Molecular Cloning

A LABORATORY MANUAL

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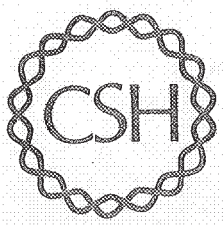

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Back cover: E. coli DH1 with fimbriae was negatively stained with phosphotungstic acid and the electron micrograph was digitized and assigned false color by computer. *Jeffrey A. Engler, Thomas R. Broker, and James I. Garrels*

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Cloning in Plasmids

In principle, cloning in plasmid vectors is very straightforward. The plasmid DNA is cleaved with a restriction endonuclease and joined in vitro to foreign DNA. The resulting recombinant plasmids are then used to transform bacteria. In practice, however, the plasmid vector must be carefully chosen to minimize the effort required to identify and characterize recombinants. The major difficulty is to distinguish between plasmids that contain sequences of foreign DNA and vector DNA molecules that have recircularized without insertion of foreign sequences. Recircularization of the plasmid can be limited to some extent by adjusting the concentrations of the foreign DNA and vector DNA during the ligation reaction. However, a number of procedures, described below, have been developed either to reduce recircularization of the plasmid still further or to distinguish recombinants from nonrecombinants by genetic techniques.

Insertional Inactivation

This method can be used with plasmids that carry two or more antibiotic-resistance markers (see Fig. 1.1). The DNA to be inserted and the purified plasmid DNA are digested with a restriction enzyme that, in this example,

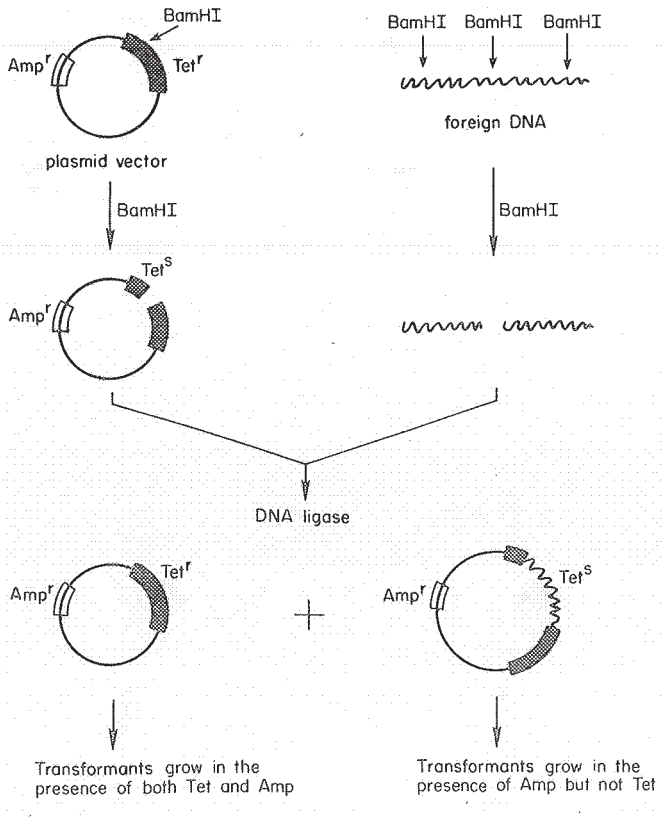

Figure 1.1
Insertional inactivation.

recognizes a unique site located in the plasmid within the tetracycline-resistance gene. After ligating the two DNAs at the appropriate concentrations, the ligation mixture is used to transform, for example, ampicillin-sensitive *E. coli* to ampicillin resistance. Some of the colonies that grow in the presence of ampicillin will contain recombinant plasmids; others will contain plasmid DNA that has recircularized during ligation without insertion of foreign DNA. To discriminate between the two kinds of transformants, a number of colonies are streaked in identical locations on plates containing ampicillin or tetracycline (see Fig. 1.2). The colonies that survive and grow in the presence of tetracycline contain plasmids with an active tetracycline-resistance gene; such plasmids are unlikely to carry insertions of foreign DNA. The colonies that grow only in the presence of ampicillin contain plasmids with inactive tetracycline-resistance genes; these plasmids are likely to carry foreign DNA sequences.

In a few cases, methods have been developed to apply positive selection to obtain bacteria that are sensitive to an antibiotic from populations that are predominantly resistant. In this way, it is possible to select for recombinant plasmids that carry an inactivated antibiotic-resistance gene as a consequence of insertion of a foreign DNA sequence. The most useful of these

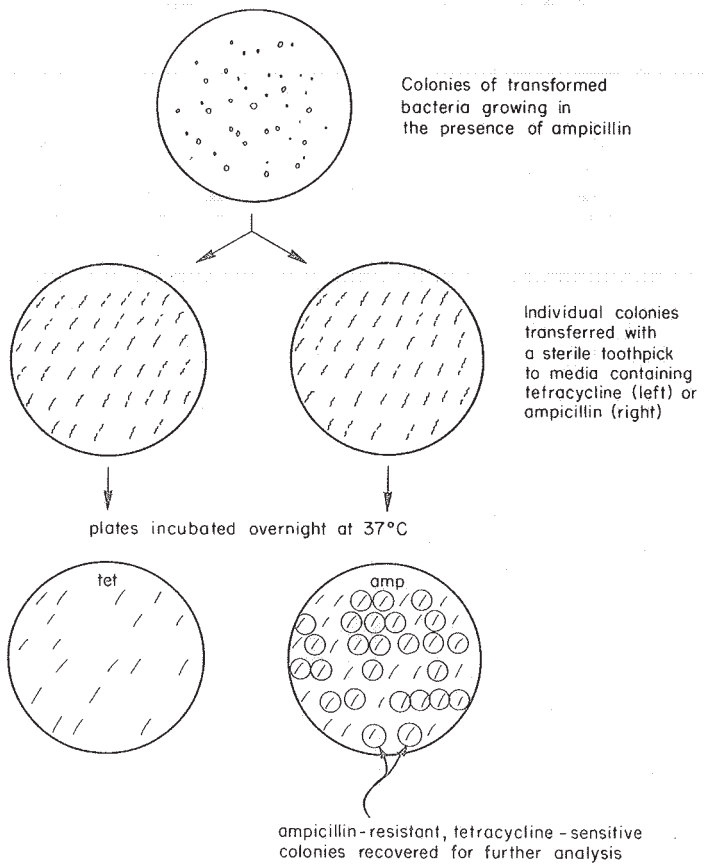

Figure 1.2

Screening for insertions of foreign DNA by inactivation of plasmid-borne, antibiotic-resistance genes.

systems is that described by Bochner et al. (1980) and Maloy and Nunn (1981), who developed media containing the lipophilic, chelating agents fusaric acid or quinaldic acid, which allow the direct positive selection of Tet^s clones from a population of Tet^s and Tet^r bacteria. For most strains of *E. coli*, approximately 90% of the colonies obtained on media containing tetracycline and fusaric acid were found to be Tet^s when plated onto media containing tetracycline alone. It is therefore possible to select from a population of bacteria transformed with pBR322 or pAT153 those cells that carry plasmids with insertions at the *Bam*HI and *Sal*I sites.

A similar technique has been developed to select for bacteria sensitive to paromomycin (Slutsky et al. 1980). This should allow the selection of derivatives of pMK16 that contain insertions at the *Sma*I or *Xho*I site (Kahn et al. 1979).

Although insertion of foreign DNA sequences within an antibiotic-resistance gene almost always leads to inactivation of that gene, at least one case is known where an insertion left the gene in a functional state. Villa-Komaroff et al. (1978) found that insertion of a segment of rat preproinsulin cDNA into the *Pst*I site of pBR322 did not inactivate the ampicillin-resistance gene. Presumably, a small piece of foreign DNA had been inserted that did not alter the reading-frame of the ampicillin-resistance gene, so that a fusion protein was formed which retained β-lactamase activity.

Directional Cloning

Most plasmid vectors carry two or more unique restriction enzyme recognition sites. For example, the plasmid pBR322 contains single *Hin*dIII and *Bam*HI sites (see Fig. 1.3). After cleavage by both enzymes, the larger fragment of plasmid DNA can be purified by gel electrophoresis and ligated to a segment of foreign DNA containing cohesive ends compatible with those generated by *Bam*HI and *Hin*dIII. The resulting circular recombinant is then used to transform *E. coli* to ampicillin resistance. Because of the lack of complementarity between the *Hin*dIII and *Bam*HI protruding ends, the larger vector fragment cannot circularize efficiently; it therefore transforms *E. coli* very poorly. Therefore, most of the colonies resistant to ampicillin contain plasmids that carry foreign DNA segments forming a bridge between the *Hin*dIII and *Bam*HI sites. Of course, different combinations of enzymes can be used, depending on the locations of restriction sites within vector and the segment of foreign DNA.

Phosphatase Treatment of Linear, Plasmid Vector DNA

During ligation, DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5′-phosphate group and the other a 3′-hydroxyl group. Recircularization of plasmid DNA can therefore be minimized by removing the 5′ phosphates from both ends of the linear DNA with bacterial alkaline phosphatase or calf intestinal phosphatase (Seeburg et al. 1977; Ullrich et al. 1977). As a result, neither strand of the duplex can form a phosphodiester bond. However, a

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