Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5433–5437, June 1991
Microbiology

Insertional mutagenesis and illegitimate recombination in mycobacteria

(shuttle mutagenesis/auxotrophs/homologous recombination/transposons/*Mycobacterium tuberculosis*)

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ABSTRACT **Mycobacteria, particularly *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium avium*, are major pathogens of man. Although insertional mutagenesis has been an invaluable genetic tool for analyzing the mechanisms of microbial pathogenesis, it has not yet been possible to apply it to the mycobacteria. To overcome intrinsic difficulties in directly manipulating the genetics of slow-growing mycobacteria, including *M. tuberculosis* and bacille Calmette–Guérin (BCG) vaccine strains, we developed a system for random shuttle mutagenesis. A genomic library of *Mycobacterium smegmatis* was subjected to transposon mutagenesis with Tn*5 seq1*, a derivative of Tn*5*, in *Escherichia coli* and these transposon-containing recombinant plasmids were reintroduced into mycobacterial chromosomes by homologous recombination. This system has allowed us to isolate several random auxotrophic mutants of *M. smegmatis*. To extend this strategy to *M. tuberculosis* and BCG, targeted mutagenesis was performed using a cloned BCG methionine gene that was subjected to Tn*5 seq1* mutagenesis in *E. coli* and reintroduced into the mycobacteria. Surprisingly for prokaryotes, both BCG and *M. tuberculosis* were found to incorporate linear DNA fragments into illegitimate sites throughout the mycobacterial genomes at a frequency of 10^{-5} to 10^{-4} relative to the number of transformants obtained with autonomously replicating vectors. Thus the efficient illegitimate recombination of linear DNA fragments provides the basis for an insertional mutagenesis system for *M. tuberculosis* and BCG.**

Mycobacterium tuberculosis is the single largest infectious cause of death in the world today, responsible for >7 million new cases of tuberculosis annually and 3 million deaths (1). Other pathogenic mycobacteria are responsible for human opportunistic infections, particularly in AIDS patients, and for a major disease in cattle. The molecular genetics of the mycobacteria has only recently begun to be developed, in part because the mycobacteria present formidable obstacles to genetic study—they clump in culture and grow very slowly. Genetic studies on mycobacteria were initiated by developing a shuttle strategy using phasmid and plasmid vectors that could be easily manipulated in *Escherichia coli* and then transferred to and stably maintained in both fast- and slow-growing species of mycobacteria (2, 3). Although the direct selection of mutants employing transposons (random insertional mutagenesis) has been a very useful approach to mutational analysis of microbial pathogenesis (4–7), at the time these studies were begun none had been described in mycobacteria. A transposable element recently identified in *Mycobacterium fortuitum* offers promise for genetic studies of mycobacteria (8), but its ability to transpose in slow-growing mycobacteria and pathogenic species is not yet known. Based on the recent demonstration of targeted mutagenesis in *Mycobacterium smegmatis* (9), we undertook to develop random shuttle mutagenesis of *M. smegmatis* employing the transposon Tn*5 seq1*, which carries the NEO marker that confers resistance to kanamycin and neomycin (10). Three auxotrophic mutants of *M. smegmatis* were isolated by this random shuttle mutagenesis. Extending this strategy to the slow-growing mycobacteria bacille Calmette–Guérin (BCG) and *M. tuberculosis* required the study of homologous recombination in these species. The BCG DNA fragment that complements the methionine auxotrophic mutation of *M. smegmatis* was isolated, insertionally inactivated with Tn*5 seq1*, and introduced into BCG for targeted mutagenesis. These studies revealed a high degree of illegitimate recombination in slow-growing species, a phenomenon not generally observed in prokaryotes.

MATERIALS AND METHODS

Bacterial Strains and DNA Manipulations. *M. smegmatis* mc^26 (2) and mc^2155 (11) have been described. BCG-Pasteur was obtained from the World Health Organization BCG Reference Laboratory at the Statens Seruminstitut (Copenhagen) and *M. tuberculosis* H37Rv was obtained from Wilbur Jones at the Centers for Disease Control (Atlanta). The methionine and pyridoxine auxotrophs of *M. smegmatis* mc^2155 (mc^2311 and mc^2313, respectively) were isolated by the random shuttle mutagenesis method as described below. *Escherichia coli* strains used were χ2338 (12), ec^2270 (χ2338, into which Tn*5 seq1* is inserted at an unknown location of the chromosome), and DH5α. Chromosomal and plasmid DNA isolation and Southern blot analysis were performed as described (7).

Random Shuttle Mutagenesis of *M. smegmatis*. The mycobacterial genomic library was constructed in pYUB36, a derivative of pBR322 (13) in which the nonessential 1.9-kilobase (kb) *Eco*RV–*Pvu* II fragment has been deleted. Chromosomal DNA of *M. smegmatis* mc^26 was partially digested with *Msp* I. After size selection, DNA inserts of 4–7 kb were ligated to *Cla* I-digested pYUB36 and used to transform *E. coli* ec^2270, and transformants were plated on L-agar medium containing both ampicillin and kanamycin at 40 μg/ml and 50 μg/ml, respectively. About 30,000 individual transformants were pooled and samples were diluted 1:1000 into 20 independent 5-ml cultures and incubated at 37°C overnight. A 200-μl sample from each overnight culture yielded ≈1000 colonies on L-agar containing a high level (i.e., 250 μg/ml) of neomycin (neomycin-hyperresistance selection), which selects for colonies resulting from transposition of Tn*5 seq1* into plasmids. Plasmid DNA from the combined pool of neomycin-resistant colonies was retransformed into χ2338 and plasmid DNA was subsequently prepared from the transformants. This Tn*5 seq1*-mutagenized plasmid library was then electroporated into one of the *M. smegmatis* strains,

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Abbreviations: BCG, bacillus Calmette–Guérin; kan^r, kanamycin resistant.

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either mc²6 or mc²155, and kanamycin-resistant (kan^r) transformants were selected on K-agar [Middlebrook 7H10 agar supplemented with Casamino acids (Difco; 5 mg/ml), diaminopimelic acid (100 μg/ml), thymidine (50 μg/ml), uracil (40 μg/ml), and adenosine (133 μg/ml), 0.2% glycerol, albumin–dextrose complex, and cycloheximide (10 μg/ml)] containing kanamycin (20 μg/ml). About 800 *M. smegmatis* transformants were screened for auxotrophy by streaking onto minimal Sauton medium without asparagine (minimal medium, MM). Three auxotrophs were obtained from this screen and their nutritional requirements were determined by plating on Sauton agar plates supplemented with one or the other of 11 pools of nutrients used for the auxanography analysis of *E. coli*.

Isolation of *M. smegmatis*, BCG-Pasteur, and *M. tuberculosis* Complementing Clones of *M. smegmatis* Methionine Auxotroph. Genomic libraries of *M. tuberculosis* H37Rv, *M. smegmatis*, and BCG were constructed by ligating 4- to 7-kb *Msp* I-digested chromosomal DNA fragments to *Cla* I-digested pYUB53, an *E. coli*/mycobacteria shuttle vector (G.V.K., unpublished data). Ligated DNAs were initially introduced into *E. coli*, and plasmids isolated from the pool of *E. coli* were electroporated into the methionine auxotroph mc²311. Clones that conferred prototrophy to mc²311 (referred to as *met*-complementing clones) were isolated. Insertional inactivation of the BCG *met*-complementing clone pYUB121 was performed in *E. coli* by neomycin-hyperresistance selection, as described above. Individual pYUB121 clones containing Tn*5 seq1* were screened for the loss of their ability to complement the methionine auxotrophic mutation of mc²311.

Electroporation. BCG and *M. tuberculosis* were subjected to electroporation as described (7) with the following modifications. BCG cultures were subcultured 1:50 in 50 ml of MADC-TW broth (3) and grown for 10 days instead of 7 days at 37°C. The harvested culture was washed first with 50 ml and then with 25 ml of ice-cold 10% (vol/vol) glycerol. After centrifugation, the final pellet was resuspended in 2.5 ml of ice-cold 10% glycerol and 0.4 ml used for each electroporation. BCG transformants were plated on Middlebrook 7H10 agar supplemented with albumin–dextrose complex, 0.2% glycerol, and cycloheximide (10 μg/ml) containing kanamycin (20 μg/ml) and methionine (50 μg/ml).

RESULTS

Random Shuttle Mutagenesis of *M. smegmatis*. Our approach to mutagenize genomic sequences (Fig. 1) involved extending the general strategy of targeted shuttle mutagenesis (14, 15) to one of random shuttle mutagenesis by constructing a plasmid genomic library of *M. smegmatis* DNA in the plasmid pYUB36, a pBR322-derived vector that is unable to replicate in mycobacteria. Random insertions in the cloned mycobacterial DNAs were generated using transposon mutagenesis in *E. coli*. We chose Tn*5 seq1* (10) because it (*i*) encodes the neo gene that confers kanamycin resistance to both *E. coli* and mycobacteria (3), (*ii*) permits selection of insertions into DNA sequences cloned into plasmid vectors using its neomycin-hyperresistance phenotype (16), and (*iii*) lacks the cryptic gene encoding streptomycin-resistance of Tn*5*, an important biohazard consideration for *M. tuberculosis* strains (17). After enrichment for plasmids containing transposon insertions by selection with neomycin, the mutagenized plasmid pool was electroporated into *M. smegmatis* and kan^r transformants were obtained at a frequency of 20–40 per μg of plasmid DNA. In contrast, no kan^r colonies were obtained with a control pBR322::Tn*5 seq1* plasmid that lacked homologous DNA sequences. As expected, Southern blot analysis of total chromosomal DNA from independent kan^r transformants digested with *Cla* I (recognition sites are absent in both Tn*5 seq1* and pYUB36) and *Sac* I (a unique site in Tn*5 seq1*), using Tn*5 seq1* as probe, demonstrated that there is only one Tn*5 seq1* insertion per transformant and that

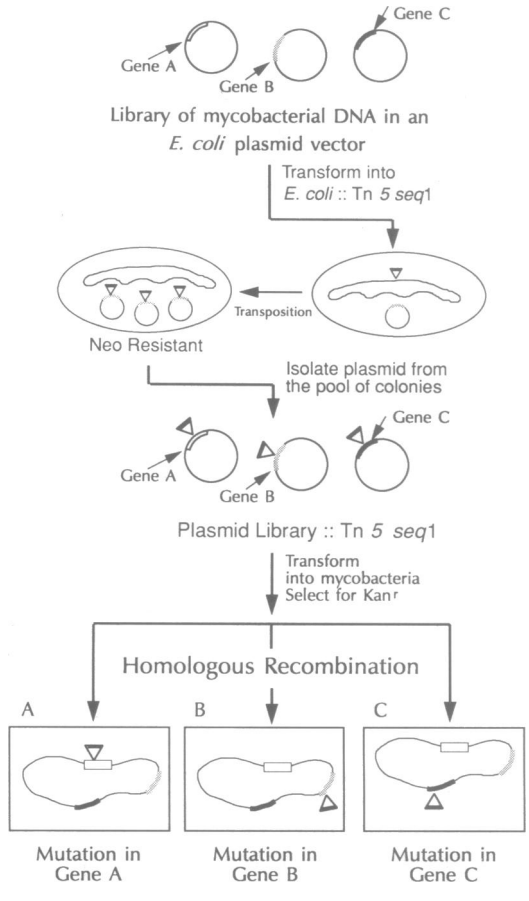

FIG. 1. Strategy for random insertional mutagenesis of mycobacteria. A plasmid genomic mycobacterium library constructed in an *E. coli* vector that is unable to replicate in mycobacteria is subjected to Tn*5 seq1* mutagenesis by selecting for a high level of neomycin (Neo) resistance. Mutagenized plasmids are then electroporated into mycobacteria. Since the recombinant plasmids cannot replicate in the mycobacterial host, stable kan^r transformants should result due to the homologous recombination with the chromosome. Replacement of the chromosomal gene by the corresponding Tn*5 seq1*-inactivated gene results in an insertional mutant, which can either be selected or screened for by appropriate means.

Tn*5 seq1* integrated at various sites within the *M. smegmatis* chromosome (data not shown).

Approximately 800 kan^r transformants that had been obtained on complete medium were purified and streaked onto minimal medium. Three auxotrophs were obtained by this screening, a methionine mutant (mc²311), a pyridoxine-requiring auxotroph (mc²313) (Fig. 2), and a third auxotroph, as yet incompletely characterized. Preliminary analysis of two of these auxotrophs indicated that Tn*5 seq1* and vector sequences were present in both and a deletion was present in one, suggesting that auxotrophic mutations could have occurred due to the recombination of incomplete genes present in the library with the chromosomal gene or by secondary rearrangements after single crossover events. Further analysis will be required to define the nature of the lesion in the auxotrophs.

Study of Recombination in BCG. Targeted mutagenesis in BCG was performed by (*i*) isolating the DNA sequences from BCG and *M. tuberculosis* genomic libraries that complemented the *M. smegmatis* methionine auxotrophic mutation (Fig. 3), (*ii*) insertionally inactivating the BCG methionine-complementing (*met*) clone with Tn*5 seq1* (Fig. 3), and (*iii*) introducing the mutagenized *met* gene into BCG by electroporation for homologous recombination. To maximize the chances of detecting double crossover events, we conducted

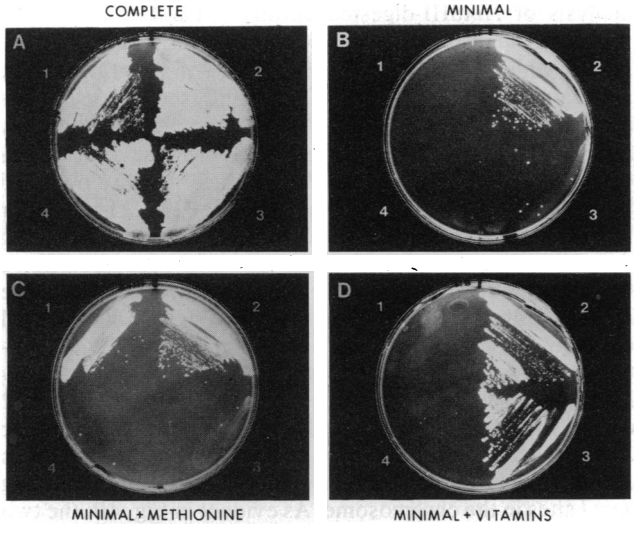

FIG. 2. Auxotrophic mutants of *M. smegmatis* generated by insertional mutagenesis. A library of insertional mutants of *M. smegmatis* was generated and subsequently screened for mutants that had lost their ability to grow on a minimal glycerol medium. The strains were grown on a rich medium (K-agar) (*A*), glycerol minimal medium (MM) (*B*), MM supplemented with methionine (*C*), or MM supplemented with pyridoxine (*D*). All media contain kanamycin. Each medium was streaked with the methionine auxotroph in sector 1, the prototroph in sector 2, the pyridoxine auxotroph in sector 3, and the uracil auxotroph in sector 4.

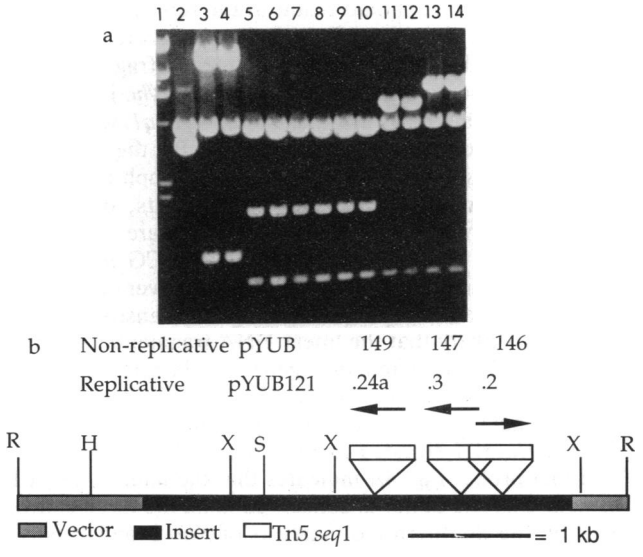

FIG. 3. (*a*) Isolation of *M. smegmatis*, BCG, and *M. tuberculosis* DNA clones that complement the *met* mutation of *M. smegmatis*. (*b*) Inactivation of the BCG *met* gene with Tn*5 seq1*. (*a*) Each lane contains individual *Xho* I-digested complementing clones analyzed by ethidium bromide-stained agarose gel electrophoresis. Lanes: 1, λ *Hin*dIII markers; 2, vector pYUB53; 3 and 4, *M. smegmatis* clones; 5–12, BCG clones; 13 and 14, *M. tuberculosis* H37Rv clones. (*b*) Schematic representation of positions of three Tn*5 seq1* insertions (pYUB121.24a, pYUB121.3, and pYUB121.2) that eliminate the complementing ability of one of the BCG *met* clones, pYUB121 (see *a*, lane 5). The *Eco*RI fragments containing Tn*5 seq1* were subcloned into pYUB127, an *E. coli* vector incapable of replicating in mycobacteria, to obtain the corresponding clones, pYUB149, -147, and -146, respectively, that were used for the study of recombination in BCG (see Table 1). H, *Hin*dIII; R, *Eco*RI; S, *Sca* I; X, *Xho* I. Note: the Tn*5 seq1* is not drawn to the scale.

the experiments with linear DNA. It was expected that homologous recombination resulting from a double crossover event of a linear DNA fragment containing the Tn*5 seq1*-inactivated methionine gene would yield kan^r methionine auxotrophs of BCG. In the experiments summarized in Table 1, very few kan^r BCG colonies above the level of spontaneous mutations were obtained from the transformation of circular molecules, whereas hundreds of colonies resulted from the transformation by linear molecules. Unexpectedly, only one methionine auxotroph of >200 kan^r transformants was obtained from the transformation of linearized pYUB146 containing a Tn*5 seq1*-inactivated methionine gene. This result suggested the existence of a high degree of illegitimate recombination when linear DNA was used.

Demonstration of Illegitimate Recombination in BCG and *M. tuberculosis*. To characterize the nature of the recombination in BCG, total chromosomal DNA from the methionine auxotroph (mc^{2}576) and two other kan^r transformants was subjected to Southern blot analysis using the 1.8-kb *Xho* I DNA fragment from the BCG *met* clone as probe (Fig. 4*A*). This probe detects a 8.5-kb fragment in the wild-type BCG chromosome. A double crossover between the chromosome

Table 1. Transformation efficiencies with circular vs. linear DNA

Plasmid	DNA	kan^r colonies on medium containing methionine, no.			
		M. smegmatis	BCG	BCG	*M. tuberculosis*
No DNA	—	0	12	12	7
pYUB53	CCC	≈10^6	3.4 × 10^5	0.93 × 10^5	3 × 10^4
pBR322::Tn*5 seq1*	CCC	0	13	—	—
	Linear	0	140	—	26
BCG *met*::Tn*5 seq1*					
pYUB146	CCC	—	16	23	—
	Linear	—	130	147	—
pYUB147	CCC	—	—	18	14
	Linear	—	—	148	27
pYUB149	CCC	—	—	22	—
	Linear	—	—	124	—
M. smegmatis met::Tn*5 seq1*, pYUB156	CCC	332	—	—	—
	Linear	196	—	—	—

CCC, covalently closed circular. Linear DNA molecules were generated by digesting the plasmids with *Eco*RI. pYUB53 is the shuttle plasmid that replicates episomally in mycobacteria and is used for determining the transformation efficiencies. The *M. smegmatis met*::Tn*5 seq1* construct is derived by subcloning the *Eco*RI fragment of the plasmid of pYUB120.5 into pYUB127, an *E. coli* vector. pYUB120.5 is derived from the *M. smegmatis met*-complementing clone, pYUB120 (Fig. 3*A*, lane 1) after Tn*5 seq1* inactivation to abolish the *met*-complementing ability. Electroporations were performed with 2–4 μg of DNA except for pYUB153 where the data is indicated in the form of number of colonies per μg of DNA (transformation efficiency). The phenotypes of all the clones were kan^r and met$^+$.

and the linear DNA should result in the replacement of the chromosomal *met* gene by the insertionally inactivated gene. In the Southern blot analysis, the 8.5-kb *Xho* I fragment of the chromosome should be replaced by two new *Xho* I fragments due to the presence of a *Xho* I site in the Tn*5 seq1*, whose total length should be 8.5 plus 3.2 kb (3.2 kb being the size of the Tn*5 seq1*). As shown in Fig. 4*A*, the auxotroph and all the prototrophs contained three *Xho* I fragments, one 8.5-kb fragment, and two fragments, A and B, that are identical to that present in the Tn*5 seq1*-inactivated BCG *met* clone. These results indicated that no double crossover occurred in the methionine auxotroph or the other kan^r transformants. It thus appears likely that the linear DNA fragment containing the Tn*5 seq1*-inactivated *met* gene has integrated illegitimately in the BCG chromosome. Further evidence supporting this conclusion was obtained by Southern blot analysis using the *Hin*dIII-digested chromosomal DNA and the same *Xho* I met probe. Fig. 4*A* indicates that the internal *Hin*dIII fragment C of the donor is conserved in all the three clones. Based on the Southern blot analysis of *Xho* I- and *Hin*dIII-digested chromosomal DNA of the kan^r transformants of BCG, it is evident that no homologous recombination has occurred between the donor DNA fragment and chromosomal sequences. In addition these results indicated that Tn*5 seq1* did not transpose into the chromosome since its position in the *met* gene of the donor was unaltered. Southern blot analysis of *Hin*dIII-digested chromosomal DNA with the probe should also detect a flanking fragment D (Fig. 4*A*), the size of which would depend on the site of integration. The variation in sizes of fragment D from different recombinants again indicates that the donor DNA fragment is randomly integrating into the BCG chromosome. A similar analysis was carried out using an additional 9 kan^r transformants obtained by the transformation of linearized pYUB146. Three donor bands (A, B, and C) were detected in all 9 clones, indicating that homologous recombination had not occurred. Since G+C-nucleotide-rich mycobacterial DNA yields very large *Hin*dIII fragments that are not resolved by standard gel electrophoresis, to establish the randomness of the integration of band D, Southern blot analysis was performed by hybridizing *Ava* I-digested chromosomal DNA of the same 12 clones with the vector probe. This probe should detect four *Ava* I fragments (Fig. 4*B*): two conserved internal fragments and fragments E and F that should vary due to the flanking *Ava* I sites in the chromosome. As evident in Fig. 4*B*, the two internal fragments were conserved and the end fragments showed different patterns in every clone, indicating that the integration had taken place at random sites. This analysis establishes that a high degree of illegitimate recombination occurs in BCG.

Several experiments were undertaken to determine if nonhomologous recombination is a feature of BCG or of the

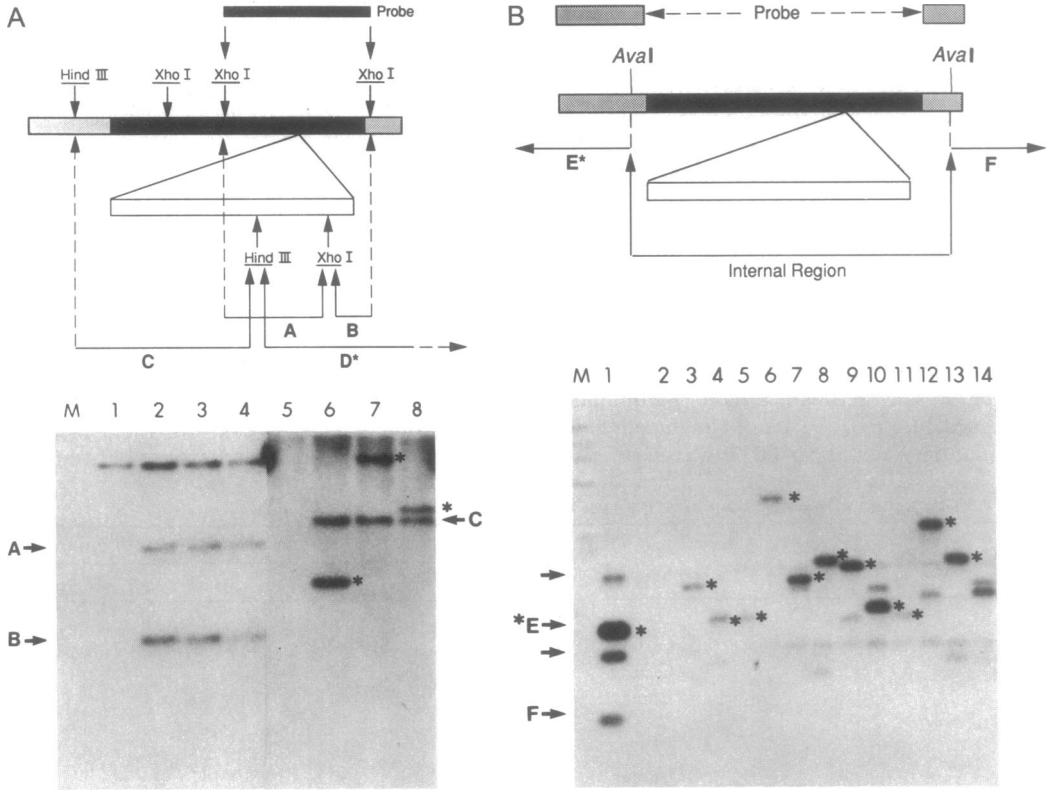

FIG. 4. Demonstration of nonhomologous recombination in BCG. (*A*) Southern blot analysis of *Xho* I- and *Hin*dIII-digested chromosomal DNA from kan^r BCG transformants using the 1.8-kb *Xho* I DNA fragment from the BCG *met* region as probes to distinguish between homologous and nonhomologous recombination. (*B*) Southern blot analysis of *Ava* I-digested chromosomal DNA from 12 BCG transformants demonstrates the randomness of illegitimate integration of pYUB146 by using vector sequences as probe. (*A*) BCG chromosomal DNAs isolated from both wild-type BCG (lanes 1 and 5) and three independent kan^r transformants, a methionine auxotroph (lanes 2 and 6), and prototrophs (lanes 3, 7, 4, and 8). Lanes 1–4 containing DNA digested with *Xho* I and lanes 5–8 contain DNA digested with *Hin*dIII. Lane M has radiolabeled λ *Hin*dIII markers. The blot is hybridized with the 1.8-kb *Xho* I fragment (see *A Upper*) from the pYUB121 into which the Tn*5 seq1* has been integrated in pYUB121.2 to abolish the methionine complementation. The * indicates differing sizes of fragment D. Only three clones are shown in this figure, but nine additional clones analyzed also demonstrated identical sizes for bands A, B, and C. The same 12 clones were analyzed for the randomness of integration by digesting with *Ava* I (see *B*). (*B*) Lane M contains radiolabeled λ *Hin*dIII markers. *Ava* I digests of donor fragment pYUB146 (lane 1), BCG-Pasteur DNA (lane 2), methionine auxotroph (lane 3), kan^r prototrophs (lanes 4–14). The blot was hybridized with the vector sequences that hybridize to the ends of the donor as indicated in *B Upper*. Note that fragment E, indicated by *, differs in size in every clone. Fragment F also varies in all the clones. The two arrows point to the two internal fragments detected by the probe that are same size in every clone.

methionine gene. When equal quantities of circular and linearized pBR322:Tn*5* *seq1* and pYUB156, which has the Tn*5* *seq1* inserted in the complementing *met* clone of *M. smegmatis*, were electroporated into *M. smegmatis*, there was no integration of either circular or linear pBR322::Tn*5* *seq1* [frequency of $<10^{-6}$, relative to the number of transformants obtained with autonomously replicating vector (Table 1], indicating that mycobacterial homologous sequences were required for integration and that illegitimate recombination did not occur in *M. smegmatis*, consistent with previous findings (9). The frequency of kan^r colonies using both linear or circular pYUB156 was about the same (Table 1). Southern blot analysis of six of these kan^r colonies of *M. smegmatis* indicated that the integration of the pYUB156 occurred in all of them in the *Eco*RI fragment of the chromosome, two of which were double crossovers lacking plasmid sequences (data not shown). We infer that these could only be obtained by homologous recombination and the results confirm that illegitimate recombination does not occur in *M. smegmatis* at observable frequencies.

In contrast, when equal quantities of circular and *Eco*RI-digested pBR322:Tn*5* *seq1* and Tn*5* *seq1*-inactivated BCG *met* clones (pYUB146, pYUB147, and pYUB149, which differ from each other with respect to the position of Tn*5* *seq1* insertion; c.f., Fig. 3) were electroporated into BCG, linear but not circular molecules integrated into the chromosome at a frequency of 10^{-5} to 10^{-4} per μg of DNA, irrespective of whether they contained mycobacterial DNA (Table 1). It could be argued that these results are dependent on the transposase function of Tn*5* *seq1*. That argument is untenable for at least three reasons: (*i*) Tn*5* is a nonreplicative transposon (18) and if it transposed in mycobacteria it should have been lost from the donor DNA, which did not occur; (*ii*) it is not obvious why transposition of Tn*5* would occur at a significantly higher frequency from linear rather than circular DNA; (*iii*) an additional experiment carried out with a pBR322 derivative, pYUB8, lacking any sequences from Tn*5* *seq1* demonstrated that its frequency of illegitimate recombination was comparable to that of pBR322::Tn*5* *seq1*. Specifically, in this experiment, circular DNA of both pBR322::Tn*5* *seq1* and pYUB8 gave only 1 transformant per μg of DNA, whereas linearized pBR322::Tn*5* *seq1* and pYUB8 gave 12 and 10 transformants per μg of DNA, respectively.

To test whether the finding of illegitimate recombination was restricted to BCG, which by virtue of being an attenuated strain might have lost some recombinational mechanisms, an analogous experiment was carried out using the virulent *M. tuberculosis* H37Rv. The results were similar to those in BCG, with only linear but not circular forms of both pYUB147 and pBR322::Tn*5* *seq1* integrating at a higher frequency into the chromosome (Table 1).

DISCUSSION

Overall the results indicate that shuttle mutagenesis has made it possible to generate random insertional mutations in fast-growing mycobacteria and has allowed the isolation of auxotrophs from a pool of insertional mutants of *M. smegmatis*. In both BCG vaccine strains and in virulent *M. tuberculosis*, illegitimate recombination using linear DNA occurs at significant frequency. It remains to be established whether homologous recombination is deficient in these mycobacteria or merely obscured by the high level of illegitimate recombination.

Illegitimate recombination, defined as any DNA rearrangement leading to covalent joining of nonhomologous previously nonadjacent linear segments that does not involve functions specified by transposable elements, is a rare phenomenon in prokaryotes (19, 20). It occurs at low frequency in yeast (21) and, relative to homologous recombination, at high frequency in *Ustilago* (22) and in all mammalian cells (23, 24). Although the molecular basis has not been fully elucidated in any system, we would suggest that these slow-growing organisms may have evolved efficient DNA repair systems capable of integrating or ligating linear DNA fragments into their chromosomes. Perhaps such a system evolved in the pathogenic mycobacteria to protect the integrity of the genome of a slow-growing organism that has to survive within the daunting radical-rich environment of the macrophage, particularly one that has a tropism for oxygen-rich regions of the lung. In practical terms, since illegitimate recombination is a process of random integration, we suggest that this mechanism can be usefully exploited for both the random insertional mutagenesis of pathogenic mycobacteria and the stable integration of foreign antigen genes into BCG vaccine strains.

We gratefully acknowledge the generous gifts of Tn*5* *seq1* by Dr. D. Berg, pYUB136 by R. Barletta. We also acknowledge the helpful discussions with Drs. R. Kucherlapati, J. Marmur, R. Young, R. Davis, T. Kieser, N. Grindley, and G. Hatfull. The data presented in this paper are part of a thesis to be submitted by G.V.K. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Sue Golding Graduate Division of Medical Sciences, Albert Einstein College of Medicine. This work was supported by Grants AI23545, AI26941, AI27235, and AI26170 from the National Institutes of Health, the Rockefeller Foundation, the Immunology of Tuberculosis Committee of the World Health Organization/UNDP Special Programme for Vaccine Development, and the Immunology and Chemotherapy Committees of the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases.

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