when synthesized with Vent DNA polymerase. Our decreased substitution rate presumably reflects the use of the higher-fidelity Pfu DNA polymerase (4).

Less satisfying was the finding of small point deletions in all clones sequenced. Different clones had different deletions, but most (60%) had only a single base missing. These deletions are presumably the result of incorporation of a non-full-length oligonucleotide, amongst the 84, during LCR. No deletions were detected in the clones produced by Au et al. (1) with gel-purified oligonucleotides, suggesting that oligonucleotide purification is an important consideration.

Several options are therefore open to the investigator working to scale up the LCR-based approach to gene synthesis at minimum expense. Costs will rise steeply if gel purified oligonucleotides are purchased, as not only does purification incur additional charges but the synthesis must also be on a larger scale. In house gel purification of oligonucleotides is possible but increases the time spent on gene synthesis. A higher annealing temperature could be tested for LCR to minimize incorporation of non-full-length oligonucleotides, and/or more clones could be sequenced, at additional expense, to identify those without deletions. Considering all these factors, and our experience, the most cost- and time-effective strategy is to simply assemble the gene from non-purified oligonucleotides and perform site-directed mutagenesis to correct a single base pair deletion.

As anticipated by Au et al. (1), the LCR-based method for gene synthesis can be applied to larger genes by increasing the number of segments. In addition, this study shows that scaling up the LCR method with non-purified oligonucleotides is a cost- and time-effective approach to in vitro gene synthesis.

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Improvement of the pBRINT-Ts Plasmid Family to Obtain Marker-Free Chromosomal Insertion of Cloned DNA in E. coli

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The expression of heterologous genes by recombinant microorganisms usually depends on the use of extrachromosomal replicating plasmid vectors that carry the gene(s) of interest. Although the usefulness of this type of vehicle has been well established in many cases, several problems related with their use have been identified, the most important being inefficient segregation and/or structural instability, as well as undesired copy number effects. An alternative approach for increasing the stabilization and controlling the copy number of heterologous genes is the chromosomal insertion of the gene(s) of interest.
We have recently described the construction and use of a plasmid vector family with a temperature-sensitive replicon, the pBRINT-\(T_s\) plasmids, which enable the insertion of cloned genes into the lacZ locus of the \(E.\) coli chromosome (4). No special intermediate strain is needed, and virtually any \(\text{RecA}\) \(\beta\)-galactosidase\(+\) (\(\beta\)-gal\(^+\)) \(E.\) coli strain is a suitable recipient to obtain chromosomal insertions using the pBRINT-\(T_s\) plasmids. Simultaneously, we have developed a simple and rapid procedure for the insertion and curing of these plasmids (4).

With pBRINT-\(T_s\) plasmids, an antibiotic resistance (Anb\(^R\)) marker for the positive selection of integrates is introduced together with the gene(s) of interest. The presence of Anb\(^R\) genes in genetically engineered bacteria might retard their use in some applications due to the increased possibility of horizontal gene transfer, which leads to the uncontrolled spread of these resistance genes to other microorganisms. Moreover, when the genetic improvement of any strain requires several successive modifications, each one requiring the introduction of a different Anb\(^R\) marker, it becomes more and more difficult to find new antibiotic genes. Therefore, it would be useful to engineer stable recombinant strains that do not contain any residual Anb\(^R\) markers.

The aim of this study was to modify the pBRINT-\(T_s\) plasmids to remove the Anb\(^R\) marker used for selection of modified cells after obtaining chromosomal insertion of the gene(s) of interest. In these new plasmids, pBRINT\(_s\)-rAnb\(^R\) family, the Anb\(^R\) selection markers are flanked by two parallel \(\text{loxP}\) sites, allowing their subsequent removal by Cre recombinase action from bacteriophage P1 (6,8).

The first step in the development of this new vector family was the construction of plasmids in which the removable Anb\(^R\) marker (i.e., Anb\(^R\) genes placed between two \(\text{loxP}\) sites, rAnb\(^R\)) was flanked by multiple cloning sites (MCS) derived from Bluescript\textsuperscript{TM} II SK\(+\) plasmid (Stratagene, La Jolla, CA, USA). The pBSL98 plasmid (1), from which the kanamycin resistance gene was removed by \(MluI\) digestion, followed by blunt-ending with T4 DNA polymerase, was used as backbone to construct this new set of plasmids. The rAnb\(^R\) cassettes chosen to construct this plasmid family encode resistance to chloramphenicol (Cat\(_2\)), gentamycin (Gen\(_4\)), and kanamycin (Kan\(_2\)). The Cat\(_2\), Gen\(_4\), and Kan\(_2\) cassettes were obtained as 1065-, 881-, and 1269-bp \(BamHI\)-Not\(_I\) DNA fragments from plasmids pLoxCat2, pLoxGen4, and pLoxKan2, respectively (6). These restriction fragments were blunt-ended with T4 DNA polymerase and then ligated to the 3089-bp \(MluI\) blunt-ended fragment of pBSL98 to obtain pBPSCat2, pBPSGen4, and pBPSKan2 plasmids. Since in these plasmids the rAnb\(^R\) cassettes are flanked by directly repeated MCS from plasmid Bluescript II SK\(+\), digestion with a restriction enzyme whose cleavage site is present in both MCS should release the rAnb\(^R\) cassettes preceded by a single MCS.

The new plasmids containing MCS-Cat\(_2\), MCS-Gen\(_4\), or MCS-Kan\(_2\) cassettes were constructed using the plnt-\(T_s\) plasmid as backbone (4). For this purpose, the MCS-Cat\(_2\), MCS-Gen\(_4\), and MCS-Kan\(_2\) cassettes were obtained as Sac\(_I\) fragments from plasmids pBPSCat2, pBPSGen4, and pBPSKan2, respectively. These DNA fragments were blunt-ended with T4 DNA polymerase and then inserted into the unique EcoRV site of plasmid plnt-\(T_s\).

![Figure 1. General structure and physical maps of the pBRINT-rAnb\(^R\) plasmids and rAnb\(^R\) genes.](image-url)
to obtain pBRINT<sub>e</sub>-Cat2, pBRINT<sub>e</sub>-Gen4, and pBRINT<sub>e</sub>-Kan2 plasmids, respectively (Figure 1).

We performed the chromosomal insertion of the pBRINT<sub>e</sub>-rAnb<sup>R</sup> plasmids following our previously described temperature-based insertion/selection procedure (4). Therefore, the nearly wild-type strain E. coli W3110, used as model host in this study, was electroporated with covalently closed circular DNA of each of the pBRINT<sub>e</sub>-rAnb<sup>R</sup> plasmids. Afterwards, one single colony transformed with each plasmid was grown in 0.5 mL LB medium without Anb for 4 h at 30°C, reincoculated into 10 mL fresh medium, and incubated additionally for 6 h at 30°C to obtain more biomass. The cultures were finally incubated overnight at 37°C, and several dilutions (10<sup>-3</sup>–10<sup>-6</sup>) were plated on solid media supplemented with X-gal, isopropyl-β-D-thiogalactoside (IPTG), and the appropriate Anb (15 µg/mL Km for pBRINT<sub>e</sub>-Cat2; 5 µg/mL Gm for pBRINT<sub>e</sub>-Gen4, and 15 µg/mL Km for pBRINT<sub>e</sub>-Kan2). After 24 h of incubation at 44°C, large Anb white colonies grew at a frequency of at least 10<sup>-3</sup> with respect to total viable cells as for the previously described pBRINT-T<sub>e</sub> plasmids. The white phenotype indicates that the plasmids have been integrated into the lacZ chromosomal gene. Then, the white colonies were screened at 44°C for sensitivity to carbenicillin (Cb<sup>s</sup>) (3) and at the same time for resistance to Cm (Cm<sup>R</sup>), Gm (Gm<sup>R</sup>), or Km (Km<sup>R</sup>) depending on the plasmid initially utilized to transform the cells. A significant portion of the tested colonies, from 2% to 20%, were Cb<sup>s</sup> at 44°C, indicating that the chromosomal insertion event by double homologous recombination occurred efficiently in these cells. These colonies were also Cb<sup>s</sup> at 30°C, which confirmed plasmid loss. The insertions were confirmed by PCR analysis as previously described (4) (data not shown). The allelic exchange frequencies obtained with the pBRINT<sub>e</sub>-rAnb<sup>R</sup> plasmids were 20%, 6%, and 2% for vectors pBRINT<sub>e</sub>-Cat2, pBRINT<sub>e</sub>-Gen4, and pBRINT<sub>e</sub>-Kan2, respectively. These efficiencies were similar to those obtained with the previously described pBRINT-T<sub>e</sub> plasmids (4).

The remaining Anb<sup>R</sup> markers used to select chromosomal insertions were mediated by site-specific recombination mediated by the Cre recombinase as already described (6). Briefly, W3110 lacZ::Cat2, lacZ::Gen4, and lacZ::Kan2 cells were transformed with the temperature-sensitive pJW168 plasmid, which carries the cre gene under the control of the PlacUV5 promoter. Transformed cells were replica-plated on LB solid medium containing X-gal without or with IPTG to induce Cre production at 30°C. One hundred percent of the colonies tested lost the Anb<sup>R</sup> marker but still remained β-gal<sup>-</sup>. This result indicates that theloxP site, which remains after Cre recombinase action, interrupts the lacZ gene. Elimination of the Anb<sup>R</sup> marker was also confirmed by PCR analysis (data not shown). Plasmid pJW168 was subsequently removed from the β-gal<sup>-</sup> cells by increasing the culture temperature to 37°C or higher, and its loss was confirmed by the loss of the Cb<sup>R</sup> phenotype.

The pBRINT<sub>e</sub>-rAnb<sup>R</sup> plasmids are useful tools designed for site-specific integration of cloned DNA into the lacZ gene, without leaving in the chromosome any Anb<sup>R</sup> markers. This is feasible due to the presence of tandemloxP sites flanking the Anb<sup>R</sup> genes in each plasmid, which allows subsequent removal of these genes by the action of the Cre recombinase. Therefore, the resulting cells do not contain any Anb<sup>R</sup> marker and can be transformed or further modified with plasmids containing the same Anb<sup>R</sup> marker previously used for the selection of integrants. The pBRINT<sub>e</sub>-rAnb<sup>R</sup> plasmids are an addition to a growing group of molecular tools designed for achieving chromosomal gene insertion and antibiotic resistance-encoding gene removal (5,7).

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