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Apramycin Resistance as a Selective Marker for Gene Transfer in Mycobacteria

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We have explored the potential of using the apramycin resistance gene as a marker in mycobacterial gene transfer studies. Shuttle plasmids available for both electroporation and conjugation studies have been constructed, and we have successfully validated the use of the apramycin resistance gene as a component of cloning vectors for *Mycobacterium smegmatis*, *M. bovis* BCG, and *M. tuberculosis*.

Mycobacterial diseases remain a major worldwide problem in infectious diseases, and this problem has recently been enhanced by the alarming appearance of multiple-drug-resistant strains. The recent development of gene manipulation in mycobacteria (11) has aided the analysis of mechanisms of drug resistance and is being employed in the study of mechanisms of pathogenesis and in the generation of potential recombinant vaccines. Gene transfer systems using shuttle cosmids (10) or phasmids (12), vectors derived from indigenous mycobacterial plasmids (13, 18, 23) and even the broad-host-range plasmid RSF1010 (8), have been developed. Most work on mycobacterial transformation has focused on *Mycobacterium smegmatis* mc^2155, a highly transformable mutant strain (24), and on *M. bovis* BCG. However, other mycobacterial species can respond differently depending on the vector and the marker gene used (7). Appropriate markers are required for direct selection of the subset of bacteria that have taken up the DNA; the kanamycin resistance (Km^r) genes from Tn*5* and Tn*903* have been used extensively (10); in addition, resistance to chloramphenicol (5), streptomycin and sulfonamide (8), hygromycin (7), and mercury salts (2) has been employed. In this study, we have explored the potential of the apramycin resistance gene (6) as a marker gene for mycobacteria.

Apramycin resistance as a selective marker. Recent studies have shown that success in mycobacterial transformation greatly depends on the selective marker used. Garbe and coworkers (7) have indicated that hygromycin resistance had certain advantages over the use of kanamycin resistance in mycobacterial transformation experiments employing a variety of hosts, including *M. smegmatis*, *M. vaccae*, *M. bovis* BCG, and *Mycobacterium* sp. strain w. This study suggested that the drug selection itself rather than other properties of the different vectors used was an important component. The hygromycin resistance (Hm^r) gene appeared to be more efficiently expressed in mycobacteria. Apramycin is an unusual aminoglycoside antibiotic with potent broad-spectrum activity that has been employed in animal husbandry but not for the treatment of human disease. Production of an aminoglycoside 3-*N*-acetyltransferase type IV [*aac*(3)-IVa] conferring cross-resistance to apramycin and a variety of other aminoglycosides has been detected in bacteria of bovine origin (4, 6, 19). Apramycin resistance has been used widely in genetic studies of streptomycetes (22). Inhibition studies performed with both *M. smegmatis* and *M. fortuitum* indicated that mycobacteria are highly sensitive to apramycin compared with hygromycin and kanamycin (not shown). The low concentrations of apramycin required confirmed the potential of apramycin as a selective agent in mycobacteria as well as in *Escherichia coli* (concentrations for apramycin and hygromycin, 30 and 50 to 200 μg/ml, respectively).

Construction of shuttle vectors containing the apramycin resistance (Am^r) gene. The strains and plasmids used in this study are listed in Table 1. Plasmid DNA from *E. coli* was isolated and analyzed by standard methods (20). Restriction enzyme digestion, ligation with T4 DNA ligase, and use of DNA polymerase I to create blunt ends were performed as recommended (Gibco BRL). Ligation products were transformed into *E. coli* DH5α by the $CaCl_2$ procedure. Ampicillin (50 μg/ml), kanamycin (25 μg/ml), hygromycin (45 μg/ml for mycobacteria; 90 μg/ml for *E. coli*), and apramycin (30 μg/ml) were added to the media as required.

Two Am^r vectors different in size and properties were constructed. To obtain bifunctional vectors for mycobacteria, the origin of replication (*ori myco*) of the *M. fortuitum* plasmid pAL5000 (18, 23) was employed. This was isolated from plasmid pYUB12 (23). In the first construction (Fig. 1), plasmid pVK1, a fusion of the pUC18 and pAL5000 replicons, was partially digested with *Sal*I and ligated to a 3.5-kb *Sal*I fragment carrying the Am^r Hm^r operon (3) to give pVK173. One of the recombinant plasmids was then partially digested with *Pst*I and ligated to the pRK2 *oriT* fragment isolated from pPM801 (16) to give plasmid pVK173T. Further analysis of pVK173T showed that a deletion upstream of the apramycin gene had occurred between the *Sal*I and *Pst*I sites.

The transfer of plasmid RSF1010 by conjugation from *E. coli* to *M. smegmatis* has been reported (8). We chose to construct mobilizable vectors by inserting *oriT*, which enables plasmid transfer in the presence of the transfer functions (*tra*) of conjugative IncP plasmids (21). pVK173T was used to confirm the efficiency of apramycin as a marker in conjugation and transformation experiments. Plasmid pPE207 (Fig. 2) was constructed from pGEM-3Zf(+) (Promega) and pYUB12. It carries only the apramycin resistance gene and replicates in both *E. coli* and mycobacteria. The ampicillin resistance (Ap^r) gene of pGEM-3Zf(+) was replaced by the *oriT* Am^r gene fragment (*Hin*dIII-*Aat*II) from pVK173T (Fig. 2). The intermediate plasmid pPE2 is a useful cloning vector for *E. coli*, since selection with apramycin avoids the appearance of satellite colonies often encountered with the use of ampicillin. The presence of the gene encoding the *lacZ* α-peptide allows recombinants in *E. coli* to

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TABLE 1. List of plasmids and strains used in this study

Vector or strain	Relevant characteristic(s)[a]	Source or reference
Mycobacterium-*E. coli* shuttle vectors		
pYUB12	Km^r	23
p16R1	Hm^r	7
pVK1	Ap^r	17
pVK173	Ap^r Am^r Hm^r	This study
pVK173T	Ap^r Am^r Hm^r oriT	This study
pPE207	Am^r oriT *lacZ* f1 ori	This study
RSF1010	Str^r Sul^r	9
E. coli vectors		
pGEM-3Zf(+)	Ap^r *lacZ* f1 ori	Promega
pPE2	Am^r *lacZ* f1 ori	This study
E. coli strains		
DH5α	*recA lacZ DM15*	Gibco BRL
S17.1	*recA pro hsdR hsdM*$^+$, integrated RP4	21
Mycobacterium strains		
M. smegmatis mc^2155	MC^26 (high transformation efficiency)	24
M. fortuitum FC-1		15
M. bovis BCG		Pasteur Institute
M. tuberculosis H37Rv		R. Stokes

[a] Abbreviations for resistance phenotypes: Am, apramycin; Ap, ampicillin; Hm, hygromycin; Km, kanamycin; Str, streptomycin; and Sul, sulfonamide.

be selected by blue and white color screening. The unique restriction sites available are *Kpn*I, *Bam*HI, *Xba*I, and *Hin*dIII. Recombinant plasmids prepared in *E. coli* were successfully transferred to mycobacteria via electroporation or conjugation.

Electrotransformation. Cells were transformed as described by Jacobs et al. (11), with 10% (wt/vol) glycerol as the electroporation buffer and a Bio-Rad electropulser. Transformants were selected on 7H10 plates (enriched with glucose for *M. smegmatis* or oleic acid-dextrose complex [Difco laboratories] for *M. bovis* BCG and *M. tuberculosis*) supplemented with 0.1% Tween 80 and the appropriate antibiotics. Plasmids pVK173T, pPE207, p16R1, and pYUB12 were introduced into *M. smegmatis* mc^2155; the transformation frequencies obtained with pPE207 were equivalent to those obtained with p16R1 and slightly better than those obtained with pYUB12 (Table 2), confirming that the Am^r gene originating in *Salmonella typhimurium* was efficiently expressed in *M. smegmatis*. The low transformation frequency obtained with pVK173T can be explained by this plasmid's larger size (>9 kb).

The shuttle plasmid pPE207 was then tested for its ability to transform other mycobacterial species. Although *M. smegmatis* is a useful model for mycobacterial genetic experiments, it is perhaps not relevant in the study of pathogenicity involving slowly growing mycobacteria. When pPE207 has been used, no transformants have been obtained with *M. fortuitum*, but successful transformation has been demonstrated with *M. bovis* BCG and *M. tuberculosis*. Despite the low transformation frequencies observed (Table 2), the results validated the potential of apramycin as a selective marker in the study of mycobacteria.

Conjugation. *E. coli* S17.1, which carries an integrated RP4, was used as the mobilizing strain (21). No exconjugants were recovered from the *M. fortuitum* strain FC-1, but the frequencies of appearance of resistant colonies in *M. smegmatis* mc^2155 were 1.6×10^{-5}, 2×10^{-5}, and 1.3×10^{-5} for RSF1010, pPE207, and pVK173T, respectively. These frequencies are in agreement with results from other studies (14). However, higher frequencies (up to 10^{-2}) have been reported for RSF1010 (8), and further experimentation with pPE207 and pVK173T would likely lead to improvement. The use of conjugative plasmids would be useful in studying gene exchange between mycobacteria and members of other genera in the environment.

Analysis of transformants and exconjugants. To confirm the presence of plasmids in the mycobacterial hosts, vectors were transferred back to *E. coli* S17.1 from *M. smegmatis* by electroduction according to the method of Baulard et al. (1). The restriction digestion profiles, identical to those of pVK173T and pPE207, indicated that no major deletion or recombination had occurred (data not shown). Transformed *M. smegmatis* containing either pVK173T or pPE207 was resistant to concentrations of apramycin up to 240 and 600 μg/ml (Table 1). In comparison, pYUB12-containing cells were resistant to

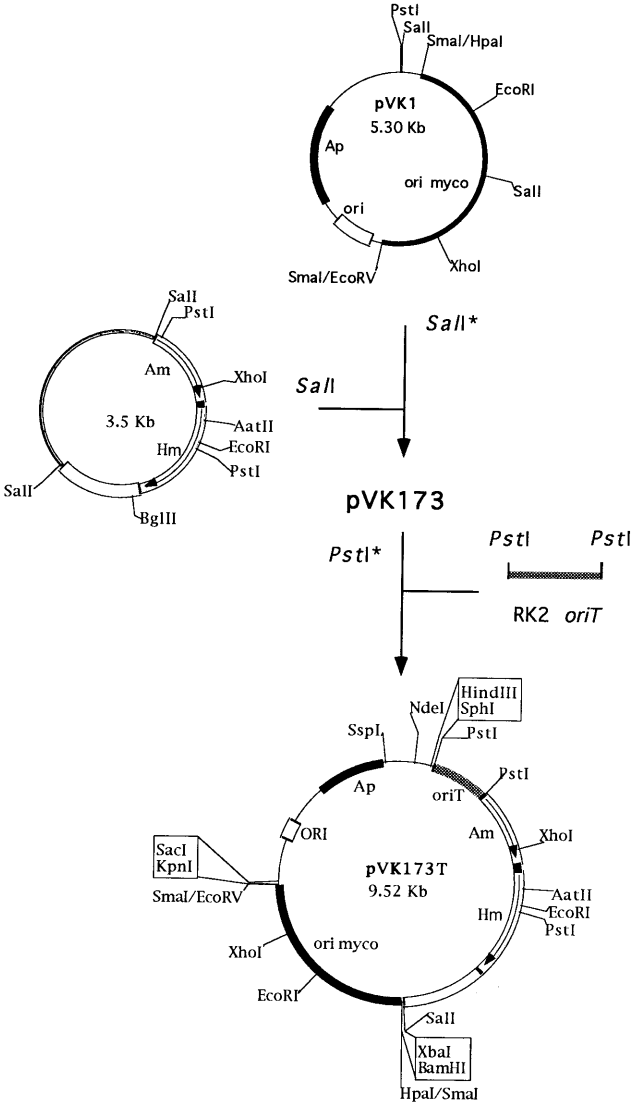

FIG. 1. Construction of pVK173T. Unique restriction sites are boxed. Hm, hygromycin; *, partial digestion.

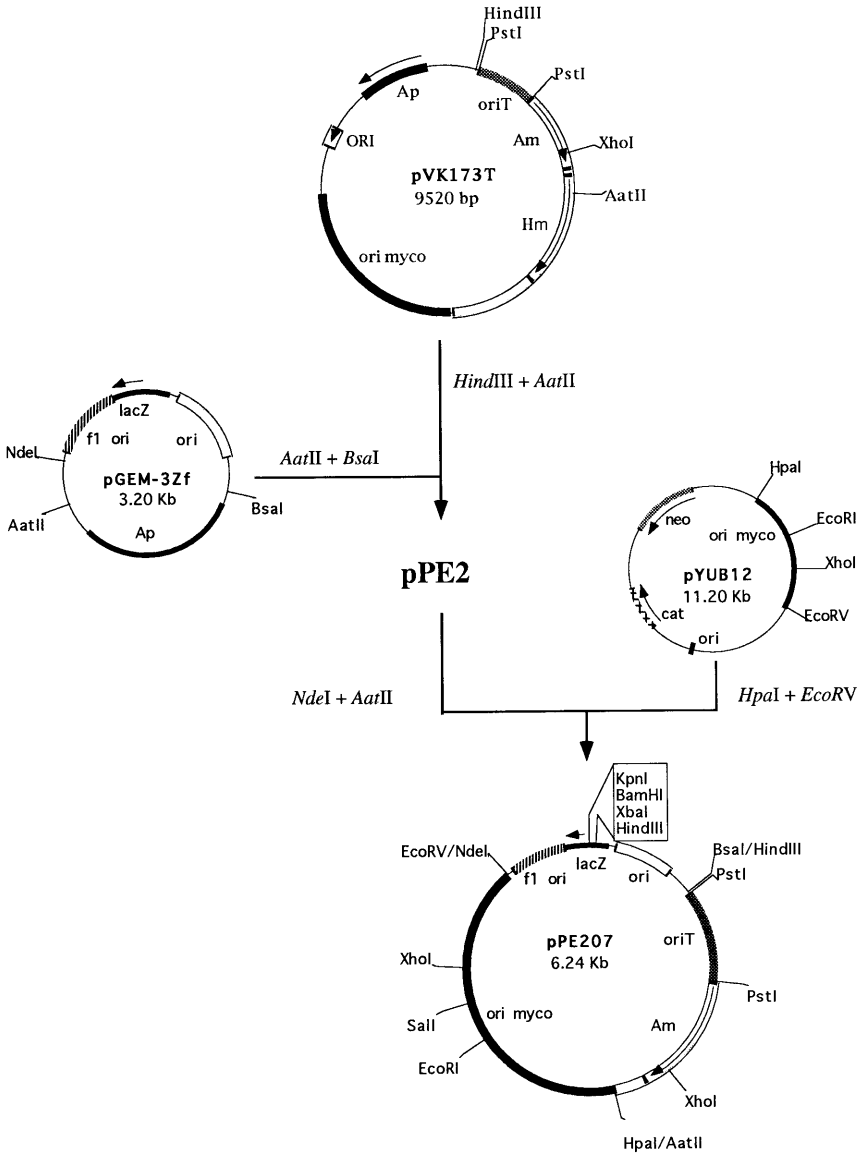

FIG. 2. Construction of pPE207. Unique restriction sites are boxed. Hm, hygromycin.

500 μg of kanamycin per ml. The *aac*(3)-IV gene used in both pVK173T and pPE207 determines resistance to tobramycin and gentamicin; this resistance was not tested in the experiments described here.

TABLE 2. Transformation frequencies for different mycobacterial species[a]

Plasmid	Transformation frequency for:			
	M. smegmatis mc^{2}155	*M. fortuitum* FC-1	*M. bovis* BCG	*M. tuberculosis* H37Rv
pPE207	1.7×10^4	0	2×10^3	1×10^2
pVK173T	2.6×10^3	0	NT	NT
p16R1	2.2×10^4	NT	NT	NT
pYUB12	1×10^4	NT	NT	NT

[a] Frequencies represent the number of transformants per microgram of DNA. Results are expressed as the mean number of colonies in duplicate experiments. NT, not tested.

pPE207 was shown to be an effective vector for cloning; the ligation of an Hm^r cassette (from p16R1) into the unique *Xba*I site in the plasmid and electroporation into *M. smegmatis* mc^{2}155 gave Ap^r Hm^r transformants.

While alternative cloning hosts (e.g., *E. coli* and *Streptomyces* spp.) continue to be effective for studying mycobacterial genes, a more efficient gene transfer system would greatly increase the potential of molecular genetic approaches in the study of mycobacteria. Plasmid-based genetic systems offer advantages such as good cloning capacity, ease of DNA manipulation, and copy number control. One serious limitation in the study of mycobacterial genetics is the limited number of markers available in the current vectors. In this work, we demonstrate that apramycin resistance is a useful selectable marker for genetic experiments in both slowly growing and fast-growing mycobacteria, using an antibiotic that is not approved for human clinical use. Interestingly, the gene encoding hygromycin resistance (*hphB*) is downstream of *aac*(3)-IVa and is cotranscribed from the same promoter (19). This dual resistance gene cas-

sette (in pVK173) has obvious advantages in gene transfer studies. For example, *hphB* could be inactivated by insertion without affecting the expression of *aac*(3)-IVa.

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