# Lecture 18

High-capacity vectors

During the development of new high-capacity lambda vectors, all of the nonessential genes were "thrown overboard." Taking this concept to an extreme, the ultimate goal is to have plasmid-like elements that can be introduced into cells by transduction or conjugation, rather than transformation.

Bacterial artificial chromosomes We have had an opportunity to learn about several methods by which DNA is taken up naturally by E. coli:

- Transformation

- Transduction

There is one more method that we should discuss, called "**conjugation**." To some extent, we have already touched on the matter in our previous discussion of the sex factor F in bacteria. As you may recall, the filamentous bacteriophage only infected male bacteria - i.e. those with pili on their surfaces. What causes a bacterium to be male or female? It is the presence or absence of the **F factor** (integrated or in plasmid form) that determines the sex. Conjugation is the process by which a cellular bridge is formed between two cells (one of which is male), and a single stranded DNA molecule is transferred from one to the other. If the F factor plasmid is transferred, the recipient becomes a "male" bacterium.
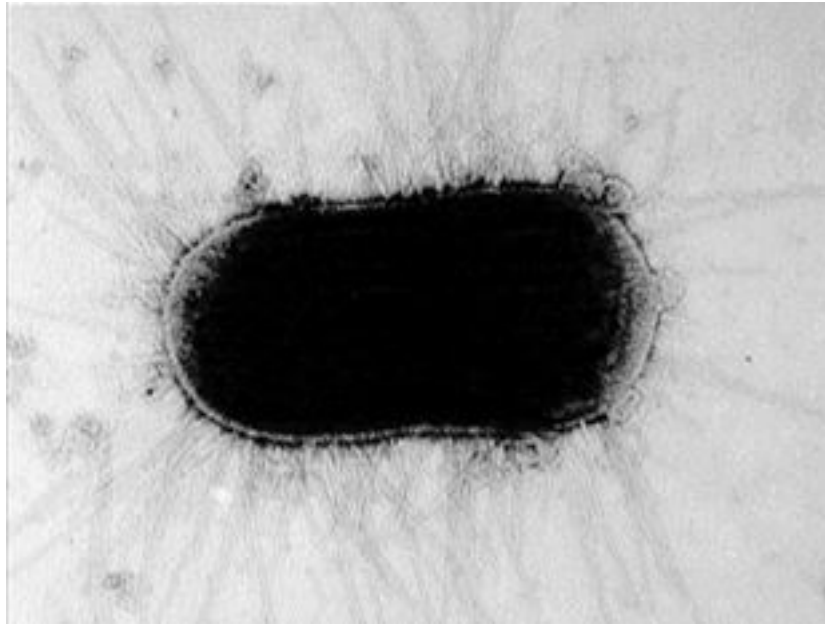
male bacterium, showing off his pili

http://mickey.lcsc.edu/~msatterw/Life/Ecol_pili.jpg

This was discovered in 1946 by Lederberg and Tatum, about 18 years after transformation was first described by Griffith. I suppose if F factor transfer had been discovered in the 1990's we would have called these "transsexual" or "transgender" bacteria, but the 1940's were a much simpler time!

The F factor plasmid is nicked at its origin and replicates as a rolling circle, causing a single-stranded DNA to be produced. It takes a bit of time for the entire F factor to be replicated, and if the bacteria are interrupted during the act, only the DNA that has made it through the cellular bridge will be transferred.

Conjugation in bacteria - transfer of an F factor
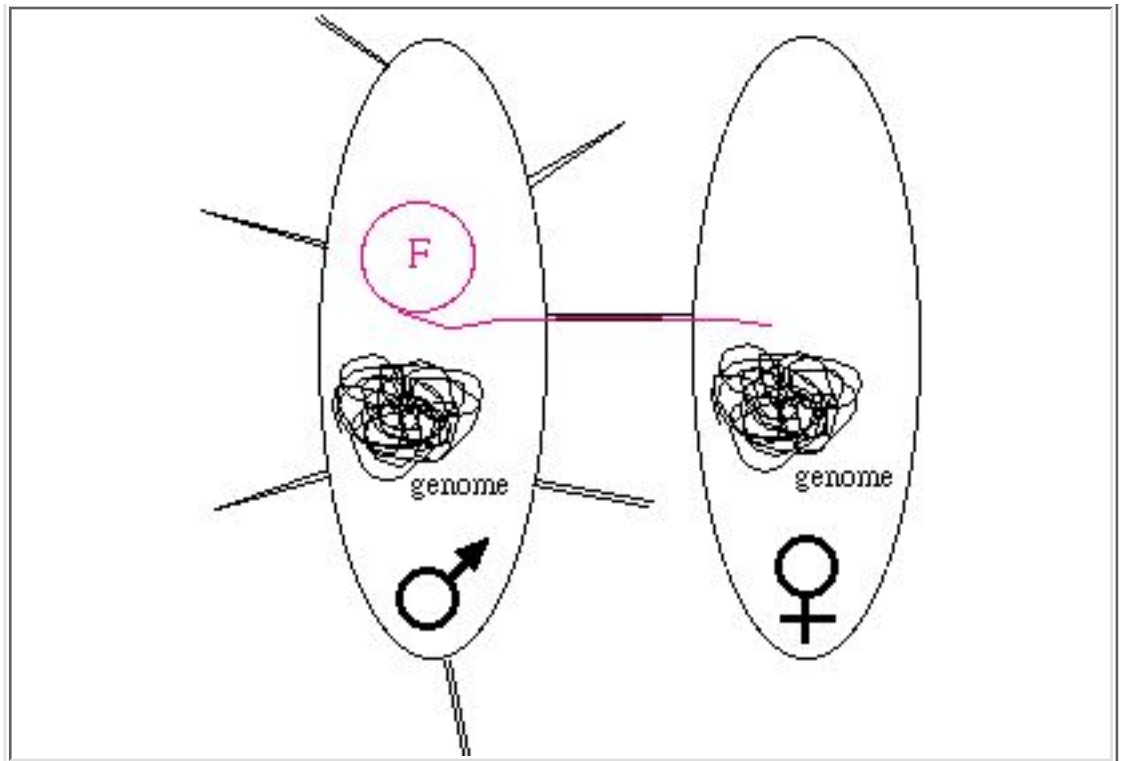
In cases where the F factor resides in the genome as an integrated copy (Hfr strain, where Hfr = high frequency recombination), then the rolling circle is the entire E. coli chromosome!

Conjugation in Hfr bacteria - transfer of a chromosome fragment
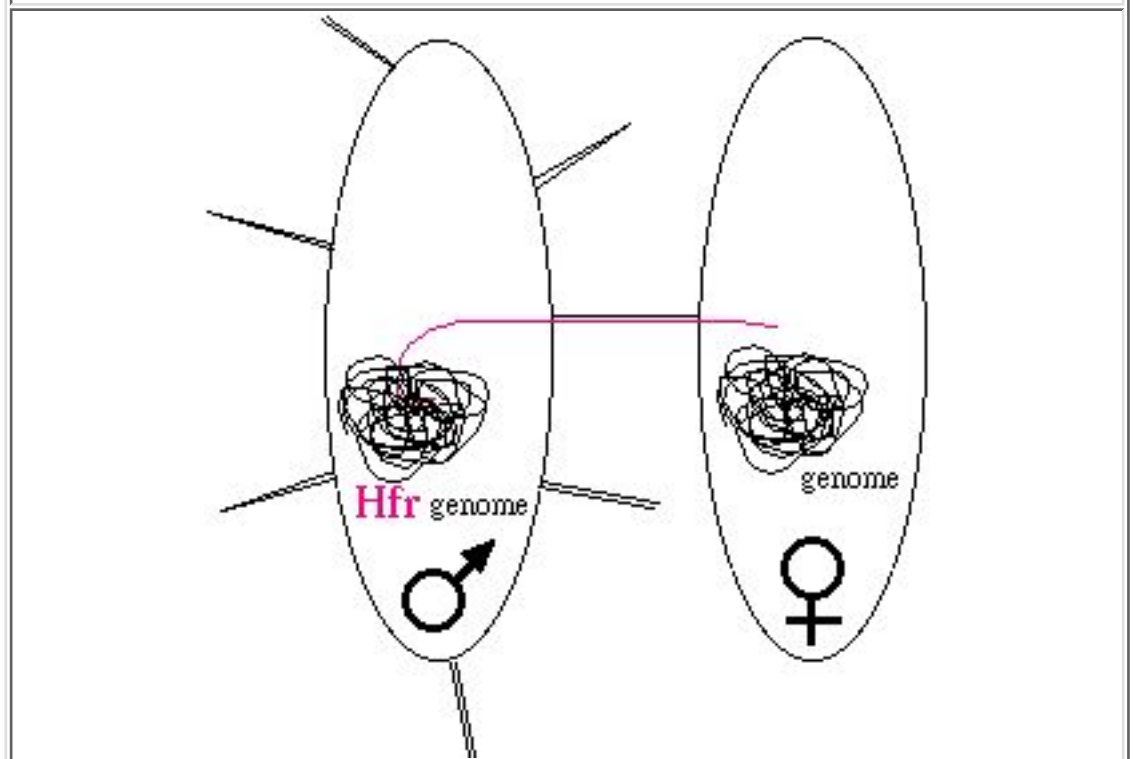
Genes replicated first from the F factor origin (i.e. those on the 3' side of the origin of replication) are more likely to be transferred to the new host because the conjugation only needs to be maintained for a few seconds. On the other hand, genes replicated last on the rolling circle are less likely to be transferred. In this way, it was possible to construct a **genetic map** of the chromosome, through purposeful interruption of the conjugating bacteria after specific time intervals, and then determining which genes were transferred at high probability. The conjugating bacteria are interrupted during the act (in the Wollman and Jacob experiment) by putting them into a blender and turning it on to "frappe". For the coupling bacteria, that's the equivalent of turning a hose on "those dogs in the yard" .

If the F factor is excised from an Hfr strain abnormally, a new plasmid is generated that may contain novel sequences. We call this an **F' factor**, to distinguish it from the previously described "F factor", which has a precise meaning. Many times in molecular biology we use bacterial strains that have particular genotypes involving F' factors. The cells that we use in transformation are:

Top10F' from Invitrogen, Inc.
F'{lacI^q Tn10(Tet^R)} mcrA delta(mrr-hsdRMS-mcrBC) phi80lacZdeltaM15 deltalacX74 deoR recA1 araD139 delta(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG

What a mess! You will notice a few old friends in this genotype, however. The indication is that an overexpressing allele of the lac repressor (lacI^q) is present on an F' plasmid. What else is there? A transposable element called Tn10, carrying a tetracycline resistance marker. If you grow Top10F' cells on tetracycline containing medium, you can make sure they will keep the F' plasmid carrying the lacI^q gene. The lacZdeltaM15 is

the C-terminal portion of lacZ that, in combination with lacZ-alpha (the N-terminal portion) in trans, can function as a beta-galactosidase enzyme. We need that gene in order to be able to use blue-white screening with some of the common cloning plasmids we've discussed. The lacZdeltaM15 was introduced into the strain by transduction, on a phage species called phi80.

To learn more about genetic exchange in prokaryotes, visit:

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| [The microbial genetics problems co-op](), put together by Stanley Maloy |
| [Kevin O'Dell]()'s course in the U.K. |

Now that we've learned a bit about F factors, you might imagine how a cloning vector could be created that was based on an F factor origin of replication. We call such engineered F' plasmids **"BACs"** or Bacterial Artificial Chromosomes. BACs are capable of carrying approximately 200 kbp of inserted DNA sequence, and the F factor origin of replication maintains their level at approximately one copy per cell. Of course, we needn't stop there! We can also use **"YACs"** which are Yeast Artificial Chromosomes, and depend on being able to replicate and be maintained in Saccharomyces cerevisiae. YACs can carry approximately 500 kbp of foreign DNA, though they are often criticized due to the problem of natural recombination in the host.

Handling DNA of this size is a real problem, as I have mentioned before, due to the potential for **shearing**. The way this is solved is to embed the cells from which a library is going to be made, in low melting point agarose. The cells can be lysed in the agarose, simply be incubating the **blocks** of agarose in sodium dodecyl sulfate (SDS), proteinase K and EDTA. Once the lysis buffer has been

washed away, the DNA in the blocks can be digested with a restriction enzyme. When you're ready to ligate the DNA into a F1-based vector, you incubate the block with an enzyme called **agarase** which digests the agarose matrix. The ligated DNA is then introduced into E. coli by transformation, using electroporation (electric shock) methods to achieve high efficiency.

Here is an example of a BAC vector, from those folks in Buffalo:

pBACe3.6

http://www.chori.org/bacpac/pbace36.htm
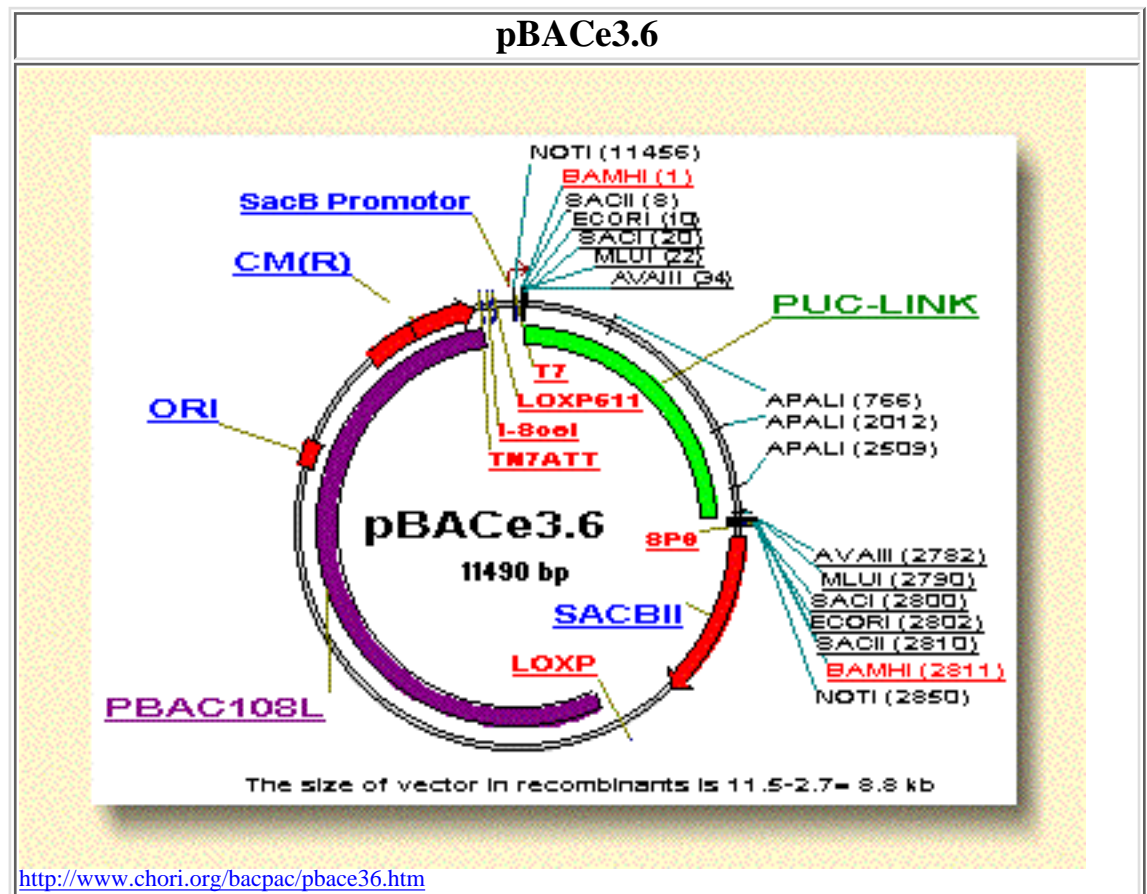
[Their description of pBACe3.6:](#)

The new vector has been named pBACe3.6. Specifically, we maintained the wildtype loxP site, added an additional mutant loxP511 site, a site for the intron encoded nuclease PI-SceI and the Tn7att site. In addition, we changed the cloning area to allow positive selection for inserts containing BAC clones through inclusion of the sacBII gene from Nat Sternberg's PI vector (Pierce et al. 1992, Proc. Natl. Acad. Sci. USA 89: 2056-2060) and disrupted the BamHI cloning site with a fragment containing a pUC plasmid.

The presence of this pUC plasmid serves dual functions: high copy number of the vector for preparing large quantities and appropriate disruption of the sacBII gene to increase viability of the vector containing strain. In addition to the BamHI site, 5 additional sites can be used for preparing BAC libraries: SacI, SacII, MluI, EcoRI and AvaIII. The EcoRI site is particularly important in view of the use of this site in the RARE cleavage procedure, thus opening the possibility for selective cloning of similar fragments from different DNA donors.

In looking at the BAC vector from RPCI, you should notice a couple of important elements:

1. A stuffer fragment (pUC-link) that separates the SacBII gene from its promoter. SacBII is a gene that provides selection against cells that express it.

2. Dual polylinkers to excise and replace the stuffer with your favorite DNA, just as with the virulent lambda vectors we discussed.

3. An origin of replication (ori) and chloramphenicol resistance gene (CM(R)) to allow stable maintenance in bacteria, as we discussed at the beginning of the course.

4. Promoters for T7 and SP6 RNA polymerases

5. An Sce I site (Sce I is a restriction endonuclease from Saccharomyces)

How do you go about using PACs and BACs in your research (the easy way)?

Obtain a gridded membrane containing spots of BAC/PAC clones. This is analogous to the "bacterial colony" lift that we discussed, except that the spots are organized and numbered.
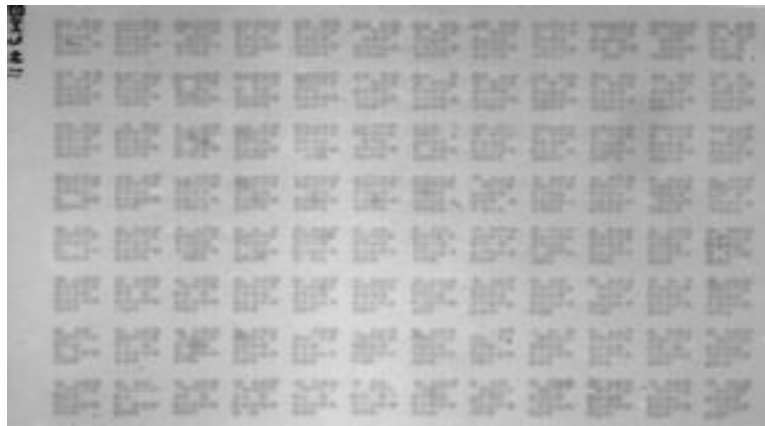
At left is an example of such a filter (not from RPCI however) from the Cambridge group's project with Trypanosoma. Note that each dot represents DNA from a different PAC!

(approximately 80 kbp of genomic sequence in each dot)

The PACs are stored as bacterial strains, in a freezer, so you can always make more.

Here are some comments from RPCI, a supplier of gridded libraries:

All of the RPCI genomic DNA libraries have been gridded onto 22x22cm positively charged nylon filters for hybridization screening purposes. Each filter contains 36,864 colonies which represent 18,432 independent clones that have been spotted in duplicate in a 4x4 clone array. Filter sets are available directly from BACPAC Resources, Department of Human Genetics, Roswell Park Cancer Institute.

Filters should be hybridized with ^{32}P-labeled (random-primed) probes ($1x10^6$ cpm/ml hybridization solution) using standard conditions. After washing to remove unbound probe, filters are wrapped in plastic film (Saran) and exposed to x-ray film (Kodak Xar, Amersham Hyperfilm) for 2 to 24 hrs. Complete documentation on interpretation of hybridization results (location of positive clone plate coordinates) is included with the filter sets.

Positive individual clones are available for $10/clone shipped using purchasers Federal Express account number. Contact Joe Catanese or Pieter de Jong with requests.

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