THE GENETICS AND USEFULNESS OF PLASMIDS

Paul A. Gulig

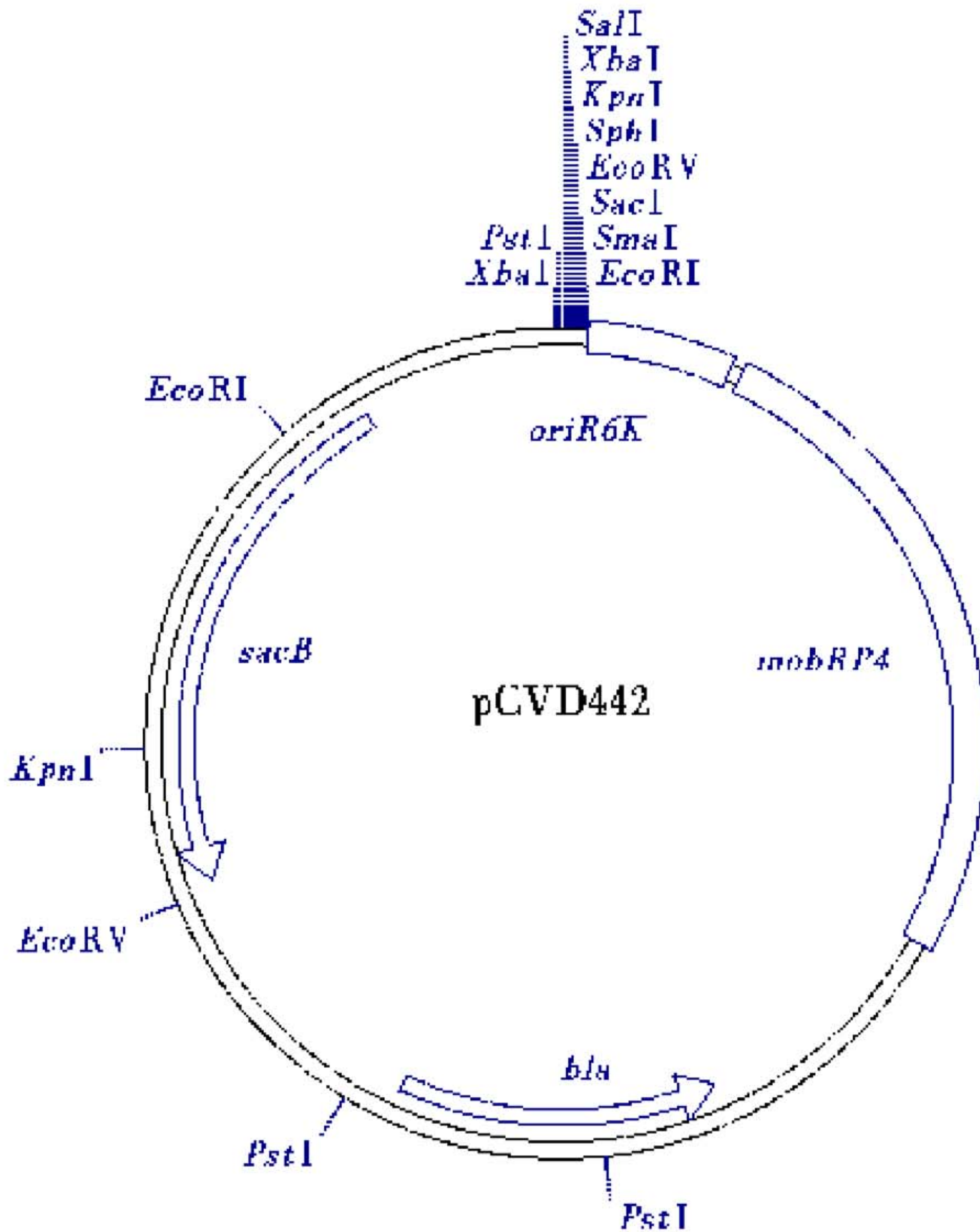
I. Plasmids - General

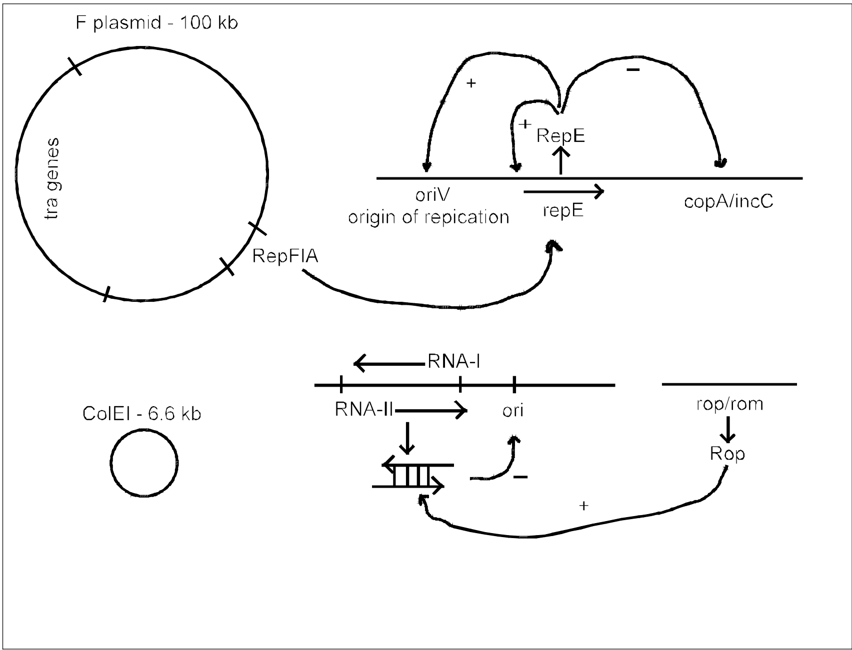
A. Extrachromosomal DNA, usually circular
B. Usually encode ancillary functions for in vitro growth
C. Can be essential for specific environments: virulence, antibiotics resistance, use of unusual nutrients, production of bacteriocins (colicins)
D. Must be a **replicon** - self-replicating genetic unit

II. Replication

A. Plasmid DNA must replicate every time host cell divides or it will be lost
B. Host cells do not “spit out” plasmid DNA
C. Two functions of replication
  1. **DNA replication**
  2. **partitioning** (making sure each progeny cells receives a plasmid)
D. **high copy** (>20) and **low copy** (<5) plasmids
E. high copy plasmids are usually small; low copy plasmids can be large
F. partitioning is strictly controlled for low copy, but loose for high copy
G. plasmid replication requires host cell functions (DNA polymerase, etc.)
H. copy number is regulated by **initiation** of plasmid replication
I. plasmids are **incompatible** when they cannot be stably maintained in the same cell because they interfere with each other’s replication.
J. Models of replication
  1. **F plasmid**
    a. large (100 kb)
    b. low copy (1-2 copies/cell)
    c. self transmissible
    d. requires protein synthesis (chloramphenicol-sensitive)
    e. *repE* gene encodes RepE protein
    f. RepE protein binds to origin of replication (*oriS*) and initiates DNA replication
    g. RepE binds to the *repE* promoter and activates transcription
    h. RepE binds to the *copA/incC* locus and causes plasmids to bind together via RepE thereby inhibiting replication (coupling)

  2**. ColEI plasmid**
    a. small (6.6 kb)
    b. high copy (20 copies/cell)
    c. non-transmissible
    d. does not require de novo protein synthesis for replication (chloramphenicol amplifiable)
    e. RNA-II is transcribed through the origin of replication, gets cut by RNaseH and serves as the primer for DNA replication
    f. RNA-I is transcribed in the opposite orientation and is complementary to RNA-II.
    g. binding of RNA-II and RNA-I prevents initiation of replication (RNA-I is a negative regulator)
    h. the Rom/Rop protein made by the *rom/rop* gene stabilize the binding of RNA-I and RNA-II (also negative regulator)


SalI
XbaI
KpnI
SphI
EcoRV
SacI
SmaI
EcoRI
PstI
XbaI
EcoRI
oriR6K
sacB
mobRP4
pCVD442
KpnI
EcoRV
bla
PstI
PstI


F plasmid - 100 kb
tra genes
RepFIA
+
+
−
RepE
oriV
origin of repication
repE
copA/incC
RNA-I
RNA-II
ori
−
rop/rom
Rop
+
ColEI - 6.6 kb

III. PLASMIDS FOR FUN AND PROFIT

Choosing a plasmid vector is like shopping for a new car. You have to take into account what you are going to use it for, how much power you want under the hood, whether it is for the road or all-terrain, how big, the color, and various other options that might make life easier (or more difficult).

A. Use. What do you want the plasmid to do for you? Express a protein, express an RNA molecule, hold a piece of DNA to use as a hybridization probe?

1. Viable expression in the bacteria. Sometimes you will want the bacteria to synthesize your favorite protein as they grow (and continue to grow). In this case you want the protein to be expressed in a **regulated** manner (so that too much is not made) and is **localized to the right place in the bacterial cell**.

a. regulated expression. If too much protein is made, the bacteria will kill themselves serving you, since they will neglect the functions they need to survive. (see promoters below). You will want to use a promoter that is **inducible** by addition of some chemical to the culture. Also, some over-expressed proteins will crystallize in the cell forming an **inclusion body**. This may not be all bad, since they are easy to purify, but then difficult to get back to soluble form.

b. proper localization. *E. coli* has a double lipid bilayer cell wall structure (inner and outer membranes). Although many eukaryotic export signals (signal or leader sequences) are recognized by bacterial translation machinery, export through one membrane results in the protein being localized to the **periplasmic space**. Since this is still "inside" the cell, your protein will accumulate there and could cause trouble. If you want your favorite protein to be exported completely outside of the bacterial cell, you will need to hookup the reading frame to an outer membrane or secreted protein signal sequence. Note, however, that if you take a non-exported protein and hook it up to a signal sequence, you may "jam-up" the export machinery and kill the cell.

2. Terminal expression. Sometimes you just want "tons" of your favorite protein, and you don't care if the bacteria die doing it. You can then use a very strong promoter that causes the bacteria to make so much protein that they die. Note that in such cases the protein may not be localized where you intended. The **phage promoters** are useful for terminal expression.

B. Expression systems. There are essentially three options here.

1. Constitutive promoters are always on at the same strength. Cloning into the middle of an antibiotic resistance gene (as in the days of old) usually fits this category. (Not very useful.)

2. Inducible bacterial promoters. These are promoters that are usually OFF, but can be turned ON by addition of the inducer factor to the culture. Some popular systems are ***lac*** **promoter** derivatives. The ***trc*** and ***tac*** promoters are very strong derivatives of the ***lac*** promoter from *E. coli*. They are highly inducible by addition of **IPTG** to cultures. Even induced, they are relatively safe against over-expression (but keep in mind the copy number of the plasmid - see below).

AraC + pBAD: As opposed to the *lac* system which is the model for induction by relieving repression, the *ara* system is the model for induction of expression by positive control. The AraC protein is a positive regulator of the pBAD promoter (which normally drives the *araBAD* genes. In the absence of arabinose, this system is very tightly OFF. In the presence of arabinose, expression is increased on the order of 1000-fold.

3. phage promoters. Bacteriophage "take over" the host cell synthesis machinery to make more phage. Some of the strongest promoters known in bacteria are phage promoters. However, the trick to getting the promoter to work is to provide the phage **RNA polymerase**. You must provide it on the vector or somewhere else in the bacterial cell (another plasmid or inserted into the chromosome of special strains). However, the polymerase must be regulated in some way, or you would never be able to clone anything into the expression site - the bacteria would kill themselves by over-expressing the protein.

e.g., T7 promoter. The phage T7 has donated its RNA polymerase and specific promoter sequence to many highly useful vectors. It is regulated by a *lac* promoter, so production of the T7 RNA polymerase is induced with IPTG, which, in turn, results in massive production of your favorite protein cloned into the vector.

4. Other expression considerations.

a. Do you need to hook up to a **ribosome binding site**? If you clone a eukaryotic reading frame, it will require a RBS upstream of the ATG.
b. Do you want your **protein exported** or not? (signal sequence at N-terminus)
c. Do you want your **protein tagged** in some way for easy purification by affinity chromatography? (e.g., Maltose Binding Protein, His-tag, etc.)

C. Screen or selection for insert. As the Stones sang, "You don't always get what you want." When your insert isn't there, it would be nice to know so you don't waste your time. There are many **screenable** markers (most popular is **LacZα complementation**) which make the bacteria **change color** when an insert is or isn't there. There are now some **selectable** markers that cause the bacteria to **die** if an insert doesn't interrupt a lethal gene.

D. Copy number. Related to regulation of expression. Many high copy number plasmids will result in over-expression of your protein (gene dosage effect) making it toxic. The pUC-based plasmids are high copy, so beware. Low copy vectors are available.

E. Size and restriction sites. A large plasmid is more likely to have numerous **restriction sites** that will limit your ability of using restriction enzymes to clone your DNA fragment into or cut it out of the vector.

F. Genetic markers. To isolate bacteria that have taken up your plasmid, you select with an antibiotic to which the vector encodes the antibiotic resistance. Some host bacteria encode antibiotic resistance markers in their chromosomes. Ampicillin resistance is very popular, but it is leaky. Kanamycin, chloramphenicol, and tetracycline resistances are better.

G. Other features. There are other features that could be relevant to selecting a vector.

1. phagemids. If you are going to sequence your insert or you need single stranded DNA, **phagemids** are handy. They incorporate bacteriophage sequences that result in the production of single stranded DNA from the vector with coating by phage proteins. The "packaged" plasmid can be purified, and the single stranded DNA used for sequencing or as a probe for hybridization, since the phage origin is strand-specific. The most popular phage is called **f1**. Getting one or the other of the strands produced is brought about by using either the "+" or "-" vectors.

2. cosmids. If you want to clone only large fragments of DNA for making libraries of entire chromosomes, **cosmids** are handy. They are based on the fact that the *E. coli* **phage λ** packages about 45 kb of DNA - no more, no less. By putting the phage DNA packaging sequence (the ***cos*** site) into a plasmid, an in vitro packaging reaction can be done to result in phage packaging only appropriately sized recombinant plasmids. The phage can then be used to efficiently introduce your plasmid into a recipient cell.

3. suicide plasmids. Used for making mutations through allelic exchange.

a. Clone the mutant allele of the gene into the plasmid.
b. Move the plasmid into the recipient strain.
c. Force the recombination of the plasmid into the recipient genome because the plasmid cannot replicate in the host cell (suicide plasmid). Plate on an antibiotic encoded by the plasmid. How do you do this? Make the plasmid depend on a positive-acting protein similar to RepE of F. Have the donor strain carry RepE, but the recipient strain does not. Most suicide plasmid use a plasmid RK2 ori that depends on the π protein (pir gene).
d. Grow the population without selection. Integration of the plasmid caused a direct repeat of the cloned sequence, and direct repeats are not stable - they resolve. If everything is equal, half of the resolutions (excisions) will have replaced the wild-type allele with the mutant allele. How do you select for the loss of the vector plasmid?
e. Use a counterselectable marker on the vector. Scusrose sensitivity is often used. The sacB gene from Bacillus is toxic to gram-negative bacteria growing in sucrose. Plate the culture on sucrose. Only those cells that have lostthe vector (excised the integrated plasmid) will survive.