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Gene Replacement in Mycobacteria by Using Incompatible Plasmids

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A simple and efficient delivery system was developed for making targeted gene knockouts in *Mycobacterium smegmatis*. This delivery system relies on the use of a pair of replicating plasmids, which are incompatible. Incompatible plasmids share elements of the same replication machinery and so compete with each other during both replication and partitioning into daughter cells. Such plasmids can be maintained together in the presence of antibiotics; however, removal of selection leads to the loss of one or both plasmids. For mutagenesis, two replicating plasmids based on pAL5000 are introduced; one of these plasmids carries a mutated allele of the targeted gene. Homologous recombination is allowed to take place, and either one or both of the vectors are lost through the pressure of incompatibility, allowing the phenotypic effects of the mutant to be studied. Several different plasmid combinations were tested to optimize loss in the absence of antibiotic selection. pAL5000 carries two replication genes (*repA* and *repB*), which act in *trans*, and the use of vectors that each lack one *rep* gene and complement each other resulted in the loss of both plasmids in *M. smegmatis* and *Mycobacterium bovis* BCG. The rate of loss was increased by the incorporation of an additional incompatibility region in one of the plasmids. To facilitate cloning when the system was used, we constructed plasmid vector pairs that allow simple addition of selection and screening genes on flexible gene cassettes. Using this system, we demonstrated that *M. smegmatis pyrF* mutants could be isolated at high frequency. This method should also be useful in other species in which pAL5000 replicates, including *Mycobacterium tuberculosis*.

Production of mutations in mycobacteria is a fundamental approach for discovering the function of genes in order to obtain knowledge concerning biology and pathogenicity. This knowledge may contribute to vaccine development by identifying virulence determinants for rational attenuation, and it may contribute to drug discovery by identifying possible targets. Mutagenesis may be achieved in a random or targeted manner. Both allelic exchange and transposon mutagenesis are rare genetic events in mycobacteria, so an efficient delivery system is required, in which the disrupted gene or transposon is introduced on a vector, homologous recombination or transposition takes place, and the vector is then lost. This has been accomplished in mycobacteria by the use of nonreplicating (suicide) plasmids (10, 14, 16), a temperature-sensitive plasmid (6), and a temperature-sensitive phage (1). In this study we took a different approach and used plasmid incompatibility. This technique has the advantage of using replicating plasmids, which results in a prolonged time in the cell for homologous recombination or transposition to occur but avoids the use of a temperature-sensitive replicon which can be difficult to lose in slowly growing species with narrow temperature ranges, such as *Mycobacterium tuberculosis*.

Plasmid incompatibility is defined as the inability of a pair of coresident plasmids to be stably maintained in the absence of external selection. This is due to sharing of one or more components of plasmid partitioning or replication systems. Plasmid loss due to incompatibility is commonly due to interference with the ability of the plasmid to correct stochastic fluctuations in its copy number (13). By imposing different kinds of selection on pairs of incompatible plasmids, copy number can be manipulated. We have previously mapped the replication functions of pAL5000 (23, 24) (Fig. 1). Two replication genes, *repA* and *repB*, encoding a putative primase and a DNA-binding protein, respectively, are essential for replication and can be supplied in *trans*. The incompatibility functions (*inc*) of pAL5000 were mapped to a *cis*-acting 120-bp fragment upstream of *repA*, which includes the origin of replication (*oriM*). The *oriM*/*inc* region is capable of conferring incompatibility to otherwise unrelated replicons.

We show here that maintenance or loss of plasmids can be controlled in both *Mycobacterium smegmatis* mc^{2}155 and *Mycobacterium bovis* BCG by using pairs of pAL5000-derived plasmids, and we developed an efficient delivery system for targeted gene replacement using incompatibility as a pressure to eliminate plasmids.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids used in this work are listed in Table 1. *M. smegmatis* strain mc^{2}155 (22) was cultured in Lemco medium (10 g of peptone per liter, 5 g of Lemco powder [Oxoid] per liter, 5 g of NaCl per liter) containing 0.05% (wt/vol) Tween 80 or on Lemco agar plates (Lemco medium containing 15 g of agar per liter). *M. bovis* BCG strain NCTC 5692 was grown in Middlebrook 7H9 liquid medium (Difco) supplemented with 10% (wt/vol) OADC (oleic acid, albumin, dextrose, catalase; Becton Dickinson) and 0.05% (wt/vol) Tween 80 or on Middlebrook 7H10 agar (Difco) supplemented with 10% (wt/vol) OADC. Antibiotics were added at the following concentrations when appropriate: kanamycin, 20 μg ml^{-1}; hygromycin, 100 μg ml^{-1}; and gentamicin, 10 μg ml^{-1}. When gentamicin and kanamycin were used in combination, the concentration of kanamycin was increased to 50 μg ml^{-1} as there is a degree of cross-resistance from gentamicin to kanamycin.

Electroporation. Competent *M. smegmatis* and *M. bovis* BCG cells were prepared as described by Parish and Stoker (15). Transformations were performed

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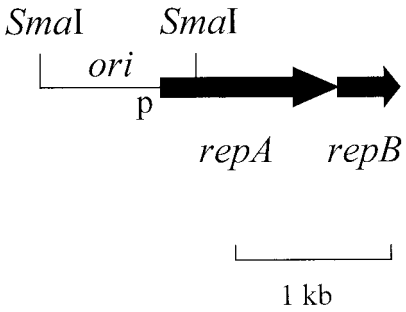

FIG. 1. Diagram of the pAL5000 replication genes. The *repA* and *repB* genes are both essential for replication. The origin and incompatibility functions map upstream of these genes, within the 1-kb *Sma*I fragment.

by using 400-μl aliquots of cells with 1 to 2 μg of DNA for *M. smegmatis* and 1 to 5 μg of DNA for *M. bovis* BCG. Cotransformants (cells transformed with two different plasmids) were selected on plates supplemented with antibiotics selecting for both plasmids.

Plasmid stability assay. A single *M. smegmatis* or *M. bovis* BCG cotransformant colony was used to inoculate 5 to 10 ml of medium (see above) with antibiotics selecting for both plasmids and incubated at 37°C with shaking until the A_{600} was approximately 0.5 (3 to 4 days for *M. smegmatis* and 3 weeks for *M. bovis* BCG). One hundred microliters of each culture was plated onto a 190-mm nonselective agar plate (medium without antibiotics) and incubated at 37°C to obtain a lawn of growth. Serial dilutions of the culture were plated onto selective and nonselective plates to obtain single cells in order to determine the proportion of antibiotic-resistant (plasmid-carrying) cells. Cells were scraped from the lawn on a nonselective plate into 1 ml of medium. Dilutions were plated onto selective and nonselective plates. Each plating was referred to as a round. This procedure was repeated through rounds of nonselective plating until no colonies could be counted on the antibiotic plates (i.e., when undiluted cells did not give rise to any colonies); no more than three rounds were necessary. Cells were always scraped from a nonselective plate having a lawn of growth. The relative stabilities of the two plasmids were monitored by calculating the reduction in plasmid numbers at each round from the colony counts and plotting the reductions in plasmid number against the rounds of plating.

The loss factor (LF) (12) was calculated by using the equation $LF = 1 - (V_1/V_2)^{1/g}$, where V_2 is the total number of cells (cells growing on nonselective medium) after g generations and V_1 is the number of cells that retained the plasmid (cells growing on selective medium) after g generations. We estimated that each round was about 23 generations (the time necessary for one cell to grow into a colony of 10^7 cells). The average loss factor was calculated from three separate experiments and was expressed as the mean ± standard deviation.

Isolation of mutants. The method used to isolate mutants was essentially the same as the method used to assess plasmid stability. However, following growth of a cotransformant in antibiotic-containing medium, 10^9 cells were initially plated onto an agar plate supplemented with gentamicin to obtain sufficient numbers of plasmid-carrying colonies for mutant isolation. Cells were then scraped and subjected to nonselective rounds of plating by plating dilutions onto plates containing gentamicin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in order to isolate white gentamicin-resistant colonies (i.e., colonies containing the mutant *pyrF* allele but lacking the plasmid-borne *lacZ* gene).

Analysis of *M. smegmatis* mutants. White colonies were initially tested for uracil auxotrophy and resistance to 1 mg of 5′-fluoroorotic acid (5′-FOA) (Sigma) per ml on minimal medium (M9 minimal salts [Difco], 20% [wt/vol] glucose, 2 ml of glycerol per liter, 0.2 mM uracil). DNA was prepared from 5-ml liquid cultures (2, 20) and used to confirm mutant generation by Southern blotting and PCR analysis.

To assess the frequency of mutant generation, serial dilutions were plated four times, and three independent experiments were performed. Phenotypically, *pyrF* mutants should have produced white colonies, been uracil auxotrophs, been

TABLE 1. Plasmids used in this study

Plasmid	Description	Relevant genotype[a]	Reference or source
pACYC184	Cloning vector	*oriE* (P15A) *cm*	5
pGOAL18	Cassette vector	*oriE amp* P_{Ag85}*lacZ* P_{hsp80}*sacB*	16
pGOAL22	Cassette vector	*oriE amp* P_{Ag85}*lacZ*	16
pINC9	*oriM*/*inc* × 2 (adjacent and in same orientation) in pNUT5	*oriE amp kan repB oriM*/*inc.oriM*/*inc*	This study
pINC52	P_{Ag85}*lacZ*/P_{hsp60}*sacB* (*Sma*I) from pGOAL18 in pINC9 (*Sca*I)[b]	*oriE amp kan repB oriM*/*inc.oriM*/*inc* P_{Ag85}*lacZ* P_{hsp60}*sacB*	This study
pINC11	*repB* PCR product and *oriM*/*inc* × 2 in pUC19	*oriE oriM*/*inc.oriM*/*inc amp repB*	This study
pINC12	P_{Ag85}*lacZ* from pGOAL22 in pINC13	*oriE oriM*/*inc.oriM*/*inc amp repB* P_{Ag85}*lacZ*	This study
pINC13	*Pac*I-containing fragment from p2NIL in pINC11	*oriE oriM*/*inc.oriM*/*inc amp repB Pac*I site	This study
pINC15	*kan* in pINC12	*oriE oriM*/*inc.oriM*/*inc amp kan repB* P_{Ag85}*lacZ*	This study
p2NIL	Manipulation vector for gene replacement	*oriE amp kan*	16
pNUT2	*hyg* in pUH36	*oriE oriM*/*inc amp hyg repA*	This study
pNUT5	*kan* and *oriM*/*inc* in pUH73	*oriE amp kan repB*	This study
pNUT12	*repA* and *oriE*/*inc* from pUH36 in pACYC184	*oriE* (P15A) *cm oriM*/*inc repA*	This study
pNUT20	pNUT12 with *Hin*dIII deletion	*oriE* (P15A) *oriM*/*inc cm repA*	This study
pNUT21	*Pac*I-containing fragment from p2NIL in pNUT20	*oriE* (P15A) *oriM*/*inc cm repA Pac*I site	This study
pNUT22	P_{Ag85}*lacZ* from pGOAL22 in pNUT21	*oriE* (P15A) *oriM*/*inc cm repA* P_{Ag85}*lacZ*	This study
pNUT23	*kan* in pNUT22	*oriE* (P15A) *oriM*/*inc cm kan repA* P_{Ag85}*lacZ*	This study
pUH36	*repA* and *oriM*/*inc* in pUC18	*oriE oriM*/*inc amp repA*	23
pUH73	*repB* in pBluescript	*oriE amp repB*	Pelle Stolt
pUH76	*oriM*/*inc* from pAL5000 as a 1-kb *SmaI* fragment in pUC19 plus *kan*	*oriE oriM*/*inc amp kan*	Pelle Stolt
pY6001	*M. smegmatis pyrF* in pUC19	*oriE amp pyrF*	8
pY6001G1	*gm* in pY6001	*oriE amp kan pyrF*::*gm*	This study
pYRANA3	*pyrF*::*gm* in pNUT12	*oriE oriM*/*inc cm repA pyrF*::*gm*	This study

[a] *oriE* is from ColE1 unless otherwise specified.
[b] See reference 16.

resistant to 5′-FOA, and grown on plates containing 10% (wt/vol) sucrose (*sacB* was carried on pINC52).

RESULTS AND DISCUSSION

Incompatibility between plasmids can provide the basis for a delivery system. The essence of any delivery system is that a DNA molecule carrying a transposon or altered gene is introduced and then lost. Efficient loss is essential, as the events being sought are rare and it is necessary to select for them. Thus, if a researcher is looking for gene replacement with an allele carrying an antibiotic resistance marker, the vector carrying this marker must be lost. This is most straightforwardly done by using a nonreplicating vector. The use of replicating vectors should theoretically increase the frequency of mutant isolation compared with that obtained with suicide vectors since (i) replicating vectors have an increased time for homologous recombination events to occur and (ii) DNA replication and recombination occur simultaneously in the cell, but replicating vectors are then more difficult to remove. Conditionally replicating vectors have been used for gene replacement with a temperature-sensitive origin of replication (4, 17). Temperature-sensitive vectors are, however, weakly thermosensitive in slowly growing members of the *M. tuberculosis* complex because of their narrow temperature range (17). These vectors therefore have to be combined with a counterselectable marker, such as *sacB*, to eliminate clones that still contain vector DNA. Growth at the permissive temperature is very slow, while colonies grown at the nonpermissive temperature also take longer to grow and have been reported to be microscopic (unpublished results).

Having shown previously that incompatibility could in principle be used (23), we carried out experiments using different plasmid pairs in order to optimize this system.

Model A: use of codependent plasmids. We previously showed that when *M. smegmatis* is transformed with two pAL5000-based plasmids carrying different markers, selection for one plasmid causes the second plasmid to be lost due to incompatibility pressure (23). This is not an ideal delivery system, in part because it requires a functional plasmid to integrate into the chromosome, which appears to be lethal for the pAL5000 replicon in *M. tuberculosis* (7).

We therefore used the fact that *repA* and *repB* act in *trans* (23) to develop a system in which we used a pair of codependent plasmids, each containing the pAL5000 minimal origin of replication (*Sma*I fragment in Fig. 1) but lacking one replication gene. This minimal origin contains regions involved in incompatibility and is referred to below as *oriM*/*inc*. As neither plasmid can replicate independently in mycobacteria, selection for one plasmid automatically selects for both, while growth in the absence of selection should result in the loss of both plasmids and thus the plasmid carrying the mutated target gene should be lost.

We constructed an Hyg^r plasmid carrying *repA* only (pNUT2) and a Kan^r plasmid that carried only *repB* (pNUT5). We called this pairing model A (Fig. 2A). The plasmids were cotransformed into *M. smegmatis* mc^{2}155 and selected on plates containing both hygromycin and kanamycin. Plasmid loss was determined during serial culture on Lemco agar in the absence of antibiotics. Plasmids were lost at a steady rate of

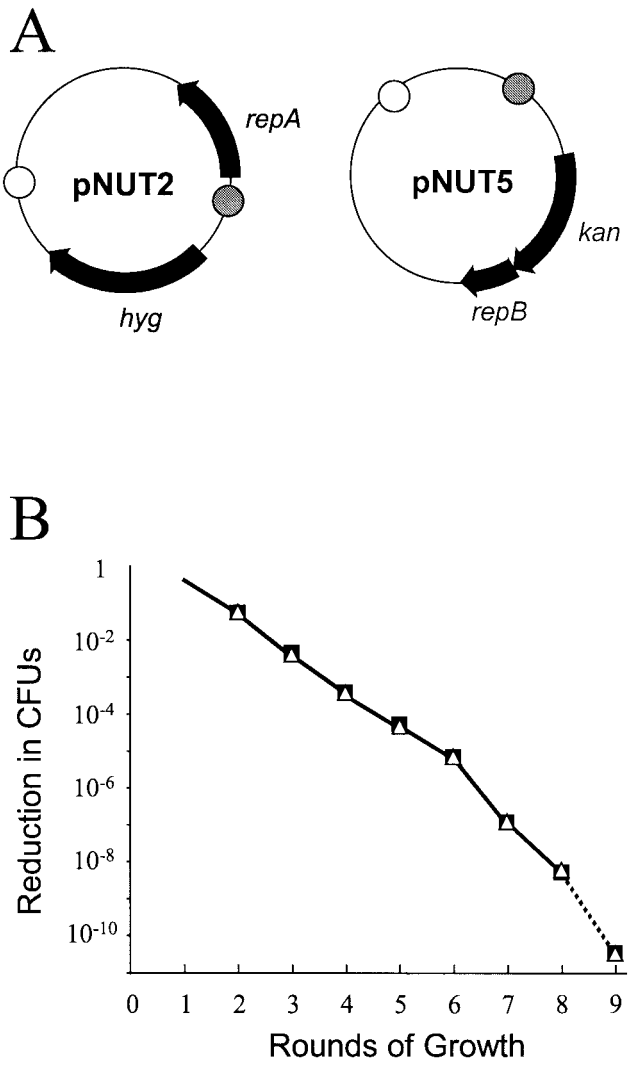

FIG. 2. Loss kinetics with model A plasmids. (A) Plasmid constructs. *kan*, kanamycin resistance gene; *hyg*, hygromycin resistance gene; *repA* and *repB*, replication genes from pAL5000; open circle, *E. coli* ColE1 *oriE*; cross-hatched circle, pAL5000 *oriM*. (B) Plasmid loss kinetics in *M. smegmatis*. Symbols: ■, Hyg^r colonies (pNUT2); △, Kan^r colonies (pNUT5). The dotted line indicates that the limit of sensitivity was reached.

10^{-1} per round with a loss factor of 0.095 ± 0.016 (Fig. 2B). When model A cotransformants were obtained, the colony size was also small but recovered after restreaking onto fresh plates, possibly because this allowed a pool of the Rep proteins to be established, although we cannot rule out the possibility that there were compensatory mutations.

Effects of different antibiotic resistance genes. A combination of antibiotic resistance genes cloned into the two incompatible plasmids was shown to be important in terms of loss rates. We have previously shown that plasmids carrying the *kan* gene are less stable than plasmids carrying *hyg* (23). In order to extend these observations, we compared the stability of pAL5000-based plasmids carrying *gm* with the stability of the same plasmids that had *gm* replaced with *kan* or *hyg* at the same site and in the same orientation; hence, the plasmids

were identical except for the resistance gene. No difference in stability between plasmids carrying *gm* and plasmids carrying *hyg* was observed. However, plasmids carrying *kan* were less stable than both of these. Therefore, it may be beneficial to include *kan* on one plasmid. A reason proposed for this is that the *hyg* gene from *Streptomyces hygroscopicus* is GC rich and so may be more compatible with mycobacteria than the transposon-derived *kan* genes having lower G+C contents (23).

Model B: addition of a second *oriM*/*inc* region. In order to increase the rate of loss, we used the observation made in previous work that a second copy of the replication origin/incompatibility region increased incompatibility if it was cloned close to the other copy in the same orientation (23). We therefore adapted model A plasmids by adding a second *oriM*/*inc* region to pNUT5, producing pINC9. We called the pairing of pINC9 (*repB kan oriM*/*inc* ×2) with pNUT2 (*repA hyg oriM*/*inc*) model B (Fig. 3A). Colonies were obtained at a frequency that was about 1% of the frequency obtained with a single replicating plasmid. The colonies were very small in the initial transformation but recovered after restreaking.

Additionally, we found using other models a significant degree of plasmid rearrangement in the ColE1 origin regions (data not shown). We therefore used different *Escherichia coli* origins (ColE1 and P15A) for the remaining experiments, and this removed the problem.

Plasmid loss was measured in the absence of selection. Both plasmids were simultaneously lost at a rate of approximately 10^{-3} per round of plating, corresponding to a loss factor of 0.213 ± 0.068 (Fig. 3B). This rate was higher than the rate seen with model A. Again, we looked for interplasmid recombination but did not observe any (data not shown).

The same experiment was repeated with *M. bovis* BCG (Fig. 3B). The plasmids were lost rapidly (approximately 10^{-2} per round of plating), although the loss factor (0.178 ± 0.066) was slightly lower than that seen in *M. smegmatis* (Fig. 3B). We concluded that we could introduce pairs of plasmids and maintain them by antibiotic selection but we could cure the plasmids efficiently by removing the selection. This provided the basis for an efficient delivery system. The most promising system was model B, and we carried out mutagenesis experiments using this model.

Targeted mutagenesis of *M. smegmatis pyrF* by using an incompatibility-based delivery system. Having shown that model B complementing plasmids were lost efficiently when selection was removed, we tested them as a delivery system for targeted mutagenesis. The *M. smegmatis pyrF* gene encodes orotidine monophosphate decarboxylase, which allows growth in media deficient in uracil but is lethal to cells grown in the presence of 5′-FOA. Mutants lacking this gene have been generated by other methods, making it a suitable test system.

A 3.2-kb *Sph*I fragment containing the *pyrF* gene interrupted by *gm* was cloned into pNUT12, producing pYRANA3 (Fig. 4A). pYRANA3 and pINC52 were cotransformed into *M. smegmatis* mc^2155, with selection for gentamicin and kanamycin. The cells were grown in selective liquid media (containing gentamicin and kanamycin) before they were plated on agar containing gentamicin. Cells were then scraped and plated onto antibiotic-free plates and taken through rounds of plating. As we were looking for relatively rare events, such as homologous recombination (11), the number of cells used to seed each plate had to be high, and we used 10^9 cells. The rationale of the screening procedure was that the selection with gentamicin would select for cells carrying the mutant *pyrF* gene, either on the plasmid or integrated into the chromosome. To eliminate the former possibility, we plated the cells in the presence of X-Gal, which distinguished between colonies carrying the pINC52 (*lacZ*$^+$) plasmid (blue) and plasmid-free colonies (white). Thus, cells were plated onto plates containing gentamicin and X-Gal at each round to identify mutants, and white colonies were picked as potential mutants for further analysis. Note that as pINC52 carries the *sacB* gene (which provides an additional screen), plasmid-carrying cells are su-

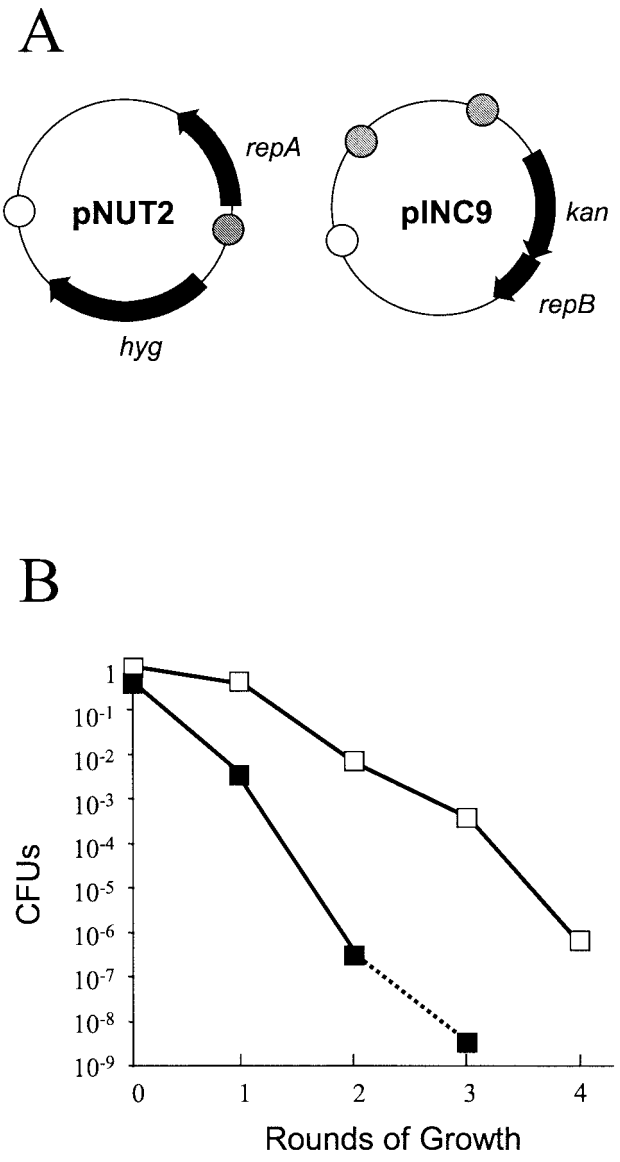

FIG. 3. Loss kinetics with model B plasmids. (A) Plasmid constructs. *kan*, kanamycin resistance gene; *hyg*, hygromycin resistance gene; *repA* and *repB*, replication genes from pAL5000; open circle, *E. coli* ColE1 *oriE*; cross-hatched circle, pAL5000 *oriM*. (B) Plasmid loss kinetics in *M. smegmatis* and in *M. bovis* BCG. Symbols: ■, Hyg^r *M. smegmatis*(pATB12); □, Hyg^r *M. bovis* BCG. The dotted line indicates that the limit of sensitivity was reached.

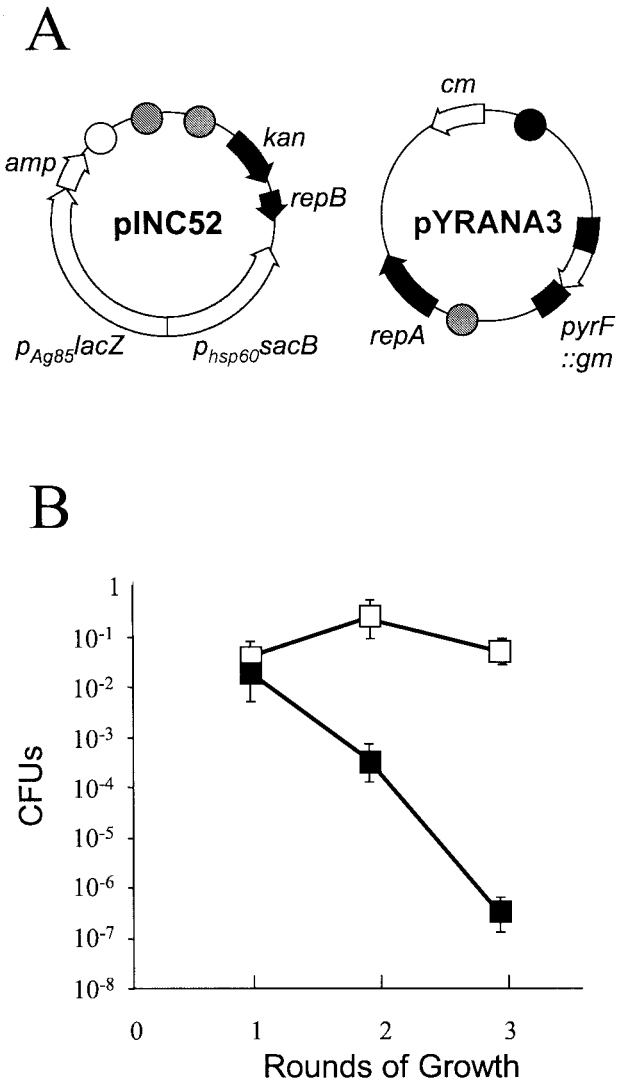

FIG. 4. Frequency of *pyrF* mutant generation in *M. smegmatis*. (A) Plasmid constructs. *kan*, kanamycin resistance gene; *cm*, chloramphenicol resistance gene; *gm*, gentamicin resistance gene; *amp*, ampicillin resistance gene; *repA* and *repB*, replication genes from pAL5000; open circle, *E. coli* ColE1 *oriE*; cross-hatched circle, *E. coli* P15A *oriE*; solid circle, pAL5000 *oriM*. (B) Plasmid loss and mutant isolation. Symbols: ■, total Gm^r colonies (plasmid loss); □, white Gm^r colonies (*pyrF* mutants). The error bars indicate standard deviations from three separate experiments.

crose sensitive, while mutants lacking the plasmid are also sucrose resistant.

After the first round of plating, white colonies were observed at an average frequency of 2.6% ± 0.9%. During the second round, the frequency of mutants increased to 22% ± 29% (one experiment yielded 64% mutants), but the frequency of mutants fell during the third round to 5.5% ± 0.50% (Fig. 4B). All of the white colonies ($n = 95$) were tested phenotypically; 90 of these had the expected phenotype of a *pyrF* mutant (uracil auxotrophs and those resistant to 5′-FOA and sucrose). Five colonies were not auxotrophic and still retained plasmids; restriction analysis showed that they had undergone rearrangements in the *lacZ* gene (data not shown). Thirty-one randomly selected auxotrophic colonies were also tested genotypically by PCR and Southern blot analysis and were confirmed to be double-crossover mutants.

Fitness of *pyrF* mutants. One surprise was that there was not a gradual accumulation of mutants when organisms were plated on gentamicin. Gm^r bacteria were seen either because they had both plasmids or because one plasmid had integrated into the chromosome as a single or double crossover. As the former was constantly decreasing and the latter should have been stable and increasing, the number of white colonies should have increased with every round. In fact, an increase was seen initially, but then there was a decrease in the subsequent round; this pattern was observed in nine separate experiments. The fact that there was not an accumulation was likely because of the greater fitness of the wild-type cells during competitive growth (21, 25). We confirmed this by growing mutant and wild-type cells in a mixed culture (data not shown). This procedure could be manipulated by making plasmid carriage more stressful. For example, we could use *kan* as the selectable marker in the target gene as it increased loss; if it was placed on the other plasmid, the competitive advantage of the wild-type cells might have been decreased. However, it is likely that most mutations result in some disadvantage, and this suggests that taking the bacteria through more than two rounds of nonselective growth is unlikely to provide great benefit.

Improved flexibility of plasmid pairs. Having shown that the plasmids could be used to efficiently isolate mutants, we addressed the lack of cloning sites in the vectors, which severely restricted their utility. Derivatives of pNUT12 and pINC52 were constructed which had an increased number of convenient restriction sites (Fig. 5). The pINC series of plasmids all contained *repB*, two *oriM*/*inc* regions that were adjacent and in the same orientation, and an *amp* gene, and *oriE* was derived from ColE1. The complementing pNUT series contained *repA*, a single *oriM*/*inc* region, and a *cm* gene, and *oriE* was derived from P15A. The two-plasmid series contained different unique restriction enzymes for cloning (Fig. 5). As two plasmids are needed for this delivery system, the mutated gene can be cloned into either plasmid depending on which plasmid carries the most convenient sites for the target gene.

In addition, a *Pac*I site was incorporated into both plasmids so that *Pac*I marker cassettes in the pGOAL series of plasmids (16) could be easily cloned onto either vector. For example, the pGOAL19 cassette contains *hyg*, $P_{Ag85}lacZ$, and $P_{hsp60}sacB$. We also constructed plasmids that contained the markers *kan* and $P_{Ag85}lacZ$/*kan* for both plasmids of the incompatible pair since these cassettes are not available as *Pac*I fragments in the pGOAL series.

In order to confirm that these plasmids were suitable delivery plasmids, combinations were tested by using *pyrF* as the target gene as described above. *pyrF*::*gm* was cloned into pINC11, and this plasmid was cotransformed with pNUT22 (Table 1). *pyrF* mutants were again isolated after one round of plating on nonselective agar, and the frequency was 4 to 16% of the colonies on a plate.

Conclusions. In this work a two-plasmid incompatibility system was developed as a delivery system for mycobacteria. Initially, the loss kinetics of different pairs of incompatible plasmids that had distinct properties, including deletions in the *rep* genes, different selectable markers, and additional copies of

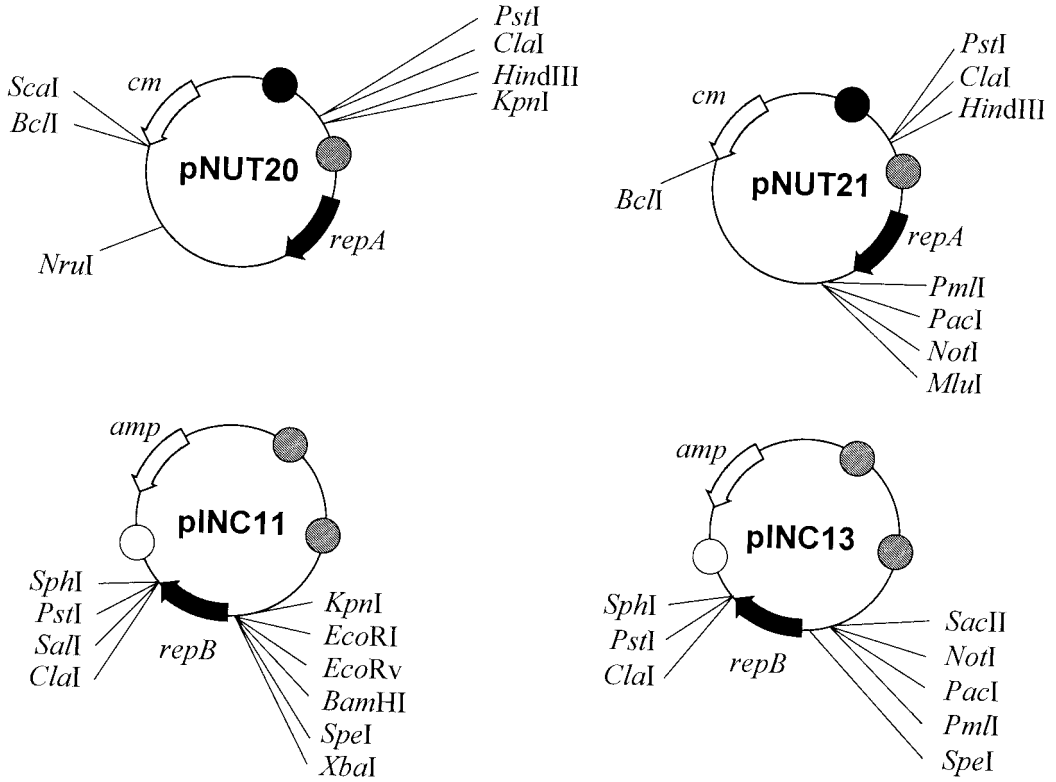

FIG. 5. Flexible vectors for cloning genes for mutagenesis. The target gene is cloned into one of these plasmids (either the pNUT or pINC series depending on which series has the most convenient restriction sites for cloning of the target gene) and mutated (or a mutated gene can be cloned in directly). If the gene is cloned in pNUT20 or pNUT21, the preparation should be cotransformed with a pINC-based plasmid (i.e., pINC12, which contains *lacZ* for screening and *repB*); if it is cloned into pINC11 or pINC13, the preparation should be cotransformed with a pNUT-based plasmid (i.e., pNUT22, which contains *lacZ* for screening and *repA*). A cotransformant can be grown in selective media and then plated onto nonselective media (to allow plasmid loss by incompatibility). Mutants can be identified by plating preparations onto plates containing X-Gal plus the relevant antibiotic (mutants produce white colonies). *cm*, chloramphenicol resistance gene; *amp*, ampicillin resistance gene; *repA* and *repB*, replication genes from pAL5000; open circle, *E. coli* ColE1 *oriE*; cross-hatched circle, *E. coli* P15A *oriE*; solid circle, pAL5000 *oriM*.

the *oriM*/*inc* region, were monitored. Both models examined showed that incompatibility could be used to efficiently eliminate plasmids from cells under nonselective conditions and therefore had the potential for use in a delivery system.

Model B was tested by using the *pyrF* gene of *M. smegmatis*, for which a high mutation frequency (10^{-1} to 10^{-2}) was obtained. This system is not only efficient and simple but rapid, since mutants were isolated during the first round of plating. Thus, *M. smegmatis* mutants could be obtained in as little as 9 days by using model B once plasmids had been constructed. Other groups have used *pyrF* in gene replacement experiments with nonreplicating plasmids and have obtained uracil auxotrophs at efficiencies of 40% (18), 1 to 10% (19), and 5% (8). Our results are comparable to these results; our average frequency was 10%, and the frequency was 64% on one occasion.

Although only used here for allelic replacement, this delivery system should also be suitable for transposon mutagenesis. Plasmid incompatibility has been used to deliver a transposon on a replicating vector in *Erwinia herbicola*. The incompatibility pressure converted a stable transposon-carrying vector into a suicide vector after introduction of an incompatible plasmid (26). In *Pasteurella haemolytica*, gene replacement was forced through extended growth of cells when a pair of incompatible plasmids was made to coexist (3). A negative selection strategy had to be used in the last stage to exclude plasmids, as transformants mainly contained two plasmids. Strong incompatibility between plasmids was also used to deliver a stable replicative plasmid into the chromosome of *Corynebacterium glutamicum* via homologous recombination, although the plasmid remained in the chromosome (9).

The approach taken in this work not only should contribute to the tools used for mycobacterial genetics since it provides a simple and efficient delivery system but also should be useful for other organisms for which plasmids have been characterized and transformation techniques have been described. As more information concerning other mycobacterial plasmids becomes available, this principle could be used in other mycobacterial species in which pAL5000 is unable to replicate, such as the members of the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex.

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