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Recombineering linear DNA that replicate stably in *E. coli*

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Abstract

The advent of recombineering technology in *Escherichia coli* has revolutionized the way recombinant DNA molecules are constructed. We present a novel application of recombineering to linearize DNA by capping their ends with individual telomeres derived from bacteriophage N15, which exists as a linear prophage in *E. coli*. The N15 telomerase occupancy site was recombined into circular DNA and resolved into individual telomeres by the phage N15 protelomerase enzyme. We demonstrate this technique by assembling linear BACs that replicate stably in their host strain *E. coli* DH10B. Correct linearization of the BACs was confirmed by restriction mapping using pulsed field gel electrophoresis. The linear BAC DNA can be easily purified using standard plasmid isolation methods and resist degradation from RecBCD nuclease *in vitro* and *in vivo* owing to the presence of telomeres. Transfection of a linear 100 kb BAC containing the human β-globin gene cluster into HT1080 cells produced accurately spliced transcripts, demonstrating that the linear DNA will be useful for subsequent functional studies. This novel recombineering technique may be particularly useful for building large linear constructs for assembling artificial chromosomes with telomeres, and may provide a unique means to clone and study large linear viral genomes that contain hairpin ends.

Keywords: Recombineering; BACs; Phage N15; RecBCD; β-Globin; Linear; Telomeres

1. Introduction

The advent of recombineering technology in *Escherichia coli* has revolutionized the way recombinant DNA molecules are constructed (Ellis et al., 2001; Narayanan et al., 1999; Zhang et al., 1998). This technology obviates the requirement for suitably placed restriction sites by enabling DNA engineering by *in vivo* homologous recombination in *E. coli*. Recombineering takes advantage of RecA-independent recombination mechanisms that function in *E. coli*. In place of RecA, recombineering operates using phage derived proteins including RecE and RecT from the *Rac* phage or Redα and Redβ from phage λ (Narayanan et al., 1999; Zhang et al., 1998).

In this recombination system, linear PCR products can be recombined into circular targets *in vivo*

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in *E. coli* without being limited by construct size. Sequences homologous to the target, as short as 42 bp, are introduced at both ends of the PCR product to impart targeting specificity during homologous recombination. These homologous sequences can be conveniently incorporated at the 5′-ends of PCR primers and hence introduced into the ends of the PCR product during amplification. The PCR product is then electroporated into *E. coli* strains that are transiently induced to express the recombineering proteins to achieve homologous recombination (Narayanan et al., 1999; Zhang et al., 1998).

A high proportion of recombineering products form precisely as predicted by exchange of shared sequences through homologous recombination, with minimal unwanted rearrangements (Ellis et al., 2001; Narayanan et al., 1999; Narayanan and Warburton, 2003; Vadolas et al., 2002; Zhang et al., 1998). Using this technology, plasmids, bacterial artificial chromsosomes (BACs), and the *E. coli* chromosome have been modified using linear double-stranded DNA and single-stranded oligonucleotides to generate various insertions, deletions, as well as fusions with reporter genes (Ellis et al., 2001; Narayanan et al., 1999; Narayanan and Warburton, 2003; Vadolas et al., 2002; Zhang et al., 1998). Subsequently, two-step knock-in/knock-out strategies were introduced, which enabled introduction of subtle base changes without leaving behind operational sequences such as antibiotic markers (Jamsai et al., 2003; Warming et al., 2005). Further improvements including the use of single-stranded oligonucleotides for recombineering resulted in up to 1% of electroporated cells being recombinants (Ellis et al., 2001). Such high efficiencies enabled identification of recombinants without the need for selection, which shortened the modification process to one step. These improvements immensely advanced the manipulation of BAC clones containing genomic loci and large viral genomes and facilitated their functional studies in *in vitro* and *in vivo* models (Valenzuela et al., 2003; Testa et al., 2003; Dolphin and Hope, 2006).

Our goal was to further advance recombineering technology to develop a novel method to purify high-quality linear DNA directly from *E. coli* without restriction digestion or gel purification (Chrast et al., 1999). Linearization is achieved in *E. coli* by capping of DNA ends with individual telomeres derived from the bacteriophage N15 telomerase occupancy site (*tos*). Bacteriophage N15 exists as a linear plasmid capped with hairpin telomeres derived from this *tos* site during its prophage stage of life-cycle (Rybchin and Svarchevsky, 1999). We demonstrate this technique by assembling linear BACs with telomeres that replicate stably in their host strain *E. coli* DH10B. The N15 *tos* was recombined into circular BAC DNA and resolved *in vivo* into individual telomeres by the phage N15 protelomerase enzyme (Deneke et al., 2000; Ravin et al., 2001). The covalently closed telomeres protect the linear DNA from nuclease degradation in the *E. coli* strain and facilitate their replication as linear plasmids.

2. Materials and methods

2.1. Bacterial strains, plasmids, and bacteriophage DNA

The *asd*$^{-}$ *zeo*^{r} *E. coli* DH10B strain was constructed by deleting the aspartate β-semialdehyde dehydrogenase (*asd*) gene in strain DH10B (Narayanan and Warburton, 2003). Plasmid pGETrec contains the recombination genes, *recE*, r*ecT*, and *gam*, which are coordinately induced by arabinose (Narayanan et al., 1999). The 100 kb globin BAC (BAC4396), which contains the complete human globin gene cluster including the LCR and the 3′ DNase hypersensitive (HS) site was described previously (Kaufman et al., 1999).

2.2. Insertion of tos sequence into BACs using homologous recombination

Homologous recombination modifications were performed according to previously described protocol (Narayanan et al., 1999) except that the N15 telomerase occupancy site (*tos*) containing cells were grown at 30 °C with shaking at 150 rpm in order to promote stability of the palindromic *tos* sequence in *E. coli* DH10B. In this work, the *tos* site was amplified as a 519 bp fragment, which includes additional flanking regions from phage N15 genomic DNA (N15 positions 24471–24989 bp; GenBank Accession No. NC_001901) using primers tosF (5′-*ctc act* AAG CTT tct aag cgc aac ggt att ac-3′) and tosR (5′-*cgt gcg* GGA TCC ttc ccc cgt ttc taa gtc tc-3′). This PCR product was digested with BamHI and HindIII and cloned into similarly digested pBeloBAC11 (New England Biolabs, Inc.) to generate the 8 kb pBelo-tos.

In order to construct a tos-Zeo cassette for insertion onto BACs, the *tos* site and the chloramphenicol (Cm) resistance gene was amplified as a cassette from pBelo-tos using primers asdTosF (5′-TCC ATA ATC AGG ATC AAT AAA ACT GCT GCA GAA ATG ATT TCA TTC ATA ACt ccg tca ctt ccc aga tcc g-3′) and asdTosR (5′-CCT GCT TTG TTA GCA CGC AGA AAG TCC GCG GCA ATT ATC AGG GAA TTT GAt cta agc gca acg gta tta c-3′) and recombined 160 bp upstream of the chromosomally located zeocin resistance gene in