The Functional Differences in the Inverted Repeats of Tn5 Are Caused by a Single Base Pair Nonhomology

Steven J. Rothstein* and William S. Reznikoff†
Department of Biochemistry
College of Agricultural and Life Sciences
University of Wisconsin-Madison
Madison, Wisconsin 53706

Summary

The inverted repeats of Tn5 are functionally different. One repeat codes for larger polypeptides, which are required for transposition. The other repeat has a better promoter for the neomycin resistance gene in the region of the repeat near the unique sequences. These dissimilarities are now shown to be caused by a single base pair difference. This change both creates a better promoter sequence and codes for part of a new UAA nonsense codon. Mutants in which the DNA sequence of a repeat is altered only at this base pair are shown to function like the opposite repeat. Furthermore, it is possible to suppress the UAA nonsense codon with an ochre suppressor, making the previously abbreviated polypeptides functional in transposition.

Introduction

Tn5 is one of a number of transposable elements that have been found to encode resistance to antibiotics. One feature they share is the presence of repeated DNA sequences at the ends of the elements, which have been found to be crucial for the transposition process (for example, see Heffron et al., 1977; Rothstein et al., 1980a). It has been postulated that the repeated DNA originated from a pair of homologous IS insertion elements, with the IS elements providing the genes necessary for transposition (MacHattie and Jackowski, 1977; Kieckner, 1977). These ancestral species would have had two identical genes for the transposition functions, a situation found for several of the transposable elements that have been isolated (see MacHattie and Jackowski, 1977). During subsequent evolution, they could undergo an alteration in one of the original IS sequences without losing the ability to transpose. This alteration could be in the form of a large deletion leaving short repeats, as is found for Tn3 (Heffron et al., 1977), or the change could have been of a more subtle nature, as will be described below for Tn5.

Tn5 is a 5700 bp piece of DNA, which encodes resistance to some aminoglycoside antibiotics including kanamycin and neomycin. It has 1500 bp inverted repeats flanking a 2700 bp unique region (Berg et al., 1975). All of the restriction endonuclease sites mapped in the inverted repeats (more than 20) were found to be in identical locations (Jorgensen et al., 1979; S. J. Rothstein and W. S. Reznikoff, unpublished results). Despite this, the inverted repeats were found to be functionally different (Rothstein et al., 1980a).

Each inverted repeat appears to code for two polypeptides. The two polypeptides that are synthesized from each repeat differ at their N-termini (Rothstein et al., 1980a). However, the polypeptides coded for by the repeat adjacent to the resistance gene (called the left repeat; see Figure 1) are smaller in molecular weight when compared to those coded for by the right repeat. Only the right repeat polypeptides are necessary for transposition. The other major difference between the two repeats is in their abilities to promote high levels of neomycin resistance. An inversion placing the resistance gene adjacent to the right repeat lowers neomycin resistance considerably. This result was understandable, since it was known that the left repeat was necessary for the expression of neomycin resistance and contained an RNA polymerase binding site near the unique region not found in the right repeat. It was therefore predicted that the left repeat near the unique region of Tn5 should contain a better promoter than the comparable sequences in the right repeat. The difference in the polypeptide coding had also been localized to the same region.

We wanted to determine the difference in the two repeats at the DNA sequence level. A sequence analysis of analogous 130 bp regions in both inverted repeats is presented. Their sequences are identical except at a single base pair. This single base pair change is shown to be sufficient to explain all the functional differences between the two inverted repeats of Tn5.

Results

A partial restriction map of Tn5 is shown in Figure 1 (Jorgensen et al., 1979). The functional dissimilarities between the two inverted repeats were known to be caused by differences in their DNA sequences between the Hind III and Bgl II sites (Rothstein et al., 1980a). It was therefore of interest to determine the sequence in this region. To accomplish this, it was necessary to have the two repeats on separate plasmids. pRZ152 was the source of left repeat DNA, while pRZ149 was the source of right repeat DNA (see Figure 5 and Rothstein et al., 1980a). Both Maxam-Gilbert and dieoxy sequencing techniques were utilized; the details of the procedures are discussed in Experimental Procedures.

The inverted repeats were sequenced from the Bgl II sites towards the Hind III sites. Figure 2 is an example of a sequencing experiment comparing the two repeats. The DNA sequences in this region, as shown in Figure 3a, are identical except for one base pair difference. At this position the left repeat has a...
T–A, while the right repeat has a G–C base pair. This sequence is identical to that found by Auerwald and Schaller (1980).

The DNA sequence surrounding the altered base pair in the left repeat was compared to the model E. coli promoter sequences of Rosenberg and Court (1979). As can be seen in Figure 3b, this region matches the model sequence quite well. Furthermore, the single base pair difference in the right repeat is at one of the most highly conserved sites in the model sequence. Mutations at this base pair have been shown to lower the activity of several promoters, including those for trp and tRNA

Rosenberg and Court, 1979). Assuming that the sequence shown in Figure 3b is the promoter for the neomycin resistance gene, it is understandable that the right repeat would not serve to promote resistance to neomycin as well as the left repeat.

The other difference between the two repeats, the abbreviation of the polypeptides coded for by the left repeat, can also be explained by this base pair change. Figure 3c shows the amino acids coded for by the DNA sequence in this region. As can be seen, the T–A base pair present in the left inverted repeat codes for part of a UAA nonsense codon not coded for by the right repeat. From their extended sequence data, Auerwald and Schaller (1980) deduced that this nonsense codon is in the correct reading frame for terminating the polypeptides coded for by the left inverted repeat.

A single base pair change thus apparently creates both a better promoter sequence and a new nonsense codon. In theory, this change is sufficient to explain the difference in the function of the repeats of Tn5.

**Construction of Tn5 Mutants**

There is a Pvu II site 92 bp away from the Bgl II site in each repeat (see Figures 1 and 3a). The DNA sequences of the two repeats between these two restriction sites are identical except for the single base pair change discussed above (Figure 3a). Thus, substituting the Pvu II–Bgl II restriction fragment from the right repeat for that of the left repeat will alter the DNA sequence of the left repeat at this one base pair. If this single base pair alteration is sufficient for determining the functional differences between the two repeats, then this hybrid repeat should now act like a right repeat. It should not be as active a promoter for the neomycin resistance gene, but should code for larger polypeptides active in transposition.

The method by which the right repeat Pvu II–Bgl II fragment was substituted for the same region from the left repeat is detailed in Figure 4. pRZ201 has the left half of Tn5 substituted into the Eco TII–Sal I sites of pBR322 and has three Pvu II sites. pRZ149 was constructed from pRZ102 (wild-type Tn5; see Figure 5) by substituting the Hae II site in lac promoter-operator fragment for the left half of Tn5. While this plasmid has five Pvu II sites, the 1750 bp Pvu II fragment carrying the 92 bp Pvu II–Bgl II region also carries the lac operator, which when present on a multicopy plasmid can be screened for by the constitutive production of β-galactosidase. Thus by digesting pRZ201 and pRZ149 with Pvu II, ligating the frag-
Tn5 Inverted Repeat Differences

Right Repeat Left Repeat
ACGTACGT

The structures of a number of other plasmids that were constructed are also shown in Figure 5; the details of their construction are given in Experimental Procedures.

The Functional Properties of the Tn5 Mutants

The mutants of Tn5 described above can be used to analyze whether a single base pair difference between the two repeats can explain their functional dissimilarities. There are three phenotypic properties that can be tested, namely, the ability to promote resistance to neomycin, the size of the polypeptides encoded and the role of these polypeptides in transposition.

Neomycin Resistance

pRZ201 has the left half of Tn5 cloned between the Eco RI and Sal I sites of pBR322, and should code for high levels of neomycin resistance. The inverted repeat present in pRZ236 differs from that in pRZ201 at a single base pair as discussed above (see Figures 3a and 5). If this change were in the promoter for the neomycin resistance gene as postulated above (see Figure 5b), then it should lower the level of neomycin resistance. The final mutant tested for its neomycin resistance phenotype is pRZ237, which has right repeat DNA between the Hind III and Bgl II sites. Since this is the region that was previously shown to be different between the two repeats, this mutant should code for a reduced level of resistance. The results are shown in Figure 6. pRZ236 and pRZ239 have very similar resistance phenotypes, and neither confer a level of resistance nearly as high as pRZ201. Therefore, the single base pair change between the Pvu II and Bgl II sites in the inverted repeats of Tn5 accounts for their functional difference with respect to the promotion of neomycin resistance.

One further point should be noted concerning the level of resistance of the Tn5 mutants. As can be seen in Figure 6, the resistance phenotype of the plasmids having pBR322 as the vector is 5–6 times higher than that seen when ColEI is the vector. For example, pRZ102, which consists of wild-type Tn5 inserted into ColEI, has a considerably lower level of resistance than does pRZ201. In the same way, when the neomycin resistance gene is adjacent to a right repeat as in pRZ141 (an inversion of Tn5 at the Bgl II sites) and is in a ColEI vector, its level of resistance is considerably lower than the corresponding mutants in pBR322, like pRZ236 or pRZ237. The reason for this effect is unknown, but may result from a difference in the copy number of the two vectors.

Peptide-Coding Properties

The right inverted repeat of Tn5 codes for two polypeptides (called 1 and 2), one or both of which are needed for transposition. They are of larger molecular weight than the corresponding pair of polypeptides coded for by the left inverted repeat (3 and 4). This
difference between the repeats has been shown to occur at the C terminal end of the polypeptides (Rothstein et al., 1980a). The presence of a nonsense codon in the left repeat, not present in the right repeat as described in Figure 3, could explain these results. To test this hypothesis, the protein-coding capacity of several of the Tn5 mutant plasmids were tested in the minicell system.

The structures of pRZ202 and pRZ233 are shown in Figure 5. They differ only in the region between the Pvu II and Bgl II sites, with pRZ202 having left repeat DNA and pRZ233 having right repeat DNA. If the nonsense codon discussed above causes the abbreviation of the polypeptides coded for by the left inverted repeat, pRZ202 should code for polypeptides 3 and 4, while pRZ233 should code for polypeptides 1 and 2. As shown in Figure 7, this turns out to be the case.
Construction of a 'Hybrid' Repeat

Transposition Properties
Since the inverted repeat polypeptides coded for by pRZ233 are the same size as those coded for by a wild-type right repeat, they should be functional in transposition. To test this prediction, the Tn5 DNA present in the plasmid, as well as that in pRZ202, was combined with pRZ154 (see Figure 5) to form a complete Tn5 genome. pRZ154 has a left-like repeat, which contains for polypeptides 3 and 4 and codes for a wild-type neomycin resistance level. pRZ242, which was constructed from pRZ202, has two repeats which should code for the smaller left repeat polypeptides (3 and 4) and should not transpose (see Figure 5). pRZ241, which was constructed from pRZ233 and differs from pRZ242 at the single base pair in question, should make all four inverted repeat polypeptides and should transpose (see Figure 5). As can be seen in Figure 7, pRZ242 does code only for polypeptides 3 and 4, while pRZ241 codes for all four inverted repeat polypeptides.

To test for transposition, pRZ241, pRZ242 and pRZ102 (wild-type Tn5) were transformed into a λimm lysogen of W3110. The transposition frequency was measured as the percentage of λ phages that transduce resistance to kanamycin, with the results shown in Table 1. pRZ241 transposes at a level ampicillin and XG. The ampicillin-resistant colonies, which turn blue on XG agar, produce β-galactosidase constitutively. In this case, the constitutivity is caused by the presence of the lac operator on a multicopy plasmid which titrates out the lac repressor protein. (a) Material that originally was right repeat DNA; (b) DNA that is from the left repeat; (c) vector DNA.

Structures of Tn5 mutants

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Neomycin Resistance Levels of Tn5 'Hybrid' Repeat Mutants

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</table>

Figure 6. Neomycin Resistance of Strains Carrying Tn5 Mutant Plasmids

Analysis of the neomycin levels required to kill 50% of the cells plated out (1000 ng/ml of the strains containing Tn5 wild type or mutant plasmids. The structures of the plasmids are shown with the following notation: (1) right repeat DNA; (2) left repeat DNA; (3) deleted DNA. The vector plasmid is also given.

Table 1. The Level of Transposition of the Tn5 Mutants

| Strain | Plasmid Presented | Frequency of Transposition (No. of transpositions/µg²) | Transposition of Wild-Type%
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<td>5.8 x 10⁻⁷</td>
<td>76</td>
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* The frequency of transposition is measured as the number of kanamycin-transducing particles per plaque-forming unit and the values shown are an average of four experiments. Plasmid structures are described in Figure 5. pRZ102, pRZ241 and pRZ242 were tested in W3110, a nonsuppressing strain. pRZ102, pRZ112, pRZ172 and pRZ174 were analyzed in the ochre suppressor strain W112 (F Δ (parA-proA-2, parB-ΔproB, strA), which inserts a glutamate at UAA codons. The % of transposition compares wild-type Tn5 (pRZ102) transposition to that of the Tn5 mutants for each strain. pRZ102 transposes at a frequency of 0.002 times lower in W112 than it does in W3110. It should be noted that a Tn5 mutant (pRZ152) which does not have any right repeat DNA and a mutant (pRZ171) which has a DNA insertion into the Hind III site of pRZ112 were also tested in W112 and were found not to transpose at any measurable frequency. This is as expected. pRZ162 has a deletion of one of the ends of Tn5 which are required for transposition. pRZ171 does not synthesize wild-type left repeat polypeptides, so that even in the ochre suppressor strain no active transposase protein is made.

Figure 7. Proteins Encoded for by Tn5 Mutant Strains

Minicells containing pRZ104 (wild-type Tn5 inserted into a different site in colE1; see Jorgensen et al., 1978), pRZ202, pRZ233, pRZ241 or pRZ242 were labeled with 35S-methionine for 30 min. The proteins were electrophoresed on a 10% SDS-polyacrylamide gel, with the resulting autoradiogram shown. The structure of the plasmids tested is presented in Figure 5. The apparent molecular weights of the Tn5 polypeptides are: 1, 58,000; 2, 61,000; 3, 63,000; 4, 49,000; and NPTII, 26,000 (Rothstein et al., 1980a).

close to that of wild-type Tn5. On the other hand, pRZ242 does not transpose at all. The nonsense codon in the left repeat therefore causes the abbreviation of the coded polypeptides, which in turn ac-

counts for the failure of these polypeptides to function in transposition.

Suppression of the Nonsense Codon

Since the left inverted repeat polypeptides are terminated by a UAA nonsense codon, it would be possible to suppress this nonsense codon in a strain carrying an ochre suppressor. To do this, E. coli strain W112, which has an ochre suppressor insertino a glutamate at UAA codons, was used. (Glutamate is the amino acid coded for in this position by the right inverted repeat, as can be seen in Figure 3c.) This strain was lysogenized with λBn and then transformed with various Tn5 plasmids whose transposition in a trans-suppressing background had already been studied (Rothstein et al., 1980a). pRZ121 contains a deletion of most of the right inverted repeat, while pRZ174 has
an insertion in the Hind III site of the right repeat. Neither Tn5 mutant transposes at a measurable frequency onto λ when in a nonsuppressing background. pRZ172 has an insertion at the Hind III site in the left inverted repeat and transposes at around 20% of the wild-type level.

As shown in Table 1, both pRZ112 and pRZ174 transpose in the suppressing background. pRZ112 appears to transpose at the same level as wild-type Tn5. It is difficult to tell exactly what this means, since pRZ102 transposes at a considerably lower frequency in this strain than in W3110 (the nonsuppressing strain used, which is not isogenic with W112). pRZ174 and pRZ172 transpose at around 75% and 33% of wild-type, respectively. It is not known why their frequencies of transposition differ, nor why pRZ172 still transposes at a lower frequency than wild-type Tn5. It does seem clear that it is possible to suppress the nonsense codon in the left repeat with an ochre suppressor.

Discussion

A model for the genetic organization of Tn5 is presented in Figure 8. Each inverted repeat appears to code for two polypeptides which differ at their N termini. The proteins coded for by the left repeat are smaller in size and only the right repeat-encoded polypeptides are required for transposition. The left repeat does have a better promoter for the neomycin resistance gene. DNA sequencing in the region of the repeats known to be different demonstrated a single base pair nonhomology that is sufficient to explain their functional dissimilarities.

The DNA sequence around the nonhomologous base pair in the left repeat matches the model promoter sequence by Rosenberg and Court (1979) quite well. Furthermore, the altered base is at one of the most highly conserved bases in the promoter sequence and mutations at this site in other promoters are known to lower the level of transcription initiation (Rosenberg and Court, 1979). It is therefore reasonable that the corresponding region in the right repeat would not be as active a promoter for the resistance gene. It can be demonstrated that altering the sequence of the right inverted repeat at this base pair leads indeed to a considerable reduction in the level of neomycin resistance.

The same base pair change also creates a nonsense codon. Auerwald and Schaller (1980) from their extended sequence predicted that this nonsense codon in the right repeat is in the correct reading frame for the inverted repeat polypeptides and would therefore account for the shorter polypeptides coded for by this repeat. This hypothesis was tested in two ways. First, the region of the right repeat known to be different only at the single base pair under discussion was substituted for the corresponding region of the left repeat. The polypeptides coded for by this "hybrid" repeat are now the same size as the wild-type right repeat polypeptides. These polypeptides are also active in transposition. Therefore, removal of the nonsense codon in the left repeat also allows the synthesis of polypeptides of larger molecular weight. The second test of the model was to demonstrate that it is possible to suppress the UAA nonsense codon with an ochre suppressor. In this genetic background the left repeat now codes for polypeptides active in transposition. Therefore, not only does the single base pair change account for the difference in the promoter activities of the two repeats, but also for their different polypeptide-coding capacities.

Assuming that the inverted repeats of Tn5 originated as a pair of homologous IS insertion elements, one can postulate a simple evolutionary scheme for the origin of this transposon. (It has recently been reported that the right inverted repeat of Tn5 does move by itself, as one would expect for an IS element [Berg et al., 1980].) A single base pair change in the repeat adjacent to the neomycin resistance gene could first make a better promoter sequence near the unique region. A deletion starting at the inside edge of the repeat would then have removed the original neomycin resistance promoter, placing this gene under the control of the "new" inverted repeat promoter. A possibly fortuitous result of this single base change would have been the creation of a new nonsense codon in the correct protein reading frame, so that the now smaller polypeptides coded for by this repeat would no longer be active in transposition.

Experimental Procedures

Materials
The media used for isolating plasmids, growing phage and minicell growth and labeling are the same as those described by Jørgensen and Feizi (1979).

The following enzymes were purchased from Bethesda Research
Laboratories: Bgl II, Sal I, Hae III, T4 DNA ligase. Hind III and Eco RI were gifts from J. Gardner and S. Stadler, respectively. 18S-methionine was purchased from Amer sham-Pharmacia (600–800 Ci/mole).

**Bacterial Strains**

C600 SF6 (Struhl et al., 1976) was used as the original recipient for transformation as well as for the isolation of plasmid DNA. DS410 (min A, min D, eme, leu Y, trp 8, rec A, thy, am r, tna, lam, mit, thi, from D. Sherratt) was the minicell producing strain used. W3110 (F - trpA33) lysogenic for lambda was transformed with the various Tn5 mutant plasmids. These strains were then used for growing phase for the transposition assays. For the suppression of the left repeat polypeptides, the Tn5 mutant plasmids were transformed into a lambda lysogen of W112 (from J. Davies). Hfr1 (Seloff and Wulff, 1971) was used as a recipient for λ-Tn5 transducing particles.

**Selection of Transformants after Ligation**

The basic ligation, transformation and selection procedures have been described elsewhere (for example, see Jorgensen et al., 1979). The selected transformants were tested for plasmid size by a modification of the procedure described by Barnes (1977). Small crude preparations of plasmid DNA capable of being cleaved by restriction enzymes were prepared using the technique of Cameron et al. (1977).

**Construction of Mutants of Tn5**

pRZ201 was constructed by digesting pHCG12G (colI-1, Hpa I) and pBR322 with Eco RI and Sal I and ligating the resulting fragments together. Ampicillin- and kanamycin-resistant colonies were selected and the plasmids present were shown to have the correct size. Purified DNA was digested with Eco RI + Sal I and with Hae III to demonstrate that the plasmid had the right structure.

pRZ202 was constructed in the same way except that pRZ414 (an inversion of the central region of Tn5 at the Bgl II sites; see Rothstein et al., 1980a) was used as the starting plasmid instead of pHCG12G. A substitution of the Eco RI-Sal I piece of pRZ414 for that of pBR322 gives transformants that are kanamycin-resistant, ampicillin-sensitive, and tetracycline-sensitive. The plasmid DNA from this strain was also digested with Eco RI + Sal I and with Hae II to show that it had the correct structure.

The construction of pRZ233 is described in the text. It was analyzed by digesting the plasmid DNA with Pvu II to make sure that the restriction sites were regenerated and its structure analyzed with several other restriction enzymes including Hae III.

pRZ236 and pRZ237 were constructed from pRZ233 and pRZ204, respectively. pRZ204 is an Eco RI-Sal I substitution of DNA from pRZ143 into PBR322. (pRZ143 is an inversion of Tn5 at the Hind III sites; see Rothstein et al., 1980a.) In order to place the resistance gene adjacent to the “hybrid” repeats, pRZ233 and pRZ204 were digested with Bgl II + Sal I and each was ligated to Bgl II + Sal cut pRZ143. Transformants resistant to ampicillin were selected and the appropriate restriction digests were done to check for the regeneration of the restriction sites and the presence of the desired structure.

To test the function of the polypeptide coded for by pRZ233 and pRZ202, a reconstruction of the Tn5 genome was carried out. pRZ241 and pRZ242 were made by digesting pRZ233 and pRZ202 respectively, with Eco RI + Sal I, and ligating them to Eco RI + Sal I-cleaved pRZ16. Kanamycin-resistant transformants harboring plasmids of the correct size were isolated and the plasmid DNA analyzed by restriction endonuclease analysis.

**Sequencing Procedure**

For sequencing Tn5, both the dideoxy chain termination (Sanger et al., 1977) and the Maxam-Gilbert procedures (Maxam and Gilbert, 1977) were utilized. The dideoxy sequencing involved cleaving the template plasmids (crZ149 for the right repeat and crZ152 for the left repeat) with Hpa II, which cleaves each of the two plasmids at a single site, and treating them with Eco III (Smith, 1979). Primers were isolated from polyacrylamide gels. To sequence starting at the Bgl II site in each repeat, Hae II-Bgl II fragments were isolated. The Hae II site in each case is in the unique region of Tn5 (see Jorgensen et al., 1979). After incubation with Klenow fragment polymerase, the four dideoxynucleotides and the four dideoxyribonucleotides, the nascent chains were cleaved with Bgl II and run on either an 8% acrylamide or 20% acrylamide gel. The same procedure was repeated using other primers including the 92 bp Pvu II-Bgl II from each inverted repeat (Jorgenson et al., 1979).

For Maxam-Gilbert sequencing, both pRZ152 and pRZ149 were cleaved with Bgl II, end-labeled with γ-32P-ATP (Maxam and Gilbert, 1977) and then reclawed with Hind III. The Bgl II-Hind III fragment was isolated from a 5% polyacrylamide and used in the chemical reactions. The resulting products were electrophoresed on both 8% and 20% polyacrylamide gels.

**Measurement of Neomycin Resistant Lysates**

The level of neomycin resistance conferred by the Tn5 plasmids in C600 SF6 was measured by a modification of the technique of Taft et al. (1979a). An identical number of cells was plated out on nutrient plates containing varying concentrations of neomycin. The number of surviving colonies was counted and the concentration of neomycin needed to kill 50% of the cells was determined graphically, this value being the EOPo of the strain.

**Minicells**

Minicells were purified as described by Roozen et al. (1971). Minicells obtained from a staphylococcal culture were washed and resuspended in 2 ml 1/4 met assay media (DFgo) in M9 salts, 0.5% glucose and 40 μg adenine per ml. They were then labeled with 50 mCi 35S-methionine for 30 min, pelleted and frozen. The pellets were resuspended in 100 μl sample buffer (Laemmli, 1970) and heated to 90°C for 2–5 min. 20 μl were electrophoresed on a 10% SDS-polyacrylamide gel (Laemmli, 1970).

**Measurement of the Level of Transposition**

The Tn5-containing plasmids to be tested for transposition were transformed into λ-blin lysogens. They were induced in 5 ml cultures and the resulting phage filtered. The phage from each Tn5-containing strain were analyzed for their ability to transduce kanamycin resistance by incubating an approximately equal number of plaque with an aliquot of Hfr1 and plating on nutrient plates containing 30 μg/ml kanamycin.

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