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Allelic Exchange in *Mycobacterium tuberculosis* with Long Linear Recombination Substrates

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Genetic studies of *Mycobacterium tuberculosis* have been greatly hampered by the inability to introduce specific chromosomal mutations. Whereas the ability to perform allelic exchanges has provided a useful method of gene disruption in other organisms, in the clinically important species of mycobacteria, such as *M. tuberculosis* and *Mycobacterium bovis*, similar approaches have thus far been unsuccessful. In this communication, we report the development of a shuttle mutagenesis strategy that involves the use of long linear recombination substrates to reproducibly obtain recombinants by allelic exchange in *M. tuberculosis*. Long linear recombination substrates, approximately 40 to 50 kb in length, were generated by constructing libraries in the excisable cosmid vector pYUB328. The cosmid vector could be readily excised from the recombinant cosmids by digestion with *Pac*I, a restriction endonuclease for which there exist few, if any, sites in mycobacterial genomes. A cosmid containing the mycobacterial *leuD* gene was isolated, and a selectable marker conferring resistance to kanamycin was inserted into the *leuD* gene in the recombinant cosmid by interplasmid recombination in *Escherichia coli*. A long linear recombination substrate containing the insertionally mutated *leuD* gene was generated by *Pac*I digestion. Electroporation of this recombination substrate containing the insertionally mutated *leuD* allele resulted in the generation of leucine auxotrophic mutants by homologous recombination in 6% of the kanamycin-resistant transformants for both the Erdman and H37Rv strains of *M. tuberculosis*. The ability to perform allelic exchanges provides an important approach for investigating the biology of this pathogen as well as developing new live-cell *M. tuberculosis*-based vaccines.

Until recently, the study of the pathogenesis of tuberculosis had been limited by the lack of genetic transfer systems for mycobacteria. Only within the last decade have recombinant mycobacteriophage vectors (15, 29), plasmid transformation systems (29), and integration-proficient vectors (18) been developed to efficiently introduce recombinant DNA into both the fast-growing mycobacteria, such as *Mycobacterium smegmatis*, and slow-growing mycobacteria, particularly *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. With these systems, it has been possible to develop BCG as a recombinant vaccine vector (2, 30), to generate luciferase reporter phages for rapid drug susceptibility analysis of *M. tuberculosis* strains (13), and to determine the molecular genetic basis for resistance to isoniazid (3, 33). Genetic complementation analyses revealed that *M. tuberculosis* H37Ra was most likely attenuated as a result of multiple mutations (26). Another complementation study revealed that an *M. bovis* strain is attenuated because of a single point mutation in a gene encoding a principal sigma factor, which is the subunit that confers upon the RNA polymerase holoenzyme the ability to recognize promoters (6).

Further analysis of the virulence factors of *M. tuberculosis* as well as the development of novel vaccines will require the generation of strains with specific chromosomal mutations. However, the slow-growing mycobacteria *M. tuberculosis*, *M. bovis*, and BCG are not well suited for traditional mutagenesis protocols and screens. In other bacterial systems, physical and chemical DNA-damaging agents routinely have been employed to enrich for strains containing mutations. Mutagenesis with DNA-damaging agents has the limitation that each chromosome may receive more than a single mutagenic lesion. This problem is readily overcome with gene transfer systems, such as conjugation, transduction, or transformation, that enable the transfer of defined chromosomal fragments and the construction of strains with single mutations. Conjugation (31) and transduction (27) have been described for *M. smegmatis*, but these systems have never been shown to function for *M. tuberculosis*, *M. bovis*, or BCG. Also essential to a mutant screen is the ability to generate a colony derived from a single mutated cell. *M. tuberculosis* as well as other mycobacteria presents problems for such analyses because these bacilli generally grow in clumps, even in the presence of a detergent. Lastly, the slow doubling time of 22 h for *M. tuberculosis*, necessitating 2 to 3 weeks for colonies to form, as well as the requirement that they be handled in biosafety level 3 containment areas, makes the screening of libraries of mutants, in which a large number of clones are unmutagenized, unattractive.

Insertional mutagenesis, a procedure in which a marker gene is used to select for a mutagenic event, can overcome the limitations mentioned above, as the selection provides a method to enrich for a pure population of mutated cells. Generally, there are two approaches for insertional mutagenesis: transposon mutagenesis and allelic exchange. Transposition was first demonstrated in *M. smegmatis* (9, 20) and recently has been achieved in slow-growing mycobacteria (21), yielding

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leucine and methionine auxotrophs of BCG. While allelic exchanges have been described for *M. smegmatis* (11, 16), efforts to achieve allelic exchange in BCG by electroporation of short linear or short circular DNA fragments have proven to be unsuccessful. In one study, a methionine biosynthetic gene interrupted with a kanamycin resistance selectable marker gene incorporated at random loci around the BCG chromosome, apparently by illegitimate recombination events (16). In another study, a uracil biosynthetic gene, also interrupted with a kanamycin resistance selectable marker gene, did appear to integrate into a homologous site in 20% of the transformants, though no allelic exchange was observed (1). A recent report describing the use of an insertionally mutated allele and a counterselectable marker presented evidence suggesting that a double crossover event could be obtained in BCG, although no stable mutant was generated (25). In this report, we describe a novel method for effecting allelic exchange in slow-growing mycobacteria by using long linear recombination substrates. We have used this method to insertionally inactivate a leucine biosynthetic gene in two different strains of *M. tuberculosis*, generating leucine-requiring auxotrophs. This general strategy could be readily applied to disrupt any desired gene in any *Mycobacterium* species.

MATERIALS AND METHODS

Bacterial strains, media, and cultivation of mycobacteria. The bacterial strains used in this study are listed in Table 1. The *Escherichia coli* strains were grown in Luria broth for amplification of recombinant clones, plasmid isolation, and transformation and in 1% tryptone–0.5% yeast extract–0.5% NaCl–0.4% maltose (TYM broth) for lambda-packaged cosmid transductions. The *M. tuberculosis* H37Rv and Erdman strains and BCG strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 0.5% Tween 80, 0.5% bovine serum albumin fraction V, 0.085% NaCl, and 0.2% glucose (M-ADC-TW broth [14]). Cultures were prepared by inoculating 1 ml from freezer stocks into 10 ml of M-ADC-TW broth in tissue culture flasks and incubating the flasks with an occasional daily shake for 7 days at 37°C. Larger cultures were prepared by inoculating 10 ml of the 7-day culture into 100 ml of M-ADC-TW broth in a 490-cm^2 roller bottle (Corning) and incubating the bottle at 37°C on a roller at 8 rpm.

Construction of plasmids. Plasmids used in this study are described in Table 2. DNA manipulations in *E. coli* were performed according to standard protocols (28). A polylinker was ligated into the cosmid vector Supercos-1 (Stratagene, La Jolla, Calif.) to generate a cosmid that could be readily excised from recombinant cosmids containing mycobacterial DNA sequences. Two single-stranded oligonucleotides (BJ 334, 5′-GATCTTTAATTAAGGATCCTGATCAGGCGCCT TAATTAA-3′, and BJ 335, 5′-GATCTTAATTAAGGCGCCTGATCAGGA TCCTTAATTAA-3′) were annealed to yield a double-stranded oligomer containing unique *Bam*HI, *Bcl*I, and *Nar*I cloning sites flanked by two *Pac*I sites. The *Bam*HI-compatible ends of the oligomers were ligated to *Bam*HI-digested Supercos-1, thereby disrupting the original *Bam*HI site in the vector. From the resultant plasmid, an *Hpa*I-*Nru*I fragment containing the *Pac*I-flanked cloning sites, double *cos* sites, *bla* for ampicillin resistance, and a ColEI origin of replication was purified and self-ligated to yield pYUB328 (Fig. 1A).

Preparation of mycobacterial chromosomal DNA. Cultures of *M. tuberculosis* or BCG (10 ml) were grown as described above and incubated for 10 to 14 days. High-molecular-weight DNA was obtained more consistently when cells were incubated for an additional day in the presence of 1% glycine (18). Chromosomal DNA was prepared as described previously (23).

Construction of genomic libraries in the *Pac*I-excisable cosmid vector pYUB328. Genomic libraries of BCG and *M. tuberculosis* were constructed in pYUB328 by using the double *cos* vector strategy as previously described (19, 32). Arms of pYUB328 were prepared by digestion with *Xba*I, alkaline phosphatase treatment, phenol-chloroform extraction, and *Bam*HI digestion. Chromosomal DNA fragments prepared by partial digestion (average size, >30 kb) were ligated to the two pYUB328 arms at a 1:10 molar ratio (insert to arms). The ligation was in vitro packaged with GigaPack Gold (Stratagene) packaging mix, and the resulting recombinant cosmids were transduced into χ2764, an in vivo λ cosmid-packaging strain (12), and selected for ampicillin resistance on plates at 30°C. Over 100,000 unique ampicillin-resistant colonies were pooled for each library, and lysates of lambda-packaged cosmids were prepared as described previously (12). Lysates containing greater than 10^{10} lambda-packaged cosmids conferring ampicillin resistance were routinely obtained.

Conjugation and selection for interplasmid recombination in *E. coli*. Conjugation was used to introduce the mutated *leuD* allele into a recombinant cosmid carrying the wild-type *leuD* allele. A 3.6-kb *Kpn*I DNA fragment containing the *leuD* gene with an insertion of the *aph* gene was first cloned into the conjugative plasmid pCVD442 (8). The pCVD442 vector is conditionally replicated in strains expressing the π protein encoded by the *pir* gene. Thus, the donor plasmid constructs in pCVD442 were initially engineered and maintained in SY327 (λ*pir*) and electroporated into S17-1λ*pir* for conjugation experiments. Donor cells were mixed with the RR1 cells, containing the recombinant cosmid, as the recipient cells. Recombinants resulting from a double crossover event in the cosmid were directly selected with kanamycin since the donor plasmid is unable to replicate in the RR1 cells that lack the *pir* gene. In addition, sucrose was used to select against donor cells and RR1 cells containing a cointegrant resulting from a single crossover between the donor plasmid and the target cosmid. The donor and recipient strains for conjugation were grown for 12 to 18 h in Luria broth with 50 μg of carbenicillin per ml. Recipient and donor cells (500 μl each) were added to 5 ml of 10 mM $MgSO_4$, vortexed briefly, and pelleted. The pellet was resuspended in 100 μl of $MgSO_4$, spotted onto Luria-Bertani agar containing 50 μg of carbenicillin per ml, and incubated at 37°C for 18 to 24 h. After incubation, the spot was pooled into 2 ml of 10 mM $MgSO_4$, and 200 μl was inoculated into 10 ml of Luria broth with 50 μg of carbenicillin per ml. After 4 h at 37°C, the cells were pelleted and plated onto Luria plates with 25 μg of kanamycin per ml and 5% (wt/vol) sucrose. After 24 h of incubation at 37°C, all the colonies were pooled and plasmid DNA was purified on a Qiagen Inc. (Chatsworth, Calif.) column. The DNA was electroporated into *E. coli* DH5α cells and plated onto Luria-Bertani plates containing 50 μg of carbenicillin and 25 μg of kanamycin per ml and 5% (wt/vol) sucrose.

Mycobacterial transformation and auxotroph screen. One-hundred-milliliter cultures of *M. tuberculosis* H37Rv and Erdman and BCG strains were grown for 7 days as described above. The cells were prepared for electrocompetence by pelleting and washing in cold 10% glycerol four to five times. The cells were resuspended in cold 10% glycerol in a final volume of 4 ml, and 400-μl aliquots were used per transformation. Only freshly prepared electrocompetent cells were used throughout the experiment. For linear-plasmid electroporations, the DNA was digested with *Pac*I (New England Biolabs) and then purified by using a QIAquick Spin PCR purification kit (Qiagen Inc.). Approximately 1 μg of DNA in a 5-μl volume was used per electroporation with a Bio-Rad gene pulser at 1,000 Ω, 25 μF, and 2.5 kV. After electroporation, the cuvettes were briefly chilled on ice and then the cells were incubated at 37°C overnight. The following day, the cells were plated on Middlebrook 7H10 agar supplemented with 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.2% glucose, 20 μg of kanamycin per ml, and 0.2% Casamino Acids (Difco) and incubated at 37°C for 3 to 4 weeks. Single colonies were patched on three independent 7H10 agar media, all containing 0.5% bovine serum albumin fraction V, 0.085% NaCl, 0.2% glucose, and 20 μg of kanamycin per ml, with or without 0.2% Casamino Acids or 50 μg of L-leucine (Sigma) per ml and analyzed 4 to 7 days later. Putative auxotrophs were picked from the leucine-supplemented master plate, resuspended in 1.0 ml of fresh medium that did not contain leucine, and vortexed. One-hundred-microliter aliquots were repatched on the three media described above; the rest of the aliquot was stored at −70°C.

Southern analysis. Samples of genomic, cosmid, and plasmid DNA were digested with *Kpn*I and then separated on a 0.4% agarose gel. The gel was processed and DNA was transferred to 0.2-μm-pore-size Biotrans nylon membranes (ICN Biochemicals, Irvine, Calif.) according to the manufacturer's procedures. The probes were labelled with [α-^{32}P]dCTP by using an oligolabelling kit (Pharmacia). The membranes were probed overnight at 65°C; this step was followed by a single wash with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (28) at 65°C for 30 min and then a stringent wash with 0.1× SSC at 65°C for 30 min. Autoradiograms were obtained after 2 to 6 h of exposure to X-ray film.

RESULTS

Construction of the excisable cosmid vector pYUB328 and genomic libraries of *M. tuberculosis* and BCG. Allelic exchange in *M. tuberculosis* and BCG has been difficult to achieve because of the high degree of illegitimate recombination which occurs in these organisms. Previous attempts to achieve this goal by using short linear DNA fragments were not successful. We reasoned that considerably long stretches of homologous DNA (approximately 20 kb) flanking the mutagenized gene would provide a more recombinogenic substrate. However, generating long linear substrates is difficult because of the problems inherent in the manipulation of large DNA fragments. Cosmid inserts could be a source of such long linear substrates if intact inserts could be removed from the cosmid vector without disrupting the insert DNA. To that end, we developed an excisable cosmid vector which allows the release of large intact segments of mycobacterial DNA from the recombinant cosmid. Pulsed-field gel analysis of *M. tuberculosis* chromosomal DNA revealed a lack of *Pac*I sites (TTAATT

TABLE 1. Bacterial strains and their descriptions

Bacteria	Description	Reference or source
E. coli K-12 strains		
DH5α	F⁻ [ϕ80*d*Δ*lacZM15*] Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 glnV44 thi-1 gyrA96 relA1*	10
RR1	F⁻ Δ(*gpt-proA*)*62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1*	4
HB101	F⁻ Δ(*gpt-proA*)*62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13*	5
χ2764	F⁻ λ (*c*I857 *b2 red*β*3 S7*)Δ(*gpt-proA*)*62 leuB1 glnV44 ara-14 galK2 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13*	13
SY327λ (*pir*)	F⁻ Δ(*lac-pro*) *argE*(Am) *rpoB nalA recA56*	22
S17-1λ (*pir*)	F⁻ *recA thi pro hsdR* RP4-2-*tet*::Mu::*kan*::Tn*7*	7
BCG strains		
Pasteur	Vaccine strain	Statens Seruminstitut
mc²797	*leuD1*::Tn*5367*	21
M. tuberculosis strains		
H37Rv	Virulent	Trudeau Institute
Erdman	Virulent	Trudeau Institute
mc²3000	H37Rv strain containing the *leuD3*::*aph* allele	This work
mc²3001	Erdman strain containing the *leuD3*::*aph* allele	This work

AA) in the guanine-plus-cytosine-rich *M. tuberculosis* genome (17). On the basis of this observation, we constructed an excisable cosmid vector, pYUB328 (Fig. 1A), with two *Pac*I sites flanking a set of unique restriction sites for cloning. Genomic libraries of BCG strains and *M. tuberculosis* H37Rv were constructed in pYUB328. Approximately 100,000 colonies were obtained for both libraries. As expected, BCG and *M. tuberculosis* H37Rv libraries constructed in pYUB328 generated large linear fragments following *Pac*I digestion. Digestion of cosmid DNA isolated from pools of clones from the respective libraries with *Pac*I (Fig. 1B, lanes 3 and 5) reveals the release of the 3.6-kb vector fragment from the intact 35- to 50-kb mycobacterial DNA insert. In contrast, digestion of the same DNA with another enzyme, *Pst*I, which cuts multiple times in mycobacterial genomic DNA and once in the vector, shows a random distribution of fragments of various lengths (Fig. 1B, lanes 2 and 4).

Introduction of an insertionally mutated allele into a cosmid. It was our hypothesis that the failure to achieve allelic exchange could be overcome by using long linear recombination substrates generated from the excisable cosmids. However, one of the limitations of engineering precisely defined mutations within a specific gene is that the large size of the cosmid makes it unlikely that restriction sites within the gene will be unique. In order to circumvent this problem and efficiently generate an insertion within a specific gene within a 40- to 50-kb *Pac*I recombinant cosmid, we developed a strategy that exploits homologous interplasmid recombination in *E. coli*. This technique consists of (i) subcloning a smaller fragment containing the gene of interest, (ii) engineering an insertion of a selectable marker gene into the subcloned gene of interest, and (iii) introducing this disrupted allele into the cosmid (by using the counterselectable suicide vector pCVD442 [8]) by homologous recombination in *E. coli*. With this system, the donor plasmid, containing the disrupted, subcloned gene, is introduced by conjugation into recipient *E. coli* cells containing the target cosmid, and recombinants are selected with the selectable marker in the mutated allele. Furthermore, double-crossover recombinants are selected for by selecting against the *sacB* gene on the pCVD442 vector, whose expression makes *E. coli* sensitive to sucrose. The end result is that the mutated allele is efficiently introduced into the *Pac*I fragment of the target cosmid.

The gene targeted for allelic exchange in *M. tuberculosis* was the homolog of the *E. coli* leucine biosynthetic gene *leuD*, which encodes the enzyme isopropylmalate isomerase. We are interested in isolating *M. tuberculosis* leucine auxotrophs because BCG leucine auxotrophs generated by transposon insertions are unable to grow in mice (21). Two- to four-kilobase *Kpn*I fragments of BCG DNA were cloned into pKSII+ as a subgenomic library, and a clone (pYUB511) (Fig. 2, lane 2) with a 2.3-kb fragment containing the wild-type *leuD* allele was identified by colony hybridization with the 6.0-kb *Kpn*I probe containing the *leuD1*::Tn*5367* allele from mc²797 (21). A kanamycin resistance cassette (*aph*) was cloned into the unique *Sph*I site within *leuD* to yield the *leuD3*::*aph* allele in pYUB512 (Fig. 2, lane 4). This *leuD3*::*aph* allele was cloned into the suicide vector pCVD442 to yield pYUB514 (Fig. 2, lane 6). A pYUB328::BCG DNA cosmid containing the wild-type *leuD* gene (pYUB515) (Fig. 2, lane 7) was identified by hybridization and used as the recipient cosmid for the allelic exchange in *E. coli*, since it had approximately 20 kb of DNA bracketing the *leuD* gene. Recombinant cosmids containing the *leu D3*::*aph* allele in place of the wild-type *leuD* allele (a schematic illustrating the recombination products is shown in Fig. 3) were isolated after conjugation by selecting for both kanamycin and sucrose resistance. Eight independent clones were examined by restriction analysis, and all were found to contain the 3.6-kb *Kpn*I fragment (the 2.3-kb *Kpn*I fragment containing *leuD* plus the 1.3-kb fragment containing *aph*) in place of the 2.3-kb *Kpn*I fragment containing the wild-type *leuD* gene. One such representative cosmid, pYUB516 (Fig. 2, lanes 8 and 9), was used in subsequent transformation experiments.

Allelic exchange in *M. tuberculosis*. To determine if long linear recombination substrates could promote allelic exchange in *M. tuberculosis*, *Pac*I digests of pYUB516, containing the *leuD3*::*aph* allele on a long DNA fragment (>40 kb), were electroporated into both the H37Rv and Erdman strains of *M. tuberculosis*. Undigested pYUB516 and both undigested and *Kpn*I-digested pYUB512 (releasing the 3.6-kb fragment containing the *leuD3*::*aph* allele) were electroporated for comparison. The long linear recombination substrate and the long

TABLE 2. Plasmids and their descriptions

Plasmid	Description	Reference or source
pYUB18	Shuttle cosmid used for evaluating transformation efficiencies	14
pBluescript II KS+	Used for subcloning restriction fragments	Stratagene
Supercos-1	Double *cos* cosmid vector	Stratagene
pCVD442	Conjugative vector dependent on π protein encoded by the *pir* gene for replication and that contains the *sacB* gene that confers sensitivity to sucrose to *E. coli* cells	8
pYUB328	*Pac*I-excisable cosmid vector	This work
pYUB511	pKSII clone with a 2.3-kb *Kpn*I BCG genomic DNA fragment identified by Southern hybridization with a (6.0-kb) *Kpn*I probe fragment from the *leuD1*::Tn*5367* BCG *leuD* auxotroph, mc^{2}797	This work
pYUB512	pYUB511 derivative containing an *aph* insertion in the unique *Sph*I site within *leuD*, thereby generating a *leuD3*::*aph* allele	This work
pYUB514	*Kpn*I fragment from pYUB512 containing *leuD3*::*aph* cloned into an *Sma*I site in pCVD442	This work
pYUB515	Cosmid with the wild-type *leuD* gene identified from the pYUB328::BCG DNA cosmid library by hybridization to the BCG *leuD1*::Tn*5367 Kpn*I probe mentioned above	This work
pYUB516	pYUB328 recombinant cosmid containing the *leuD3*::*aph* allele within a large *Pac*I fragment generated by recombination between pYUB514 and pYUB515	This work

circular substrate (undigested pYUB516) yielded kanamycin-resistant recombinants in both strains of *M. tuberculosis*, while both the smaller linear and circular pYUB512 substrates yielded no recombinants (Table 3). The failure to obtain transformants with the short pYUB512 substrates, which were comparable to substrates used previously (16), likely resulted from a lower overall transformation frequency.

The transformants were plated on media containing amino acids to allow growth of both leucine auxotrophs, obtained by allelic exchange, and any other auxotrophs that might have arisen as a result of illegitimate recombination. A large set of kanamycin-resistant transformants were tested for auxotrophy, and 17 of 287 (6%) and 11 of 176 (6%) kanamycin-resistant transformants for *M. tuberculosis* H37Rv and Erdman, respectively, were auxotrophs. Although long linear and long circular recombination substrates yielded similar numbers of transformants per microgram of DNA, auxotrophs were obtained only from transformation with the long linear substrate. When patched onto minimal medium containing leucine alone, all 28 auxotrophs were found to require leucine, suggesting that these recombinants had resulted from homologous allelic exchange. One each of the H37Rv and Erdman leucine auxotrophs was expanded and stocked as mc^{2}3000 and mc^{2}3001, respectively.

Southern analysis of the kanamycin-resistant transformants provided conclusive evidence that allelic exchange had occurred in *M. tuberculosis* (Fig. 4). Chromosomal DNA from both the parent *M. tuberculosis* H37Rv strain and kanamycin-resistant transformants was digested with *Kpn*I and probed with the 3.6-kb *Kpn*I fragment containing the *leuD3*::*aph* allele. A 2.3-kb fragment is seen in the lanes containing wild-type genomic DNA (Fig. 4, lanes 5) and wild-type cloned genes, i.e.,

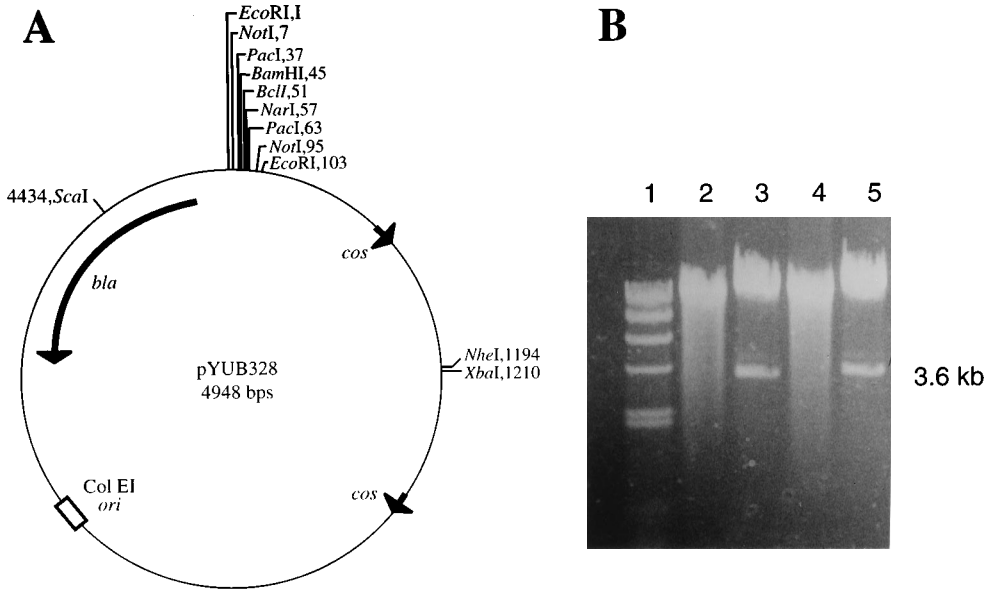

FIG. 1. Excision of pYUB328 from mycobacterial genomic libraries. (A) Schematic of pYUB328. pYUB328 contains *Pac*I sites flanking the unique restriction sites *Bam*HI, *Bcl*I, and *Nar*I; the ColE1 origin of replication (*ori*); *bla*, a gene encoding a β-lactamase that confers ampicillin resistance in *E. coli*; and *cos*, a sequence containing the λ *cos* site. (B) Ethidium bromide-stained gel of DNA of cosmid libraries constructed in pYUB328. Lane 1, λ *Hin*dIII marker; lane 2, BCG library in pYUB328 digested with *Pst*I; lane 3, BCG library in pYUB328 digested with *Pac*I; lane 4, *M. tuberculosis* H37Rv library in pYUB328 digested with *Pst*I; lane 5, *M. tuberculosis* H37Rv library in pYUB328 digested with *Pac*I.

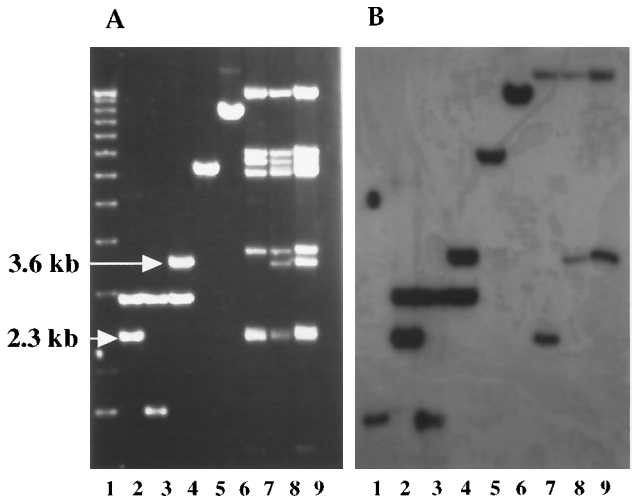

FIG. 2. Analysis of interplasmid recombination. (A) Ethidium bromide-stained gel of plasmids listed below. (B) Southern hybridization of the gel with DNA from lane 2 (pYUB511) used as an [α-^{32}P]dCTP-labelled probe. Lane 1, 1-kb ladder; lane 2, pYUB511 digested with *Kpn*I; lane 3, pYUB511 digested with *Kpn*I and *Sph*I; lane 4, pYUB512 digested with *Kpn*I; lane 5, pCVD442 digested with *Sph*I; lane 6, pYUB514 digested with *Sph*I; lane 7, pYUB515 digested with *Kpn*I; lanes 8 and 9, pYUB516 digested with *Kpn*I. See Results for details.

plasmid pYUB511 (lanes 1) and cosmid pYUB515 (lanes 3). However, the disrupted allele gives rise to a 3.6-kb fragment (2.3-kb *leuD* fragment plus 1.3-kb *aph* gene) after *Kpn*I digestion of pYUB512 (Fig. 4, lanes 2) and pYUB516 (lanes 4). The four kanamycin-resistant leucine auxotrophs of *M. tuberculosis* H37Rv examined (Fig. 4, lanes 6 to 9) contained this 3.6-kb hybridizing fragment and not the 2.3-kb fragment, indicating that the wild-type *leuD* gene had been replaced with the disrupted allele. All four kanamycin-resistant, nonauxotrophic transformants appeared to have the wild-type *leuD* allele (Fig. 4, lanes 10 to 13). Two of the clones exhibited only the 2.3-kb wild-type fragment, suggesting that these are spontaneous kanamycin-resistant mutants (Fig. 4, lanes 10 and 11), whereas two others showed additional hybridizing bands, suggesting illegitimate integrations of the substrate (lanes 12 and 13).

DISCUSSION

The inability to perform allelic exchanges in *M. tuberculosis* has been a major limitation in the genetic analysis of this important pathogen. The functions of individual genes can best be determined by the comparison of the phenotypes of strains that are genetically identical except at the gene in question. The ability to perform allelic exchange in *M. tuberculosis* would allow not only studies of gene function but also the generation of new vaccine strains having precisely defined mutations.

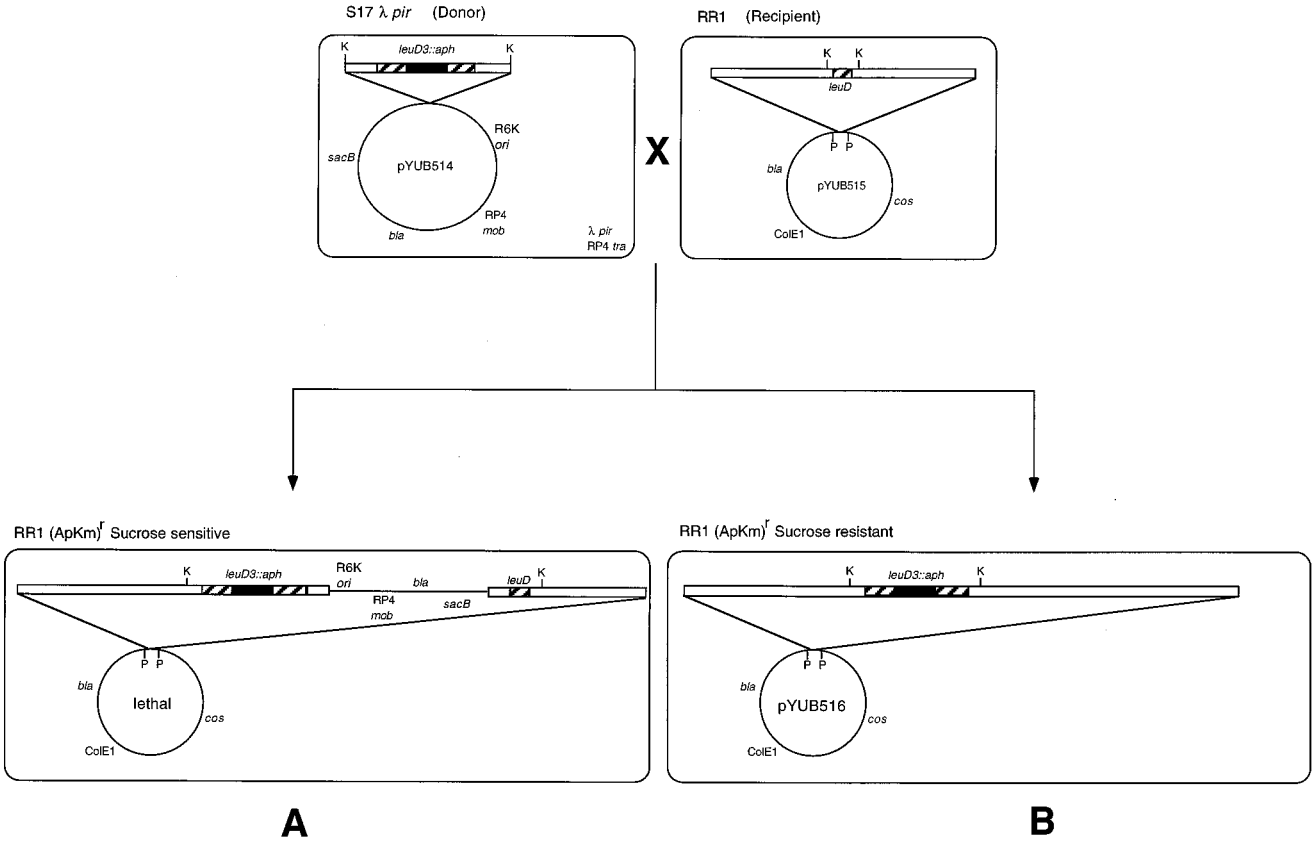

FIG. 3. Schematic of recombination in *E. coli*. The donor plasmid pYUB514, containing the subcloned *leuD3*::*aph* allele, was introduced via conjugation into recipient RR1 cells containing the target cosmid pYUB515. If a single homologous recombination event occurred between the donor and target, the resultant cointegrate would be lethal to the cell in the presence of sucrose because of the *sacB* gene (A). However, in the case of a double crossover event, the resultant cosmid would lack the *sacB* gene and thus cells with this cosmid would be resistant to sucrose (B). Selection for resistance to ampicillin, kanamycin, and sucrose yields RR1 clones containing cosmids with the *leuD3*::*aph* allele replacing wild-type *leuD*, as shown in panel B for pYUB516. The intact insert with the *leuD3*::*aph* allele can be released from pYUB516 by *Pac*I digestion, and the long linear insert DNA can be used as a substrate for recombination in mycobacteria. Abbreviations for restriction endonuclease sites: K, *Kpn*I; P, *Pac*I. The *leuD* alleles are represented by hatched boxes, while the *aph* gene is shown as a solid box. The wild-type *leuD* gene present in pYUB515 is not shown to scale because of the size of the cosmid insert relative to pYUB514. Note that the *Kpn*I sites shown in pYUB514 were filled in prior to cloning.

Previous attempts to achieve allelic exchange in strains of the *M. tuberculosis* complex showed that electroporated linear DNA was preferentially inserted into the mycobacterial genome by illegitimate recombination (1, 16). Kalpana et al. used a short (<6-kb) linear DNA fragment encoding a BCG methionine biosynthetic gene disrupted by a kanamycin resistance gene as a recombination substrate. In numerous experiments, electroporation of this construct into BCG and *M. tuberculosis* yielded stable kanamycin-resistant transformants; however, Southern analysis revealed that the DNA fragment had integrated at random sites around the genome (16). Aldovini et al. used a short linear DNA fragment containing the *uraA* gene into which a kanamycin resistance gene was inserted, and they obtained random integrations in 8 of 10 transformants analyzed. Even though the substrate had integrated into a homologous site in 2 of the 10 transformants, allelic exchange had not occurred (1). Recently, Norman et al. reported gene replacement in BCG achieved by using a replicating plasmid where counterselection against the single crossover event was dependent upon a functional *katG* gene in an isoniazid-resistant mutant (25). Although PCR products from a single transformant suggest that a double crossover homologous recombination event had occurred, no stable plasmid-free transformant resulting from an allelic exchange was obtained.

The recombination experiments with *E. coli* provide numerous possible explanations of why the short substrates failed to yield allelic exchange in BCG and *M. tuberculosis*. These include (i) the lack or defectiveness of recombination enzymes, (ii) overactive exonuclease activity, (iii) infrequent positioning of sequences that promote recombination (equivalent to chi sites in *E. coli* [24]), and (iv) a recombination system that must be induced. Our strategy to overcome a number of these possible limitations and to achieve allelic exchange in *M. tuberculosis* was to use long linear recombination substrates because they may be more slow to degrade than short recombination substrates, they may provide the necessary recombination junctions, and they may be generally more recombinogenic. Linear substrates may be more recombinogenic than circular molecules because some recombination enzymes may require double-stranded ends of the DNA substrate (24). We showed that long linear DNA fragments could be readily obtained from genomic libraries constructed in the excisable cosmid vector pYUB328 and identified a clone containing the BCG *leuD* gene with 20 kb of flanking DNA. The efficient homologous recombination system of *E. coli* was then exploited to introduce a precisely defined insertional mutation within the *leuD* gene. *Pac*I cleavage generated long linear recombination substrates which were electroporated into both the H37Rv and Erdman strains of *M. tuberculosis*. Leucine auxotrophic mutants resulting from allelic exchange were obtained in 6% of the transformants of both strains; however, recombinants were only obtained with long recombination substrates but not with short substrates. Thus, the frequency of transformation with long linear recombination substrates is sufficiently high to obtain the desired allelic exchange with relatively few transformants, but it is likely dependent upon the lengths of DNA flanking the mutated allele. Further studies will be necessary to evaluate these parameters, but the *leuD3*::*aph* allele on the *Pac*I fragment of pYUB516 is an ideal substrate for identifying conditions that promote efficient allelic exchange.

The availability of genomic libraries in excisable cosmids

TABLE 3. Transformation and allelic-exchange frequencies of *M. tuberculosis* Erdman and H37Rv

DNA substrate	Transformant and no. of leucine-requiring mutants[a]			
	M. tuberculosis H37Rv		*M. tuberculosis* Erdman	
	Kan^r	Kan^r Leu^-/Kan^r	Kan^r	Kan^r Leu^-/Kan^r
No DNA	0	0	0	0
pYUB18	$>10^4$	0	$>10^4$	0
pYUB512 (undigested)	0	0	0	0
pYUB512 (*Kpn*I digested)	0	0	0	0
pYUB516 (undigested)	193	0/193	122	0/122
pYUB516 (*Pac*I digested)	349	17/287	210	11/176

[a] Kan^r, kanamycin-resistant phenotype; Kan^r Leu^-, kanamycin-resistant and leucine-requiring phenotype. Approximately 1 μg of DNA was used for each transformation experiment.

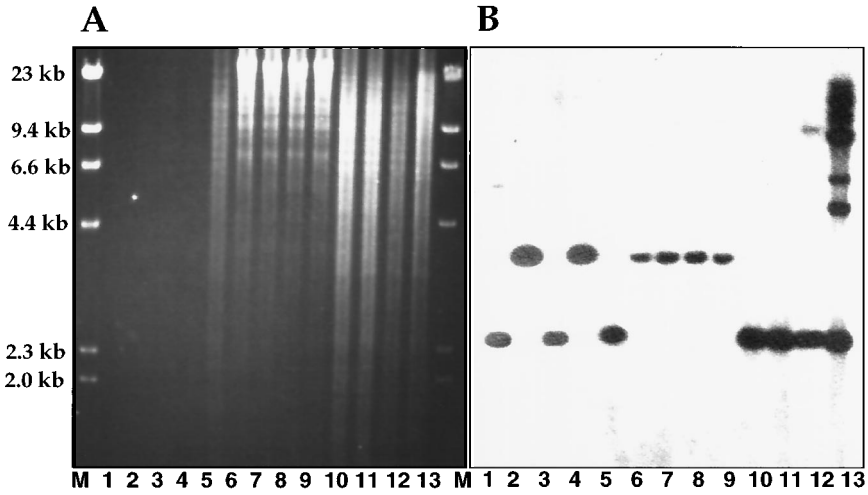

FIG. 4. Southern analysis of leucine auxotrophs of *M. tuberculosis*. (A) Ethidium bromide-stained gel of digested DNAs. (B) Southern hybridization of the gel. Chromosomal DNAs were isolated from kanamycin-resistant transformants, cleaved with *Kpn*I, and probed with [α-^{32}P]dCTP-labelled *leuD3*::*aph*. Plasmids and chromosomal DNA were digested with *Kpn*I. Lanes M, λ*Hin*dIII marker; lanes 1, pYUB511; lanes 2, pYUB512; lanes 3, pYUB515; lanes 4, pYUB516; lanes 5, *M. tuberculosis* H37Rv; lanes 6 to 9, leucine auxotrophs of *M. tuberculosis* H37Rv (note that mc^{2}3000 is in lanes 6); lanes 10 to 13, nonauxotrophic kanamycin-resistant clones of *M. tuberculosis* H37Rv.

offers a wide range of possibilities in addition to providing substrates for allelic exchange in *M. tuberculosis*. These cosmid libraries provide (i) ideal substrates for a canonical set of cosmids that has been ordered for the *M. tuberculosis* library and which will facilitate genome sequencing; (ii) extremely versatile substrates for interchange of integrative or extrachromosomal vectors with any selectable marker, because of the ability to precisely excise the insert from the vector with *Pac*I restriction enzyme; (iii) a simple means of excising an insert from a cosmid that is integrated onto the chromosome, because of the absence of *Pac*I sites elsewhere on the chromosome; and (iv) recombination substrates for linkage analysis (3). In addition, it is possible to generate transposon insertions in the *Pac*I cosmids in *E. coli* that either mutate desired genes or provide a selectable marker gene linked to a gene of interest. Such constructs have already proven to be invaluable in performing linkage analyses to establish that a specific allele of *inhA* conferred resistance to both isoniazid and ethionamide (3).

While further efforts for optimizing the conditions for homologous recombination are necessary, it is not too early to envision the wide range of applications that may result from the observation made in this study. Although BCG is one of the most widely used vaccines, its variable efficacy necessitates the development of newly attenuated *M. tuberculosis*-based vaccines. Making precisely defined nonrevertible mutations in the *M. tuberculosis* genome is an essential step in the development of such vaccines. Additionally, mutants such as the leucine auxotroph are powerful tools for genetically dissecting the virulence mechanisms of *M. tuberculosis.*

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ADDENDUM

While the manuscript was in review, another group (Reyrat, J.-M., et al., Proc. Natl. Acad. Sci. USA **92:**8768–8772, 1995) described allelic exchange at the *ureC* locus of BCG achieved by using short linear recombination substrates. The reasons for the differences between experiments with short linear and long linear recombination substrates are unknown. Application of our system will hopefully reveal more information about the recombination machinery in slow-growing mycobacteria.

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