DNA Sequencing by Tn3 Insertion in Vitro: Comparison of the Shotgun and Ordered Methods

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Abstract

We sequenced an *E. coli* 6.6 kb fragment by using in vitro transposition reaction of Tn3. Tn3 inserted randomly into the target plasmid and the ends of the transposon can be used as movable primer sites. Our selection system eliminates cointegrates and leaves only the simple insertions which makes it suitable for sequencing. We applied both the shotgun method and the ordered method in order to compare them. The shotgun method required more clones but produced the total sequence quickly by the use of a suitable software such as DNAMAN. Moreover, the redundancy of sequencing involved in shotgun method has the advantage of making the result more reliable. The ordered method requires information about the order and separation of transposon insertion sites in different clones in order to select the plasmids to be used for sequencing, but resulted in minimum redundancy. However, the amount of preliminary work required in the ordered method before actual sequencing far outweighs the benefit in the reduction in the number of clones necessary to be sequenced. The *E. coli* sequence we obtained turned out to be a recombinant between the standard database sequence (AE000441) and another sequence published by Jensen's group (X00781) within the *rph* gene.

In vitro transposition of a transposon can be used as a tool in the sequencing of a DNA that is too long to be sequenced in a single step from a single primer, when the transposon has little insertion site specificity; ends of the transposon can be used as "movable primer sites" inserted in different locations in the DNA. Several systems are now commercially available that are based on this principle.

Maekawa *et al.*¹ has described an in vitro transposition system for Tn3. We modified their system to be suitable as a tool for use in sequencing a large DNA fragment. The modification consists in the choice of a selection system which allows only direct simple insertions of Tn3 to be selected, whereas Maekawa *et al.*'s system allows not only simple insertions but also cointegrates to survive. Cointegrates contain two copies of the transposon, which preclude their use as a sequenc-

ing aid. Our system can be used either as a shotgun method, because Tn3 can insert at random in DNA; or alternatively a preliminary ordering step can be included using appropriate restriction enzymes.

In this paper we used this system to determine the sequence of a 6.6 kb *E. coli* *Bam*HI DNA fragment derived from strain DOO *crp*⁻², cloned into the plasmid vector pKF3³ to give the plasmid pFJK1. We tested both the shotgun method and a sequencing procedure including a preliminary ordering step by using restriction enzymes, in order to compare their merits and demerits.

We used delta Tn3 transposon⁴ that is derived from Tn3. Most of the transposase gene is deleted in delta Tn3 contained in plasmid pMM200⁴. A cell extract was prepared from *E. coli* strain Kml196 harboring plasmid pMM240⁴ that overproduces Tn3 transposase. Cointegrates cannot survive at 42°C as they have the *kil* gene

Fig. 1. The shotgun method. A) Distribution of transposon insertion sites in plasmid pFJK1. (Black Bar) *E. coli* 6.6 kb DNA. (Solid black arrow) Genes within the cloned *E. coli* DNA; *rph*: RNase PH, *yicC*: putative alpha helix protein, *dinD*: DNA-damage-inducible protein, *yicG*: orf of a hypothetical protein, *yicF*: putative enzyme, *gmK*: guanylate kinase. Cm^R: chloramphenicol acetyl transferase (data obtained from *E. coli* genome database). The numbers in the parentheses are the coordinate in our system of the site of insertion or, in the case where insertion occurred in the vector part, the vector coordinate; spikes inside the plasmid circle indicate that the transposon inserted in orientation from right to left, while those outside the circle indicate left to right orientation with respect to the coordinate of pKF3. Directions of arrows indicate directions of transcription. 1 indicates the start site of the coordinate system of the plasmid going clockwise. B) Sequence assembly graph was obtained using DNAMAN Sequence Assembly menu; parameters used were minimum overlap, 50 bases; identity between bases of the overlapping sequences, 85%. The upper black line is the total sequence assembled from the different partial sequences. The names of the plasmids used are shown on the left. Primers R2 and F3 for plasmid pKF3 were used to sequence the ends of the 6.6 kb *E. coli* fragment. Sequences obtained from each delta Tn3 insertion with the Tn3 L primer were converted into the reverse complementary sequences and after removing the 5 bp overlap, joined to the sequences obtained with the Tn3 R primer, so that the direction of the arrows represents the orientation of insertion of delta Tn3 in each clone.

of pMM200. We used a restriction enzyme (*Tth*III) to eliminate the donor plasmid from the reaction mixture after transposition. This is to avoid the lethal effect of simultaneous transformation by an unreacted donor plasmid.

DNA sequencing was done with two primers corresponding to the ends of the transposon sequence and reading the sequence outward from the transposon. Joining the two sequences from the two primers for each clone gave about 1000-1600 bp sequences. Sequencing reactions were performed with BigDye terminator cycle sequencing kit (PE Applied Biosystems) and analyzed by ABI 377 automated DNA sequencer (PE Applied Biosystems). The transposon specific primers were Tn3 IRR (5'-GACCAAAATCCCTTAACG-3') and Tn3 IRL (5'-AGAAAACGTTGCTTAACG-3'). These primers are located near the right or left end within delta Tn3. For sequencing the junctions of the cloned 6.6 kb *E. coli* DNA with the vector pKF3, primers R2 (5'-GGACGTTTTACACCGTATTTC-3') and F3 (5'-TGCAAGAACTCTGTTATC-3') were used. The sequence assembly and data analyses were done by DNAMAN software for Macintosh (Lynnon Biosoft).

The transposition reaction mixture (40 μ l) contained 1.2 μ g (30 μ g/ml) donor plasmid pMM200, 0.7 μ g target DNA (pFJK1), the cell extract¹ containing Tn3 transposase (protein conc. 80 mg/ml), 25 mM HEPES-NaOH buffer (pH 7.6), 60 mM KCl, 12 mM Mg-acetate, 5 mM dithiothreitol, 100 μ g/ml tRNA, 50 μ g/ml BSA (bovine serum albumin), 2 mM ATP, 100 μ M each of the 4 dNTPs each, 40 μ M NAD, 20 mM phosphocreatine, 3% PVA, 100 μ g/ml creatine phosphokinase (CPK). The reaction was carried out at 30°C for 2.5 hrs and stopped by adding 160 μ l of 50 mM Tris-HCl buffer (pH 8.0) containing 10mM EDTA. Transposition reaction products were recovered by using QIAGEN-Mini, dissolved in 20 μ l water and digested by *Tth*III restriction enzyme to eliminate the transposon donor pMM200, followed by phenol treatment and ethanol precipitation with 4 μ g tRNA as carrier. After drying, the DNA was dissolved in 10 μ l water. 4 μ l of this DNA was transformed into 80 μ l of competent *E. coli* DOO *crp*⁻ cells² according to Dower *et al.*³. The culture was shaken for 1hr at 30°C and was plated on LB plates containing chloramphenicol (6 μ g/ml) and ampicillin (50 μ g/ml) followed by over-

night incubation at 42°C. Thirty-three Ap^R and Cm^R bacterial clones containing a plasmid, all with single transposon insertion were obtained. From these 33 plasmids we chose 17 plasmids at random and performed sequencing from the transposon primers in order to test the shotgun method. The site of insertion of transposon in each clone within the total sequence was deduced which was distributed more or less evenly (Fig. 1A). The sequence information from 17 clones was more than sufficient to assemble the total sequence of the *E. coli* DNA fragment (Fig. 1B).

To test the ordered sequencing method, restriction analyses of the 16 plasmids which were not used for the shotgun method were performed with three restriction enzymes *Bam*HI, *Sac*I and *Pst*I to identify the approximate transposon insertion sites of each clone prior to sequencing. We knew from the known sequence data of pKF3 and delta Tn3 and preliminary digestion of pFJK1 that a plasmid obtained by delta Tn3 insertion should give three, two and three fragments, for *Bam*HI, *Sac*I, and *Pst*I respectively. The lengths of restriction fragments obtained for each of these enzymes are dependent on the site of delta Tn3 insertion; we could calculate the approximate site of insertion of delta Tn3 for each clone using the restriction analyses data; actually we calculated all the 4 hypothetical outcomes of the possible positions, starting with the data of one enzyme, *Bam*HI for example, and compared the experimentally observed data for the other two enzymes with the calculated results, to choose the right position because delta Tn3 could insert in one of two orientations and the relative orientation of the restriction fragments has two alternative possibilities. To determine the position, at least 3 restriction enzymes, viz., *Bam*HI, *Sac*I and *Pst*I, had to be employed. The result also showed that in all the clones there were only single delta Tn3 insertions. Plasmids from two of the 16 clones contained the transposon in the vector part outside the cloned 6.6 kb *E. coli* DNA, but in the other 14 the transposon was located within the *E. coli* DNA. We used the insertion sites data to select the clones distributed suitably to give the total sequence. For the sequencing we chose only nine clones, and assembled the total sequence of the cloned *E. coli* DNA by using the Sequence Assembly function of the DNAMAN software (Fig. 2B). Thus ordering before sequen-

Fig. 2. The ordered method. A) Distribution of transposon insertion sites in plasmid pFJK1 used for the ordered method; those shown with dotted lines indicate clones not used for sequencing. Other explanations are similar to Fig. 1A. B) Sequence assembly graph for the ordered method. Sequences obtained from each delta Tn3 insertion with one Tn3 primer were converted into the reverse complementary sequences and after removing the 5 bp overlap, joined to the sequences obtained with the other primer, so that the direction of the arrows represents the direction of increase of coordinate from left to right in each clone. Other explanations are similar to Fig. 1B.

cing, though helpful to minimize redundancy of sequencing reaction, is very time-consuming and laborious. The location of the sites of insertion of delta Tn3 in plasmid pFJK1 in the 9 clones used in the actual sequencing in the ordered method as well as those not used (dotted lines) are shown in Fig. 2.

Thus we obtained the 6.6 kb sequence from both the shotgun and ordered methods. We have found that the

coordinates of the ends of the 6.6 kb sequence are 810306 and 816979 of the *E. coli* chromosomal DNA in the coordinate system of F.R. Blattner in Genbank (Accession No. AE 000441 & AE 000442). Our sequences obtained by both of the methods were completely identical. This sequence is identical to the database sequence except at one position; we got an extra G between the position 810642 and 810647 which changed a

Fig. 3. Comparison of our sequence with AE000441⁸ (shown in white in black) and X00781⁷ (shown in black in gray) sequences. The DNA sequence data are taken from Genbank/ EMBL/ DDBJ database (AE000441 or X00781). We count base position from the first base of the *rph* coding sequence. In the *rph* gene coding sequence we found one C insertion and one C deletion in comparison with X00781 which change the amino acids Gly Gly to Ala Trp as shown within the box. At the end of the *rph* coding sequence we found an extra G in comparison with AE000441, resulting in the addition of 10 amino acids, just as in X00781. Stop codons are shown with stars. The invertedly identical 9 bp sequences bracketing the 4 bp loop shown in the boxes constitute the attenuator for *pyrE*. Our sequence is therefore the result of a recombination event somewhere between these regions of two sequences corresponding to AE000441 and X00781.

run of 4 G's into a run of 5 G's.

As the two sequences obtained from the two methods showed the same difference from the database sequence it seems probable that the piece of DNA we sequenced actually had this difference from the database sequence. This would result in a frameshift of the *rph* gene coding sequence, in which this base is located, resulting in an *rph* protein 10 amino acids longer than that produced by the database sequence. As a matter of fact, Jensen⁷, have reported that *E. coli* K12 strains W3110 and MG1655, which are used to construct the database sequence, both have a single GC pair deletion near the end of the *rph* gene resulting in premature termination of the RPH protein and attenuation in *pyrE* expression, in contrast to other strains which are without this deletion (Accession number X00781 and V01578). Thus our DNA is similar to this active form of the *rph* gene. However, there are two consecutive amino acid substitutions at position 123 and 124, from Ala-Trp in the strains studied by Jensen, on the one hand, to Gly-Gly in our strain, W3110 and MG1655, on the other (Fig. 3). A closer look at the nucleotide sequences reveals that one can be generated by deletion and insertion of one C each from the other, i.e., two counteracting frameshift mutations separated by only 4 base pairs. It is thus probable that they do not affect the activity of RPH. Thus our sequence can be considered to have re-

sulted from a recombination between the AE000441 sequence and X00781 sequence (Fig. 3).

This new Tn3 based DNA sequencing strategy has allowed us to sequence the 6.6 kb fragment simply and rapidly. One single transposition reaction was sufficient to produce sufficient number of colonies necessary to get the total sequence both for the shotgun and ordered methods. In terms of speed the shotgun method is superior though one has to sequence more clones to get the total sequence. But it is faster and less tedious than the ordered method. The ordered method is more systematic as it selects the minimum essential number of clones prior to the actual sequencing. It thus requires a smaller number of clones to be sequenced, but the preliminary process, though straightforward, is laborious and time-consuming even in the case of a DNA of the size we chose to sequence, i.e., about 6.6 kb. If we had to sequence a longer DNA, the assembly function of the software we used would work as successfully for the shotgun method as in the present case, but the ordering process using restriction enzymes (or PCR) could increase in complexity in a forbidding way! Thus, as long as we stick to the ordered strategy, the use of transposon insertion is inferior to the deletion method⁶, which can order partial sequences naturally in a single agarose gel electrophoresis by the size of plasmids; but the availability of an efficient computer software for sequ-

Table 1 Comparison of Shotgun and Ordered Sequencing Strategies

	Shotgun	Ordered
Clones at the start	17	16
Number of clones sequenced	17	9
Total number of base pairs used for assembly	18740	10260
Assembled sequence (bp)	6674	6674
Redundancy *	2.8	1.5

*The average number of times a position was sequenced.

ence assembly from partial sequences makes the shotgun method with transposon insertion especially advantageous. Table 1 summarizes a comparison of the shotgun and ordered methods. In conclusion, the availability

of an inexpensive yet powerful software has made the shotgun method the method of choice for use with the transposon insertion.

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