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Efficient Homologous Recombination in Fast-Growing and Slow-Growing Mycobacteria

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Although homologous recombination is a major mechanism for DNA rearrangement in most living organisms, it has been difficult to detect in slowly growing mycobacteria by a classical suicide vector approach. Among the possible reasons for this are the low levels of transformation efficiency, the relatively high levels of illegitimate recombination, and the peculiar nature of the *recA* gene in slowly growing mycobacteria. In this report, we present an efficient homologous recombination system for these organisms based on the use of replicative plasmids which facilitates the detection of rare recombination events, because the proportions of recombined molecules increase over time. Intraplasmid homologous recombination in *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG was easily selected by the reconstitution of an interrupted kanamycin resistance gene. Chromosomal integration via homologous recombination was selected by the expression of the kanamycin resistance gene under the control of a chromosomal promoter that was not present in the plasmid before recombination. This technique was termed STORE (for selection technique of recombination events). All the clones selected by STORE had undergone homologous recombination, as evidenced by PCR analyses of the kanamycin-resistant clones. This technique should be applicable to all organisms for which homologous recombination has been difficult to achieve, provided the gene of interest is expressed.

Homologous recombination is a powerful mechanism by which DNA fragments can be inserted, deleted, or altered at specific sites in the genome, but it has been notoriously difficult to demonstrate in slowly growing mycobacteria (7). If such a mechanism could be efficiently used in slowly growing mycobacteria, it would help tremendously in understanding the molecular mechanisms of diseases such as tuberculosis and leprosy. In addition, the recent development of *Mycobacterium bovis* BCG as a live vaccine vector for the presentation of heterologous antigens (2, 22) would also benefit from the possibility of inserting foreign genes into the chromosome at precise positions to ensure the persistence of the heterologous genetic information in the recombinant vaccine strains.

Although homologous recombination is relatively efficient in fast-growing mycobacteria, such as *Mycobacterium smegmatis* (10), previous attempts to achieve homologous recombination by various selection methods resulted mainly in the detection of illegitimate recombination in slowly growing *M. bovis* BCG and *Mycobacterium tuberculosis* (11). In these studies nonreplicating plasmids were used. A significant increase in the level of transformation efficiency of linear DNA allowed for the detection of homologous recombination events in BCG (1). However, by this approach, only single homologous recombination events were observed, apparently resulting from an unclassical strand invasion mechanism. More recently, and while this paper was in preparation, Norman et al. (17) described a method for the replacement of a BCG target gene by homologous recombination, indicating that slowly growing mycobacteria are not completely refractory to homologous recombination.

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The results of these studies suggest that the low-level transformation efficiency of slowly growing mycobacteria and the use of nonreplicating plasmids may have constituted major handicaps for the detection of homologous recombination. We therefore decided to dissociate the recombination events from the transformation efficiencies by the use of replicative plasmids. Since recombination has been proposed to occur simultaneously with DNA replication in some instances (23), we reasoned that the use of replicative plasmids might significantly increase the recombination frequency. However, the use of replicating plasmids requires a recombination phenotype distinct from the transformation phenotype.

In this study we describe a powerful selection system for intraplasmid and plasmid-chromosomal homologous recombination in mycobacteria. With replicating plasmids, relatively high levels of homologous recombination were obtained in fast- and slowly growing mycobacteria, and 100% of the selected clones had undergone homologous recombination.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All cloning steps were performed with *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.). The mycobacterial strains used were *M. bovis* BCG (vaccine strain 1173P2) and *M. smegmatis* mc^2155 (21), an efficient plasmid transformation mutant of *M. smegmatis* mc^6 (generously provided by T. Kieser, Norwich, United Kingdom). Cultures of *M. bovis* BCG or *M. smegmatis* were grown at 37°C in Sauton medium (20) supplemented with 10 μg of $HgCl_2$ per ml, 15 μg of streptomycin per ml, or 20 μg of kanamycin per ml when appropriate. Mycobacterial transformation was performed as previously described (12). After transformation, the mycobacteria were plated onto Middlebrook 7H10 agar (Difco) supplemented with 15 μg of streptomycin per ml, 20 μg of kanamycin per ml, or 12 μg of $HgCl_2$ per ml as indicated in Table 1. MICs of kanamycin, as defined by the lowest concentrations of kanamycin at which no bacterial growth is detected, were estimated as previously described for mercury compounds (3).

Plasmids and DNA manipulation. pBluescript $SKII^+$ and pUC4K were purchased from Stratagene and Pharmacia LKB (Uppsala, Sweden), respectively. pHP45Ω (18) was kindly provided by H. Krisch (Geneva, Switzerland). pRR3ΔKan is a pAL5000 derivative described previously (12), and pEN102 is described elsewhere (5). The latter plasmid provides mercury resistance to both slowly and fast-growing mycobacteria and is a derivative of pMR001 (3). Re-

striction analyses of plasmids from mycobacteria were done after electroduction into *E. coli*, as described by Baulard et al. (4).

Restriction enzymes, T4 DNA polymerase, polynucleotide kinase, Klenow fragment, and other DNA modifying enzymes were purchased from Boehringer (Mannheim, Germany), Pharmacia LKB, and New England Biolabs (Beverly, Mass.). All DNA manipulations were performed under standard conditions, as described by Sambrook et al. (19).

Plasmid constructions. pRC016 was constructed as follows. A 0.7-kb *Hin*dII-*Hin*dIII DNA fragment containing the 5′ region of the kanamycin resistance (Km^r) gene from pUC4K was cloned into the corresponding sites in pBluescript $SKII^+$ to generate pRC002. A 2-kb *Hin*dIII fragment isolated from pHP45Ω and containing the streptomycin resistance (Sm^r) gene was cloned into the unique *Hin*dIII site of pRC002. The resulting pRC003 was then cut with *Eco*RV and *Bam*HI and religated with a 0.9-kb *Nru*I-*Bam*HI fragment from pUC4K containing the 3′ region of the Km^r gene. This plasmid, named pRC007, was then cut by *Hin*dII, and the 3.6-kb fragment was cloned into the filled-in *Bam*HI site of the mycobacterium-*E. coli* shuttle plasmid pEN102 containing the mercury resistance ($HgCl_2^r$) genes. The resulting shuttle vector pRC016 therefore contains a disrupted Km^r gene with a duplicated central 463-bp region that constitutes the target site for intra- or intermolecular homologous recombination.

The construction of pStore4 was as follows. The complete BCG *hsp60* gene was amplified by PCR from chromosomal DNA extracted from BCG. The oligonucleotides used had the following sequences: 5′-ATTGCGGACGGCC AAACCTACG-3′ and 5′-CTCGCCGGGGTCAGAAATCCAT-3′. The amplified product was then cloned into the *Sma*I site of pBluescript $SKII^+$ to generate pStore1. The promoter region of the Km^r gene from Tn*903* was eliminated ($Km^r\Delta P$) by PCR with an oligonucleotide overlapping the ribosomal binding site (5′-TTCAATACAAGGGGTGTTATGAGC-3′) and an oligonucleotide corresponding to sequences behind the stop codon (5′-TTCGAGGGAGCCACGGT TGATGAG-3′). A transcriptional fusion between the *hsp60* gene and the $Km^r\Delta P$ fragment was then generated by cloning this PCR product, which had previously been phosphorylated, self-ligated, and then digested with *Eco*RI, into the *Eco*RI site of pStore1. The resulting plasmid, named pStore2, was then digested with *Xba*I and *Eco*RV, blunt ended with Klenow fragment, and purified on agarose. The purified 3-kb fragment containing the *hsp60-Km*$^r\Delta P$ fusion was then ligated into the unique *Sca*I site of pMR002 to yield pStore3. The pMR002 shuttle vector is a pRR3ΔKan derivative in which the 2-kb *Sma*I fragment isolated from pHP45Ω and containing the Sm^r gene was cloned into the unique blunt-ended *Pst*I site. The 0.65-kb *Bam*HI fragment, which contains the promoter and the 5′ end of the *hsp60* gene, was then deleted from pStore3 to yield pStore4. In contrast to pStore3 and as expected, pStore4 did not confer Km^r to *E. coli*, *M. smegmatis*, or BCG.

Preparation of mycobacterial DNA. Mycobacteria were grown to late log phase in Sauton medium and harvested by centrifugation at 6,000 × *g* for 5 min. The pellet was weighed, resuspended in 10% glycerol (1 ml/200 mg), aliquoted in 1-ml fractions, and centrifuged again at 6,000 × *g* for 10 min. The semidried mycobacterial pellets of approximately 200 mg were then subjected to 3 consecutive 10-s microwave heatings (900 W) and resuspended into 1 ml of buffer 1 (3% sodium dodecyl sulfate, 1 mM $CaCl_2$, 10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 200 μg of proteinase K per ml). After the addition of 200 mg of white Fontainebleau sand (grain size, 150 to 210 μm in diameter Rhône-Poulenc, France), the suspension was vigorously shaken for 10 min, incubated for 30 min at 56°C, and shaken for an additional 15 min. The suspension was centrifuged for 15 min at 14,000 × *g*, and the supernatant was adjusted to 2 mM (final concentration) EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid]. The DNA was precipitated by the addition of 0.8 volume of isopropanol and 0.3 M sodium acetate (final concentration) and resuspended in 0.5 ml of TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA). After heating the DNA solution for 5 min at 68°C, 1 volume of phenol-chloroform-isoamyl alcohol (49/49/2) was added and the solution was vigorously mixed and then centrifuged at 14,000 × *g* for 10 min. One volume of water-saturated ether was added to the aqueous phase, and after mixing, the solution was centrifuged again at 14,000 × *g* for 5 min. Finally, sodium acetate (0.3 M final concentration) and 2.5 volumes of ethanol were added to the aqueous phase and incubated at 4°C for at least 10 min. After centrifugation for 30 min at 14,000 × *g*, the DNA was air dried, dissolved in 50 μl of TE buffer, and stored at −20°C until further use.

Nucleotide sequence determination. Nucleotide sequences were determined with double-stranded DNA by the dideoxyribonucleotide chain-termination method with [α-^{35}S]dCTP (1,000 Ci/mmol; Amersham France, Les Ulis, France) and a DNA T7 sequencing kit (Pharmacia LKB), as recommended by the supplier.

RESULTS

Intraplasmid homologous recombination in *E. coli*. To test the use of replicating plasmids for the detection of intra- or interplasmid homologous recombination, pRC016 was constructed. This plasmid contains a disrupted Km^r gene with a 463-bp DNA duplication; the duplicate sections are separated by a 2-kb DNA fragment which contains the Sm^r gene (Fig. 1). In addition, pRC016 contains the $HgCl_2^r$ genes so that transformants can be selected with mercury. Homologous recombination can subsequently be detected by resistance to kanamycin, which can occur only through intra- or interplasmid homologous recombination as illustrated in Fig. 1.

Since one of the reasons for the apparent low levels of homologous recombination detected by previous studies could be related to the unusual nature of the *recA* gene of the members of the *M. tuberculosis* complex (7), we first tested pRC016 in *E. coli* in a $RecA^-$ background. After overnight incubation at 37°C, the colony counts indicated that the recombination rates were in the range of 10^{-3} to 2.10^{-4} (Table 1).

Plasmid DNA of eight independent clones was then isolated and used to retransform *E. coli* XL1-Blue. All transformants were Km^r and Sm^s, suggesting that intraplasmid homologous recombination had occurred. Plasmid DNA from one colony of each transformed group was then isolated and digested with *Hin*dIII or *Eco*RI. As shown in Fig. 2A, digestion with *Eco*RI resulted in the appearance of three fragments of 2.85, 3.4, and 5.6 kb in size. The first and the third fragments were also found in the original pRC016, whereas the second fragment had replaced the original 2.75-kb and 3.25-kb fragments of pRC016. Digestion with *Hin*dIII resulted in three fragments of 5.95, 4.9, and 1.1 kb. Two additional *Hin*dIII fragments of 2 and 0.5 kb were detected in the original pRC016. These fragments originally contained the Sm^r gene and the duplicated region of the Km^r gene. Therefore, these restriction analyses confirmed that intramolecular homologous recombination had occurred.

Intraplasmid homologous recombination in mycobacteria. Since the introduction of pRC016 into *E. coli* XL1-Blue already resulted in a significant proportion of recombined pRC016 conferring resistance to kanamycin, transformation of *M. smegmatis* or *M. bovis* BCG with pRC016 prepared from *E. coli* XL1-Blue generated a background of approximately 10^{-3} to 10^{-4} Km^r mycobacteria. To avoid this interference, four independent $HgCl_2^r$ clones were selected after transformation of *M. smegmatis* and BCG with pRC016. These clones contained the original pRC016 that had not yet undergone homologous recombination, as determined by restriction analysis after electroduction into *E. coli*. After cultivating these clones in liquid medium containing 10 μg of $HgCl_2$ per ml for 7 days (BCG) or for 2 days (*M. smegmatis*), recombination events were enumerated by counting the CFU on Km- or $HgCl_2$-supplemented plates (Table 1). Approximately the same proportion (10^{-3} to 10^{-4}) of Km^r colonies among the $HgCl_2^r$ colonies was obtained with BCG as was obtained with *M. smegmatis*. The plasmid DNA of four independent Km^r BCG colonies from each of the four original BCG clones (16 total) was analyzed by *Eco*RI digestion after electroduction into *E. coli*. As shown in Fig. 2B, all clones had the same restriction pattern, which was indistinguishable from that observed after intramolecular recombination in *E. coli* XL1-Blue (compare Fig. 2B with 2A). All Km^r clones were Sm^s, confirming that intramolecular recombination of pRC016 occurs in both BCG and *M. smegmatis* (Fig. 1A). These results show that, like *E. coli* XL1-Blue, both fast- and slowly growing mycobacteria can undergo homologous recombination at high frequency.

Homologous recombination between replicative plasmids and the chromosome in fast-growing and slowly growing mycobacteria. The high levels of homologous recombination detected in both fast-growing and slowly growing mycobacteria when replicative plasmids are used, compared with the levels seen in previous studies (1, 11), suggest that replication increases the frequency by which plasmid-plasmid recombination is detected in mycobacteria. This encouraged us to adapt the concept to plasmid-chromosome recombination.

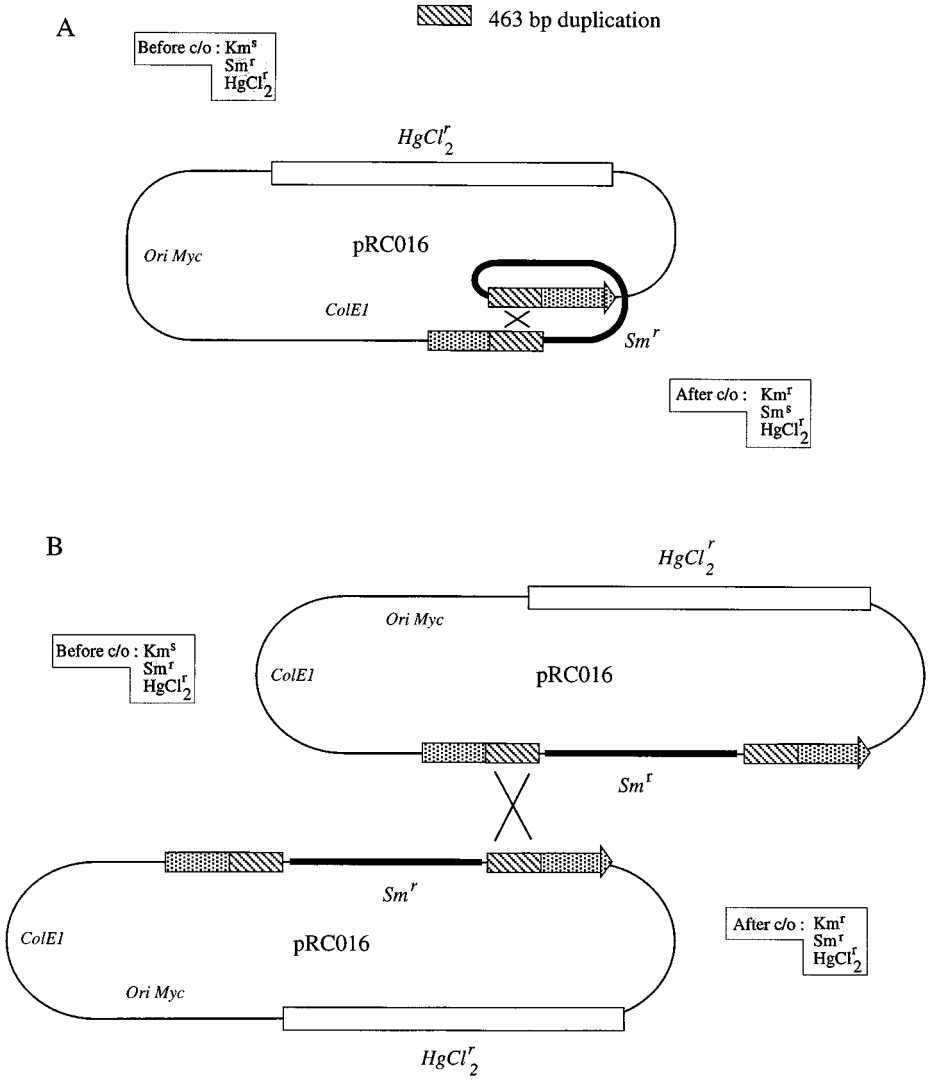

FIG. 1. Intra- and interplasmid homologous recombination. Two different recombination events can theoretically occur to create a functional Km^r gene. Intramolecular recombination of pRC016 (A) results in the loss of the Sm^r gene, whereas intermolecular recombination of pRC016 (B) retains the Sm^r gene in the recombined plasmid. The $HgCl_2^r$ genes are maintained after both events. The antibiotic-resistant and -sensitive phenotypes before and after recombination are indicated. The stippled arrows and boxes depict the Km^r genes, which contain duplicate 463-bp regions from the original plasmid. The open boxes designate the $HgCl_2^r$ genes, and the thick black lines designate the Sm^r genes. The approximate positions of the mycobacterial and ColE1 origins of replication are indicated as *OriMyc* and *ColE1*, respectively.

Even if the rate of homologous recombination between a plasmid and a chromosome is significantly lower than the transformation rate, the use of replicative plasmids offers three advantages over the use of nonreplicating plasmids. (i) Plasmid copy numbers higher than 1 increase the probability of recombination. (ii) Replicative plasmids allow for dissociation of the transformation efficiency from the recombination efficiency, and the continued presence of a plasmid in a bacterial cell allows detection of even very rare recombination events. (iii) Since homologous recombination has been suggested to occur during replication (23), the use of replicative plasmids should increase the recombination rate.

Although the use of replicating plasmids provides these theoretical advantages for the detection of even rare recombination events, conventional assays to select for recombination via replicative plasmids cannot be used. In conventional homologous recombination assays using suicide vectors, the recombined clones are simply selected by their resistance to a marker that is introduced into the chromosome via the nonreplicating plasmid. In contrast, selection for recombination with a replicative plasmid requires a recombination phenotype distinct from the transformation phenotype. Therefore, a system was developed in which resistance to one compound was used to select for transformation and resistance to another compound could only be detected when homologous recombination had occurred. The principle is illustrated in Fig. 3. The 3′ two thirds of the BCG *hsp60* gene without its promoter was cloned in a Sm^r *E. coli*-mycobacterium shuttle plasmid. The promoterless Km^r gene was inserted after the stop codon of the mycobacterial *hsp60* gene. The Km^r gene is not expressed in the replicative form of this plasmid, named pStore4, but it should be expressed after recombination occurred between the *hsp60* region of pStore4 and the homologous allele in the mycobacterial chromosome (Fig. 3). Thus, homologous recombination

TABLE 1. Homologous recombination of pRC016 in *E. coli* XL1-Blue and in BCG[a]

Strain	Medium	CFU in 1 μl of culture with clone:				
		1	2	3	4	5
E. coli XL1-Blue	LB	$6 \cdot 10^5$	$3.4 \cdot 10^5$	$2.2 \cdot 10^5$	$6 \cdot 10^5$	$4 \cdot 10^5$
	LB + $HgCl_2$	$5 \cdot 10^5$	$3 \cdot 10^5$	$1.4 \cdot 10^5$	$4 \cdot 10^5$	$3.6 \cdot 10^5$
	LB + Km	$6 \cdot 10^2$	$2 \cdot 10^2$	$4 \cdot 10^2$	$1.4 \cdot 10^2$	$2 \cdot 10^2$
M. bovis BCG	M7H10	$2.5 \cdot 10^4$	$2.5 \cdot 10^5$	$5 \cdot 10^4$	$2.5 \cdot 10^5$	
	M7H10 + $HgCl_2$	$2 \cdot 10^4$	$2 \cdot 10^5$	$5 \cdot 10^4$	$2.5 \cdot 10^5$	
	M7H10 + Km	20	25	25	25	

[a] Plasmid pRC016 was introduced into *E. coli* XL1-Blue, and the transformation mixture was first incubated in 1 ml of 2YT liquid medium in the presence of 10 μg of $HgCl_2$ per ml. After a 30-min incubation at 37°C, the bacteria were spotted in serial 10-fold dilutions onto Luria-Bertani (LB) agar plates supplemented either with 10 μg of $HgCl_2$ per ml or with 20 μg of kanamycin per ml. After overnight incubation at 37°C, approximately 10,000-fold more $HgCl_2^r$ colonies were detected than Km^r colonies. Five independent $HgCl_2^r$ colonies were picked, incubated for 2 h at 37°C in 1 ml of Luria-Bertani medium containing 10 μg of $HgCl_2$ per ml, spotted in 10-fold serial dilutions onto Luria-Bertani agar with or without 20 μg of kanamycin per ml or 10 μg of $HgCl_2$ per ml, and incubated at 37°C overnight. Plasmid pRC016 was purified from *E. coli* XL1-Blue and introduced into BCG by transformation. The transformation mixture was plated onto Middlebrook 7H10 agar supplemented with 12 μg of $HgCl_2$ per ml and incubated for 10 days at 37°C. Four individual $HgCl_2^r$ colonies were grown in 10 ml of Sauton medium containing 10 μg of $HgCl_2$ per ml for 7 days at 37°C, spotted in 10-fold serial dilutions onto Middlebrook 7H10 agar with or without 20 μg of kanamycin per ml or 12 μg of $HgCl_2$ per ml, and incubated at 37°C for 14 days.

can easily be selected by plating pStore4-transformed bacteria on a kanamycin-supplemented medium. We named this method selection technique of recombination events (STORE).

The STORE system was first tested in *M. smegmatis* mc^2155. After transformation with pStore4, approximately 10% of the Sm^r clones were also Km^r. Electroduction experiments with Km^r clones produced several hundred Sm^r *E. coli*, but no Km^r colonies were detected. This result showed that the Km^r phenotype in *M. smegmatis* was not conferred by the plasmid as an autonomous replicon. Individual Km^r *M. smegmatis* clones were then picked and cultivated for 5 days in the presence of kanamycin, and the chromosomal DNA was then subjected to PCR analysis using primers that yield amplification products only after the plasmid has recombined at the *hsp60* locus (Fig. 4A). The first PCR oligonucleotide (O-1) corresponded to the *hsp60* 5′ chromosomal region, while the second one (O-2) hybridized to the plasmid DNA, such that only insertion of the plasmid at the *hsp60* locus generates an amplification product. A DNA fragment of the expected size was amplified from Km^r *M. smegmatis* and partially sequenced from both ends, as shown in Fig. 4B. The sequence revealed a composite *M. smegmatis*-BCG *hsp60* gene adjacent to the Km^r gene, confirming that recombination had occurred at the *hsp60* locus.

When the same experiment was conducted with BCG, several hundred Sm^r clones were obtained but no Km^r clones could be detected immediately after transformation. Four Sm^r clones were grown in liquid medium supplemented with streptomycin for 10 days and then plated onto kanamycin-containing medium. Two weeks later, several hundred Km^r colonies were observed. One Km^r clone from each plate was grown in liquid medium containing kanamycin and analyzed by PCR using the primers described above. As shown in Fig. 5, a 1.6-kb DNA fragment was amplified from all four BCG clones tested. Determination of the DNA sequences of both ends of this fragment from one of the four clones confirmed that it corresponds to the *hsp60* DNA. No PCR product could be detected when chromosomal DNA from Km^s BCG was used as a negative control, indicating that homologous recombination had occurred in all four analyzed clones. Southern blot analyses on one selected clone (data not shown) indicated that the Km^r gene was inserted into a single locus in the BCG chromosome and that the mycobacterial origin of replication of the inserted plasmid was lost, suggesting that the origin is unstable when integrated in a mycobacterial chromosome. These results clearly show that homologous recombination between plasmids and chromosomes can be obtained at high frequencies in slowly growing as well as in fast-growing mycobacteria when replicative vectors are used.

The *hsp60* promoter is considered to be very strong. It was thus of interest to investigate whether homologous recombination into a gene with a weaker promoter can also be selected by the STORE technology. We therefore determined the MIC of kanamycin for the recombinant BCG and the nonrecombinant BCG. Recombinant BCG still grew at 250 μg of kanamycin per ml but did not grow at 500 μg/ml, whereas the nonrecombinant BCG did not grow at kanamycin concentrations as low as 2 μg/ml. Therefore, the STORE technology should theoretically be able to select for homologous recombination in genes that contain promoters at least 100-fold weaker than the *hsp60* promoter.

DISCUSSION

The rearrangement of DNA is one of the fundamental properties of life and is of great biological significance. Several different mechanisms can underlie DNA rearrangements. Among those, recombination between homologous sequences is shared by virtually all living organisms, but homologous recombination has been difficult to achieve in the *M. tuberculosis* complex. One of the reasons for the previous lack of detectable homologous recombination in these organisms has been suggested to be the unusual nature of their *recA* gene (7). The *M. tuberculosis* RecA protein contains an internal sequence named intein that is removed by a novel mechanism of protein splicing (8). The RecA proteins of all the species of the *M. tuberculosis* complex as well as that of *Mycobacterium leprae* contain inteins (9), whereas an intein is lacking in RecA of *M. smegmatis*, a species in which significant levels of homologous recombination have been observed (10). In addition, even spliced *M. tuberculosis* RecA catalyzes homologous recombination very inefficiently (13).

However, not all mechanisms of recombination between homologous sequences are *recA* dependent (6). Recombination between tandem duplications can occur by a *recA*-independent slipped-strand mispairing mechanism (14) or by a sister-strand exchange mechanism (15). However, these mechanisms are linked to DNA replication and would not have been detected in previous approaches that used nonreplicative suicide DNA to uncover homologous recombination in slowly growing mycobacteria. This assumption is supported by the very recently

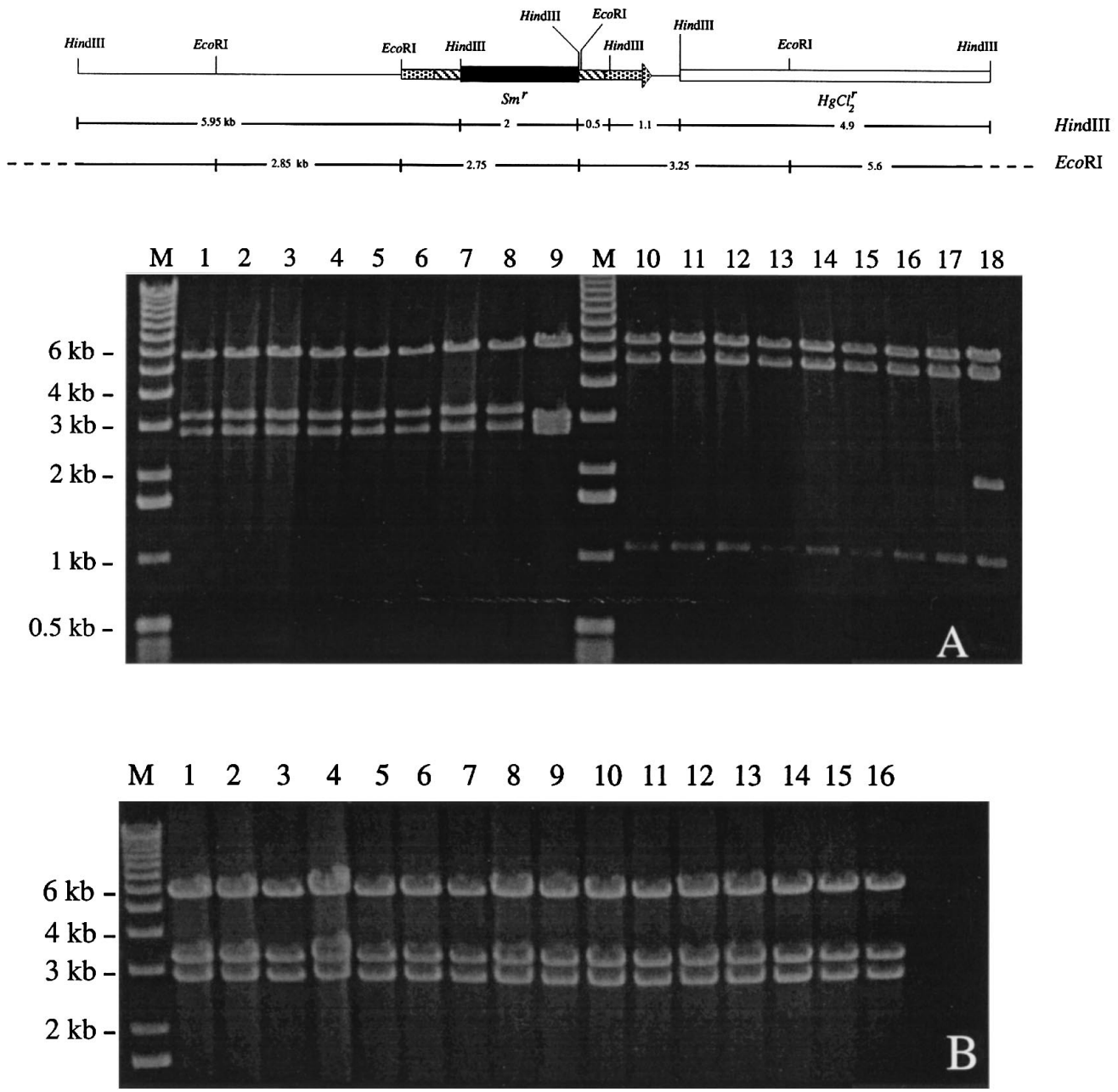

FIG. 2. Restriction analyses of recombined pRC016. The drawing at the top of the figure shows a linear restriction map of pRC016. Only *Hin*dIII and *Eco*RI sites are indicated. Symbols are as described in the legend to Fig. 1. The numbers on the second and third lines represent the sizes, expressed in kilobases, of the DNA fragments obtained after restriction of nonrecombined pRC016 with *Hin*dIII and *Eco*RI, respectively. (A) *Eco*RI (lanes 1 to 8) and *Hin*dIII (lanes 10 to 17) restriction analyses of pRC016 derivatives that have recombined in *E. coli* XL1-Blue. Lanes 9 and 18 contain nonrecombined pRC016 restricted with *Eco*RI and *Hin*dIII, respectively, and lanes M contain the size markers (1-kb ladders). (B) *Eco*RI restriction analyses of pRC016 derivatives that have recombined in BCG (lanes 1 to 16). Lane M contains the size markers (1-kb ladder).

described use of replicating plasmids to detect homologous recombination in *M. bovis* BCG (17).

Besides allowing for the detection of *recA*-independent recombination, the use of replicative plasmids also allows the uncoupling of recombination events from the transformation step. After transformation of the *E. coli recA* mutant XL1-Blue with pRC016, a plasmid containing a duplication of 463 bp in the *aph* gene separated by an approximately 2-kb intervening sequence containing a Sm^r gene, Km^r clones that contained rearranged plasmids which had undergone homologous recombination could be recovered. The frequency of these events was estimated to be in the range of 10^{-3} to 2.10^{-4}. Similar rates were found for *M. smegmatis* and *M. bovis* BCG, and restriction analyses of the recombined plasmids indicated that the rearrangements in the mycobacteria were indistinguishable from those found in *E. coli* XL1-Blue. All the analyzed plasmids had undergone intramolecular recombination, and no interplasmid recombination was detected. This is consistent with the findings of Matfield et al. (16) that intramolecular recombination may

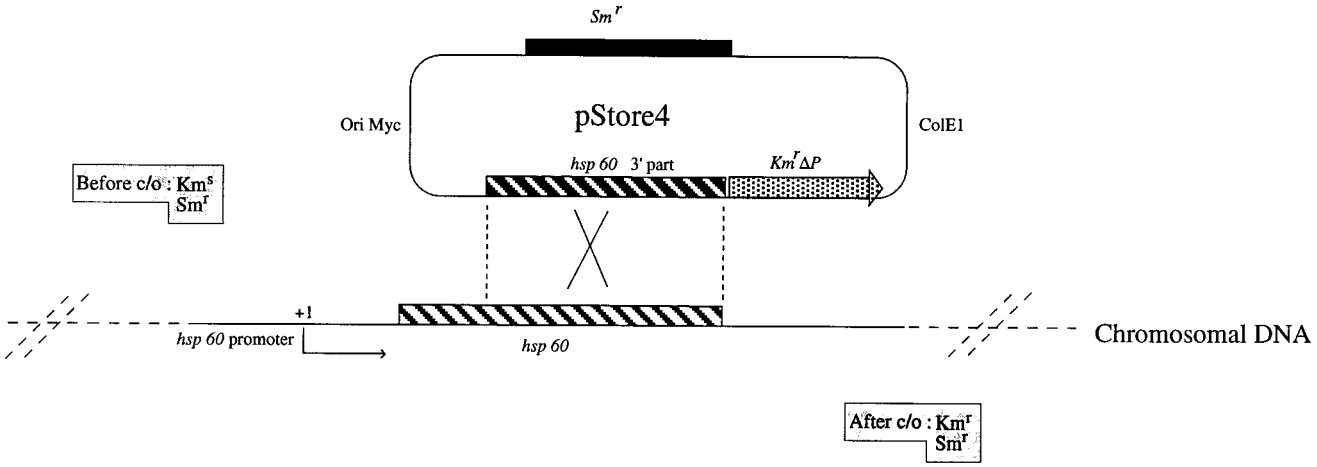

FIG. 3. STORE principle. The replicative form of pStore4 confers only Sm^r. Homologous recombination between the 3′ region of the *hsp60* gene on pStore4 (box hatched with thick black lines) with the homologous region in the chromosome (box hatched with thick white lines) will place the promoterless Km^r gene (*Km^{r}ΔP*) under the control of the *hsp60* promoter in the chromosome. The antibiotic-resistant and -sensitive phenotypes before and after recombination (c/o) are indicated. Symbols are as described for Fig. 1.

in some cases be much less *recA* dependent than intermolecular plasmid recombination.

Homologous recombination between replicating plasmids and a mycobacterial chromosome was also detected at significantly high levels by the STORE technology described in this paper. This technology uses the replication of plasmids to increase the detection frequency of homologous recombination in a chromosome, which therefore becomes independent of the transformation yield and can be easily selected for, even when the recombination rate is very low. The probability of detecting homologous recombination increases with each generation, and cultures are progressively enriched with recombined clones.

This is best described by the following mathematical model. The model has been simplified in that it applies only to irreversible homologous recombination between a plasmid and a chromosome. This approximation is justified by the fact that in the case of rare recombination events, the reversible recombination becomes negligible. The first recombination event becomes detectable when the number of cells (c) equals the inverse of the recombination rate ($1/T$). This number depends on the number of generations (n), starting from a single cell, in the following equation: $n = \log c/\log 2$. If R_n is the measurable proportion of recombined molecules after n generations, then

$$
\begin{aligned}
R_n &= T && \text{(at the } n\text{th generation)}\\
R_{n+1} &= T + (1 - T)T && \text{(at the } n + 1 \text{ generation)}\\
&= 1 - (1 - T)^2 &&\\
R_{n+2} &= 1 - (1 - T)^2 + \{1 - [1 - (1 - T)^2]\}T && \text{(at the } n + 2 \text{ generation)}\\
&= 1 - (1 - T)^3 && \text{and}\\
R_{n+x} &= 1 - (1 - T)^x && \text{(at the } n + x\text{th generation)}
\end{aligned}
$$

Since $0 < T < 1$, it follows that $R > T$.

If $T < 0.01$, then $R_{n+x} \approx T \cdot x$. In that case, R increases linearly with the number of generations. In addition, R increases proportionally with the copy number of the plasmid (y): $R_{n+x} \approx T \cdot x \cdot y$. Thus, this model shows that for any given rate of irreversible homologous recombination event the detectable proportion of recombined molecules increases with each generation, provided that the recombination rate remains constant over time. This was observed here for both slowly growing and fast-growing mycobacteria. Over a period of 2 weeks we observed a gradual enrichment of the mycobacterial cultures with Km^r colonies (not shown), indicating that, as expected, the probability of recombination of the plasmid into the chromosome is significantly higher than that of the reverse recombination. This allows one to detect homologous recombination events even in organisms for which homologous recombination or transformation rates are still lower than those of the myobacteria studied here. Therefore, this technology can in principle be adapted to any procaryotic cell impervious to classical suicide gene disruption technique.

The use of a recombination phenotype different from the transformation phenotype described here decreases the probability of obtaining illegitimate recombination, since the STORE technology requires the presence of a functional promoter upstream of the homologous region. This is best achieved by homologous recombination as shown here. Although previous reports indicated that illegitimate recombination was far more frequent than homologous recombination in slowly growing mycobacteria (11), using the STORE technology we found that all Km^r clones of both *M. smegmatis* and *M. bovis* BCG had undergone homologous recombination. Since *hsp60* sequences of BCG could recombine with those of *M. smegmatis*, a sequence identity of 100% is apparently not required. Interestingly, the introduction of mismatches have been found to drastically reduce *recA*-dependent homologous recombination in *E. coli* (24).

One advantage of the method described in this work is that the use of the STORE technology allows us to detect homologous recombination even when the plasmid is still present in the bacterial cell as an autonomous replicon. In contrast, the method described by Norman et al. (17) requires the elimination of the plasmid and can be used only with mycobacterial strains that carry recessive chromosomal resistance determinants to antibiotics, such as isoniazid. Although isoniazid-resistant derivatives can easily be selected with many mycobacterial species and strains, the fact that this antibiotic is one of the most widely used antituberculosis drugs limits the applicability of this approach. In principle, the STORE technology can be readily applied to any mycobacterial strain. This should be useful not only for gene replacement in mycobacteria but

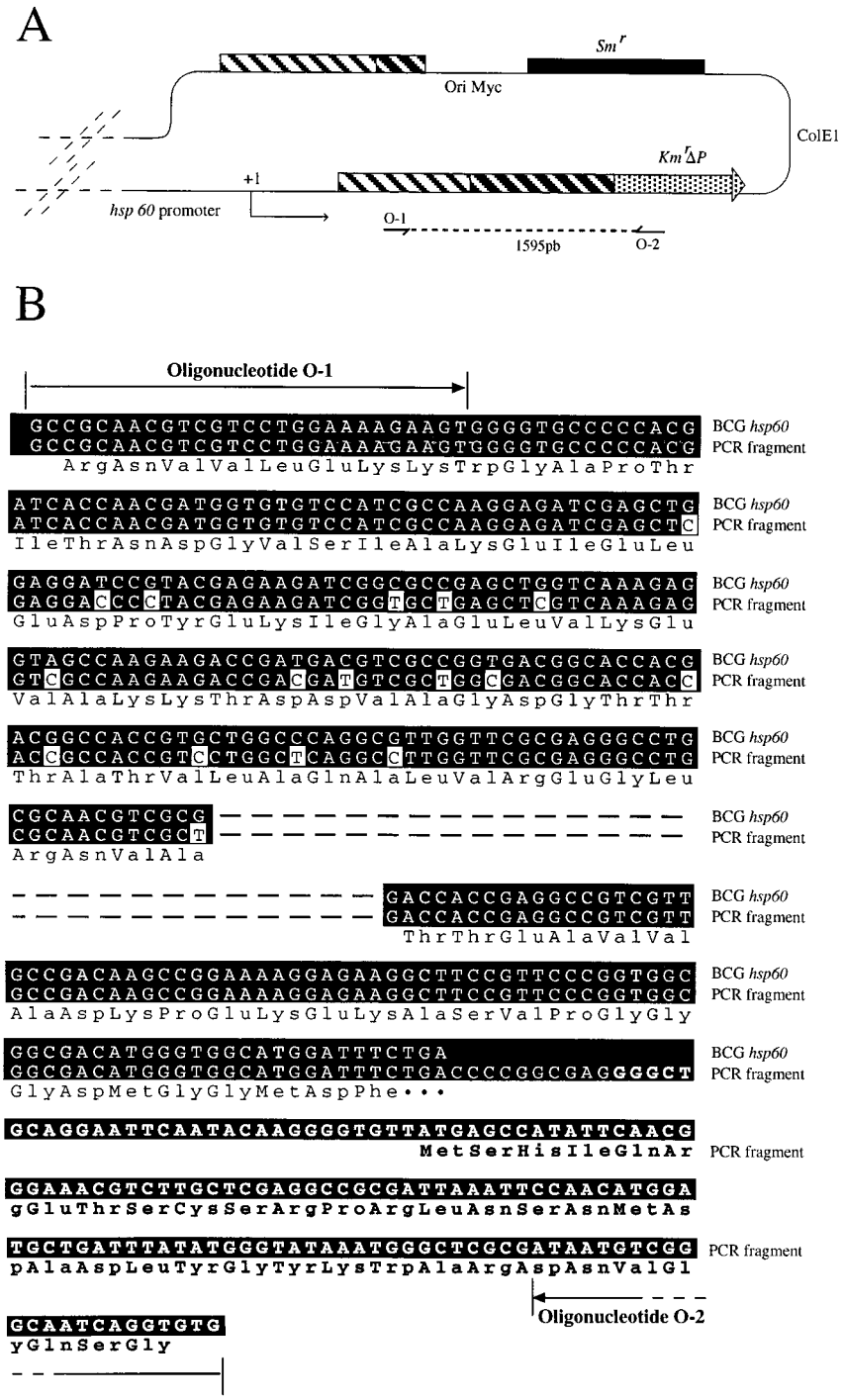

FIG. 4. Integration of pStore4 into the *M. smegmatis* chromosome by homologous recombination. After integration of pStore4 into the *M. smegmatis* chromosome (A), the promoterless Km^r gene (*Km^{r}ΔP*) was under the control of the *hsp60* promoter and therefore yielded a Km^r phenotype. After recombination had occurred, a 1,595-bp DNA fragment was amplified from the *M. smegmatis* chromosome with the oligonucleotides O-1 and O-2, as depicted by small half-arrows. These oligonucleotides had the following sequences: O-1, 5′-GCCGCAACGTCGTCCTGGAAAAGAAGT-3′, and O-2, 5′-CACACCTGATTGCCCGACATTAT-3′. This DNA fragment could be amplified only after integration of pStore4 into the chromosome by homologous recombination. Symbols are as described for Fig. 1 and 3. (B) Comparison of the partial sequence of the *M. smegmatis-M. bovis hsp60* hybrid gene obtained after PCR amplification with that of the *M. bovis* BCG *hsp60* gene. Both ends of the PCR-amplified 1,595-bp DNA fragment were partially sequenced. The broken double line indicates an unknown sequence of the hybrid *hsp60* gene. The amino acid sequences are shown in the triple letter code. · · · denotes the stop codon. Nucleotide differences between the *M. smegmatis* and BCG *hsp60* genes are highlighted in open boxes. All of them correspond to silent alterations. The amino acid sequence in boldface letters is that encoded by the Km^r gene fragment. The stippled line represents approximately 1.1 kb of unsequenced DNA. The positions of oligonucleotides O-1 and O-2 are indicated.

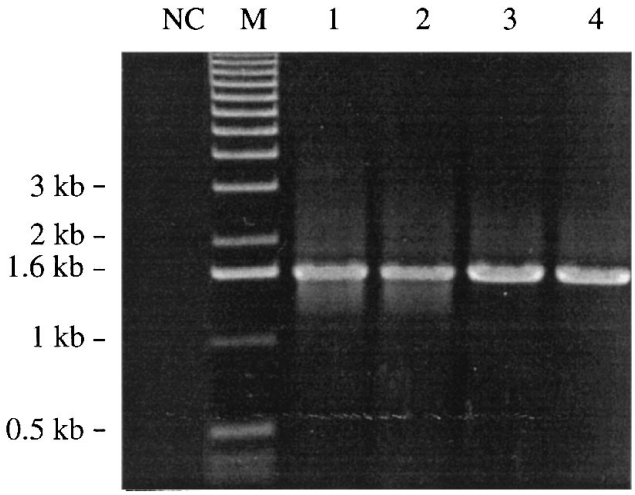

FIG. 5. PCR analysis of *M. bovis* BCG DNA after chromosomal integration of pStore4 by homologous recombination. PCR analysis was carried out on four independent Km^r mycobacterial clones with the oligonucleotides O-1 and O-2 depicted in Fig. 4. All four clones yielded approximately 1.6-kb DNA fragments (lanes 1 to 4). Lane M contains the size markers (1-kb ladder), and lane NC contains the negative control consisting of a PCR mixture with Km^s BCG DNA.

also for stable integration of heterologous genes in the chromosome of live mycobacterial vaccine strains.

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