INFECTION AND IMMUNITY, Mar. 1995, p. 1004–1012 Vol. 63, No. 3
0019-9567/95/$04.00+0
Copyright © 1995, American Society for Microbiology

In Vivo Growth Characteristics of Leucine and Methionine Auxotrophic Mutants of *Mycobacterium bovis* BCG Generated by Transposon Mutagenesis

RUTH A. MCADAM,† TORIN R. WEISBROD, JEAN MARTIN, JOSEPH D. SCUDERI, AMANDA M. BROWN, JEFFREY D. CIRILLO,‡ BARRY R. BLOOM, AND WILLIAM R. JACOBS, JR.*

Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

Received 20 October 1994/Accepted 19 December 1994

Insertional mutagenesis in *Mycobacterium bovis* BCG, a member of the slow-growing *M. tuberculosis* complex, was accomplished with transposons engineered from the *Mycobacterium smegmatis* insertion element IS*1096*. Transposons were created by placing a kanamycin resistance gene in several different positions in IS*1096*, and the resulting transposons were electroporated into BCG on nonreplicating plasmids. These analyses demonstrated that only one of the two open reading frames was necessary for transposition. A library of insertions was generated. Southern analysis of 23 kanamycin-resistant clones revealed that the transposons had inserted directly, with no evidence of cointegrate formation, into different restriction fragments in each clone. Sequence analysis of nine of the clones revealed junctional direct 8-bp repeats with only a slight similarity in target sites. These results suggest that IS*1096*-derived transposons transposed into the BCG genome in a relatively random fashion. Three auxotrophs, two for leucine and one for methionine, were isolated from the library of transposon insertions in BCG. They were characterized by sequencing and found to be homologous to the *leuD* gene of *Escherichia coli* and a sulfate-binding protein of cyanobacteria, respectively. When inoculated intravenously into C57BL/6 mice, the leucine auxotrophs, in contrast to the parent BCG strain or the methionine auxotroph, showed an inability to grow in vivo and were cleared within 7 weeks from the lungs and spleen.

The high human and monetary costs of tuberculosis, caused by *Mycobacterium tuberculosis* and *Mycobacterium bovis* in both developing and industrialized nations (4, 5, 39, 43), necessitate urgent research into the growth and metabolism of mycobacteria. Among the priorities in tuberculosis research is the development of a more effective vaccine since the efficacy of the strain used currently, i.e., *M. bovis*-derived bacillus Calmette-Guérin (BCG), has varied widely in controlled trials and there are concerns about its safe use in vaccinating human immunodeficiency virus-infected individuals (3, 11). Live attenuated strains provide the best protection, and results with other bacterial pathogens have shown that by attenuation, it is possible to limit replication of the bacterium in vivo while stimulating immunity to the natural agent (8). Although significant strides have been made in developing genetic tools for the molecular manipulation of mycobacteria in recent years (reviewed in references 17, 19, and 49), the lack of an efficient method of isolating mutants in the slow-growing strains has been a major obstacle. The isolation and characterization of mutations in the genomes of *M. tuberculosis* and BCG are not only required for the construction of attenuated strains but also crucial to the analysis of the functions of their genes. Such mutants could be used to elucidate the mechanisms of invasion, survival, and persistence of the pathogenic mycobacteria as well as to promote the understanding of mycobacterial metabolism and to lead to the development of new drugs to fight mycobacterial diseases.

Conventional strategies to generate mutants of slow-growing mycobacteria are inadequate. The tendency of mycobacteria to clump because of their waxy surfaces makes it difficult to generate colonies arising from a single cell and, thus, limits the usefulness of chemical and physical mutagens. In addition, efforts aimed at performing allelic exchanges to generate gene replacements with selectable marker genes have thus far been unsuccessful (1, 22). Moreover, attempts to introduce homologous, linear DNA fragments into BCG and *M. tuberculosis* revealed that the homologous fragments were incorporated preferentially via illegitimate recombination at random chromosomal sites rather than at the homologous site. Although this approach generated auxotrophic mutants, the frequency of insertion is too low to develop useful libraries of mutants (22).

Transposon mutagenesis systems offer an efficient means of generating libraries of insertion mutants in bacteria (2, 23). Specialized transposons also enable the study of transcriptional or translational regulation (40) and the identification of secreted proteins (30). An insertional mutagenesis strategy that includes appropriate selectable marker genes could overcome the limitation caused by clumping of mycobacteria. Numerous transposons and insertion elements have been described for the mycobacteria (reviewed in reference 33). Recently, a kanamycin-resistant derivative of Tn*610* was shown to insert at random in the chromosome of *Mycobacterium smegmatis* (32), and with a temperature-sensitive plasmid (14), Tn*610* was used to generate auxotrophic mutants (15). Nevertheless, despite this success, transposon mutagenesis in the *M.*

* Corresponding author. Mailing address: Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-2888. Fax: (718) 518-0366. Electronic mail address: jacobs@aecom.yu.edu.

† Present address: Department of Molecular Genetics, Central Veterinary Laboratory, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom.

‡ Present address: Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305.

TABLE 1. Bacterial strains, plasmids, and transposable elements

Strain, plasmid, insertion element, or transposon	Genotype or description	Reference and/or source
E. coli K-12 strains		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17 glnV thi-1* λ$^-$ *gyrA96 relA1* Δ(*lacZYA-argF*)U169	16; GIBCO/BRL
CV512	*leuA371*	41; *E. coli* Stock Center
CV514	*leuB401*	41; *E. coli* Stock Center
CV522	*leuC222*	41; *E. coli* Stock Center
CV524	*leuD211*	41; *E. coli* Stock Center
M. bovis BCG strains		
M. bovis BCG	Pasteur strain	Statens Seruminstitut
mc^{2}789	*sbpA2*::Tn*5367*	This study
mc^{2}796	*zzz-1*::Tn*5366*	This study
mc^{2}797	*leuD1*::Tn*5367*	This study
mc^{2}798	*leuD2*::Tn*5366*	This study
mc^{2}826	*zzz-2*::Tn*5367*	This study
mc^{2}827	*zzz-3*::Tn*5367*	This study
mc^{2}828	*zzz-4*::Tn*5367*	This study
mc^{2}829	*zzz-5*::Tn*5368*	This study
mc^{2}830	*zzz-6*::Tn*5368*	This study
mc^{2}831	*zzz-7*::Tn*5368*	This study
Plasmids		
Bluescript II KS$^{+/-}$	pUC derivative, Amp^r	Stratagene
pMV261	Multicopy, extrachromosomal mycobacterial expression vector	42
pMV361	Single-copy, L5 integration-proficient mycobacterial expression vector	42
pYUB8	pBR322 derivative containing *oriE*, *aph*, and *tet* genes	22
pYUB53	pYUB8 derivative containing *oriE*, *oriM*, *aph*, and *tet* genes	22
pYUB285	Δ*oriM*, contains *oriE* and *tet* genes and Tn*5367*	This study
pYUB297	Δ*oriM*, contains *oriE* and *tet* genes and Tn*5368*	This study
pYUB305	Δ*oriM*, contains *oriE* and *tet* genes and Tn*5369*	This study
pYUB312	Δ*oriM*, contains *oriE*, *aph*, and *tet* genes	This study
pYUB419	pMV261::*leuCD*	This study
pYUB425	pMV361::*leuCD*	This study
Insertion element or transposon		
IS*1096*	*M. smegmatis* insertion element	7
Tn*5366*	IS*1096* derivative containing *aph* gene	This study
Tn*5367*	IS*1096* derivative containing *aph* gene	This study
Tn*5368*	IS*1096* derivative containing *aph* gene	This study
Tn*5369*	IS*1096* derivative containing *aph* gene	This study

tuberculosis complex has not yet been reported. Following the discovery of the insertion element IS*1096* in *M. smegmatis* (7), we have investigated its potential as a transposon for insertional mutagenesis of mycobacteria.

In this report, we describe the construction of transposons from the insertion element IS*1096*, the generation of a library of insertion mutants in BCG, the isolation and characterization of transposon-induced auxotrophic mutations, and their growth capabilities in vivo.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used and relevant characteristics are listed in Table 1. *Escherichia coli* strains were grown in L broth and when transformed with plasmids were grown in media containing either ampicillin (100 μg/ml) or kanamycin (50 μg/ml) as described previously (29). Lyophilized *M. bovis* BCG was cultured in 10 ml of M-ADC-TW broth (20) for 7 days and stored at −70°C in seed lots. Inoculation for the first passage was at 1/100 into roller bottles, and cells were grown for 7 to 10 days before passage or use. Cells were passaged no more than six times. Following transformations, cells were plated on Middlebrook 7H10 agar containing ADC enrichment (basal 7H10 medium) and 20 μg of kanamycin per ml or on complex 7H10 medium containing ADC enrichment, 0.5 or 1% Casamino Acids, tryptophan (20 μg/ml), and kanamycin (20 μg/ml).

Plasmid constructions. Plasmids and their features are described in Table 1. Transformation of *E. coli* was by electroporation (20) or the $CaCl_2$ procedure using pretreated competent cells (Bethesda Research Laboratories). The plasmids used in the transposition experiments were derived from modification of pMV261 (42) and then insertion of the transposon. In the first step, the *Not*I-*Pst*I fragment of pMV261 was replaced with the *tet*^{r} gene obtained by PCR from pBR322. This generated pYUB272, which consists of origins of replication in *E. coli* and mycobacteria (*oriM*) and adjacent *Eco*RI and *Hin*dIII sites. IS*1096*, flanked by *lacZ* sequences (100 to 200 bp on each side), had been cloned in both orientations into the multicloning site of pGEM7Zf$^+$ (Promega) (7) to create plasmids pYUB234 and pYUB235, enabling excision of the insertion element with *Eco*RI and *Hin*dIII.

Transposons were derived by inserting an aminoglycoside phophotransferase (*aph*) gene from Tn*903* into IS*1096*. The *aph* gene was PCR amplified from pKD348 (generously supplied by Keith Derbyshire) to include a *trp* transcriptional terminator (6) at the 3′ end. Plasmids pYUB297 and pYUB305 contain the transposons Tn*5368* and Tn*5369*. These transposons were assembled on pYUB234 by insertion of the *aph* gene into the *Sal*I and *Nco*I sites, respectively, and were cloned into pYUB272 with *Eco*RI and *Hin*dIII. Tn*5366* and Tn*5367*, having an *aph* gene in the *Mlu*I site (in opposite orientations), were created after, rather than before, ligation of the IS*1096* element to pYUB272. The transposon delivery plasmids derived from these constructions are pYUB284 and pYUB285. Insertion of the *aph* gene into the *Mlu* site necessitated a partial digestion since there is also an *Mlu*I site in pYUB272. The construct obtained from *aph* insertion into this second site provided a plasmid having the *aph* gene outside the IS element (pYUB312), which could then be used as a control to monitor any illegitimate recombination of plasmids.

Finally, to obtain a delivery plasmid unable to replicate in mycobacteria, the mycobacterial origin (*oriM*) was inactivated in each of the constructs by internal

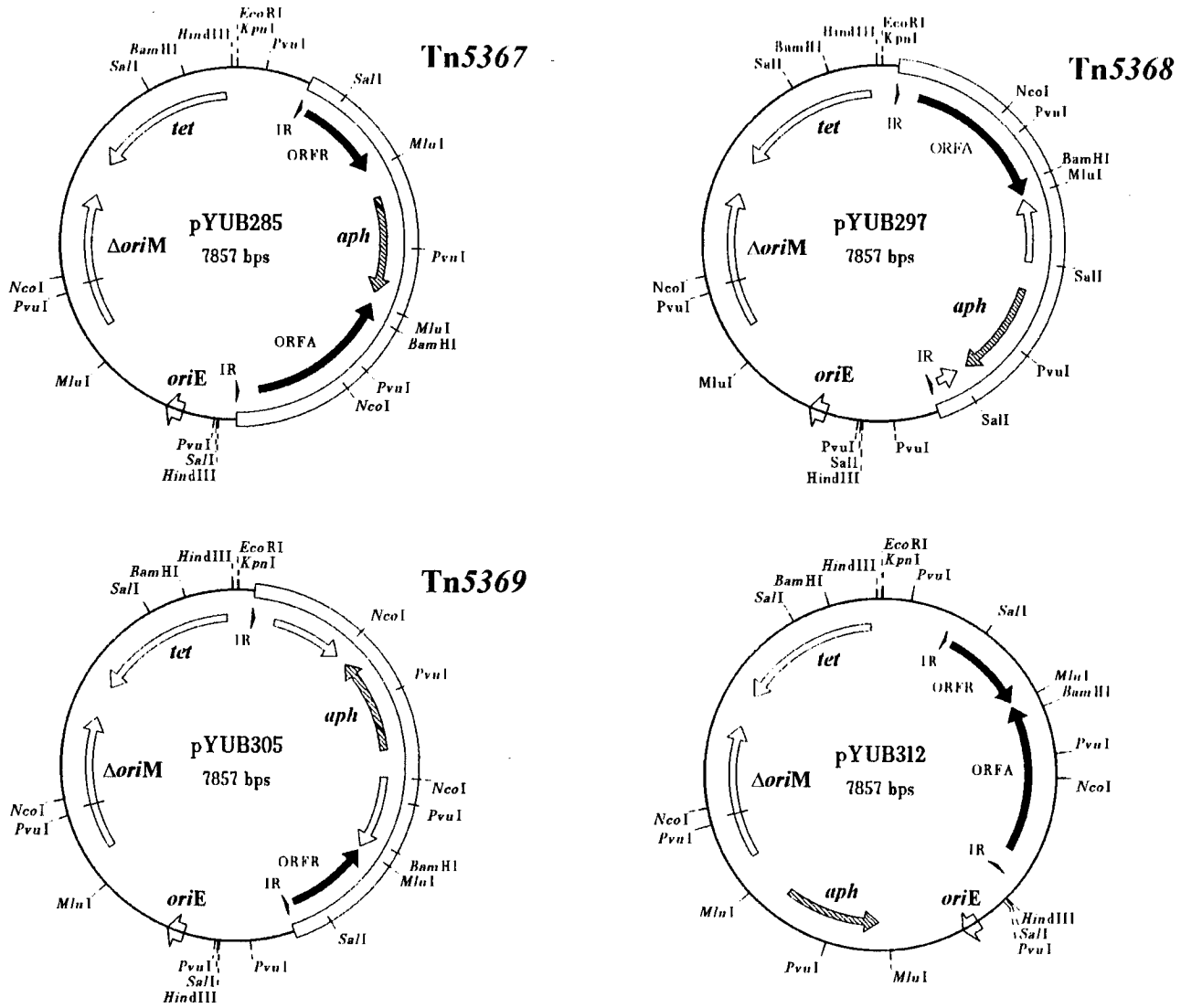

FIG. 1. IS*1096*-derived transposons and plasmid derivatives. The IS element has two major ORFs, namely, ORFA and ORFR (marked by filled arrows), corresponding to the genes *tnpA* and *tnpR* (7), respectively. These genes are bounded by inverted repeats (IRs). Transposons were constructed by inserting an *aph* gene (shaded arrow), between the ORFs, into the *Mlu*I site (Tn*5366* and Tn*5367*), into a *Sal*I site within ORFR (Tn*5368*), or in an *Nco*I site within ORFA (Tn*5369*). All plasmids contain an *aph* gene for kanamycin resistance, which constitutes part of the transposons in pYUB285, pYUB297, and pYUB305. They also have a disabled *oriM* (represented by a single arrow) which contains a 556-bp deletion, marked by a bar. pYUB312 contains the *aph* gene outside the IS element, so transformants will be seen only if integration of the plasmid has occurred. Plasmid pYUB284 containing Tn*5366* is not shown.

deletions with *Dra*III, which removed 556 bp of DNA and a significant part of an open reading frame (ORF) in the origin (25, 38, 48). The *Dra*III deletion internal to the *oriM* was performed by digestion and then by T4 DNA polymerase treatment. The plasmid constructs are shown in Fig. 1.

Transformation of *M. bovis* BCG with transposon delivery constructs. Plasmids were prepared from *E. coli* by both alkaline lysis-sodium dodecyl sulfate (29) and column (Quiagen) methods. BCG cells, after being washed in 10% glycerol, were transformed by electroporation as described previously (20), after which 4 ml of a medium (M-ADC-TW broth containing 0.5% Casamino Acids and 20 μg of tryptophan per ml [complex medium]) was added; this was followed by incubation overnight at 30 or 37°C and plating on complex 7H10 agar containing kanamycin (20 μg/ml). Colonies were counted after 3 weeks of incubation at 37°C. In addition to the transposon delivery plasmids, three additional plasmids were used as controls. The efficiency of transformation was determined with an *oriM*-containing vector, either pMV261 or pYUB53, and any illegitimate integration was monitored by use of either one of two plasmids, namely pYUB312 (with the *oriM* deletion but the *aph* gene outside the insertion element) or pYUB8 (which has no insertion element nor mycobacterial origin but has an *aph* gene). A control of cells alone having no plasmid was also electroporated and plated in fashion identical to that of the samples to estimate the level of spontaneous resistance to kanamycin (or background).

Southern blotting and hybridization. Single BCG colonies were grown in 10 ml of M-ADC-TW medium containing kanamycin and expanded 1:50 for preparation of DNA. Whole DNA was prepared from 50-ml cultures by a 10-fold scale-up of the cetyl-trimethylammonium bromide method (46). DNA concentration was estimated by agarose gel electrophoresis, and approximately 2 μg was digested with restriction enzyme and run on a 0.7 or 1% agarose gel at 40 V overnight. The DNA was transferred to a nylon membrane (ICN), and hybridization was performed with plasmid pYUB285 labelled with [α-^{32}P]dCTP as a probe (8). The sizes of fragments hybridizing on *Kpn*I and *Bam*HI Southern blots were estimated by use of the mobilities of standard DNA markers run on each gel.

Isolation of integrated transposons and sequence analyses. *Kpn*I-digested fragments containing the integrated transposon were cut from an agarose gel and cloned into Bluescript II KS$^+$ (Promega) by using kanamycin selection. Outward primers (646 5′-CCTCGATCACAGCGCGAA-3′ and 647 5′-CCATCATCG GAAGACCTC-3′) based on the sequence of IS*1096* were used with the Sequenase version 2.0 and the Longranger (United States Biochemical) acrylamide gel reagents for conventional sequencing. Alternatively, sequences were generated on an Applied Biosystems DNA sequencer. Sequence alignments were generated with the TfastA program in the Genetics Computer Group—Wisconsin package.

Auxotroph isolation, characterizations, and reversion analyses. Colonies ob-

tained on pYUB284 (Tn*5366*), pYUB285 (Tn*5367*), and pYUB297 (Tn*5368*) transformation were picked with wooden sticks into 96-well plates containing complex 7H9 medium. They were grown and washed twice in basal (M-ADC-TW) medium before being replicated, by use of a 96-prong template (Dankar, Boston, Mass.), onto agar plates with and without amino acid supplement. Promising candidates were streaked again from the original 96-well plate to establish their auxotrophy. Auxanographic analysis was performed on washed cultures as described previously (22) with amino acid supplements utilized for analysis of *E. coli* (9). Reversion analyses were performed by growing the three auxotrophic mutants mc^{2}789, mc^{2}797, and mc^{2}798 in M-ADC-TW broth containing 0.5% Casamino Acids and kanamycin to the early stationary phase. Cells were sonicated briefly and diluted in M-ADC-TW broth, and samples were plated on basal and complex media.

Complementation of the leucine auxotrophs with the *E. coli leuCD* genes. We cloned the *E. coli leuCD* by PCR cloning into the mycobacterial expression vectors pMV261 and pMV361 (42). The carboxy terminus of the *leuB* gene was cloned in frame with the *hsp60* amino terminus by engineering a unique *Pst*I site at position 3557 (sequence accession number D10483) with the oligonucleotide 5′ TATAT-CTGCAG-TGCGTTACAGCCTGGAT. A unique *Sal*I site was engineered 35 bp downstream from the stop codon of the *leuD* gene by use of the oligonucleotide 5′ TATAT-GTCGAC-GATGACGTGGACGATAGC. The oligonucleotides were mixed with DN5α chromosomal DNA and amplified with Vent Polymerase (New England Biolabs, Waltham, Mass.) by use of a 2-min denaturation at 94°C (one cycle), and a 1-min denaturation at 94°C, a 1-min annealing at 60°C, and a 2.5-min polymerization at 72°C (30 cycles). The PCR-generated fragment was the expected size of 2.3 kb and was cleaved with *Pst*I and *Sal*I prior to cloning into pMV261 to generate pYUB419. The *Pst*I-*Sal*I fragment from pYUB419 containing the *leuC* and *leuD* genes was cloned into pMV361 to generate pYUB425. Both pYUB419 and pYUB425 were electroporated into mc^{2}797 and mc^{2}798, and the transformed cells were plated on 7H10 minimal agar containing kanamycin or 7H10 agar containing leucine and kanamycin. For both plasmids, the numbers of transformants were equal on both media.

Growth kinetics of BCG auxotrophic mutants in mice. Cultures of the parent BCG and the BCG auxotrophs were grown in 50 ml of M-ADC-TW broth supplemented with 0.5% Casamino Acids in 490-cm^2 roller bottles at 37°C. When the cells reached densities of approximately 5×10^7 CFU/ml, the cells were frozen in 1-ml aliquots. After the cells were frozen, titers were determined from a sample. The cells were resuspended in phosphate-buffered saline (PBS) to a final volume of 5×10^6 CFU/ml. Immediately prior to injection, the cells were sonicated briefly to break up clumps with a Braun-U sonicator at an output of 70 W and with a microprobe for 7 s. For each auxotroph, a nonauxotrophic insertion mutant, and the parent BCG, 25 mice (C57BL/6 male mice, 6 to 8 weeks old) were infected intravenously by retro-orbital puncture with 10^6 CFU/0.2 ml. At days 1, 7, 14, 21, and 45, five mice were sacrificed, and their spleens and lungs were removed. Each spleen was added to 4.5 ml of PBS or each set of lungs was added to 9 ml of PBS and homogenized in a Glass/Col homogenizer. The homogenate was serially diluted, and 0.1-ml samples were plated onto three different media, namely, (i) complex 7H10, (ii) complex 7H10 with kanamycin, and (iii) basal 7H10 agar, to determine the numbers of viable CFU. Colony counts on each complex medium were equivalent, while there was no growth of the auxotrophs on basal 7H10 agar. The titers from each of five mice were averaged, and standard deviations were determined.

RESULTS

Genetic analysis of IS*1096*. IS*1096* represents an ideal candidate insertion element to be engineered into a transposon for mutagenesis since it transposes randomly in *M. smegmatis* and is not present in either BCG or *M. tuberculosis* (7). IS*1096* appears to have two genes (*tnpA* and *tnpR*), and the translated ORFs (ORFA and ORFR, respectively) were found to share only weak homologies with known transposases or resolvases. To determine whether one or both ORFs were required for transposition, we cloned a kanamycin resistance (*aph*) gene into each of the two ORFs as well as between them to create three separate transposon constructs (Fig. 1). Tn*5366* is identical to Tn*5367* but has the *aph* gene in the reverse orientation. The transposons were cloned into a plasmid capable of replicating in *E. coli* but unable to replicate in mycobacteria. Tn*5368* has the *aph* gene inserted into *tnpR*; Tn*5369* has the insertion in *tnpA*; and in Tn*5367*, the *aph* gene does not disturb either ORF (Fig. 1).

To test for transposition, the plasmids containing the various transposon constructs were electroporated into BCG and plated on complex medium containing kanamycin. In five separate experiments using independent BCG cultures, between 10^2 and 10^3 kanamycin-resistant colonies were obtained per μg of plasmid for the transposons Tn*5366*, Tn*5367*, and Tn*5368*. The numbers of kanamycin-resistant colonies following electroporation with IS*1096*-derived transposons were consistently 5- to 10-fold above the numbers of spontaneous kanamycin-resistant colonies, suggesting that transposition had occurred. In contrast, in the same experiments where transposition was observed, significant numbers of kanamycin-resistant colonies were not obtained following electroporation of one or more of the control plasmids, i.e., either pYUB8 (22), which has the *aph* gene and no *oriM*, or pYUB312, in which the *aph* gene was cloned outside of the IS*1096*. In addition, in two experiments, transformation with pYUB305 (containing Tn*5369* in which *aph* is inserted in *tnpA*) yielded numbers of colonies at background levels. It must be noted that significant numbers of transpositional transformants were found in only 5 of more than 20 separate experiments, suggesting that improvements in the delivery systems are required. Nevertheless, these results established that *tnpA* is essential for transposition and likely to encode the transposase and that *tnpR* is dispensable.

Southern analyses of IS*1096*-derived transposons in BCG. To confirm that the kanamycin resistance resulted from the transposition, DNA was isolated from 19 random colonies arising from transformation with plasmids carrying Tn*5367* and Tn*5368*. Southern analysis was performed with three different restriction enzymes and plasmid pYUB285 as a probe. The results for six of such clones, strains mc^{2}826 through mc^{2}831, are shown in Fig. 2. Since no *Kpn*I site exists in the transposon, one fragment hybridizes in each clone (Fig. 2A). To verify that insertion was random, *Bam*HI, which cuts once within the transposon, was also used. When probed with plasmid, two bands corresponding to fragments obtained from insertion of the transposon into the chromosome are visible (Fig. 2B). To determine whether any plasmid sequences which might reveal the presence of cointegrate intermediates or an illegitimate recombination event were present, the chromosomal DNAs were digested with *Pvu*I and hybridized (Fig. 2C). Digestion of the delivery plasmids with *Pvu*I yields several fragments, including one internal to the transposon (see Fig. 1). Thus, if transposition had occurred by simple insertion rather than replication and cointegrate formation, the expected bands from the transposon-containing clones should include this internal fragment and two additional bands resulting from the insertion of the transposon into the chromosome. Three bands were seen with Tn*5367* insertions in the mutants mc^{2}826, mc^{2}827, and mc^{2}828; each had the expected internal band as well as two unique bands of differing sizes. Results for the clones transformed with Tn*5368* showed that two of the clones (mc^{2}829 and mc^{2}831) gave three bands (mc^{2}831, a possible doublet), but the third (mc^{2}830) displayed only one additional band along with the expected internal fragment, suggesting that deletion of part of *tnpA* must have occurred. The conclusion to be drawn from these experiments is that the absence of extra bands excludes the possibility of plasmid integration. All 19 clones showed unique patterns with this combination of enzymes, which suggests that the IS*1096*-derived transposons inserted in a relatively random fashion in BCG.

Sequence analysis of insertion junctions. Further proof of transposition was obtained by sequencing across the junctions of insertion of the transposons with primers 646 and 647, reading outwards from the IS*1096* element. When the six clones mc^{2}826 through mc^{2}831 described above were examined, an 8-bp direct repeat to each side of the inverted repeats was found. The sequences of these duplicated target sites, including the junctional sequences of the three auxotrophs mc^{2}789, mc^{2}797, and mc^{2}798 described below, are given in Fig.

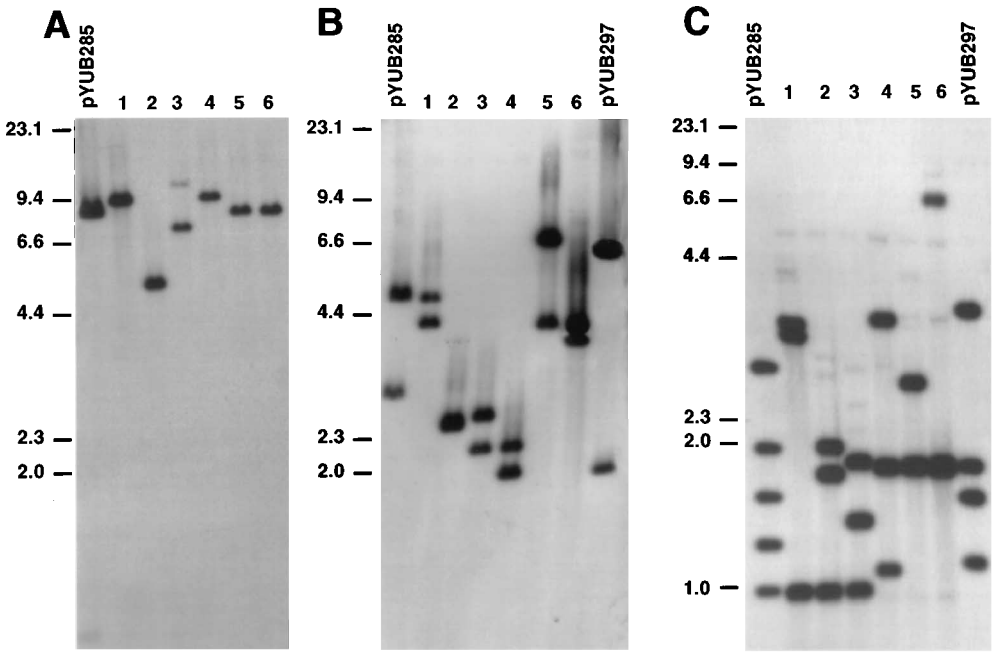

FIG. 2. Southern blots of six representative BCG clones resulting from transposition with Tn*5367* (lanes 1 to 3) and Tn*5368* (lanes 4 to 6). Lanes: 1, mc^{2}826; 2, mc^{2}827; 3, mc^{2}828; 4, mc^{2}829; 5, mc^{2}830; 6, mc^{2}831. DNA was digested with *Kpn*I (A), *Bam*HI (B), and *Pvu*I (C). Blots were probed with pYUB285, and mobilities are indicated by markers in kilobase pairs. Note that lane 3 in panel A is a partial digest.

3. There is a weak consensus at the insertion junctions of XXX(A/T)(A/T)X(G/C)X, where A or T always stands at positions 4 and 5, and it is also noted that there is a preference in the target site for an AT-rich center and GC-rich ends. When 50-bp sequences of flanking DNA were compared (data not shown), no similarities between clones could be discerned.

Isolation and characterization of auxotrophic mutants of BCG. To establish the potential usefulness of the IS*1096*-derived transposons for the *M. tuberculosis* complex, we sought to isolate defined auxotrophic mutations. Nine hundred twenty-three kanamycin-resistant colonies were arrayed in 96-well plates, grown, washed, and tested for auxotrophy by patching onto plates lacking amino acid supplements. Promising candidates were tested by auxanography, and three auxotrophs were found, one for methionine (mc^{2}789) and two for leucine (mc^{2}797 and mc^{2}798). The two leucine auxotrophs were generated in separate experiments with different transposons and thus were not siblings. The growth of all three auxotrophs could be supported in liquid or solid basal media by the addition of the relevant amino acid. Reversion analysis demonstrated that all three auxotrophic mutations reverted at frequencies between 1×10^{-7} to 2×10^{-7} (Table 2).

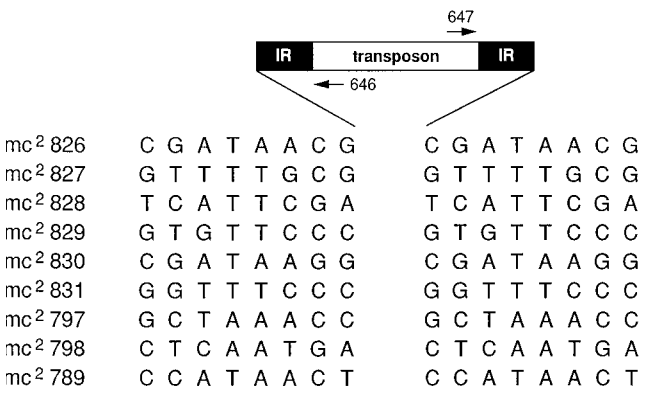

FIG. 3. Duplicated DNA sequences found on both sides of the transposon following transposition. An 8-bp direct repeat is present in each clone, presumably from duplication of target DNA. Sequences are derived from mc^{2}826 to mc^{2}831 shown in Fig. 2 and the auxotrophic strains mc^{2}797, mc^{2}798, and mc^{2}789.

Molecular characterizations of the auxotrophic mutations. To test whether the transposon insertions caused the auxotrophic phenotypes, the transposon insertions were characterized in greater detail. Chromosomal DNAs isolated from the auxotrophic mutants (mc^{2}789, mc^{2}797, and mc^{2}798) and one uncharacterized prototrophic insertion mutant (mc^{2}796) were compared by Southern blot analysis using *Kpn*I and *Bam*HI and a transposon delivery plasmid as a probe. As can be seen in Fig. 4, the two leucine auxotrophs show identically sized fragments of 12 kb with *Kpn*I, implying that the two transpositions are close to each other. They do, however, give rise to different-sized *Bam*HI fragments on blotting, indicating that the mutants are not identical and that there is at least one *Bam*HI site between them. Each auxotrophic mutant had a single transposon insertion.

Sequence analysis of auxotrophic mutants. The determination of the sequence flanking the insertion in methionine auxotroph mc^{2}789 revealed 35% identity and 57% similarity for 105 amino acids with a sulfate-binding protein of a cyanobacterium, *Anacystis nidulans* (Fig. 5A), and 19% identity and 45% similarity to the *sbpA* gene of *Salmonella typhimurium* (data not shown). The sequence of the DNA surrounding the transposon insertions was determined for all three auxotrophs. Transposon insertions in the two leucine auxotrophs were found to be 219 bp apart, with one *Bam*HI site between. Homology searches of the database revealed that the transposons had inserted into a gene whose ORF has 43% identity and 61% similarity for 191 amino acids with the *leuD* gene of

TABLE 2. Reversion and complementation analysis of *M. bovis* BCG auxotrophs

Strain	Genotype	Phenotype	Reversion frequency	Complementation	
				pYUB419	pYUB425
BCG-Pasteur	Wild type	Prototroph	NA[a]	NA	NA
mc^{2}789	*sbpA1*::Tn*5367*	Methionine auxotroph	1.0×10^{-7}	No	No
mc^{2}797	*leuD1*::Tn*5367*	Leucine auxotroph	1.6×10^{-7}	Yes	Yes
mc^{2}798	*leuD2*::Tn*5366*	Leucine auxotroph	2.0×10^{-7}	Yes	Yes

[a] NA, not applicable.

E. coli (Fig. 5B). The clone mc^{2}797 has a transposon insertion at a position near the start of the gene (*leuD1*::Tn*5367*), whereas clone mc^{2}798 (*leuD2*::Tn*5366*) is further downstream (Fig. 5B).

Complementation of the leucine auxotrophs by *E. coli leuC* and *leuD* genes. For further confirmation that the transposon insertions had generated mutations in the *leuD* gene, we complemented the clones mc^{2}797 and mc^{2}798 with the well-characterized *E. coli* genes. We chose to use the combination of the *leuC* and *leuD* genes because it is known that the LeuC and LeuD proteins interact in *E. coli*, forming the isopropylmalate dehydratase, and it is not known whether a hybrid BCG LeuC-*E. coli* LeuD molecule would be functional. The genes were cloned by PCR and *E. coli* chromosomal DNA into the episomal mycobacterial expression vectors pMV261 and pMV361 to form pYUB419 and pYUB425, respectively. Since the BCG *hsp60* promoter is active in *E. coli* (42), transformation of pYUB419 and pYUB425 into *E. coli* demonstrated that both plasmids could complement the *E. coli leuC* (strain CV522) and *leuD* (CV524) mutations but failed to complement either the *E. coli leuA* (CV512) or *leuB* (CV514) auxotrophic mutations as expected. In addition, both the episomal construct pYUB419 and the integrating construct pYUB425 complemented the leucine auxotrophies of both mc^{2}797 and mc^{2}798 (Table 2), thus supporting that the transposon insertions had caused auxotrophic mutations by insertionally inactivating the BCG homolog of the *leuD* gene.

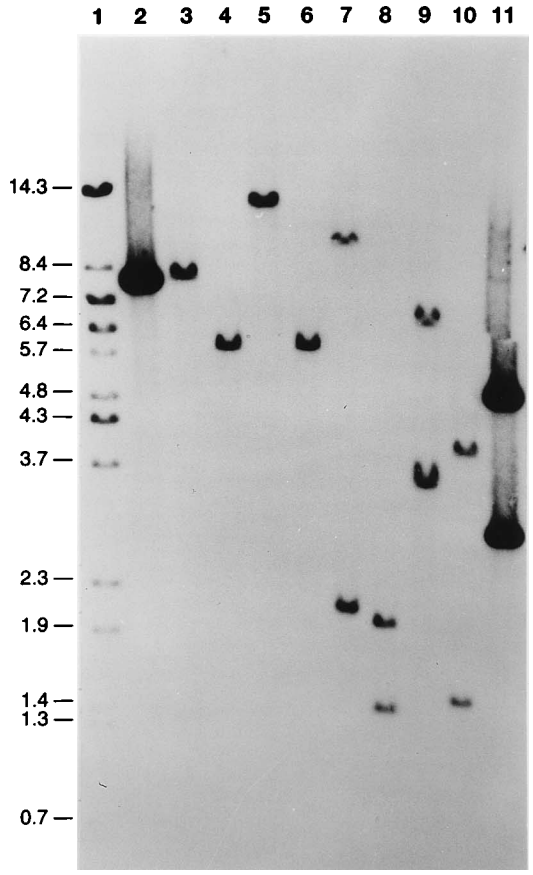

FIG. 4. Southern analysis of auxotrophic mutants of BCG. Chromosomal DNAs were isolated from mc^{2}789 (lanes 3 and 7), mc^{2}797 (lanes 4 and 8), mc^{2}796 (lanes 5 and 9), and mc^{2}798 (lanes 6 and 10), digested with either *Kpn*I (lanes 2 to 6) or *Bam*HI (lanes 7 to 11), electrophoresed on agarose gels, and hybridized with radiolabelled pYUB285. Lane 1 contains the molecular size standards (10^3 bp), and lanes 2 and 11 contain pYUB285.

Growth characteristics of the leucine and methionine auxotrophs in mice. The mouse serves as a useful model for assaying the virulence of *M. tuberculosis* complex strains (35). The growth of the transposon-induced auxotrophs in C57BL/6 mice were compared with that of wild-type BCG following intravenous infection with 10^6 CFU. The methionine auxotroph showed growth characteristics in the lungs and spleen similar to those of the BCG parent (Fig. 6), with an initial decrease, a growth phase, and slow decline. In striking contrast, both leucine auxotrophs showed no evidence for growth in vivo and were cleared from both lungs and spleen within 7 weeks.

DISCUSSION

The characterization of auxotrophic mutants of bacteria has contributed fundamentally to our understanding of the pathways of primary bacterial metabolism. Furthermore, the study of auxotrophs of pathogenic bacteria has made a special impact by allowing the isolation of strains which are avirulent and yet immunogenic. One of the highest priorities in tuberculosis research is to develop new vaccines from attenuated strains. These vaccines could, in principle, be created from virulent *M. tuberculosis* or *M. bovis* or from the already attenuated *M. bovis* BCG strain by genetic insertion of genes encoding immunostimulatory products. A more severely attenuated BCG strain is also currently being sought for the immunization of human immunodeficiency virus-infected individuals.

The auxotrophs described in this study were obtained by transposon mutagenesis using transposons derived from the *M. smegmatis* insertion element IS*1096*. We have shown that these transposons are active in BCG, and they insert relatively randomly, creating an 8-bp duplication in the target DNA, and show no evidence of cointegrate formation, suggesting that they transpose by a simple "cut-and-paste" method of transposition. For IS*1096*-derived transposons to transpose, only one of the ORFs encoded by *tnpA* appears to be required. Further evidence supporting this conclusion comes from the recent isolation of insertion elements IS*1001* from *Bordetella pertussis* (45), IS*AE1* from *Alcaligenes eutrophus* (24), and IS*31831* from *Corynebacterium glutamicum* (47), all of which

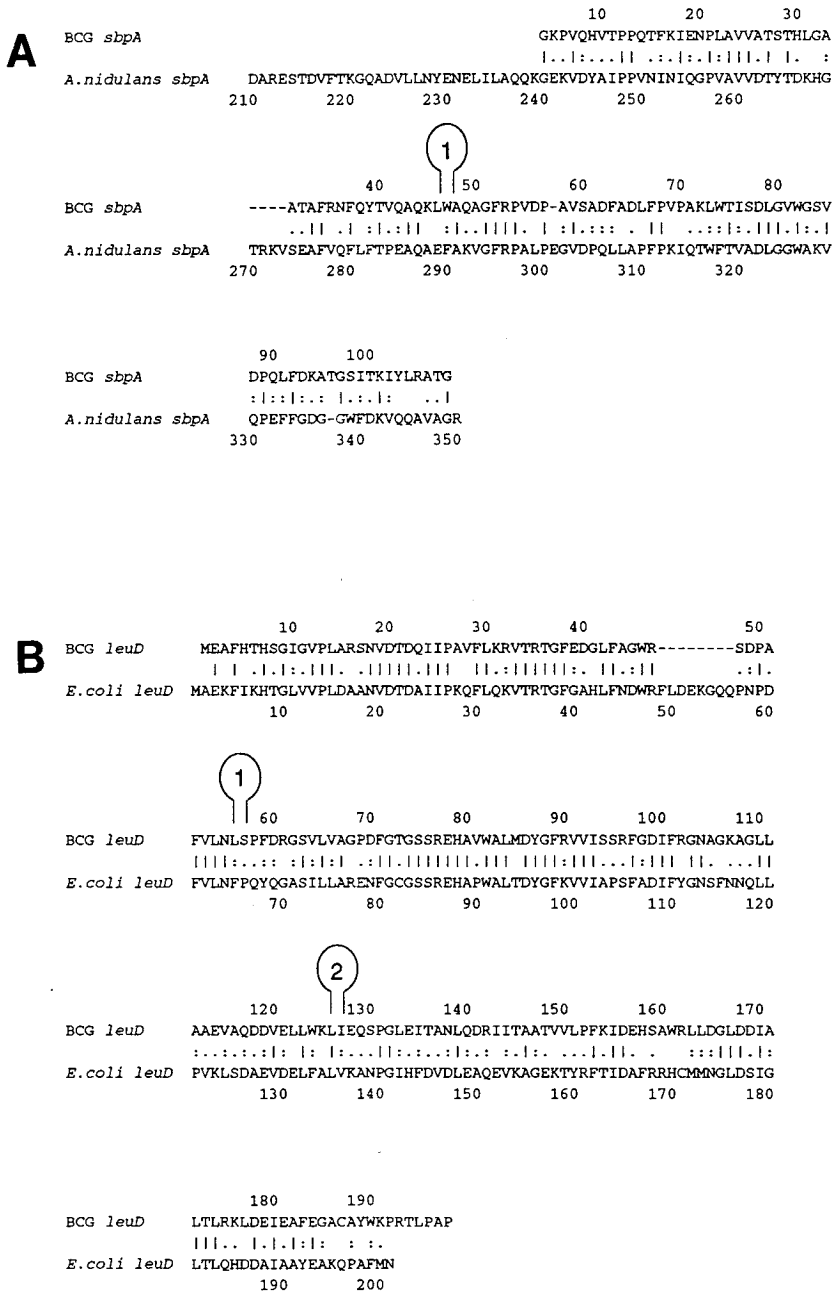

FIG. 5. Analysis of the genes containing the transposon insertions. Sequences flanking the transposon insertions in the auxotrophic BCG mutants aligned with homologs. (A) Homology alignment of the *sbpA* gene of BCG with the *sbpA* gene of *A. nidulans*. The lollipop containing number 1 shows the site of insertion of the transposon. (B) The homology alignment of the *leuD* gene of BCG with the *E. coli leuD* gene. The numbers in the lollipops show the sites of insertion for *leuD1* and *leuD2*, respectively.

have a single ORF which shares homology with ORFA of IS*1096*, suggesting that mini-transposon derivatives could be readily designed. Furthermore, the auxotrophs derived from transposon insertion have low reversion frequencies and appear to be stable after animal passage. Thus, transposons derived from IS*1096* coupled to an efficient delivery system have great potential for general use in insertional mutagenesis in mycobacteria.

In our experience, the use of electroporation to transform BCG with a nonreplicating plasmid is not sufficiently reliable as a delivery method. We and others (1, 12) have noted inconsistencies in transformation frequencies. A more suitable approach would be to use conditionally replicating plasmids, conjugative plasmids, or phage. Guilhot et al. have recently used an efficient plasmid-based system to generate a library of transposon insertions in *M. smegmatis* (15). However, because of the narrow temperature growth range of *M. tuberculosis* and *M. bovis*, this system is not readily applicable to the slow-growing mycobacteria. Although conjugation has been reported between *E. coli* and *M. smegmatis*, no conjugation with slow-growing mycobacteria has yet been achieved (13). We have recently been successful in constructing shuttle phasmid deriv-

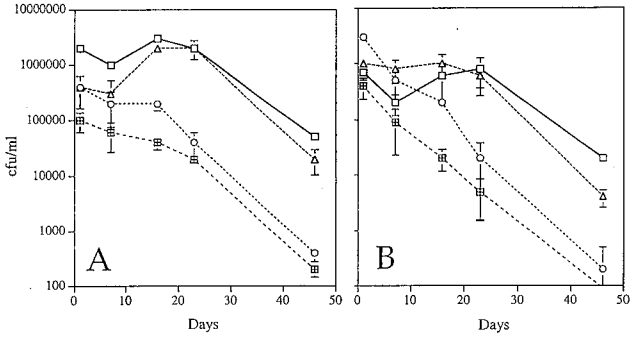

FIG. 6. Growth kinetics of BCG auxotrophic mutants in mice. Mean numbers of CFU present in the spleen (A) or lungs (B) of mice following infection with the BCG and BCG auxotrophs are shown. Symbols: □, BCG; △, mc^{2}789 *met-2*::Tn*5367*; ⊞, mc^{2}797 *leuD1*::Tn*5367*; ○, mc^{2}798 *leuD2*::Tn*5366*.

atives of mycobacteriophage L5 (44). The combination of the knowledge of the entire sequence of L5 (18) and shuttle phasmid technologies (21) should offer a powerful means of delivering transposons efficiently to mycobacteria.

Three auxotrophs were obtained, one requiring methionine for growth and two requiring leucine. The transposon in the methionine auxotroph had inserted into an ORF with high homology to a family of sulfate-binding proteins. The sulfate uptake pathway and metabolism into cystine have been studied extensively in *S. typhimurium*, and the three-dimensional structure of the sulfate-binding protein has been determined (36). The sulfate-binding protein binds to sulfate, is taken up by an ATP transporter, and enters the cell through a pair of channel-forming proteins. Since methionine is a sulfur-containing amino acid, it could be supposed that an insertion in sulfate-binding protein has affected methionine synthesis in the BCG auxotroph. This observation requires further investigation since other bacteria such as *S. typhimurium* obtain methionine from cysteine and not directly from sulfate. Furthermore, cystein auxotrophy in *S. typhimurium* does not map to the sulfate-binding protein gene. Interestingly, *Streptomyces* spp. and fungi have a somewhat different sulfate utilization pathway and are capable of converting methionine to cysteine (27). Whether mycobacteria in general, or BCG specifically, have further differences in their sulfur-containing amino acid biosynthesis pathways is under investigation.

Two of the auxotrophs have a requirement for leucine which is caused by transposon insertions proximal to each other in an ORF. Sequence analysis and homology searches indicated that this gene encodes the BCG homolog of the product of the *leuD* gene in *E. coli*. Complementation of the auxotrophs was achieved with a DNA fragment from *E. coli* encoding the *leuC* and *leuD* genes, placed under the control of the *hsp60* promoter. The gene product of the *leuD* gene in *E. coli* is one subunit of the heterodimer isopropylmalate dehydratase. The other subunit is provided by the product of the *leuC* gene positioned upstream in the same operon. We have not yet determined whether the *E. coli leuD* gene alone will complement the auxotrophy, but these results show that the *E. coli leuD* gene product is functional in BCG. Further analysis of the leucine pathway in BCG is of interest since a complementing *leuD* gene could be used as a selectable marker for further genetic manipulations of the leucine auxotrophs. It would also be of interest to determine whether the leucine pathway genes are assembled in a single operon and subject to transcriptional attenuation as seen in *E. coli* (41).

Members of the *M. tuberculosis* complex multiply intracellularly in cell culture as well as in animal models. BCG also gains access to the intracellular environment but undergoes limited replication. When BCG is introduced via the intravenous route in C57BL/6 mice, after an initial increase in number, it is steadily cleared. We found that after an initial lag in the number of CFU, the methionine auxotroph behaved similarly to the parental BCG in mice and was cleared at a similar rate. The leucine auxotrophs, however, were cleared rapidly. Our observations suggest that whereas methionine (or a substrate for methionine biosynthesis) is available to BCG intracellularly, leucine must be limiting at that site, preventing replication of the bacterium. Piez and Eagle reported the leucine level in the cytoplasm of the HeLa cell which is adequate for the bacterial requirement (37). Interestingly, we know of no leucine auxotrophic mutants of pathogenic bacteria that have been reported to be attenuated. Attenuation of intracellular bacterial pathogens is usually associated with purine, pyrimidine, and aromatic amino acid auxotrophies (10, 26, 31). BCG has not been observed free in the cytoplasm intracellularly, so having gained access to the inside of the cell, it may reside in a specialized phagosome where leucine is not available. Leucine and other auxotrophic mutants obtained in *M. tuberculosis* could be worthwhile probes of the intracellular trafficking of *M. tuberculosis* in host cells, which has been reported to gain access to the cytoplasm (34).

The marked difference in numbers of CFU between the leucine auxotroph and the parental BCG observed in mice after 2 weeks provides a host strain and complementing gene for the elegant in vivo expression technology system (28). Thus, the construction of genomic libraries in the appropriate complementing vector should be useful for the identification of genes of *M. tuberculosis* which are preferentially activated intracellularly in vivo as well as in cell culture. In addition, the combination of auxotroph and complementing gene also should provide a balanced lethal selection (8) in vivo to ensure that recombinant plasmids expressing foreign antigens are stably maintained in recombinant BCG vaccine strains when grown in mammalian hosts.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant Al26170.

We wish to thank Rupa Udani for growing cultures, Mary Avalee for DNA sequencing, and V. Balasubramanian for computer-drawing expertise.

REFERENCES

1. **Aldovini, A., R. N. Husson, and R. A. Young.** 1993. The *uraA* locus and homologous recombination in *Mycobacterium bovis* BCG. J. Bacteriol. **175:** 7282–7289.
2. **Berg, C. M., D. E. Berg, and E. A. Groisman.** 1989. Transposable elements and the genetic engineering of bacteria, p. 879–925. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
3. **Bloom, B. R., and P. E. M. Fine.** 1994. The BCG experience: implications for future vaccines against tuberculosis, p. 531–557. *In* B. R. Bloom, Tuberculosis, pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
4. **Bloom, B. R., and C. J. L. Murray.** 1992. Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.
5. **Centers for Disease Control.** 1993. Tuberculosis morbidity—United States, 1992. Morbid. Mortal. Weekly Rep. **42:**696–704.
6. **Christie, G. E., P. J. Farnham, and T. Platt.** 1981. Synthetic sites for transcription termination and a functional comparison with tryptophan operon termination sites *in vitro*. Proc. Natl. Acad. Sci. USA **78:**4180–4184.
7. **Cirillo, J. D., R. G. Barletta, B. R. Bloom, and W. R. Jacobs, Jr.** 1991. A novel transposon trap for mycobacteria: isolation and characterization of IS*1096*. J. Bacteriol. **173:**7772–7780.
8. **Curtiss, R., III.** 1990. Attenuated *Salmonella* strains as live vectors for the expression of foreign antigens, p. 161–188. *In* G. C. Woodrow and M. M.

Levine (ed.), New generation vaccines. Marcel Dekker, Inc., New York.
9. **Curtiss, R., III.** 1981. Gene transfer, p. 243–265. *In* P. Gerhart (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
10. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that can not survive within a macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
11. **Fine, P. E. F.** 1989. The BCG story: lessons from the past and implications for the future. Rev. Infect. Dis. **11**(Suppl. 2)**:**5353–5359.
12. **Garbe, T. R., J. Barathi, S. Barnini, Y. Zhang, and D. B. Young.** 1994. Transformation of mycobacterial species using hygromycin-resistance as a selectable marker. Microbiology **140:**133–138.
13. **Gormley, E. P., and J. Davies.** 1991. Transfer of plasmid RSP1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. J. Bacteriol. **173:**6705–6708.
14. **Guilhot, C., B. Gicquel, and C. Martin.** 1992. Temperature-sensitive mutants of the *Mycobacterium plasmid* pAL5000. FEMS Microbiol. Lett. **98:**181–186.
15. **Guilhot, C., I. Otal, I. van Rompaey, C. Martin, and B. Gicquel.** 1994. Efficient transposition in mycobacteria: construction of *M. smegmatis* insertion mutant libraries. J. Bacteriol. **176:**535–539.
16. **Hanahan, D.** 1983. Studies of transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
17. **Hatfull, G. F.** 1993. Genetic transformation of mycobacteria. Trends Microbiol. **1:**310–314.
18. **Hatfull, G. F., and Sarkis, G. J.** 1993. DNA sequence, structure, and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. Mol. Microbiol. **7:**407–417.
19. **Jacobs, W. R., Jr., and B. R. Bloom.** 1994. Molecular strategies for identifying virulence determinants of *Mycobacterium tuberculosis*, p. 253–268. *In* B. R. Bloom (ed.), Tuberculosis, pathogenesis, protection, and control. American Society for Microbiology Press, Washington, D.C.
20. **Jacobs, W. R., Jr., G. V. Kalpana, J. D. Cirillo, L. Pascopella, R. A. Udani, W. D. Jones, Jr., R. G. Barletta, and B. R. Bloom.** 1991. Genetic systems for the mycobacteria. Methods Enzomol. **204:**537–555.
21. **Jacobs, W. R., Jr., M. Tuckman, and B. R. Bloom.** 1987. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature (London) **327:**532–536.
22. **Kalpana, V. G., B. R. Bloom, and W. R. Jacobs, Jr.** 1991. Insertional mutagenesis and illegitimate recombination in mycobacteria. Proc. Natl. Acad. Sci. USA **88:**5433–5437.
23. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
24. **Kung, S. S., J. Chen, and W. Y. Chow.** 1992. Molecular and genetic characterization of an *Alcaligenes eutrophus* insertion element. J. Bacteriol. **174:** 8023–8029.
25. **Labidi, A., E. Mardis, B. A. Roe, and R. J. Wallace, Jr.** 1992. Cloning and DNA sequence of the *Mycobacterium fortuitum* var *fortuitum* plasmid pAL5000. Plasmid **27:**130–140.
26. **Leung, K. Y., and B. B. Finlay.** 1991. Intracellular replication is essential for virulence of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **88:**11470–11474.
27. **Lydiate, D. J., C. Mendez, H. M. Kieser, and D. A. Hopwood.** 1988. Mutation and cloning of clustered *Streptomyces* genes essential for sulphate metabolism. Mol. Gen. Genet. **211:**415–423.
28. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
31. **Marquis, H., H. G. A. Bouwer, D. J. Hinrichs, and D. A. Portnoy.** 1993. Intracytoplasmic growth and virulence of *Listeria monocytogenes* auxotrophic mutants. Infect. Immun. **61:**3756–3760.
32. **Martin, C., J. Timm, J. Rauzier, R. Gomez-Lus, J. Davies, and B. Gicquel.** 1990. Transposition of an antibiotic resistance element in mycobacteria. Nature (London) **345:**739–743.
33. **McAdam, R. A., C. Guilhot, and B. Gicquel.** 1994. Transposition in mycobacteria, p. 199–216. *In* B. R. Bloom, Tuberculosis, pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
34. **McDonough, K. A., V. Kress, and B. R. Bloom.** 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. Infect. Immun. **61:**2763–2773.
35. **Orme, I. M., and F. M. Collins.** 1994. Mouse model of tuberculosis, p. 113–134. *In* B. R. Bloom (ed.), Tuberculosis, pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
36. **Pflugrath, J. W., and F. A. Quiocho.** 1988. The 2 A resolution structure of the sulfate-binding protein involved in active transport in *Salmonella typhimurium*. J. Mol. Biol. **200:**163–180.
37. **Piez, K. A., and H. Eagle.** 1958. The free amino acid pool of cultured human cells. J. Biol. Chem. **231:**533–545.
38. **Rauzier, J., J. Moniz-Pereira, and B. Gicquel-Sanzey.** 1988. Complete nucleotide sequence of pAL5000, a plasmid from *Mycobacterium fortuitum*. Gene **71:**315–321.
39. **Raviglione, M. C., P. Sudre, H. L. Rieder, S. Spinaci, and A. Kochi.** 1993. Secular trends of tuberculosis in Western Europe. Bull. WHO **71:**297–306.
40. **Slauch, J. M., and T. J. Silhavy.** 1991. Genetic fusions as experimental tools. Methods Enzymol. **204:**213–248.
41. **Somers, J. M., A. Amzallag, and R. B. Middleton.** 1973. Genetic fine structure of the leucine operon of *Escherichia coli* K-12. J. Bacteriol. **113:**1268–1272.
42. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351:**456–460.
43. **Sudre, P., G. ten Dam, and A. Kochi.** 1992. Tuberculosis: a global overview of the situation today. Bull. WHO **70:**149–159.
44. **Udani, R., S. Bardarov, and W. R. Jacobs, Jr.** Unpublished observation.
45. **van der Zee, A., C. Agterberg, M. Van Agterveld, M. Peeters, and F. R. Mooi.** 1993. Characterization of IS*1001*, an insertion sequence element of *Bordetella parapertussis*. J. Bacteriol. **175:**141–147.
46. **van Soolingen, D., P. W. M. Hermans, P. E. W. De Haas, D. R. Soll, and J. D. A. Van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.
47. **Vertes, A. A., M. Inui, M. Kobayashi, Y. Kurursu, and H. Yukawa.** 1994. Isolation and characterization of IS*31831*, a transposable element from *Corynebacterium glutamicum*. Mol. Microbiol. **11:**739–746.
48. **Villar, C. A., and J. Benitez.** 1992. Functional analysis of pAL5000 plasmid in *Mycobacterium fortuitum*. Plasmid **28:**166–169.
49. **Young, D. A., and S. T. Cole.** 1993. Leprosy, tuberculosis and the new genetics. J. Bacteriol. **175:**1–6.