'incorporazione e l'espressione del DNA clonato**
- **di introdurre mutazioni nel DNA e di studiarne gli effetti**

ESSENZIALE: CARRIERS = PLASMIDI

A General Strategy to study or use recomobinant DNA



Plasmids



A plasmid is a **small DNA molecule (1-200kb) orignally** hosted by bacteria, **physically separated** from chromosomal DNA and can **replicate independently ("replicons")**.

Plasmids exist "**naturally**"

Copy number:
1 – 1000nds

Shape:
Cicular, doublestrandend
Some linear plasmids exist

Present in:
Bacteria
but also sometimes in archea and eukaryotic cells (yeast)

Advantage to bacteria: - plasmid often carry genes that give a selecive advantage
- plasmid can be passed on to other bacteria: horizontal gene transfer

What is the difference to viruses/bacteriphages?

- **plasmids are not packaged into capsids**
- **virus/phages does not give selective advantage**

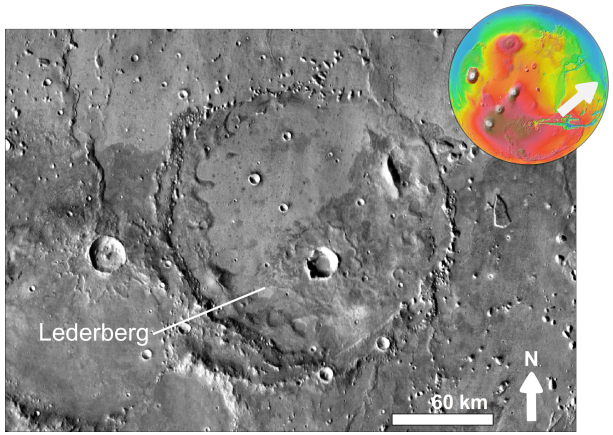
1. INTRODUCTION PLASMIDS

History

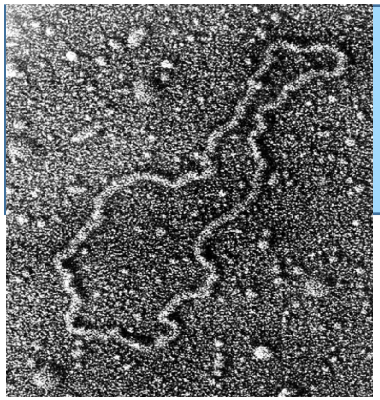
The term plasmid was introduced in 1952 by the American molecular biologist **Joshua Lederberg** to refer to "**any extrachromosomal hereditary determinant**" in bacteria

The original definition also included bacterial viruses; thus refinement:

Genetic elements that exist exclusively or predominantly outside of the chromosome and can replicate autonomously.



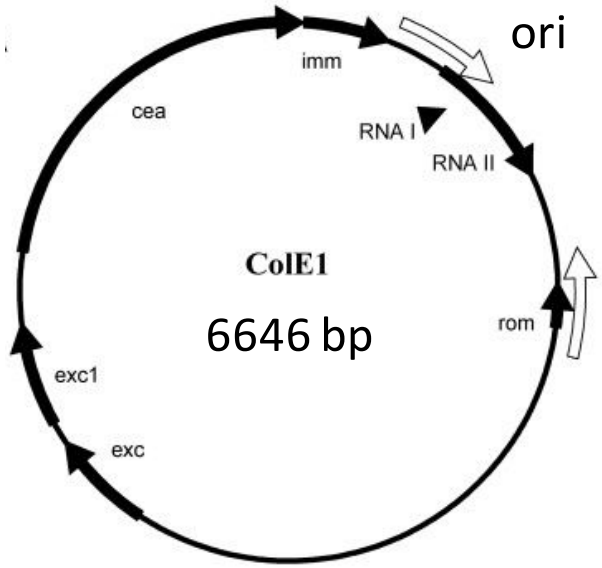
1958 Nobel Prize in Physiology or Medicine for discovering that bacteria can mate and exchange genes (bacterial conjugation)



Plasmids

Natural

Present as "wildtype" plasmids in bacteria

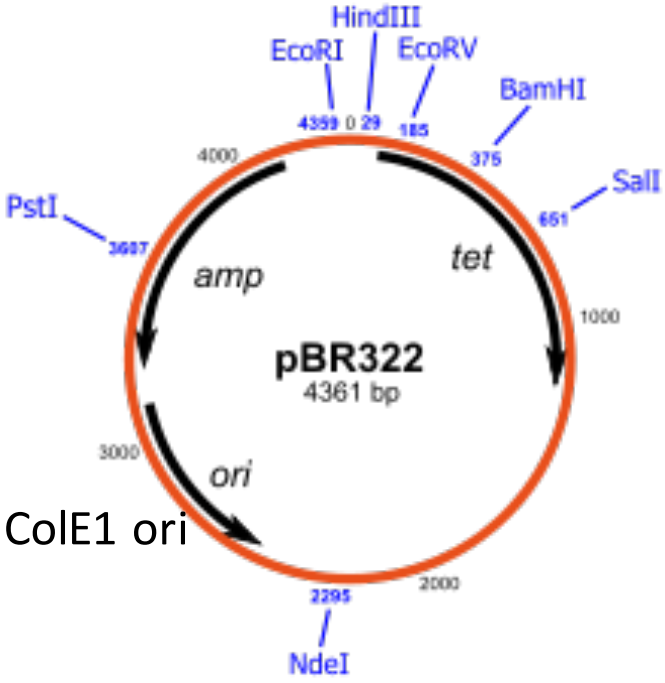


Bacteria

Engineered

optimized plasmid for laboratory use in..

- Specific resitrction sites
- marker genes
- is a recombinant DNA molecule



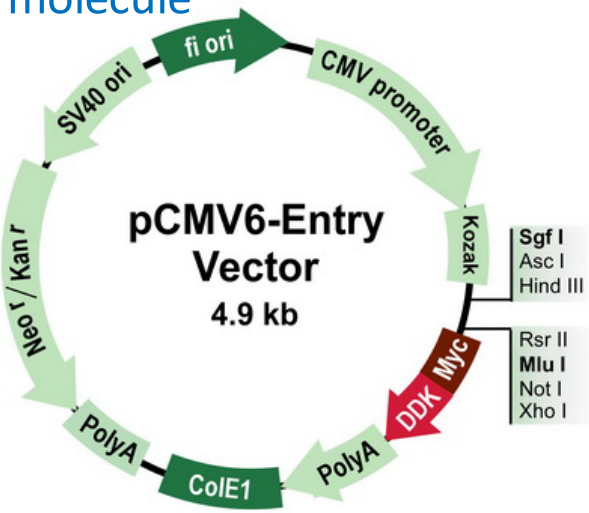
ColE1 ori

Bacteria

Engineered

optimized plasmid for laboratory use in..

- Specific resitrction sites
- Marker genes
- Promoters for expression in eukaryots
- Is a recombinant DNA molecule

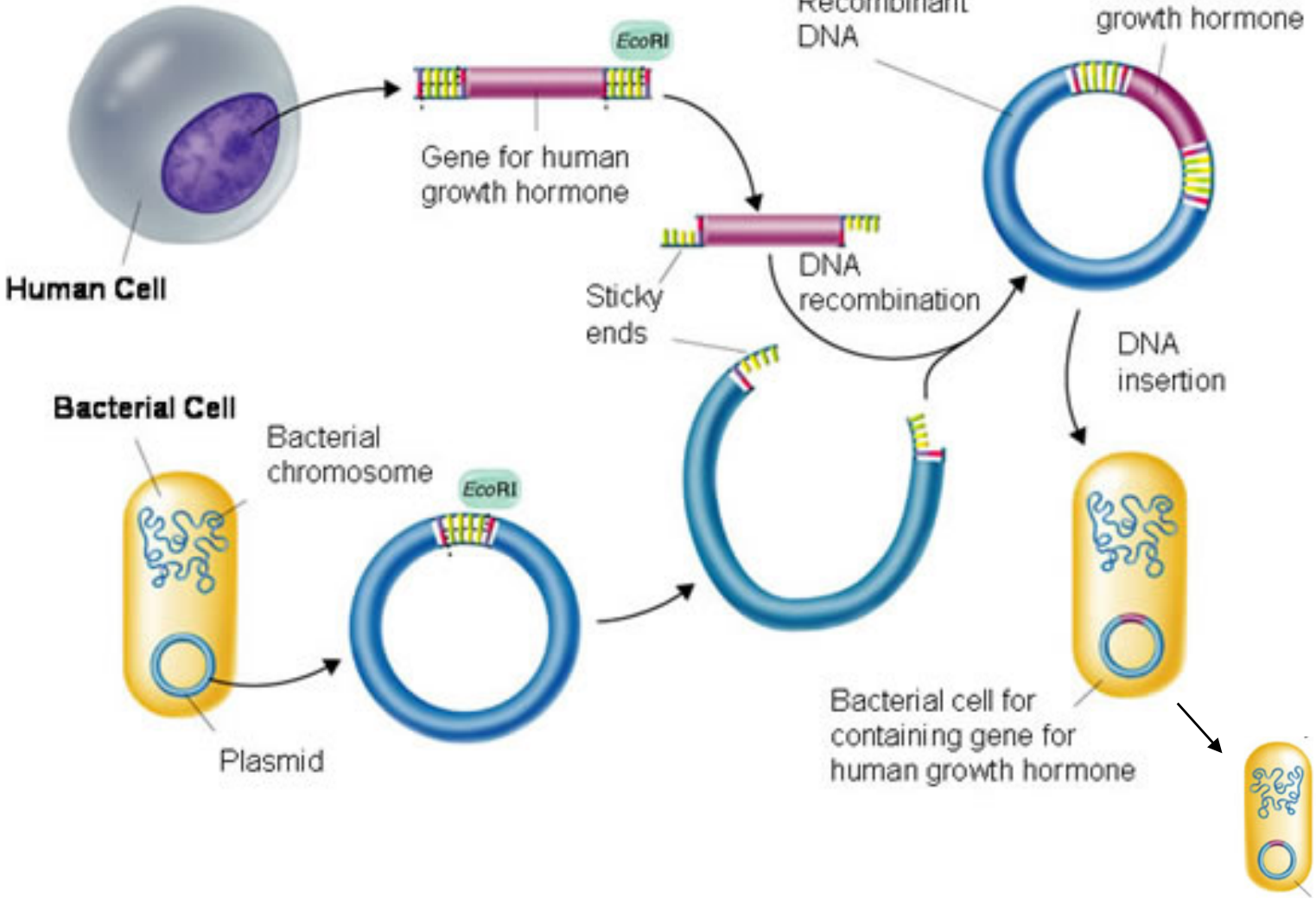


Bacteria

Mammalian cells

Plasmids

Why interesting for molecular biology? Recombinant DNA technology



Take segment of human DNA (genomic, cDNA)

Take and linearize bacterial plasmid DNA

Insert into plasmid ="recombinant DNA" (ligation of termini using ligase)

Re-insert obtained plasmid into bacteria that does not have own plasimds

Bacteria proliferate
Pplasmid in bacteria replicate and can reach 1-1000 copies

Amplified human DNA can be studied

Plasmids - Vectors

VECTORS:

- Are recombinant DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed.
- Typical vectors are: plasmid, cosmid, lambda phages, retrovirus, adenovirus, lentivirus,…
- Vectors can be circulare but also linear

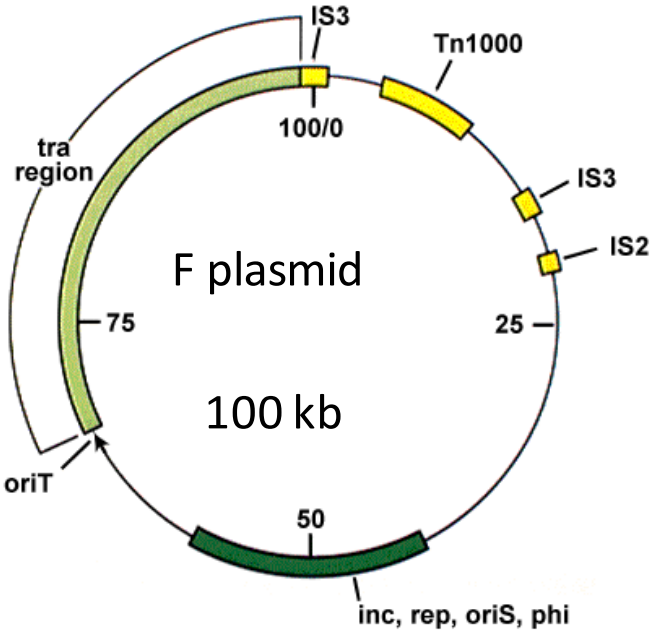
Sono disponibili vari tipi di vettori di clonaggio

Vettore	Caratteristiche	Isolamento del DNA	Contenuto massimo di DNA	
Plasmide	Alto numero di copie	Fisico	10 kb	Natural, engineered
Fago	Infetta batteri	Attraverso l'impacchettamento nel fago	20 kb	Natural, engineered
Cosmide	Alto numero di copie	Attraverso l'impacchettamento nel fago	48 kb	Engineered
BAC	Basato sul plasmide F	Fisico	300 kb	Engineered
YAC	Origine + centromero + telomero	Fisico	>1 Mb	Engineered

Lenti-, Adeno, Retroviruses — Natural, engineered

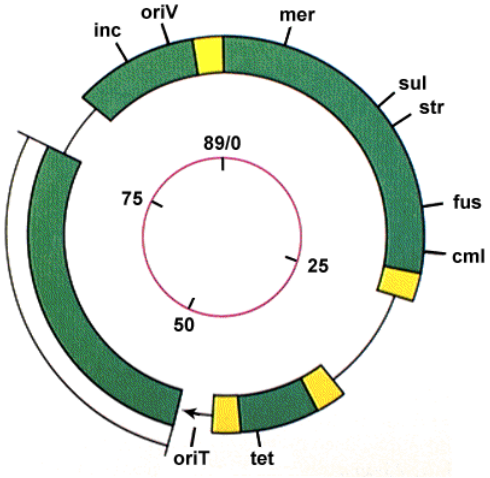
Natural Plasmids

Natural Plasmids - Grouped after their properties



- **F-plasmids**: encode tra region for horizontal gene transfer (conjugation), (trans**F**er); F^+ (plasmid donor); F^- plasmid recipient

- **R- plasmid:** **Encode genes for resistance** against antibiotics and/or heavy metals. (Ampicilin, Kanamycin



- **Col – plasmids:** - **produces colicins** (antibacterial)
- **Catabolic plasmids:** -have properties to **use odd carbon/ energy source (** many *Psuedomonas* have such plasmids
- **Virulent plasmids:** **-** Encode toxins, pathogenic**.**
- **Cryptic plasmids:** **- no known** property

Natural Plasmids

Natural Plasmids – grouped according to other features

- Classification based on possibility to do horizontal gene transfer

- Conjugative plasmids (F plasmids): able to do horizontal gene transfer (geni *tra*)

-Non-conjugative plasmids
• Plasmidi R, • Plasmidi Col, • Plasmidi degradativi, • Plasmidi della virulenza:

- Classification based on copy number

- **High copy number plasmids** (relaxed plasmids); Plasmidi ad alto numero di copie (rilassati; 10 - >100)

- **Low copy number plasmids** (stringent plasmids); Plasmidi a basso numero di copie (stringenti 1-4)

Natural Plasmids

Natural Plasmids – Essential and non essential features:

ESSENTIAL FEATURES: Assure the replication and maintenance of plasmids in the host cell:

- Replication:
  - -uses the replication system (enzymes, proteins) of the host cell
  - - event of initiation, elongation and termination independent from host DNA
  - - occurs during the entire cell cycle of the host
  - -All plasmids contain the "**ori" region** that encodes information for the replication of the plasmid
- Copy number control:
  - -a certain amount of copies present per cell
  - - controlled by the initiation frequency
  - - low (1-4) to high (10 - >100)
- Partitioning system for vertical transfer:
  - - for low (and medium) copy number
  - - genes that control the safe passage of plasmid to daughter cells
  - - High copy plasmids have stochastic segregation to daughter cell
- Host specificity/range:
  - - low to broad

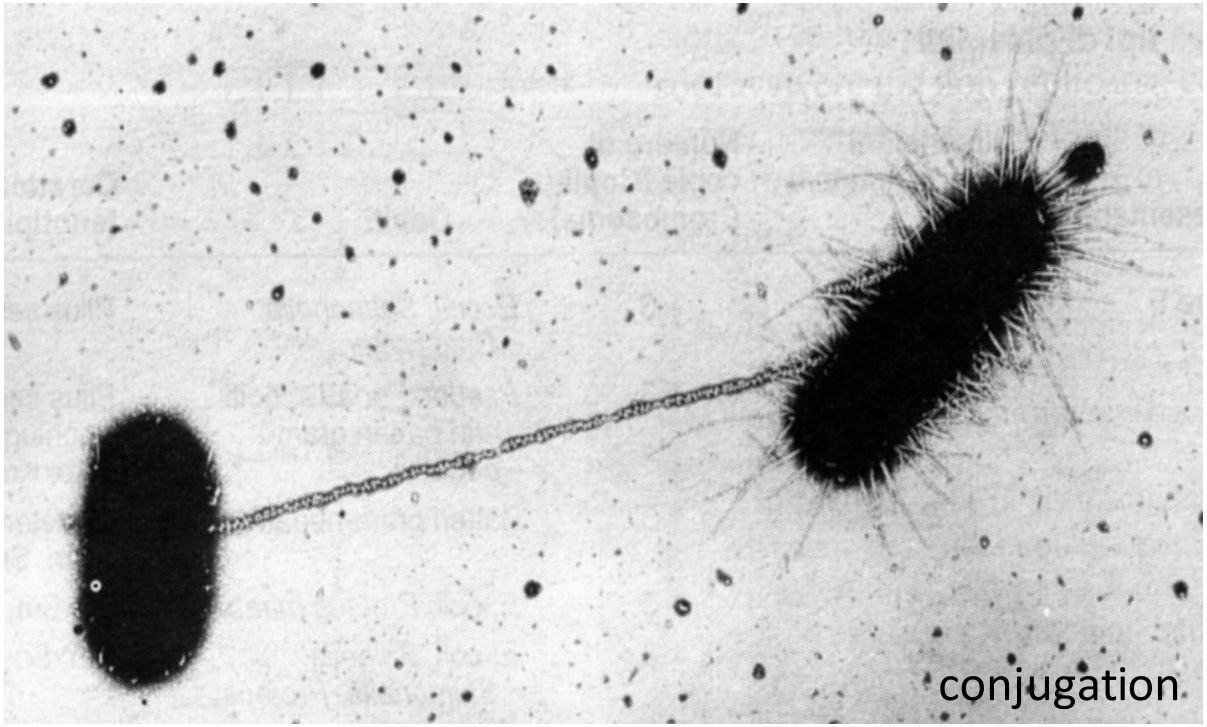
Natural Plasmids

Natural Plasmids - Grouped after their essential and non-essential genes:

NON ESSENTUAL FEATURE – mechanism for horizontal transfer of plasmids

- Important genes
  - *pili*-genes
  - *ori*T
  - *tra/ mob* genes

- Replicated plasmid is transferred via pilus from one cell to another cells (=horizontal transfer)



Pili sessuali: presenti in numero di 1-10 per cellula, sono spessi 9-10 nm

Natural Plasmids

Natural Plasmids - Grouped after their essential and non-essential genes:

NON ESSENTUAL FEATURE: plasmid encodes gene that gives surviving value for host/plasmid

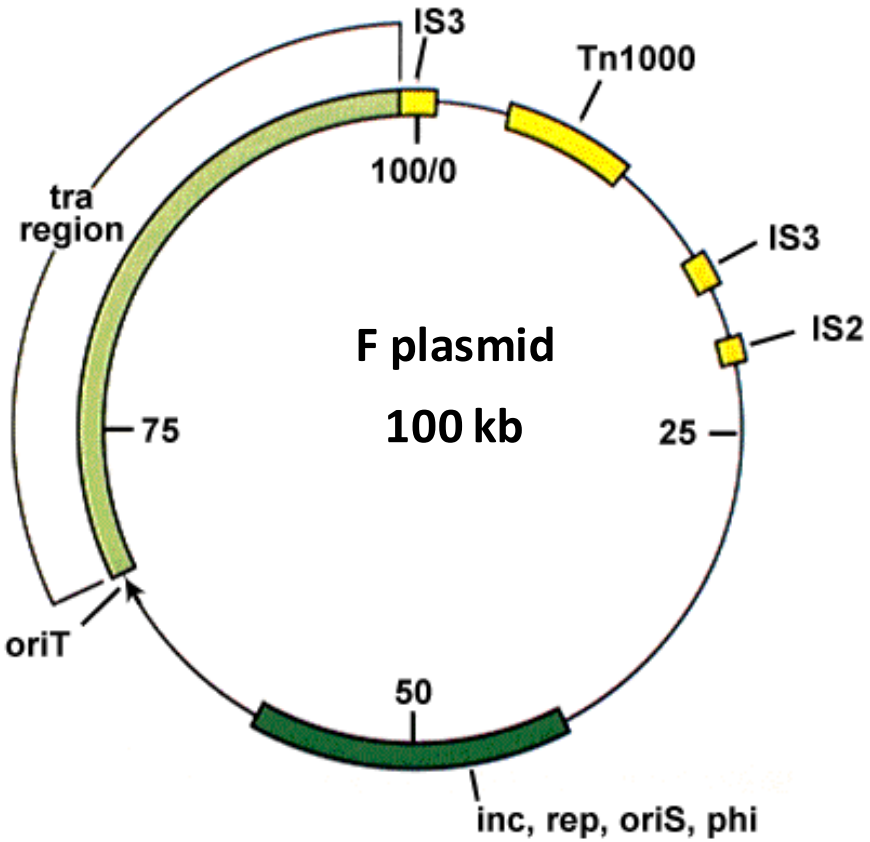
- Resistance against antibiotics
- Host defence against foreign DNA
- Production of antibacterial substances (colicins)
- genes for pathogenesis/virulence
- genes to be able to use special energy/carbon sources, e.g. phenol

Esempi di fenotipi conferiti da plasmidi

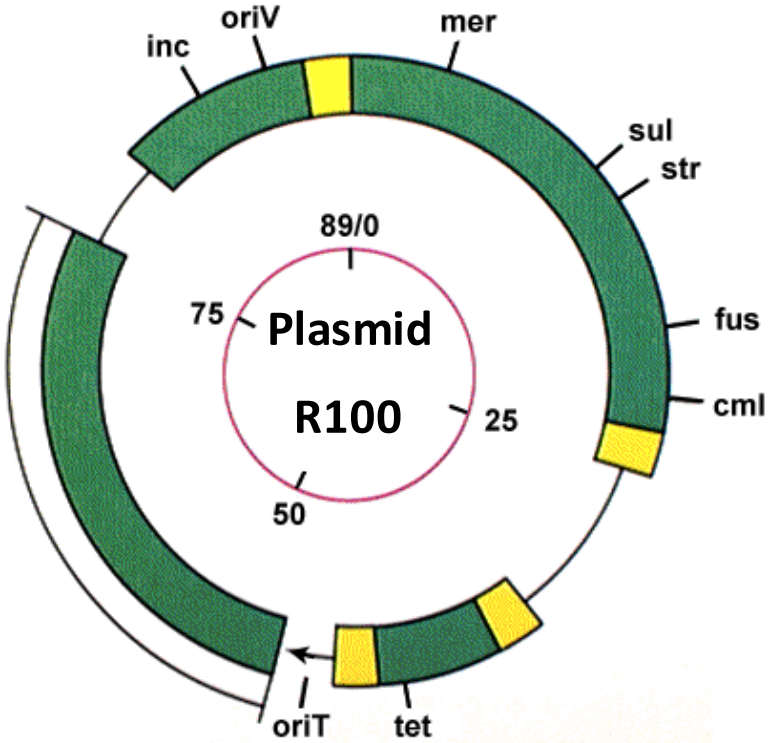
Produzione di antibiotico ► SCP1 ► *Streptomyces coelicolor*
Antibiotico-resistenza ► RP4 ► *Pseudomonas aeruginosa*
Resistenza al batteriofago ► pNP40 ► *Lactococcus lactis*
Produzione di batteriocina ► p9B4-6 ► *Lactococcus lactis*
Trasferimento coniugale ► F ► *Escherichia coli*
Cristallo proteico insetticida ► pHD2 ► *Bacillus thuringiensis*
Competenza ecologica nel suolo ► pRtrW14-2c ► *Rhizobium leguminosarum*
Produzione di emolisina ► pJH1 ► *Enterococcus faecalis*
Degradazione dell'erbicida ► 2,4-D pJP4 ► *Alcaligenes eutrophus*
Fermentazione del lattosio ► pLM3601 ► *Lactococcus lactis* subsp. *cremoris*
Resistenza ai metalli pesanti ► pMERPH ► *Pseudomonas sp.*
Fissazione dell'azoto ▸ pIJ1007 ▸ *Rhizobium leguminosarum*
Nodulazione ▸ pPN1 ▸ *Rhizobium trifoli*
Degradazione di alcaloidi ▸ pRme41a ▸ *Rhizobium meliloti*
Formazione di tumori ▸ Ti plasmid ▸ *Agrobacterium*
Produzione di proteasi ▸ pLM3001 ▸ *Lactococcus lactis*
Produzione di feromoni ▸ pAD1 ▸ *Enterococcus faecalis*
Produzione di sideroforo ▸ pDEP10 ▸ *Escherichia coli*
Tolleranza a NaCl ▸ pRtrW14-2b ▸ *Rhizobium leguminosarum*
Degradazione del toluene ▸ Tol plasmids ▸ *Pseudomonas putida*

Plasmid Maps

Natural plasmids



Note: F plasmid can also integrate into host genome
= primitive transposon (IS2, IS3 sites)



90 kb

Note: Plasmids that can integrate into genome
are also called episomes

NIH Guidelines for use of bacteria and recombinant DNA

- **BASIC RULE**
  - **Specified handling and construction processes**
  - **Microorganisms containing recombinant DNA are prohibited to bring outside of the laboratory**
  - **Vectors that sexually move to "unsafe" bacteria was prohibited**
  - **Tra region and mob region must be non-functional**
  - **Nic/bom region must be non-functional (nic/bom containig plasmids can be mobilzed by mob encoding plasmids**

The roles of some tra-gene encoded proteins:[4]	
Pili Assembly and Production	*traA, traB, traE, traC, traF, traG, traH, traK, traL, traQ, traU, traV, traW,*
Inner Membrane Proteins	*traB, traE, traG, traL, traP*
Periplasmic Proteins	*traC, traF, traH traK, traU, traW*
DNA transfer	*traC, traD, traI, traM, traY*
Surface Exclusion Proteins	*traS, traT*
Mating Pair Stabilization	*traN, traG*

2. REPLICATION OF PLASMIDS

1. Plasmid replication requires host DNA replication machinery.
2. Most wild plasmids carry genes needed for transfer and copy number control.
3. All self replication plasmids have a ***oriV*: origin of replication**
4. Some plasmids carry and ***oriT*: origin of transfer** (for horizontal gene transfer) These plasmids will also carry functions needed to be mobilized or ***mob* genes**.
5. Plasmid segregation is maintained by a ***par* locus**-a partition locus that ensures each daughter cells gets one plasmid. Not all plasmids have such sequences.
6. There are 5 main "**incompatibility" groups** of plasmid replication. Replication is limited to particular bacterial species. Not all plasmids can live with each other.
7. Agents that disrupt DNA replication destabilize or cure plasmids from cells.

Replication of Plasmids

Natural plasmid

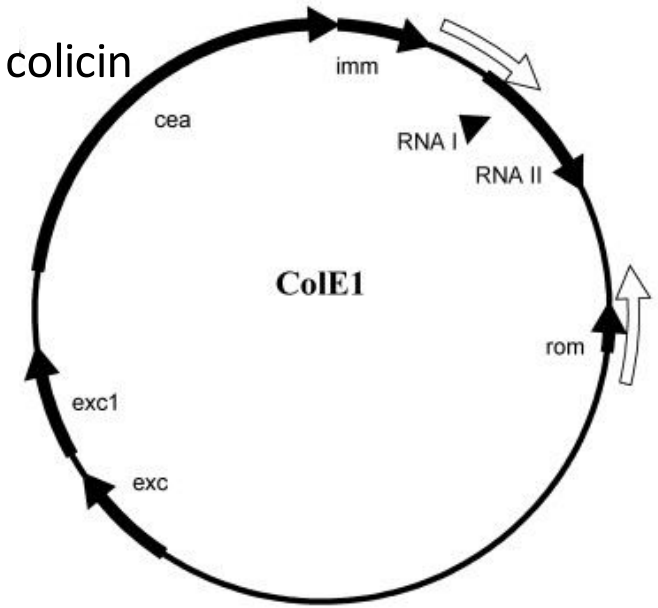


Table 11-1 Examples of some plasmids and their properties

Plasmid	Size (Kb)	Number of copies per chromosome	Self-transmissible	Phenotypic features
Col plasmids				
ColE1	6.4	10–15	No	Colicin E1 disrupts energy gradient, host immunity to Colicin E1
ColE2	7.6	10–15	No	Colicin E2 is a DNase, host immunity to Colicin E2
ColE3	7.6	10–15	No	Colicin E3 is a ribosomal RNase, host immunity to Colicin E3
F plasmid	94.5	1–2	Yes	F-pilus, conjugation
R plasmids				
R100	106.7	1–2	Yes	Cam^r Str^r Sul^r Tet^r
RK2	56.0	5–8	Yes	Broad host range
pSC101	9.0	<5	No	Low copy number, compatible with ColE1-type plasmids, Tet^r
Phage plasmid				
λdv	6.4	50	No	λ genes *cro, cI, O, P*
Recombinant plasmids				
pBR322	4.4	20	No	Medium copy number, ColE1-type replication, Amp^r
pUC18	2.7	200–500	No	High copy number, ColE1-type replication with a mutation that increases the copy number, Amp^r
pACYC184	4.0	10–12	No	Cam^r Tet^r

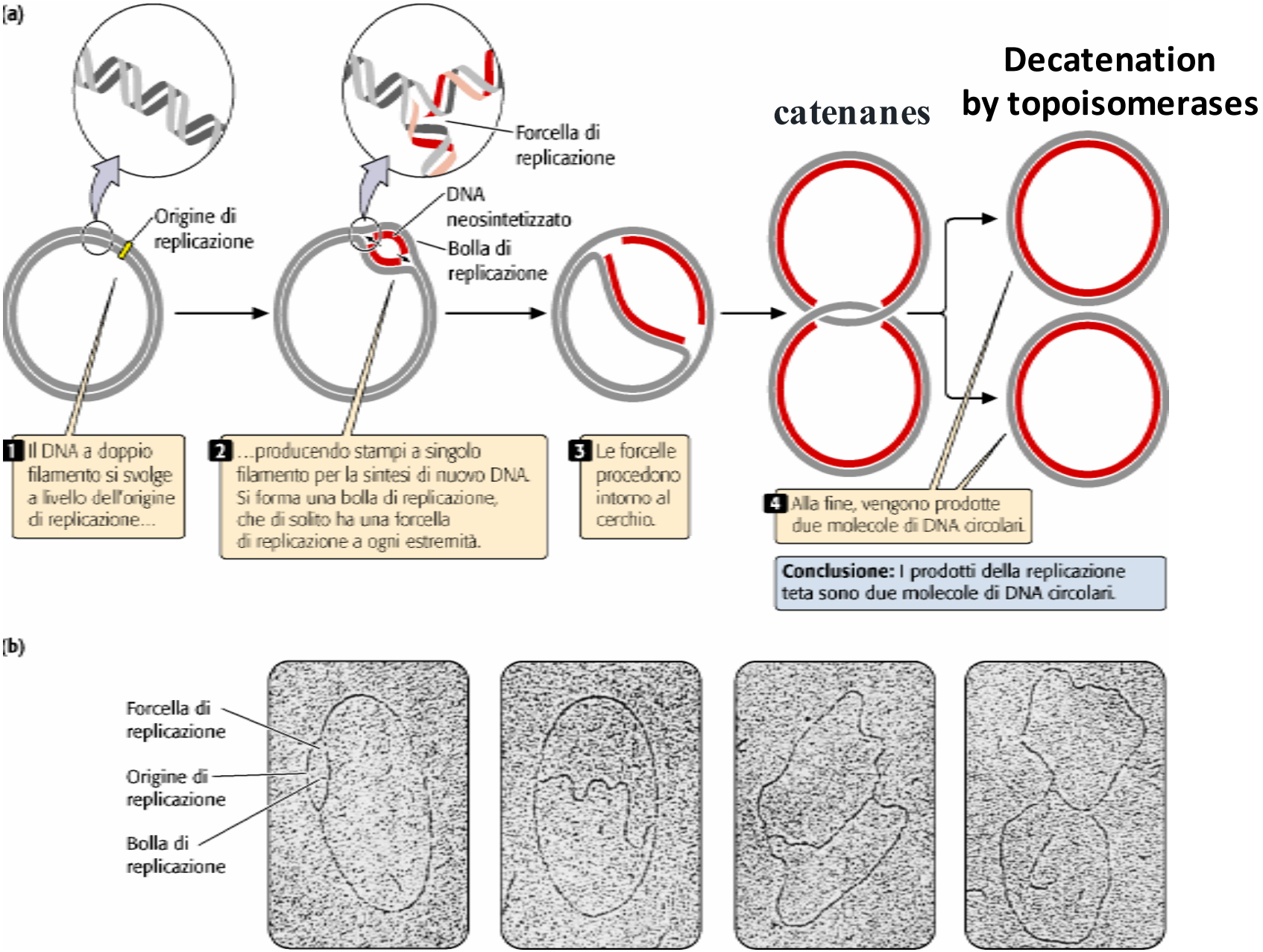
Replication origins of plasmids control:

- **Il numero di copie / Copy number (High/low copynumber plasmids)**
- **Lo spettro d'ospite / Host spectrum (Broad/Narrow host spectrum) – host proteins needs!!**
- **I gruppi di incompatibilità / Incompability group (some plasmids cannot co-exist in bacteria**

Colicin: secreted to environment; bind receptor to pass through membrane and attack physiology (membrane depolarization, DNase, RNase) of cell. Plasmid holding bacteria express resistance protein from plasmid

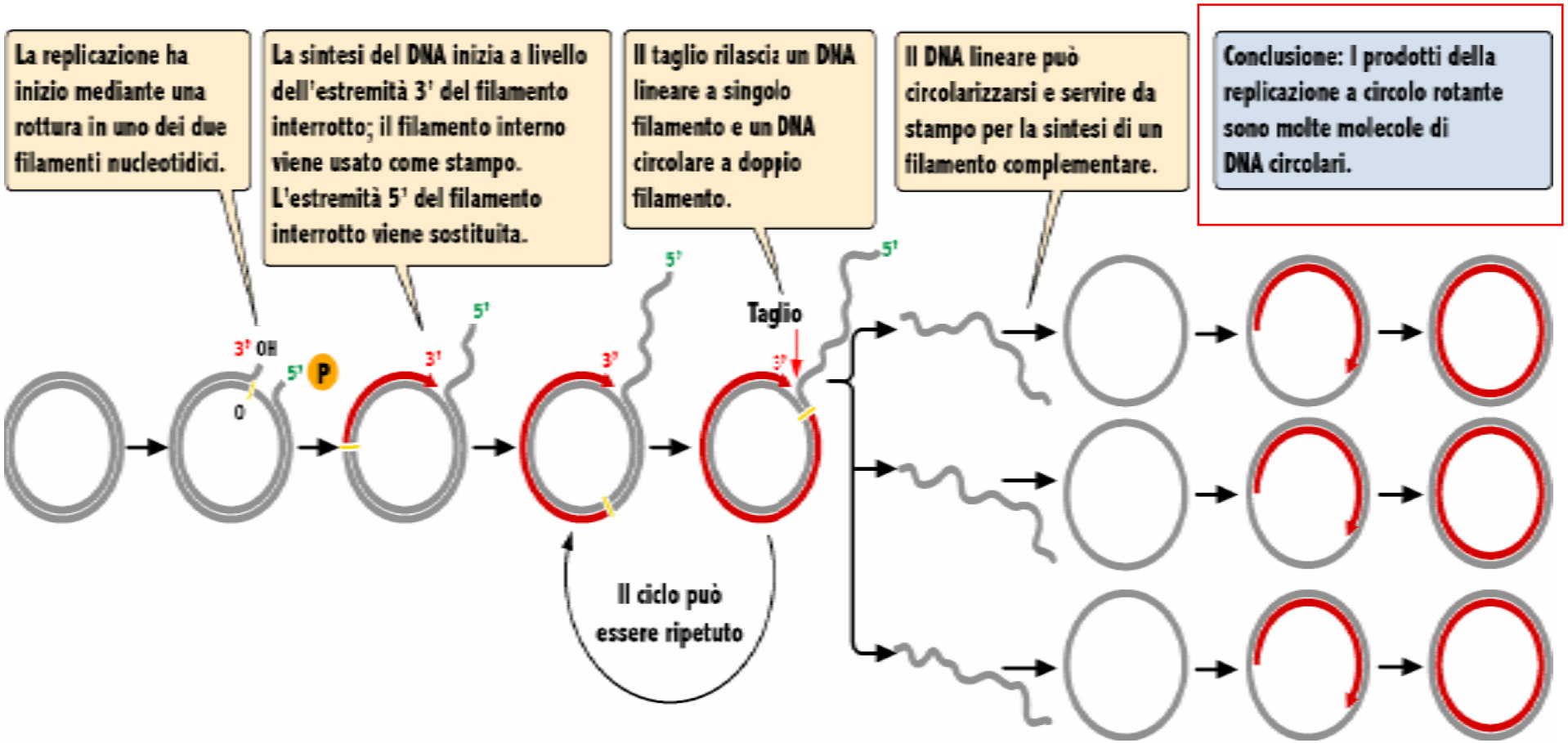
Replication of Plasmids

Type 1: La replicazione theta



Replication of Plasmids

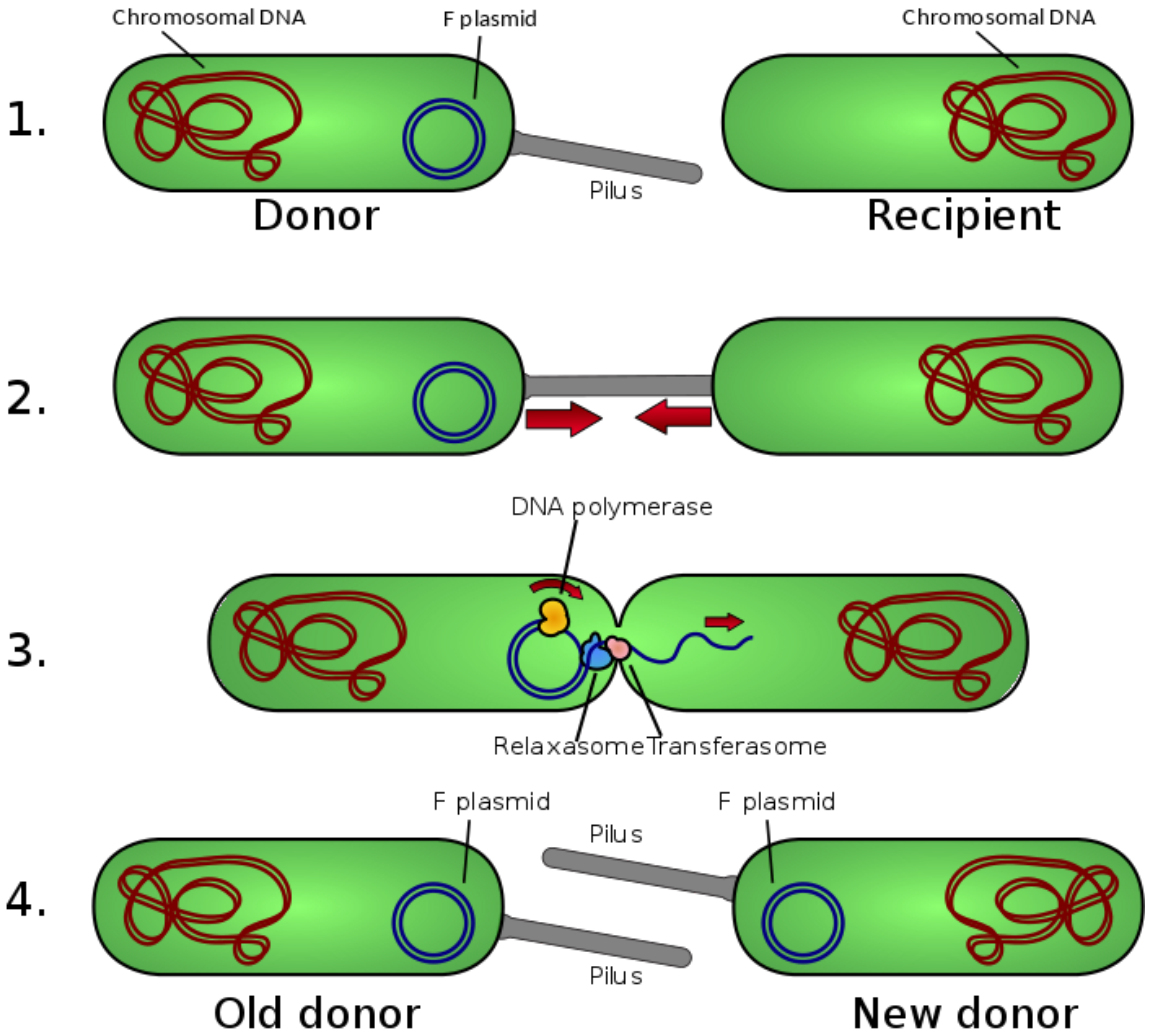
Type 2: La replicazione a circolo rotante (rolling circle)



Replication of Plasmids

Type 2: La replicazione a circolo rotante (rolling circle)

Rolling circle DNA replication is linked with horizontal transfer of plasmids (mobility)



The F-plasmid:
Donor cell produces pilus.

Pilus attaches to recipient cell and brings the two cells together.

The mobile plasmid is nicked and a single strand of DNA is then transferred to the recipient cell.

Both cells synthesize a complementary strand to produce a double stranded circular plasmid and also reproduce pili; both cells are now viable donor for the F-factor.

The F-plasmid is an episome (a plasmid that can integrate itself into the bacterial chromosome by homologous recombination) with a length of about 100 kb. It carries its own origin of replication, the oriV, and an origin of transfer, or oriT.[4] There can only be one copy of the F-plasmid in a given bacterium, either free or integrated, and bacteria that possess a copy are called F-positive or F-plus (denoted F+). Cells that lack F plasmids are called F-negative or F-minus (F−) and as such can function as recipient cells.

oshua Lederberg and Edward Tatum in 1946

Classic semiconservative replication of plasmids - oriV

engeneered plasmid

Natural plasmid

Common Vectors	Copy Number+	ORI	Incompatibility Group	Control
pUC	~500-700	pMB1 (derivative)	A	Relaxed
pBR322	~15-20	pMB1	A	Relaxed
pET	~15-20	pBR322	A	Relaxed
pGEX	~15-20	pBR322	A	Relaxed
pColE1	~15-20	ColE1	A	Relaxed
pR6K	~15-20	R6K*	C	Stringent
pACYC	~10	p15A	B	Relaxed
pSC101	~5	pSC101	C	Stringent
pBluescript	~300-500	ColE1 (derivative) and F1**	A	Relaxed
pGEM	~300-500	pUC and F1**	A	Relaxed

OriV from natural plasmids that proved to ensure good plasmid replication: classic: pMB1, ColE1

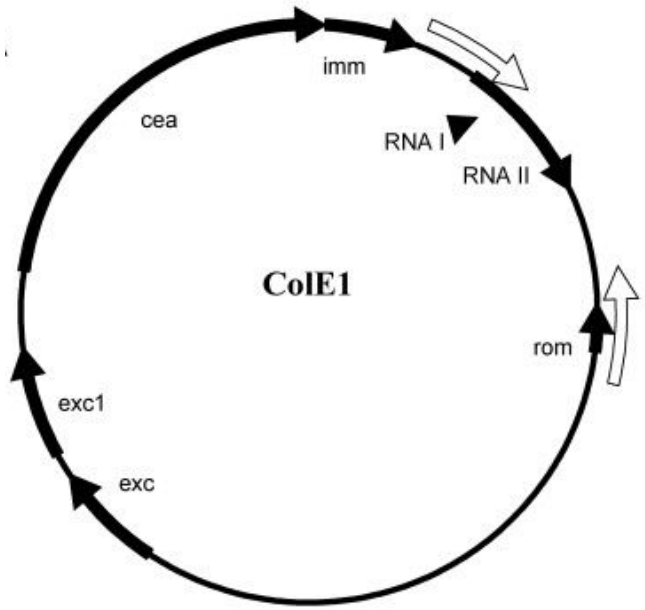
Plasmids (vectors) commonly used in the laboratory contain oriV from native plasmids.

OriV sequencens can be improved by mutation (pUC contains pMB1 oriV with 1 or 2 mutations

Note: pMB1 is a close relative of the ColE1 plasmid

Replication and copy number control of plasmids

Example: ColEI found in bacteria. Its name derives from the fact that it carries a gene for colicin E1 (the **cea** gene); Colicins are released into the environment to reduce competition from other bacterial strains. ColE1 also codes for immunity from this product with the **imm** gene. In addition, the plasmid has a series of mobility (mob) genes. Replication is controlled by the **expression of RNAs** across the oriV and the plasmid encoded **Rop** protein



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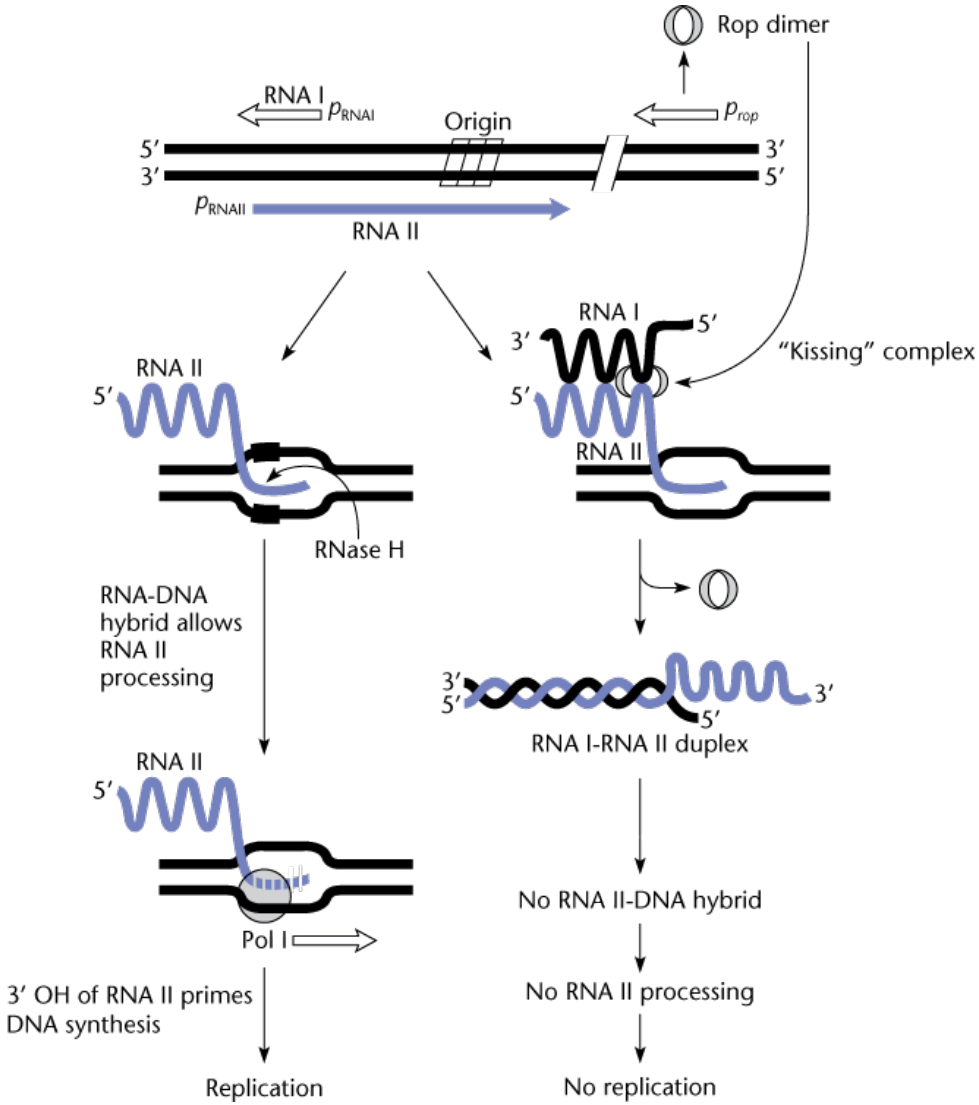
pColE1	~15-20	ColE1	A	Relaxed
pR6K	~15-20	R6K*	C	Stringent
pACYC	~10	p15A	B	Relaxed
pSC101	~5	pSC101	C	Stringent
pBluescript	~300-500	ColE1 (derivative) and F1**	A	Relaxed
pGEM	~300-500	pUC and F1**	A	Relaxed

Replication and copy number control of plasmids

ColEI oriV is used for many laboratory plasmids



Question: What happens to ColEI when bacteria grow (short term) in media with inhibitors of translation

ColE1 Replication control - an example of primer control of replication

1.Transcription by RNA II produces RNA primers for start of DNA replication.

2.RNA II remains as RNA:DNA hybrid that is processed by host RNaseH1 (digest RNA component of RNA:DNA hybrids)

3.Processed RNA II acts as a primer for host DNA Pol I → plasmid copies increase.

4.As the concentration of plasmid increases, plasmid encoded Rop protein and RNA I increase

5. At a specific Rop + RNA I threshold a Rob dimer stabilizes the RNA I-II RNA:RNA duplex; no RNA II:DNA hybrid formation

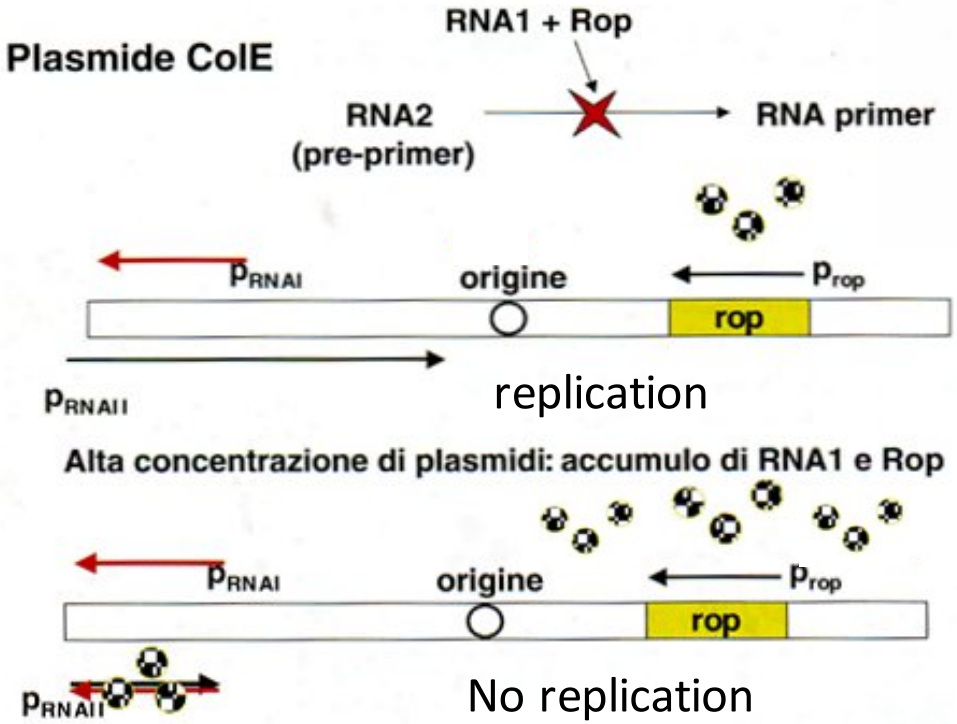
6.No RNA primers available prime DNA replication..

7.Copy number controlled

Replication and copy number control of plasmids

How to increase copy number of laboratory plasmids??

INTRODUCTION OF MUTATIONS IN Rop



La funzione di Rop

Rop controlla negativamente la replicazione plasmidica:
aumenta il tasso di legame di RNAI ad RNAII.

Rop assente → Replicazione plasmidica più frequente

pBR322 rop⁺ 15 copie/cellula

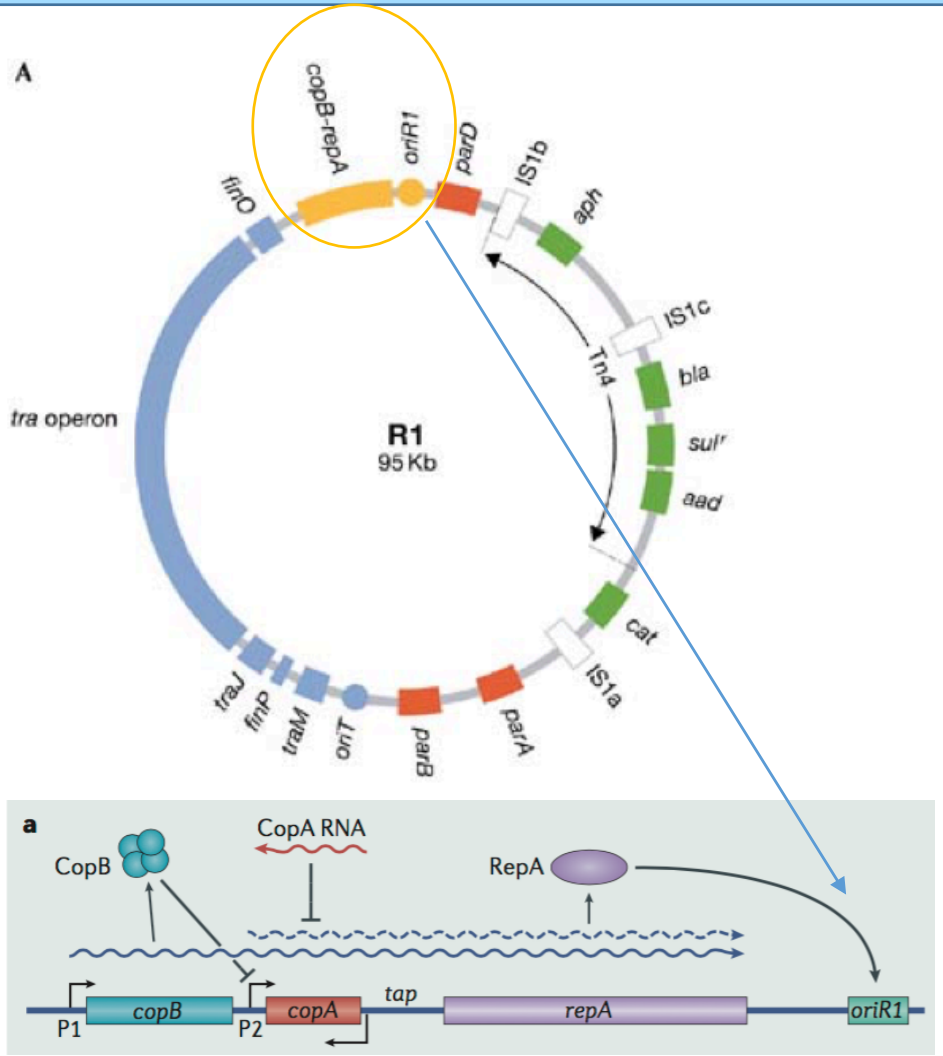
derivativo di pBR322
costruito per delezione

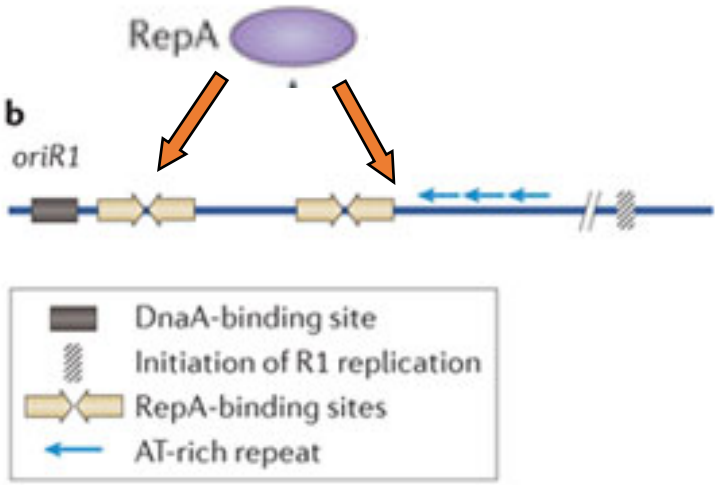
pUC18 Δrop 50-100 copie/cellula

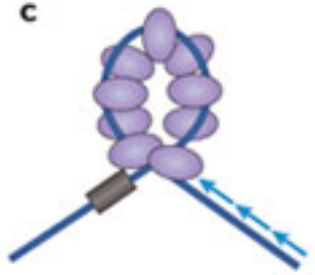
Note:
pBR322 is an engeneered plasmid containing the pMB1 oriV with wt rop. (<20 copies)
pUC18 contains pMB1 oriV with rop deletion → copy number increase. (<700 copies)

Note: pMB1 is a close relative of ColE1 sharing the same oriV function
(both are in the same compatibility group)

Replication and copy number control of plasmids







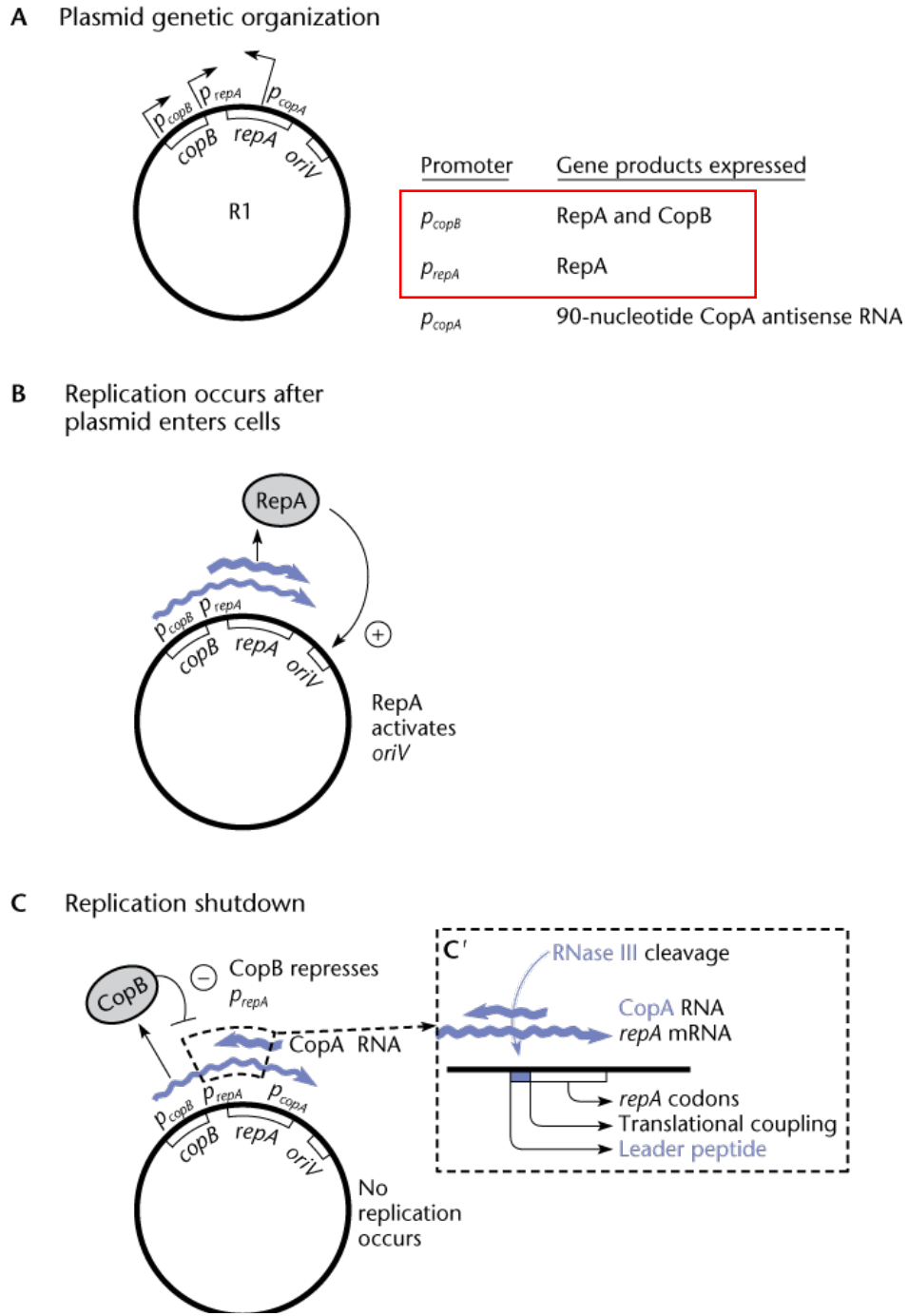
(**A**) A map of R1 showing antibiotic resistance genes (green), insertion sequences (white), its basic replicon (yellow), conjugation genes (blue) and stability systems (red).

R1 plasmid: replication controlled by the plasmid encoded RepA protein

Plasmid R1 provides a well-studied model for replication systems of enteric plasmids. In this plasmid, the replication initiator RepA binds to the origin site, oriR1, which lies downstream of repA (see the figure, part a). This oriR1 site contains binding sites for RepA flanked by a DnaA box at one end and three AT-rich repeats at the other (see the figure, part b). DnaA is not essential for replication of this plasmid, but seems to have an accessory role. DNA loop formation, mediated by RepA (see the figure, part c), is thought to drive DNA melting at the AT-rich region, which allows DnaC to load the replicative DNA helicase, DnaB. Replication initiates 400 nucleotides downstream of this site.

Replication and copy number control of plasmids

Example:
R1 plasmid



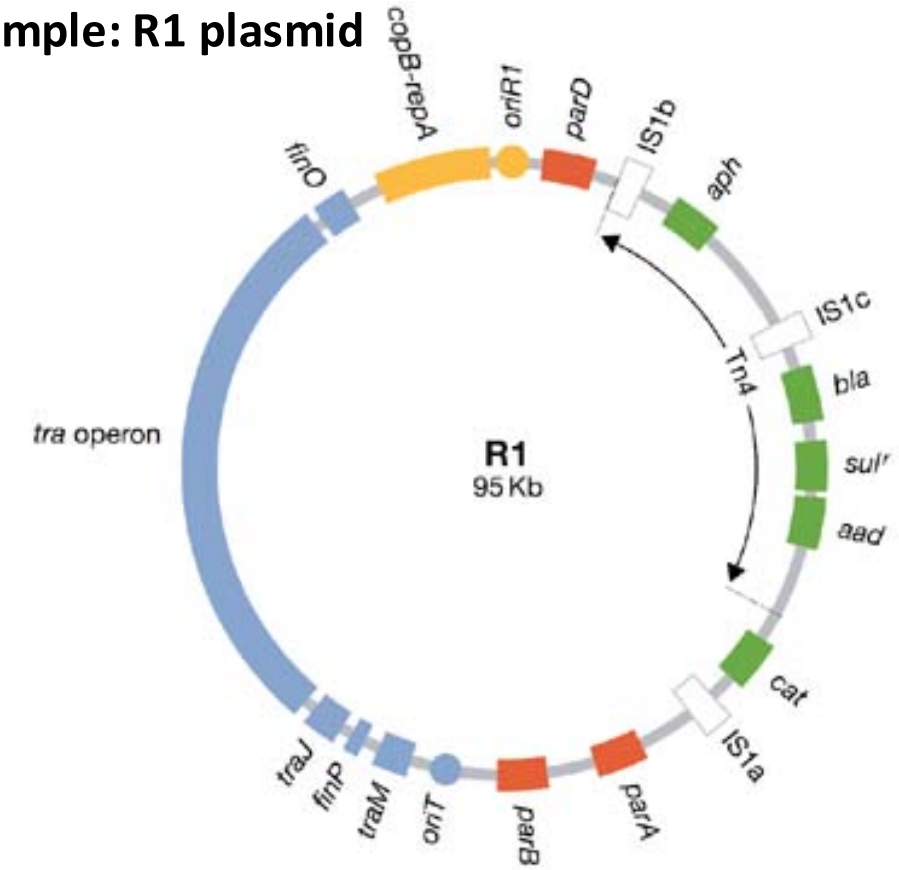
The events upon entry into a cell

1. Pomoter PcopB primes the transcription of RepA and copB mRNA. mRNA + protein levels continuously increase.
2. CopB expression increase **and CopB represses RepA expression at PrepA**
3. From, pCopA now a 90base antisense RNA is produced
4. short RNA CopA binds to 5-end of the RepA mRNA, forming dsRNA
5. This is recognized by host RNAaseIII and degraded.
6. No RepA proteins → no initation at oriV
7. **ATTENTION:** Bacteria devides; CopB reduced to 50% → go to point 1

→ → concentration of RepA protein is maintained by rate of RNA:RNA duplex formation.

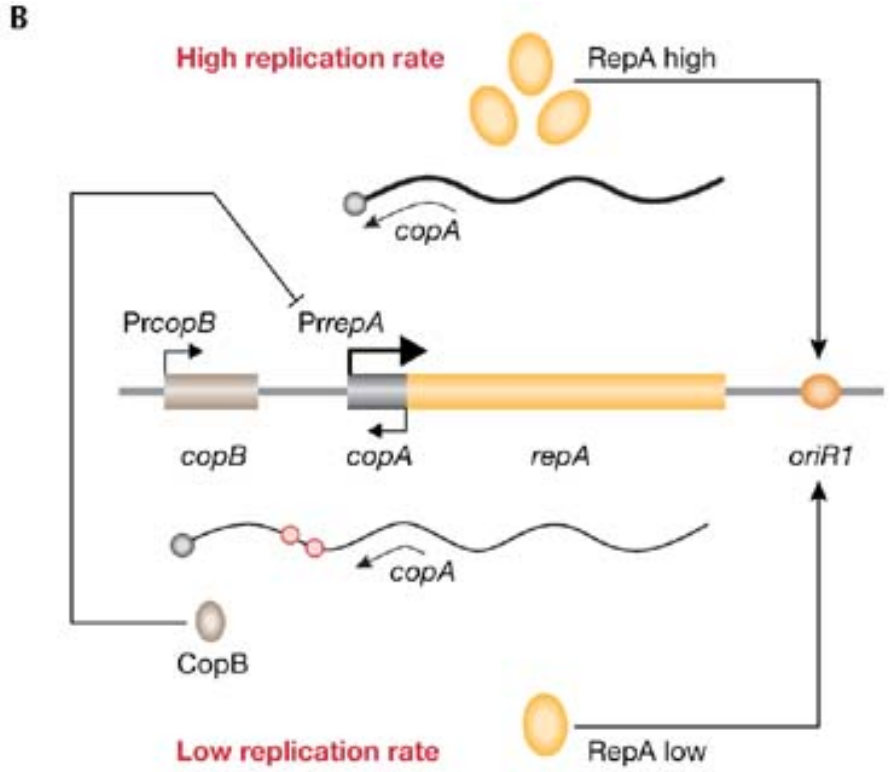
Replication and copy number control of plasmids

Example: R1 plasmid



Guillermo de la Cueva-Méndez, and Belén Pimentel EMBO Rep. 2007;8:458-464

Note: here, R1 oriV is called "oriR1"



R1 and copy-number control. (**A**) A map of R1 showing antibiotic resistance genes (green), insertion sequences (white), its basic replicon (yellow), conjugation genes (blue) and stability systems (red). (**B**) Pr*copB* produces some RepA as well as CopB, a repressor of Pr*repA*, which keeps R1 copy number low. In the absence of CopB, stronger Pr*repA* increases RepA and R1 copy number. Antisense RNA *copA* limits translation of RepA and is less effective when Pr*repA* is active. Red circles on RNA denote UUACU sites. *Cop*, copy-number control gene; ori, origin of replication; Pr, promoter; Rep, replication initiation factor.
UUACU sites: can be cleaved by RNAse (additional mechanisms of regualtion; not releant for our lecture)

3. MAINTENANCE OF PLASMIDS IN BACTERIA

A. Plasmid partition systems – for low copy plasmids (Example R1 plasmid)

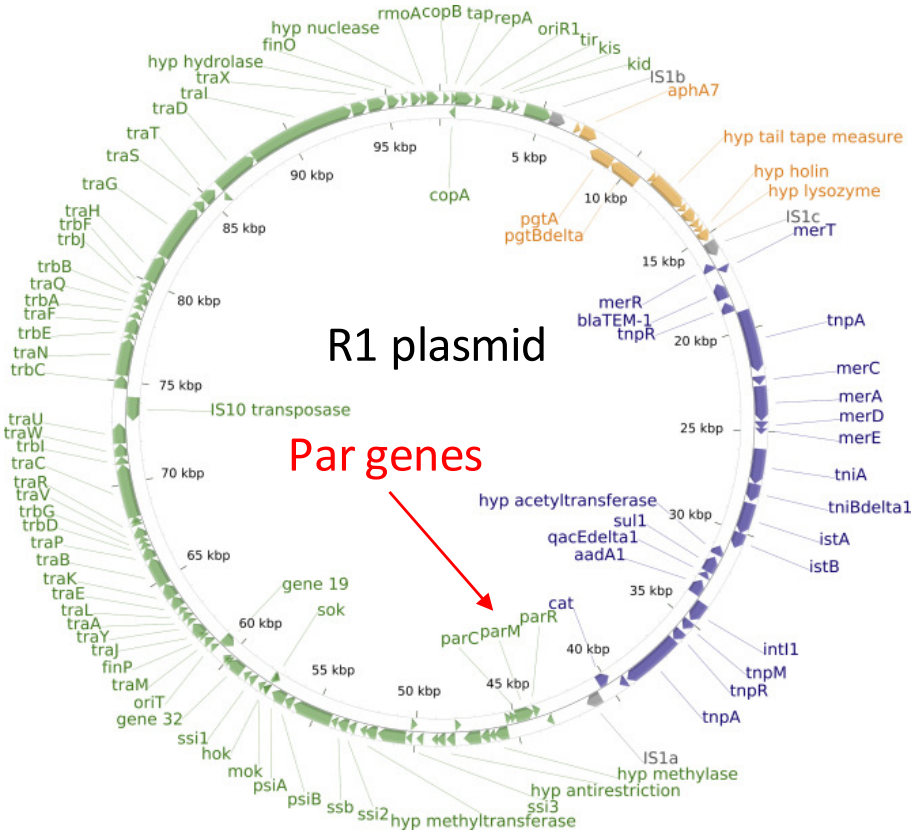
B. Toxin – Antitoxin systems

C. High (medium) copy number plasmids

Plasmid copies are paired around a centromere-like site and then separated in the two daughter cells. Partition systems involve three elements, organized in an auto-regulated operon:

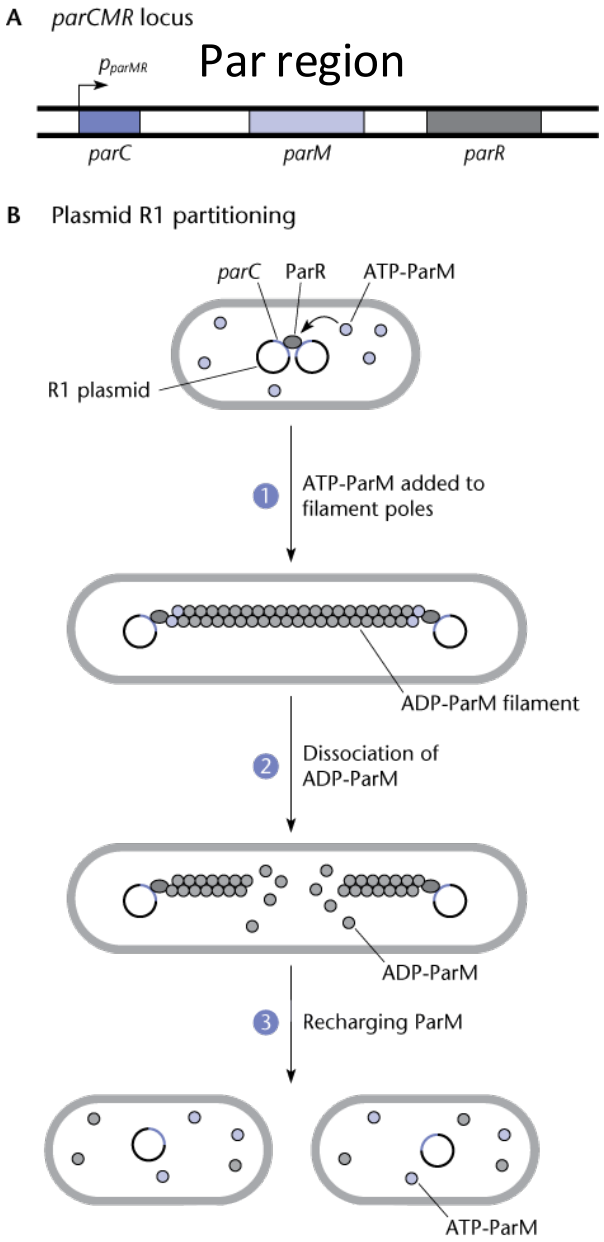
1. A centromere-like DNA site
2. Centromere binding proteins (CBP)
3. The motor protein

The centromere-like DNA site is required in cis for plasmid stability. It often contains one or more inverted repeats which are recognized by multiple CBPs. This forms a nucleoprotein complex termed the partition complex. This complex recruits the motor protein, which is a nucleotide triphosphatase (NTPase). The NTPase uses energy from NTP binding and hydrolysis to directly or indirectly move and attach plasmids to specific host location (e.g. opposite bacterial cell poles).



Maintenance of plasmids in bacteria

A. Plasmid partition systems – for low copy plasmids (Example R1 plasmid)



Stabilità segregativa (funzione *par*)

ParM binds to DNA-binding proteins, called ParR that bind centromer like DNA sequences on plasmid (parC)

Sister plasmid segregation is achieved through bidirectional insertional polymerization of the ParM-ATP forming filaments.

ATP hydrolysis results ADP-ParM → depolymerization of filament.
Bacteria can complete cell division.

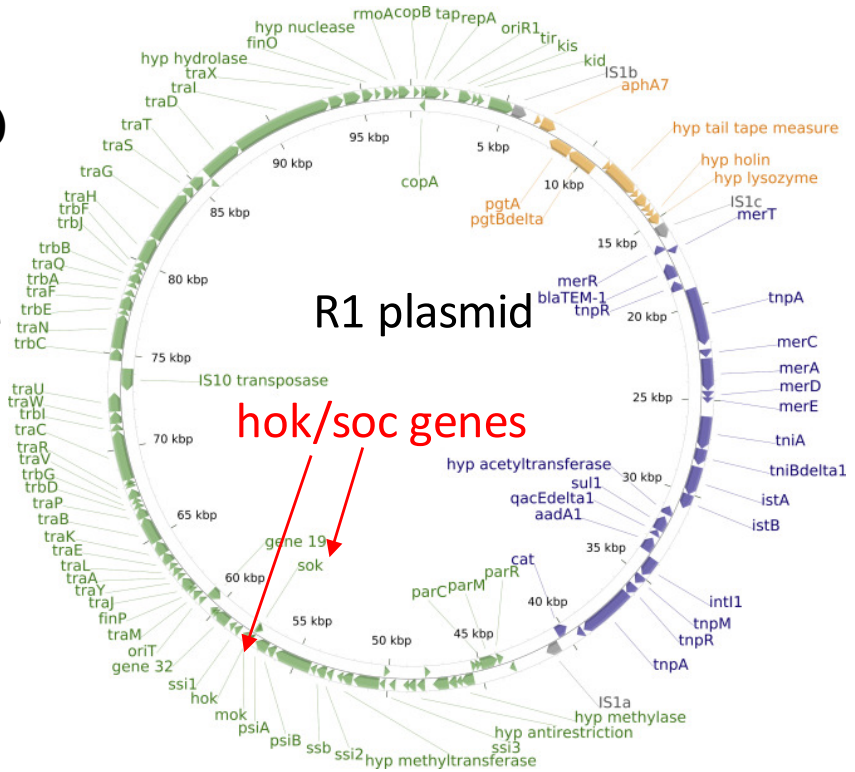
Maintenance of plasmids in bacteria

B. Toxin – Antitoxin systems

hok –sok system of R1 plasmid

Il plasmide R1(o R100) porta un gene letale *hok* (host cell killing) che codifica per una tossina in grado di provocare depolimerizzazione delle membrana.
Sull'elica complementare del DNA di *hok* viene trascritta il mRNA del gene *sok* che ha una una regione di 128 nt complementare con hok (non SD di *hok*). Repressione della traduzione di hok.
I 2 RNA hanno diversa emivita 20 min e 1 min.

- **Hok non viene mai tradotto per azione del mRNA di sok e la cellula con R1 rimane pertanto vitale.**
- **Se una cellula non eredita R1 in seguito a divisione allora mRNA sok che ha una lunga emivita verrà tradotto perchè mRNA sok avendo un emivita più breve non sarà più presente.**



mRNA hok emivita 20 min

depolimerizzazione delle membrana. Proteina 52 amminoacidi,

mRNA sok emivita 1 min

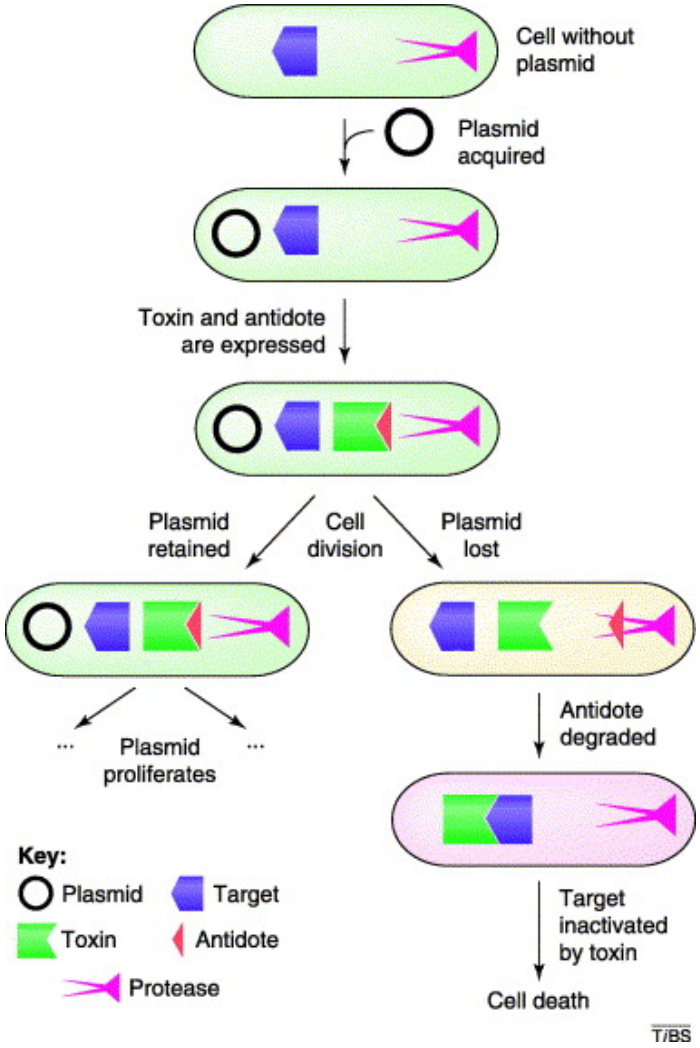
Plasmide R1=R100

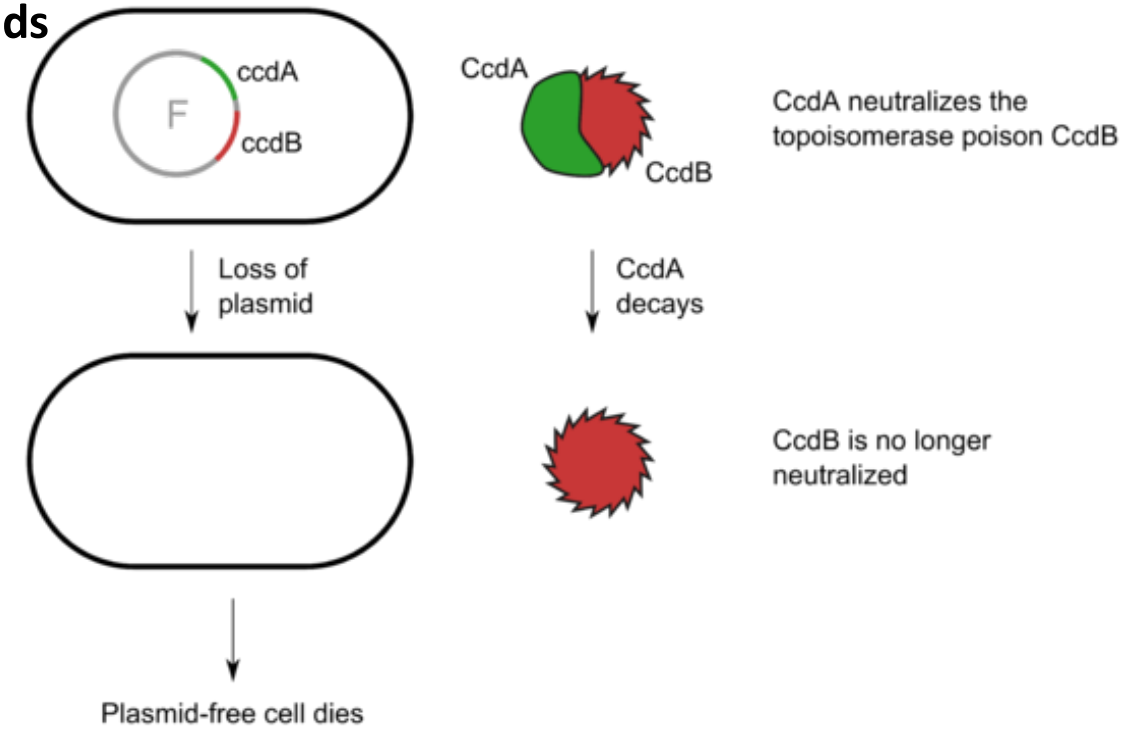
Maintenance of plasmids in bacteria

A. Plasmid partition systems

B. Toxin – Antitoxin systems (F1 plasmid)

C. High (medium) copy number plasmids





Il plasmide F sintetizza un sistema basato su tossina-antitossina in grado di eliminare le cellule che, in seguito ad un errore nella divisione cellulare non hanno ricevuto almeno una copia del plasmide F. La proteina **CcdB è una tossina stabile (con bersaglio la DNA girasi)** la cui funzione viene bloccata dal legame con un **antitossina CcdA più facilmente degradabile**. Se il plasmide è presente la continua sintesi di CcdA inibisce CcdB. Se non vi è plasmide invece CcdA verrà degradata + velocemente di CcdB che rimarrà quindi libera e potrà inibire la girasi provocando la morte delle cellule

Maintenance of plasmids in bacteria

A. Plasmid partition systems

B. Toxin – Antitoxin systems

C. High (medium) copy number plasmids

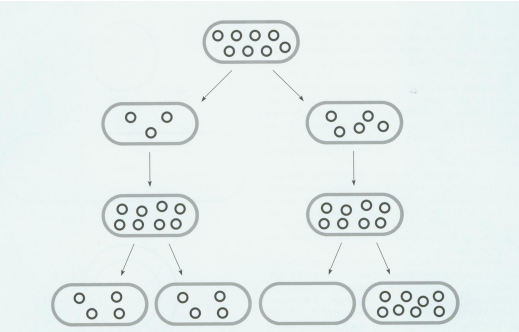
Typically used in laboraotry (in E. coli)

I plasmidi ad alto numero di copie si ripartiscono secondo due modalità:

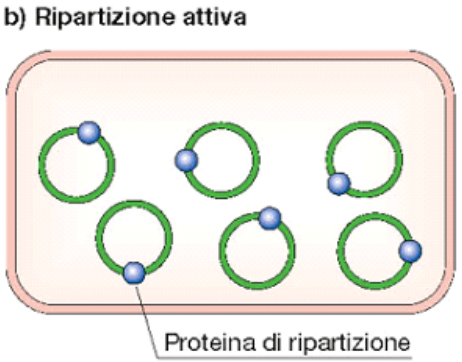
1. **ATTIVA**
2. **STOCAISTICA** o casuale

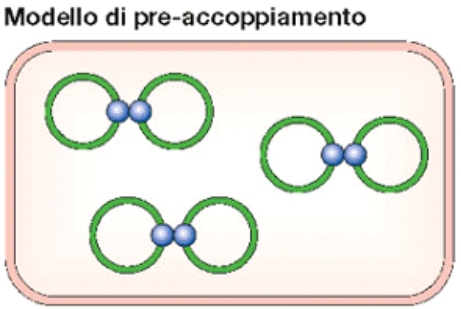
1. RIPARTAZIONE ATTIVA: Nel caso della ripartizione attiva i plasmidi vengono riconosciuti da una proteina che dimerizzando forma delle coppie di plasmidi.
La struttura DNA –proteina-DNA si localizzerà a livello del sito di divisione garantendo cosi la corretta divisione tra le cellule

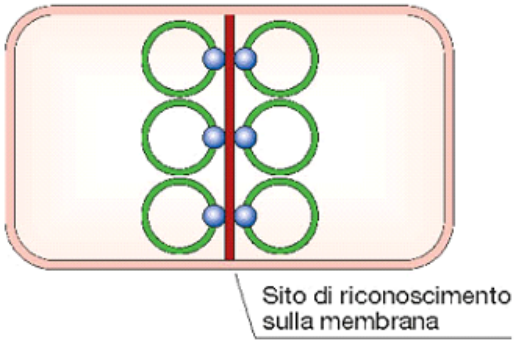
2. RIPARTAZIONE STOCAISTICA



Plasmids contain
Antibiotics resistance genes!!



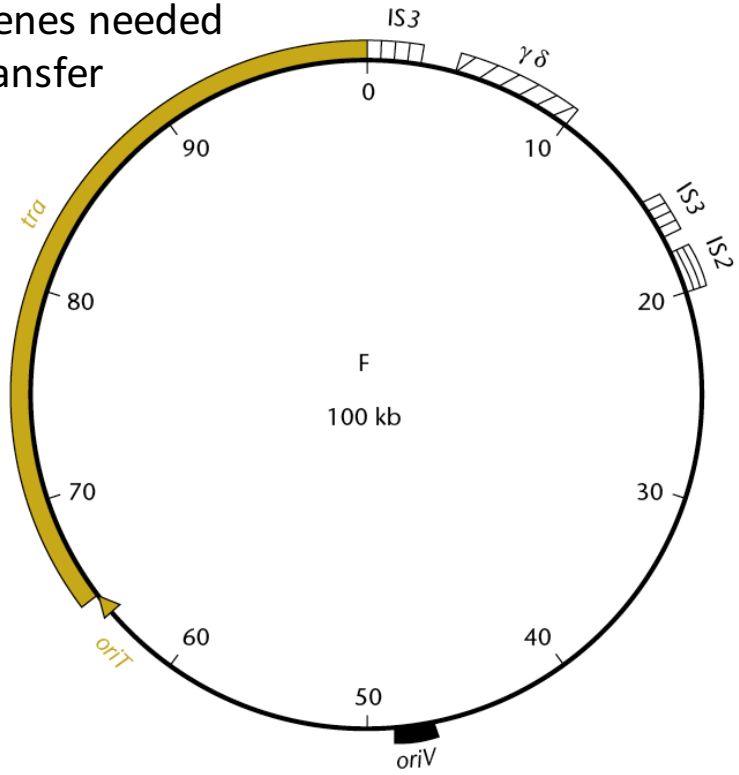




4. HORIZONTAL TRANSFER OF GENETIC INFORMATION

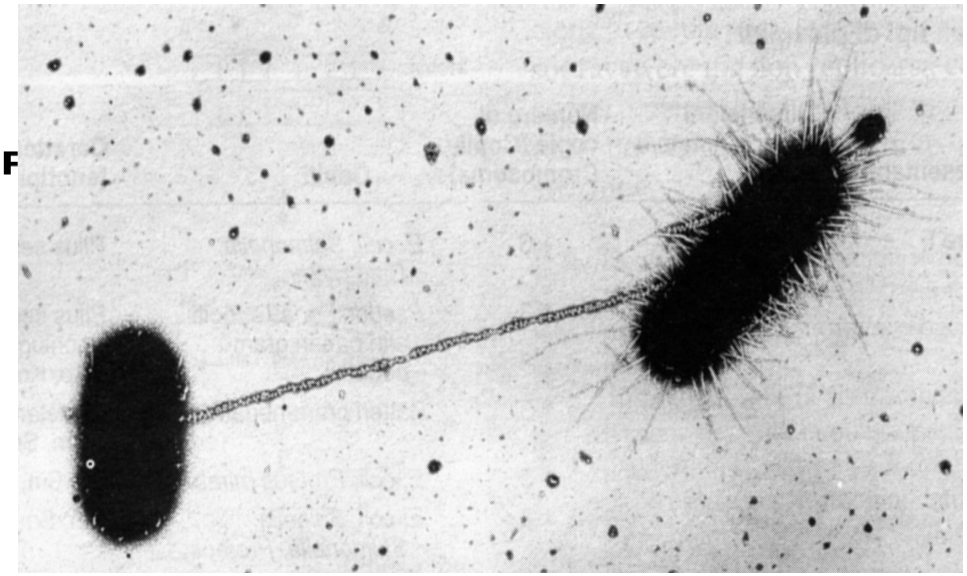
F plasmid

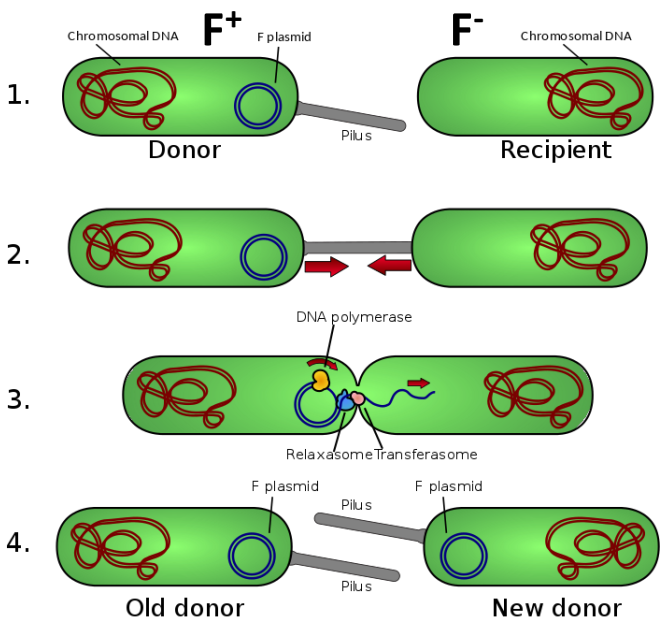
tra-region:
30+ genes needed for transfer



tra and trb locus encode proteins required for **conjugation** such as the pilin gene and regulatory genes, which together form pili on the cell surface

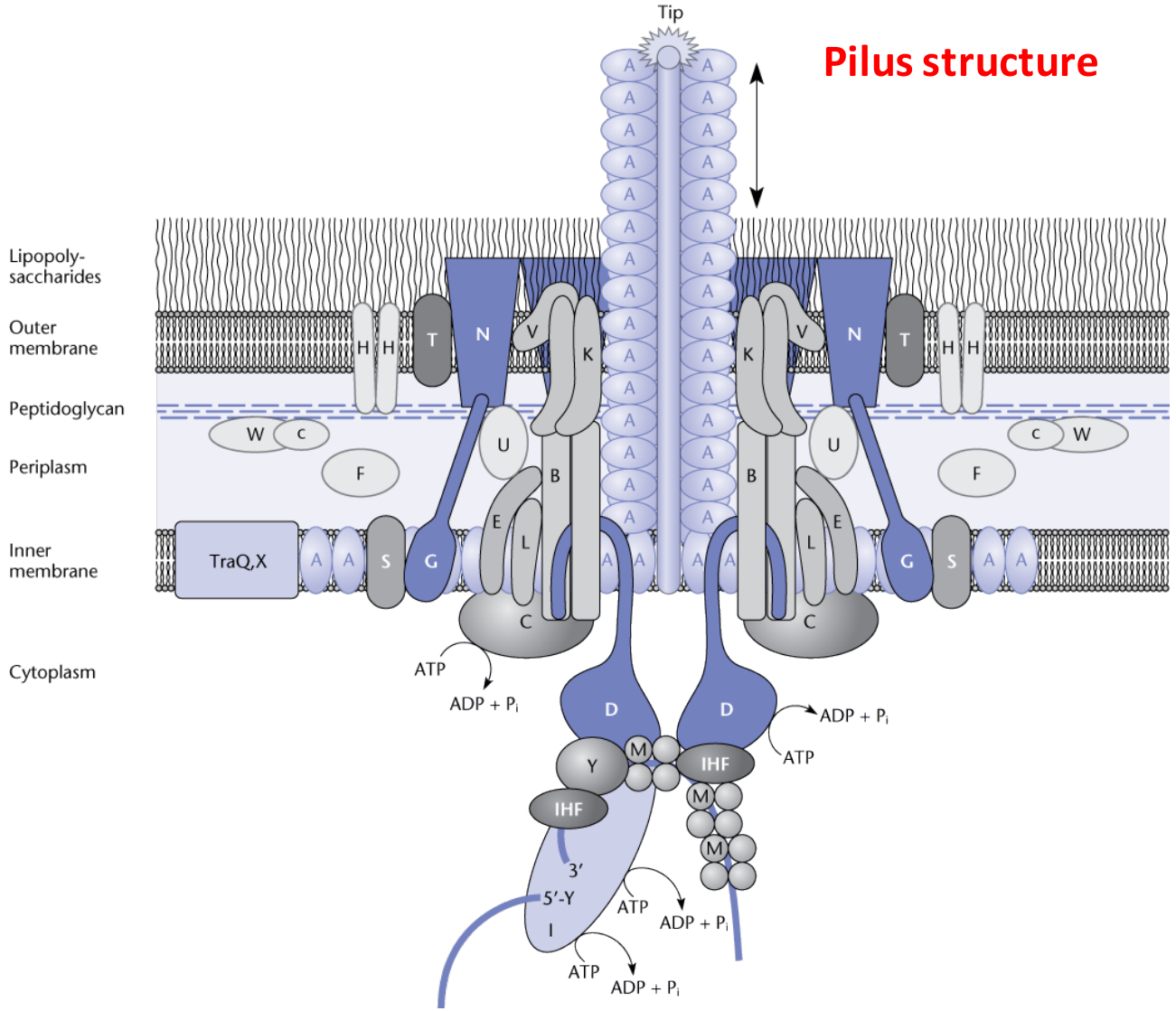
oriT: required for DNA transfer; location of nick for rolling cycle replication



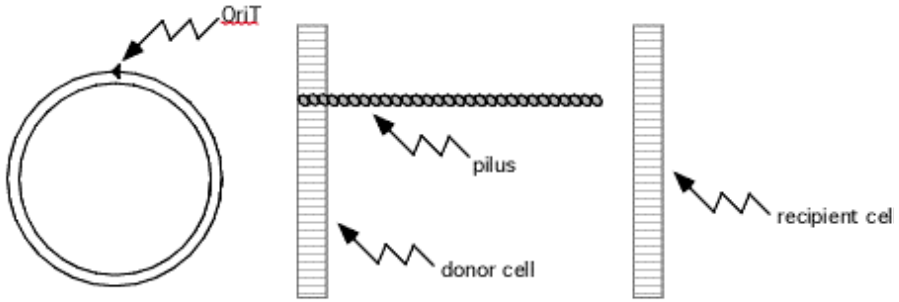


Horizontal transfer of genetic information

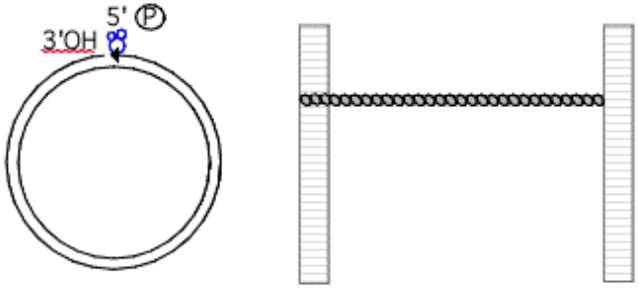
F plasmid



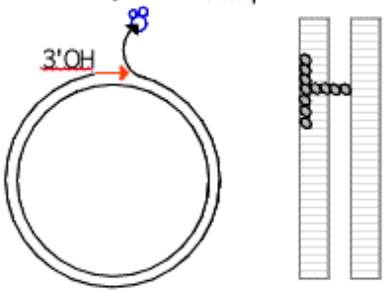
Horizontal transfer of genetic information



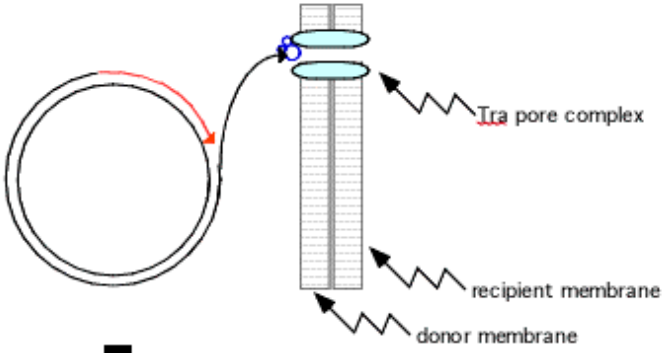
Contact between donor and recipient cells.
DNA relaxase ( ) nicks at oriT and covalently binds to 5' Ⓟ



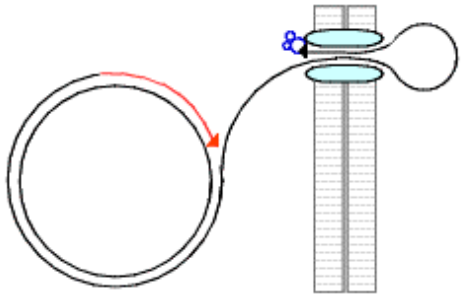
Pilus retracks, bringing donor and recipient into close proximity
and Tra proteins form a pore complex that spans the membranes



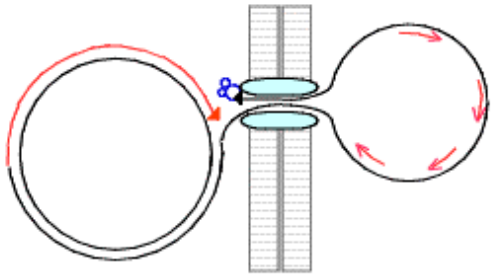
Rolling circle DNA replication initiates at 3'OH and proceeds 5' to
Membranes brought into close proximity to form mating bridge.
Relaxase interacts with membrane Tra pore complex



DNA replication pushes the ssDNA into the recipient cell



Lagging strand DNA replication in recipient cell converts ssDNA to dsDNA



Upon complete replication of plasmid, the old and new oriT sites
"collide", and nicking between oriT sites occurs

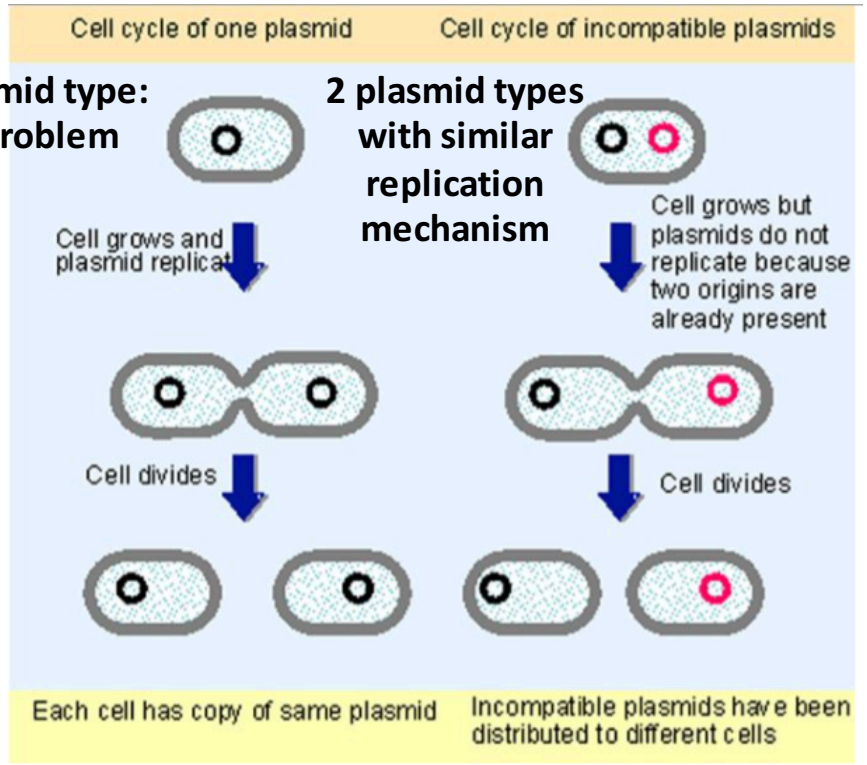
5. PLASMID INCOMPATIBILITY GROUPS

Incompatibilità tra plasmidi

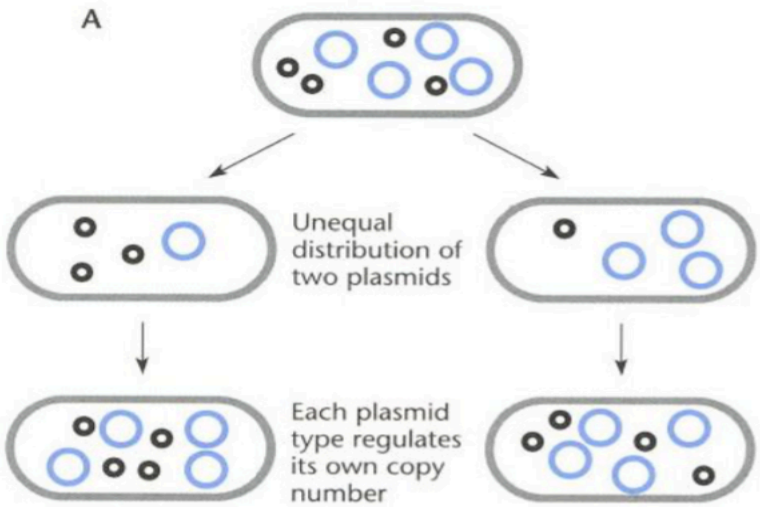
1.Not all plasmids can live together. –> plasmids are subdivided in **incompatibility groups**
2.Plasmids that are able to coexist in the same cell do not interfere with each other's **replication** (oriV) or **partitioning**

→ Plasmid with different oriV regulation can coexist (ColEI and R1 plasmid)
→ Plasmid with different partitioning system can co-exist

3. **A single cell can have as many Inc group plasmids as it can tolerate and replicate**!



2 plasmid types with different replication mechanism



Plasmid incompatibility

Laboraotry + natural plasmids

Common Vectors	Copy Number+	ORI	Incompatibility Group	Control
pUC	~500-700	pMB1 (derivative)	A	Relaxed
pBR322	~15-20	pMB1	A	Relaxed
pET	~15-20	pBR322	A	Relaxed
pGEX	~15-20	pBR322	A	Relaxed
pColE1	~15-20	ColE1	A	Relaxed
pR6K	~15-20	R6K*	C	Stringent
pACYC	~10	p15A	B	Relaxed
pSC101	~5	pSC101	C	Stringent
pBluescript	~300-500	ColE1 (derivative) and F1**	A	Relaxed
pGEM	~300-500	pUC and F1**	A	Relaxed

Note: pMB1 and ColE1 origins have same mechanism if initiation

Note: pMB1 and R6K have origins with different mechanism if initiation

Plasmid incompatibility

Laboraotry + natural plasmids

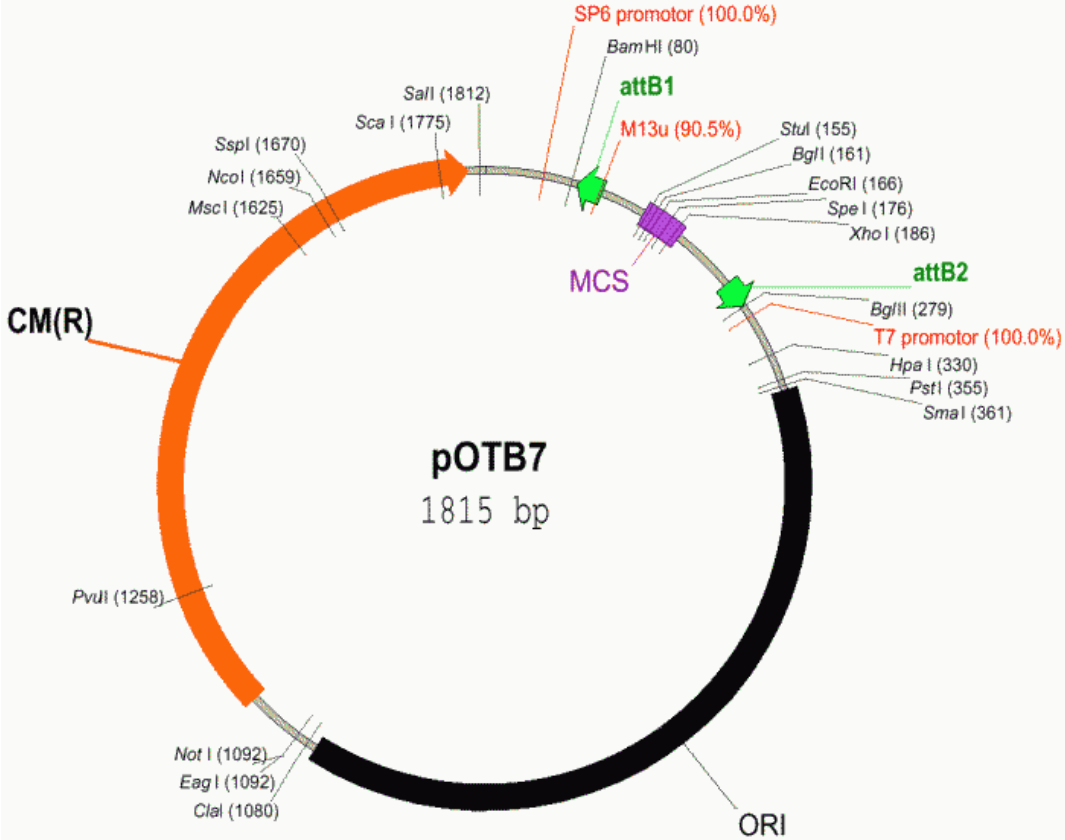
Common Vectors	Copy Number+	ORI	Incompatibility Group	Control
pUC	~500-700	pMB1 (derivative)	A	Relaxed
pBR322	~15-20	pMB1	A	Relaxed
pET	~15-20	pBR322	A	Relaxed
pGEX	~15-20	pBR322	A	Relaxed
pColE1	~15-20	ColE1	A	Relaxed
pR6K	~15-20	R6K*	C	Stringent
pACYC	~10	p15A	B	Relaxed
pSC101	~5	pSC101	C	Stringent
pBluescript	~300-500	ColE1 (derivative) and F1**	A	Relaxed
pGEM	~300-500	pUC and F1**	A	Relaxed

THIS IS IMPORTANT IN CASES 2 DIFFERENT PROTEINS NEED TO BE EXPRESSED FROM A PLASMID IN BACTERIA

→ PLASMIDS NEED TO BE IN DIFFERENT INCOMPABILITY GROUPS

6. INTRODUCTION IN LABORATORY PLASMIDS

- Origin of replication
- Antibiotic resistance gene (Amp, Kan, Tet, Chl)
- Multiple cloning site



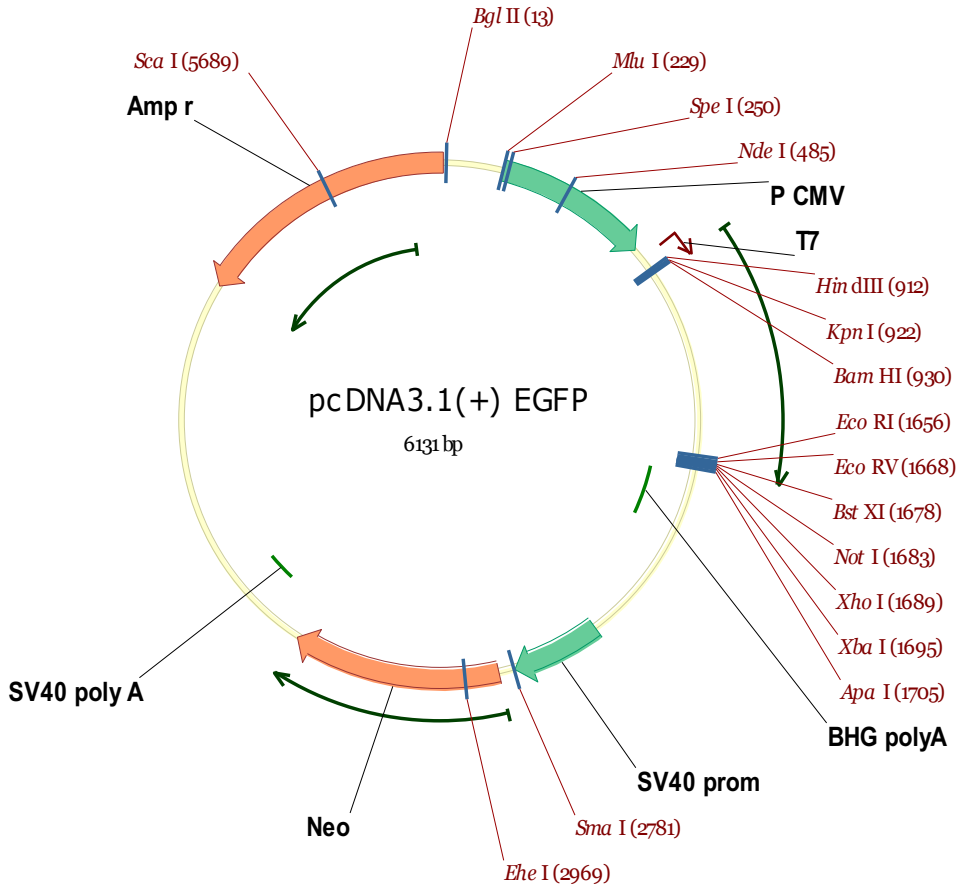
Map of pOTB7 vector showing Chloramphenicol resistance gene (CMR), replication origin (ORI) and multiple cloning site (MCS)

TO MAINTAIN PLASMID IN BACTERIA, CELLS ARE GROWN ON AGAR CONTAINING CHRLORAMPHENICOL ONLY BACTERIA THAT CARRY PLASMID CAN SURVIVE

LABORATORY PLASMIDS = VECTORS

Optional plasmids elements

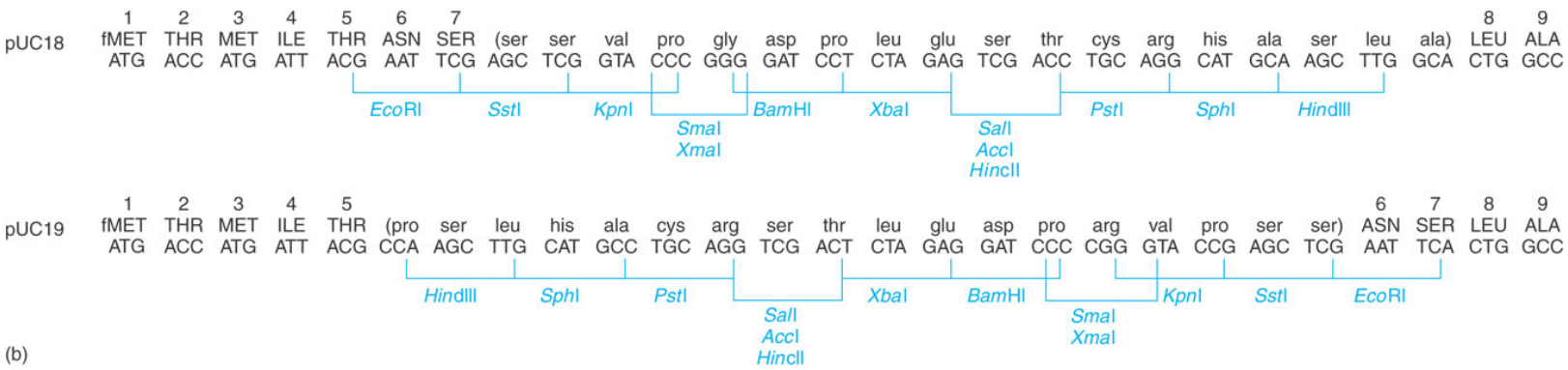
- Multiple cloning site
- Promoter for cloned sequence
- Reporter gene
- Tag
- Regulatory sequences for eukaryotic transcription
- Cassette for blue-white colony selection (lacZ)



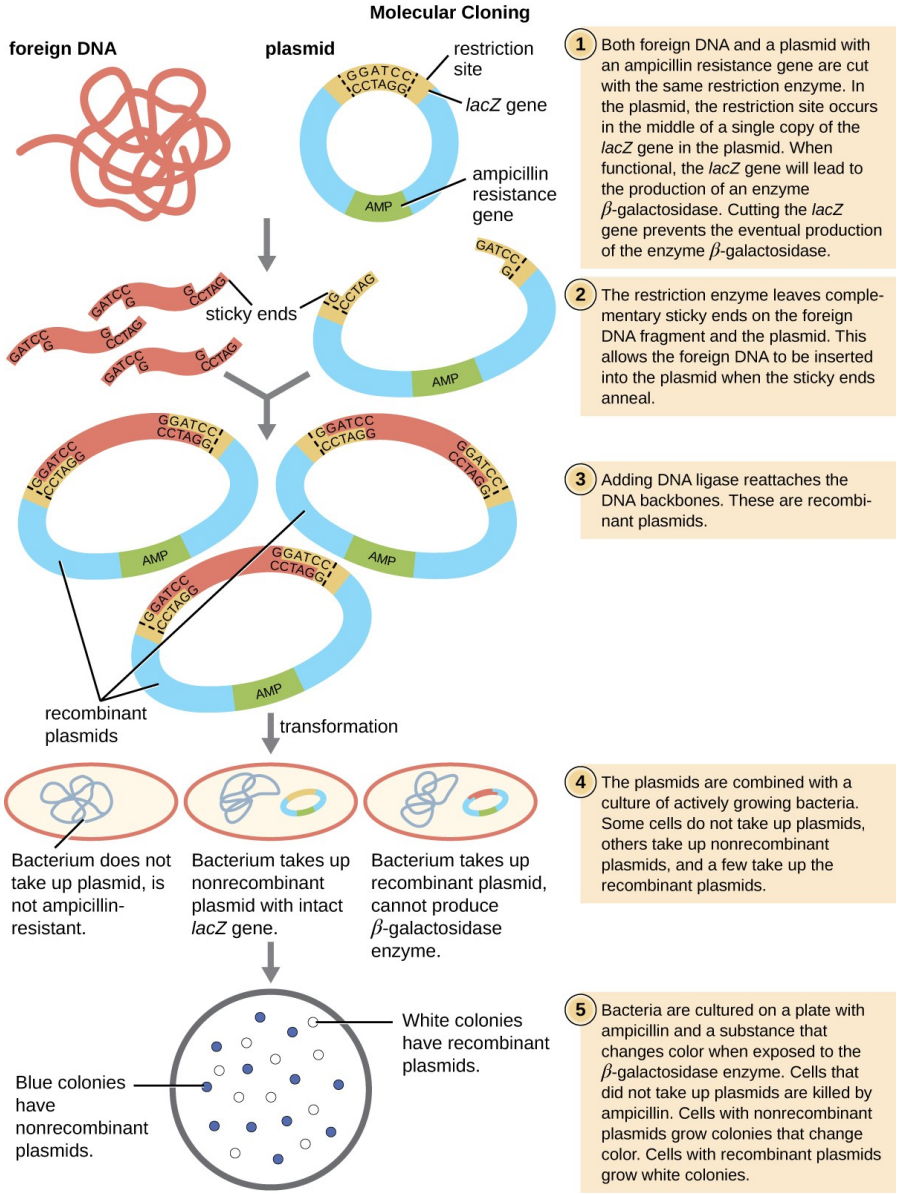
MULTIPLE CLONING SITE: ADVANTAGE

- **Unique sites (usually)**
- **Insert excision facilitated**
- **Restriction endonuclease mapping and subcloning made easier**

Figure 4.6



CLONING AND BLUE - WHITE SELECTION



1. Both foreign DNA and a plasmid with an ampicillin resistance gene are cut with the same restriction enzyme. In the plasmid, the restriction site occurs in the middle of a single copy of the *lacZ* gene in the plasmid. When functional, the *lacZ* gene will lead to the production of an enzyme *β*-galactosidase. Cutting the *lacZ* gene prevents the eventual production of the enzyme *β*-galactosidase.
2. The restriction enzyme leaves complementary sticky ends on the foreign DNA fragment and the plasmid. This allows the foreign DNA to be inserted into the plasmid when the sticky ends anneal.
3. Adding DNA ligase reattaches the DNA backbones. These are recombinant plasmids.
4. The plasmids are combined with a culture of actively growing bacteria. Some cells do not take up plasmids, others take up nonrecombinant plasmids, and a few take up the recombinant plasmids.
5. Bacteria are cultured on a plate with ampicillin and a substance that changes color when exposed to the *β*-galactosidase enzyme. Cells that did not take up plasmids are killed by ampicillin. Cells with nonrecombinant plasmids grow colonies that change color. Cells with recombinant plasmids grow white colonies.

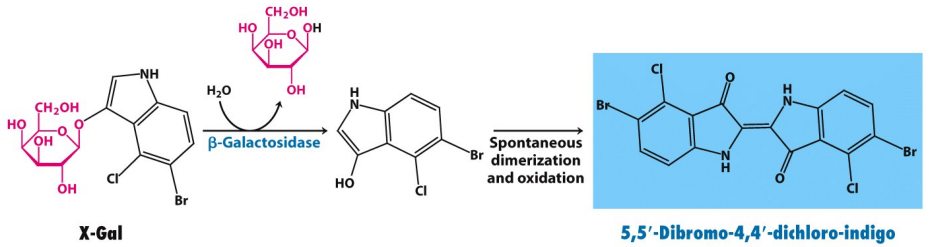
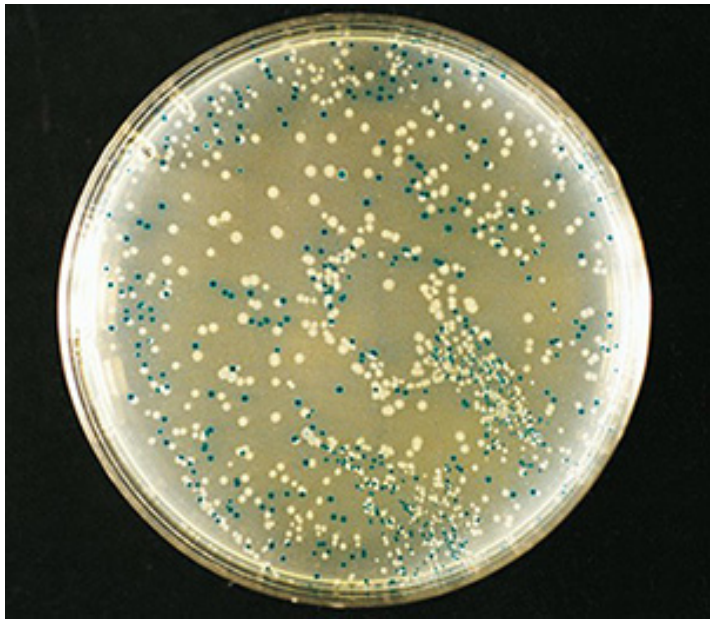


Figure 31.5
***Biochemistry*, Seventh Edition**



Agar containing Ampicillin and X-GAL
Blue: no insert
White: insert

A DEFINED VECTOR FOR EACH APPLICATION

- Cloning and sequencing of DNA and cDNA fragments
- Generation of genomic and cDNA libraries
- Expression of recombinant proteins
- Generation of mutant proteins
- Analysis of regulatory sequences
- Gene targeting