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Rapid modification of bacterial artificial chromosomes by ET-recombination

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ABSTRACT

We present a method to modify bacterial artificial chromosomes (BACs) resident in their host strain. The method is based on homologous recombination by ET-cloning. We have successfully modified BACs at two distinct loci by recombination with a PCR product containing homology arms of 50 nt. The procedure we describe here is rapid, was found to work with high efficiency and should be applicable to any BAC modification desired.

Bacterial artificial chromosomes or BACs (1) have become a widespread and powerful resource in molecular biology and a number of methods have been developed to manipulate them. However, these methods are not always readily applicable to every BAC modification desired, since some require the construction of specific plasmids (and thus the presence of suitable restriction sites around the region to be manipulated) whereas others only allow modification at certain loci (2–4). Furthermore, the need to screen through many colonies in order to obtain the desired recombinant makes these methods potentially quite elaborate and genetic instability may be a serious problem.

Recently, we developed a strategy that allows specific DNA engineering independently of the presence of suitable restriction sites (5). This method is based on homologous recombination mediated by the *rec*E and *rec*T proteins, and is thus referred to as ET-cloning. We previously described the pBAD-ETγ plasmid (5), which allows ET-cloning in *rec*BC$^+$ strains due to the constitutive expression of the recBC-inhibiting Gam protein (6). Here, we extend this technique to modify a BAC in its host strain and use a new plasmid, pBAD-αβγ, in which *rec*E and *rec*T have been replaced by their respective functional counterparts of phage lambda (Figs 1A and 2A). Using this plasmid, the overall efficiency of specific recombination was found to be 1–3-fold higher as compared to pBAD-ETγ (data not shown).

To show that BACs can be manipulated by ET-cloning, we obtained a BAC from Research Genetics that carries the mouse homolog of the human AF4 gene (7 and data not shown). In the first approach, we replaced the chloramphenicol resistance gene (cmR) which is resident in the AF4-BAC vector by the zeocin resistance gene derived from pZero1 (Invitrogen). The 5′ oligo we designed for this experiment consisted of a 50 nt homology arm (which allows recombination with the homologous sequence 5′ of the cmR gene on the AF4-BAC), followed by a 21 nt stretch which primes the PCR amplification of the zeocin gene. The 3′ oligo was similarly constructed, with a stretch of 50 nt that is homologous to the 3′ region of the AF-4 BAC cmR, followed by a 21 nt stretch that primes the 3′ end of the PCR amplification of the zeocin gene. The PCR reaction, *Dpn*I digestion and DNA purification were done as previously described (5). After transformation of the BAC packaging strain HS996 (a phage-resistant derivative of DH10B, Research Genetics) with pBAD-αβγ, we followed the procedure which is available from our webpage (http://www.embl-heidelberg.de/ExternalInfo/stewart/index.html) except that the cells used for ET-cloning were arabinose-induced for 1 h and subsequently harvested at an OD_{600} of 0.20–0.25. Following this procedure, we obtained an average of 130 colonies in three independent experiments from which we analyzed a total of 18 colonies. All except one, which failed to grow in liquid medium, were correct. Figure 1B shows the results from six independent colonies. Notably, no unintended rearrangements of the BAC are evident from the total *Eco*RI restriction fragment pattern. This experiment was successfully repeated using a different BAC, showing that the procedure is not specific to the AF-4 BAC (data not shown).

To show that this BAC modification method is not marker- or locus-specific, we replaced the protein E sequence that is present in the vector of the AF4-BAC by the kanamycin resistance gene Kan (which originates from the Tn903 transposon) flanked by FRTs. The FRTs were included in the oligonucleotides between the 50 nt homology arm and the 21 nt region that primes the PCR amplification (Fig. 2). In three independent experiments we obtained an average of 100 colonies that were resistant to both chloramphenicol and kanamycin. Of these, seven BAC DNA preparations were digested with *Hin*dIII and checked by Southern hybridization with the Kan PCR product that was also used for recombination. As shown in Figure 2B, all of the BAC preparations analyzed showed the expected bands. The Kan gene was then removed by Flp excision after transformation of the modified BAC into 294-FLP cells (8, Fig. 2A). As is shown in Figure 2B, the excision took place in all of the tested BAC clones. Thereby, we constructed an AF-4 BAC clone which contains a single FRT at the original protein E locus.

The method that is described here should allow modification of BACs, as well as P1s and PACs (5), at any desired site, and is not dependent on the presence of specific restriction sites flanking the locus to be modified. The construction of specific plasmids is not

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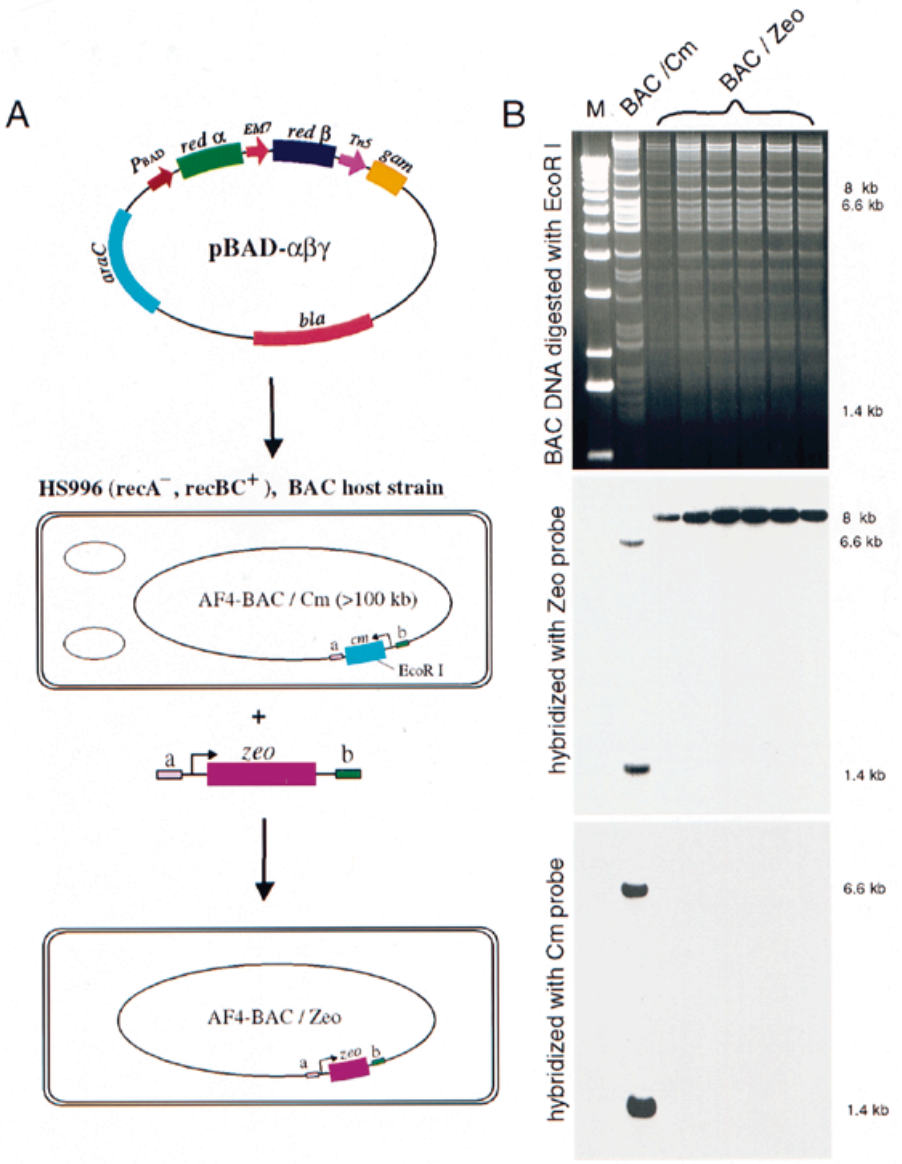
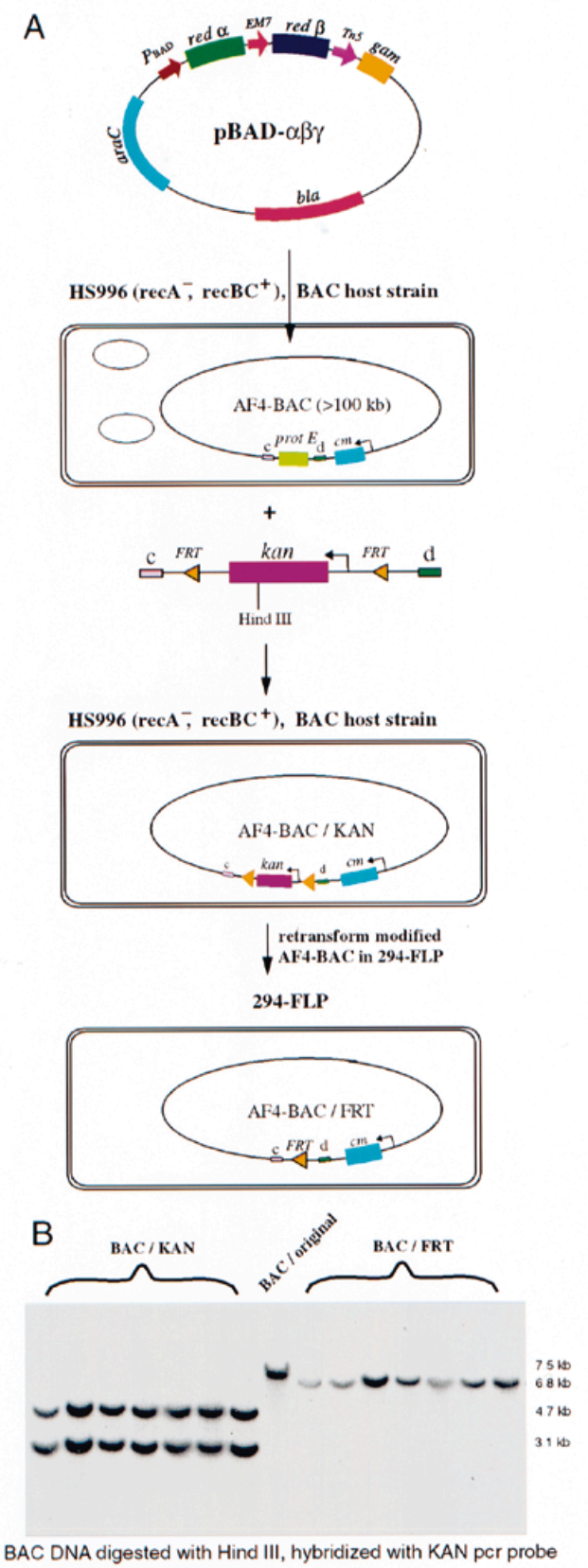


Figure 1. (**A**) Outline of the strategy to replace the CmR gene of the AF4-BAC by the Zeocin resistance gene. Sequences labeled a and b depict the 50 nt homology regions between the PCR product and the targeted AF4-BAC. First, pBAD-αβγ was transformed into the BAC host cells. Subsequently, Redα expression was induced with arabinose, competent cells were prepared and then transformed with the shown PCR product and plated onto 30 µg/ml zeocin plates. (**B**) Analysis of BAC DNA preparations. The upper panel shows the *Eco*RI digestion pattern of the original AF-4 BAC (lane BAC/Cm) and six independent modified BACs (lanes BAC/Zeo). The recombined AF-4 BAC contains the zeocin gene instead of the original CmR gene, thereby deleting the *Eco*RI site which is present in the CmR gene. We were unable to observe the recombination-specific bands because of the presence of comigrating additional bands in the same region. The middle panel shows the results of Southern hybridization with a probe that was made from the Zeo PCR product shown in Figure 1A. Strong hybridization to the 8 kb Zeo-containing fragment in the modified BAC/Zeo and weaker hybridization to the 6.6 and 1.4 kb fragments in the original BAC due to the presence of the homology arms a and b in the probe are evident. In the lower panel we used a CmR-specific probe, which resulted in hybridization only to the original BAC. M, 1 kb ladder (New England Biolabs).

required. Furthermore, the recombination reaction occurs in the BAC host strain and thus in a *rec*A⁻ background, which potentially reduces the risk of undesired intramolecular recombination. This risk is also limited because the expression of Redα requires arabinose induction, thereby limiting the recombinogenic window to that period in which the ET cloning step is being performed. Our results so far, although limited to only three BACs, indicate that the method does not provoke unwanted recombination between repetitive elements resident in the BAC (data not shown). Conveniently, we found that the pBAD-αβγ plasmid was rapidly lost in the absence of continued ampicillin selection. Consequently, BAC DNA preparations could be made without obvious contamination by this plasmid. Since the method we present here is quickly performed, appears to work with high fidelity and does not appear to be BAC- or locus-specific, it can potentially become the method of choice for the modification of BACs.



BAC DNA digested with Hind III, hybridized with KAN pcr probe

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Figure 2. (**A**) Outline of the strategy to replace the protein E gene of the AF4-BAC by a single FRT. Sequences labeled c and d depict identical sequences on the PCR product and the targeted AF4-BAC. First, the protein E gene was replaced by the Kan gene flanked by FRTs, using 25 μg/ml chloramphenicol and 30 μg/ml kanamycin plates for selection. Then, the Kan gene was excised by Flp recombination in 294-FLP cells, resulting in a single FRT at the locus of the original protein E sequence. (**B**) Analysis of seven independent BAC DNA preparations. All the bands shown were obtained after *Hin*dIII digestion and hybridization with a probe that was made from the KAN PCR product shown in Figure 2A. The recombined AF-4 BAC (lanes BAC/KAN) contains the Kan gene flanked by FRTs instead of the protein E sequence, thereby introducing an extra *Hin*dIII site which results in two distinct bands. After Flp recombination, the FRT containing BAC (lanes BAC/FRT) displays a single band which is smaller than the original BAC (lane BAC/original) due to the deletion of the protein E sequence.