Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 10961–10966, September 1997
Microbiology

Conditionally replicating mycobacteriophages: A system for transposon delivery to *Mycobacterium tuberculosis*

STOYAN BARDAROV*, JORDAN KRIAKOV*, CHRISTIAN CARRIERE*†‡, SHENGWEI YU*, CARLOS VAAMONDE*, RUTH A. MCADAM†§, BARRY R. BLOOM*, GRAHAM F. HATFULL¶, AND WILLIAM R. JACOBS, JR.*‖

*Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY, 10461; †Central Veterinary Laboratory, Molecular Genetics Unit, Surrey KT15 3NB, United Kingdom; and ¶Pittsburgh Bacteriophage Institute, University of Pittsburgh, Department of Biological Sciences, 365A Crawford Hall, Pittsburgh, PA 15260

Edited by John R. Roth, University of Utah, Salt Lake City, UT, and approved July 17, 1997 (received for review March 10, 1997)

ABSTRACT **Transposon mutagenesis provides a direct selection for mutants and is an extremely powerful technique to analyze genetic functions in a variety of prokaryotes. Transposon mutagenesis of *Mycobacterium tuberculosis* has been limited in part because of the inefficiency of the delivery systems. This report describes the development of conditionally replicating shuttle phasmids from the mycobacteriophages D29 and TM4 that enable efficient delivery of transposons into both fast- and slow-growing mycobacteria. These shuttle phasmids consist of an Escherichia coli cosmid vector containing either a mini-Tn*10*(kan) or Tn*5367* inserted into a nonessential region of the phage genome. Thermosensitive mutations were created in the mycobacteriophage genome that allow replication at 30°C but not at 37°C (TM4) or 38.5°C (D29). Infection of mycobacteria at the nonpermissive temperature results in highly efficient transposon delivery to the entire population of mycobacterial cells. Transposition of mini-Tn*10*(kan) occurred in a site-specific fashion in *M. smegmatis* whereas Tn*5367* transposed apparently randomly in *M. phlei*, Bacille Calmette–Guérin (BCG), and *M. tuberculosis*. Sequence analysis of the *M. tuberculosis* and BCG chromosomal regions adjacent to Tn*5367* insertions, in combination with *M. tuberculosis* genomic sequence and physical map data, indicates that the transpositions have occurred randomly in diverse genes in every quadrant of the genome. Using this system, it has been readily possible to generate libraries containing thousands of independent mutants of *M. phlei*, BCG, and *M. tuberculosis*.**

In April 1993, tuberculosis was declared a global health emergency—the first such designation in the history of the World Health Organization. The distinction is regrettably justified because tuberculosis remains one of the largest burdens of disease and death in the world (1, 2) due in part to the increased susceptibility of HIV-infected individuals and the ominous emergence of multidrug resistant strains in both industrialized and developing countries. It is our view that effective new tuberculosis control and prevention strategies will require additional knowledge of the causative agent and its interaction with the human host.

To systematically delineate virulence determinants, identify the metabolic pathways, and discover novel drug targets for *Mycobacterium tuberculosis*, a methodology generating libraries of mutants will be essential. Although mutant isolation and gene transfer strategies have been successfully used for fast-growing nonvirulent mycobacteria, such as *M. smegmatis* (3, 4), determining the genetic basis of phenotypes for *M. tuberculosis* has been frustrated by the lack of a natural gene transfer system in this pathogen. Furthermore, traditional mutational analyses based on the characterization of colonies arising from single cells after treatment with DNA-damaging agents is of limited value for slow-growing mycobacteria because the frequency of mutants is very low, multiple mutations occur in the same cells, and the mycobacteria tend to clump.

Transposon mutagenesis has been successfully used in diverse genera of bacteria (5, 6). The first transposition events in *M. smegmatis* were reported using Tn*610* (7), followed by transposons engineered from insertion sequences (IS)*900* and IS*986* (8, 9). Transposition in Bacille Calmette-Guérin (BCG) (12) was reported using a transposon constructed from the insertion element IS*1096* (10). Remarkably, the only reports of successful isolation of auxotrophic mutants for mycobacteria of the *M. tuberculosis* group used insertional mutagenesis systems: illegitimate recombination (11), transposon mutagenesis (12), and allelic exchange (13). A very promising approach to deliver transposons into *M. smegmatis* is the use of a conditionally replicating vector that is able to replicate at 30°C but not at 37°C (14). A library of 30,000 Tn*611* insertion mutants was obtained from three independent experiments yielding 80 auxotrophic mutants with 15 different phenotypes. However, this system had not yet been applied to the slow-growing mycobacteria such as *M. tuberculosis*.

Conditionally replicating phage systems have proven to be very efficient systems for transposon mutagenesis in numerous bacterial species (5). One of the great advantages of a phage delivery system is that essentially every cell in the bacterial population can be infected with the transposon-carrying phage, generating large numbers of independent mutants. Shuttle phasmid vectors, chimeric molecules that replicate in *Escherichia coli* as plasmids and in mycobacteria as phages, were the first recombinant DNA vectors engineered for mycobacteria (15). Various phasmids, constructed from different mycobacteriophages, such as TM4, L1, and D29, have proven useful for the development of transformation systems for mycobacteria (16) and for the development of luciferase reporter phages for rapid diagnosis and drug susceptibility testing of *M. tuberculosis* clinical isolates (17, 18).

In this report, we describe the isolation of conditionally replicating D29 and TM4 shuttle phasmid vectors that can replicate in *M. smegmatis* at 30°C but fail to replicate and lyse the host cells at 38.5°C (D29-based phasmids) or 37°C (TM4-based phasmids). With these phasmids engineered to deliver the transposons Tn*5367* or mini-Tn*10*(kan), we were able to generate efficiently transposon libraries of *M. phlei*, BCG, and *M. tuberculosis* mutants.

MATERIALS AND METHODS

Bacterial Strains, Media, and Culture Methods. The mycobacterial strains used in this study are listed in Table 1. The

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Abbreviations: BCG, Bacille Calmette–Guérin; kan^r, kanamycin resistance; MOI, multiplicity of infection; IS, insertion sequence.

‡Present address: Institute de Biologie, Laboratoire de Virologie Moleculaire, 2 Boulevard Henri IV, 34060 Montpellier, Cedex, France.
§Present address: Glaxo Welcome Research and Development, Medicine Research Center, Hertford Shire SGI 2NY, U.K.
‖To whom reprint requests should be addressed. e-mail: jacobs@aecom.yu.edu.

Table 1. Mycobacterial strains used in this study

Species	Strain	Genotype or description	Reference or source
M. aurum	mc^{2}18		W. Jones, CDC
M. chelonae	mc^{2}21		W. Jones, CDC
M. phlei	mc^{2}19	Phage host	W. Jones, CDC
M. phlei	ATCC354	NTCC54	ATCC
M. phlei	ATCC11758	TMC1548, type strain	ATCC
M. phlei	ATCC27086	Jahasz F89	ATCC
M. phlei	ATCC27206	SN109	ATCC
M. fortuitum	ATCC6841	TMC1529	ATCC
M. fortuitum	ATCC35755	TMC1545	ATCC
M. flavescence	ATCC14474	TMC1541	ATCC
M. smegmatis	mc^{2}155	*ept-1*	ref. 23
M. smegmatis	mc^{2}1255	*ept-1 rpsL4*	ref. 20
M. smegmatis	ATCC359	Alias *M. butiricum*	ATCC
M. smegmatis	ATCC11759	Penso strain Milch	ATCC
M. smegmatis	ATCC23032	Runyon Special 9	ATCC
M. smegmatis	ATCC27204	SN2	ATCC
M. smegmatis	ATCC27205	SN38	ATCC
M. smegmatis	ATCC35797	TMC1515	ATCC
M. smegmatis	ATCC37798	TMC1533 "R. Koch"	ATCC
M. vaccae	ATCC15483	TMC1526 SN920	ATCC
M. vaccae	ATCC23014	SN923	ATCC
M. tuberculosis	Erdman	Virulent isolate	F. Collins, FDA
BCG		Pasteur strain	Staten Serum Institut

CDC, Centers for Disease Control; ATCC, American Type Culture Collection; FDA, Food and Drug Administration.

E. coli strains, DH5α or HB101, used for cloning hosts were grown as described (19). For preparation of electrocompetent cells for transfections, *M. smegmatis* mc^{2}155 or mc^{2}1255 were grown in Luria–Bertani plus Tween 80 0.05% broth (20). Mycobacteriophages were propagated in mc^{2}155 as described (21). When required, the following antibiotics were used at the specified concentrations: carbenicillin (50 μg/ml) and kanamycin (25 μg/ml for *E. coli;* 20 μg/ml for mycobacteria); BCG, *M. tuberculosis* Erdman strain, and other mycobacteria were grown in Middlebrook 7H9 broth enriched with albumin (fraction V) 0.5%, dextrose 0.2%, sodium chloride 0.85%, and Tween 80 0.05% (M-ADC-TW) broth (21). For transposon delivery experiments, BCG and *M. tuberculosis* were grown in M-ADC-TW broth without glycerol.

Isolation of Temperature-Sensitive Mycobacteriophages. D29 shuttle phasmids were propagated in *M. smegmatis* mc^{2}155. Samples of phages (10^9 pfu/ml) were mutagenized with hydroxylamine as described (22) using conditions that yielded 0.1% viable surviving phages. These were plated on *M. smegmatis* mc^{2}155 and incubated for 24 h at 30°C until very small plaques appeared. Plates

Table 2. Phages, cosmids, shuttle plasmids, and transposons used in this study

	Description	Reference
Transposons		
Tn*5367*	IS*1096*-derived transposon containing kan^r gene	12
Mini-Tn*10*(kan)	Derivative of Tn*10*-containing *aph* gene	5
Cosmids		
pYUB328	ColE1 *amp^r* exciseable double *cos* vector	13
pYUB552	Minimal (2.4-kb) pYUB328 cosmid derivative	This work
pYUB553	pYUB552::Tn*5367*	This work
pYUB554	pYUB552::mini-Tn10(kan)	This work
Phages		
λ	*cI857*, *Sam10*	
D29	Wild type	15
TM4	Wild type	15
PH101	*ts* mutant of TM4 that fails to form plaques at 42°C	This work
Shuttle phasmids		
phAE60	D29 Δ (45440-48108 bp)::pYUB328	This work
phAE65	*ts* mutant of phAE60 that fails to replicate at 42°C	This work
phAE70	*ts* mutant of phAE65 that fails to replicate at 38.5°C	This work
phAE71	*ts* mutant of phAE65 that fails to replicate at 38.5°C	This work
phAE72	*ts* mutant of phAE65 that fails to replicate at 38.5°C	This work
phAE73	*ts* mutant of phAE65 that fails to replicate at 38.5°C	This work
phAE76	*ts* mutant of phAE65 that fails to replicate at 38.5°C	This work
phAE87	PH101::pYUB328 that fails to replicate at 37°C	This work
Conditionally replicating transposon delivery shuttle plasmids		
phAE77	phAE70::pYUB553	This work
phAE78	phAE70::pYUB554	This work
phAE94	phAE87::pYUB553	This work

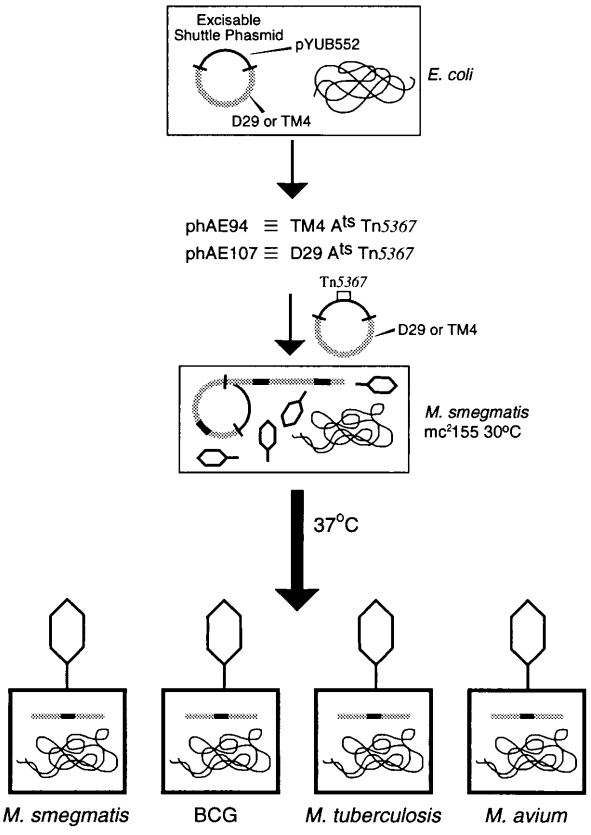

FIG. 1. Transposon delivery in mycobacteria using conditionally replicating shuttle phasmids. Shuttle phasmids are mycobacteriophage molecules into which an *E. coli* cosmid has been inserted in a nonessential region. These molecules can thus replicate in *E. coli* as cosmids and replicate in mycobacteria as phages. The cosmid is flanked by restriction sites that are not found in the shuttle phasmid, thus allowing for simple ex*cis*-ion of the cosmid. A cosmid containing a transposon is readily cloned into the phage backbone by cosmid cloning in *E. coli*. Cosmids are isolated from *E. coli* and transfected into *M. smegmatis* cells by electroporation. At permissive temperatures (30°C), shuttle phasmids undergo growth as a lytic mycobacteriophage to high titers. Infection of various mycobacteria with the mycobacteriophages containing the transposons results in the delivery to every cell in a population of mycobacterial cells. The temperature-sensitive mutations in the mycobacteriophages prevent phage propagation, which allows for transpositions into the mycobacterial chromosome to occur. The broad host ranges of D29 and TM4 mycobacteriophages allow for delivery of transposons to a variety of different mycobacterial species, including BCG and *M. tuberculosis*.

were then shifted at 42°C and incubated for another 24–36 h. Phage plaques that remain very small after this incubation period were screened for their abilities to form plaques at 30, 37, and 42°C. Phage clones forming plaques at 30°C, but not at 42°C, were clone-purified, amplified to a high titer, and analyzed for reversion frequency. Shuttle phasmids from either D29 or PH101 were prepared as described (15, 17, 18).

Recombinant DNA Methodologies. Cosmids, phasmids, transposons, and phages used in this study are listed in Table 2. DNA manipulations were done essentially as described (19). High molecular weight chromosomal DNA from BCG or *M. tuberculosis* for generating cosmid libraries was purified as described (13).

Transposon Mutagenesis. Fast-growing mycobacteria were cultured in LBT to an OD at A_{600} of 1.0 ($\approx 2 \times 10^8$ cfu/ml). BCG and *M. tuberculosis* were grown for 7–10 days after inoculation of 10 ml of the starter culture into a 100-ml roller bottle culture ($A_{600} = 1.0$) in M-ADC-TW without glycerol plus 0.4% casamino acids. Ten milliliters of cultures was concentrated by centrifugation and resuspended in 1 ml of MP buffer (50 mM Tris·HCl, ph 7.6/150 mM NaCl/2mM $CaCl_2$) (21). The cells were prewarmed at the nonpermissive temperature and then were mixed with 2×10^{10} pfu/ml [multiplicity of infection (MOI) = 10]. The cell–phage mixture was incubated at the nonpermissive temperature for 30 min for the fast-growing mycobacteria or 4–6 hours for the slow-growing mycobacteria. After completion of the adsorption time, 2 ml of prewarmed "adsorption-stop buffer" (MP buffer containing 20 mM sodium citrate and 0.2% Tween 80) was added to prevent further phage infections. Samples of this mixture were plated on prewarmed tryptic soy agar plates for the fast-growing mycobacteria or M-ADC-TW-based agar media containing 0.1% Tween 80, 0.4% casamino acids, 40 μg/ml L-tryptophan, and 20 μg/ml kanamycin for the slow-growing mycobacteria. Transposition frequency was estimated as a number of kanamycin-resistant (kan^r) colonies per total number of input cells.

Table 3. Transposition of mini-Tn*10*(kan) and Tn5367 in fast-growing mycobacteria

Species	Strain	Copies of IS*1096*	Transpositions	
			Mini-Tn10(kan) via:phAE78	
M. smegmatis	mc^2155	11	3600	
			Tn5367 via:	
			phAE77	phAE94
M. smegmatis	mc^2155	11	>10	>10
M. smegmatis	ATCC359	3	80	215
M. smegmatis	ATCC11759	6	0	0
M. smegmatis	ATCC23032	9	330	345
M. smegmatis	ATCC27204	5	0	0
M. smegmatis	ATCC27205	14	0	0
M. smegmatis	ATCC35797	11	85	260
M. smegmatis	ATCC35798	9	285	425
M. aurum	mc^218	None	0	0
M. chelonae	mc^221	None	0	0
M. phlei	mc^219	None	2	2000
M. phlei	ATCC354	None	0	3
M. phlei	ATCC11758	None	0	0
M. phlei	ATCC27086	None	0	0
M. phlei	ATCC27206	None	0	1
M. fortuitum	ATCC6841	None	0	0
M. vaccae	ATCC15483	None	0	0
M. vaccae	ATCC23014	None	0	0

Southern Blotting and DNA Sequencing. Southern blotting was done by the alkali-denaturing procedure. DNA was transferred to Biotrans nylon membranes (ICN) by the capillary method. Hybridization and detection were done as recommended by the manufacturer (enhanced chemiluminescence, Amersham). Sequence analysis was performed using the Applied Biosystems Prism Dye Terminator Cycle Sequencing Core kit with AmpliTaq DNA polymerase (Perkin–Elmer) and an Applied Biosystems 377 automated DNA sequencer. Chromosomal DNA sequence at the junction of the transposon insertions was obtained by PCR sequencing outwards from the transposon using primers HOPS1 (5′-GGCGTAGGAACCTCCATCATC-3′) and HOPS2 (5′-CTTGCTCTTCCGCTTCTTCTCC-3′) with cosmid DNA as a template. The sequence thus obtained (400–600 bp) was used to blast-search the *M. tuberculosis* Sanger Sequence database or GenBank database.

RESULTS

Development of Conditionally Replicating Shuttle Phasmids. Our goal was to develop conditionally replicating mycobacteriophage vectors for the delivery of transposons to a broad range of mycobacteria, including the slow-growing *M. tuberculosis* and BCG (Fig. 1). We chose to construct a phasmid vectors with the exciseable cosmid pYUB328 (13) because such vectors provide an easy means to introduce transposons into nonessential regions of a mycobacteriophage.

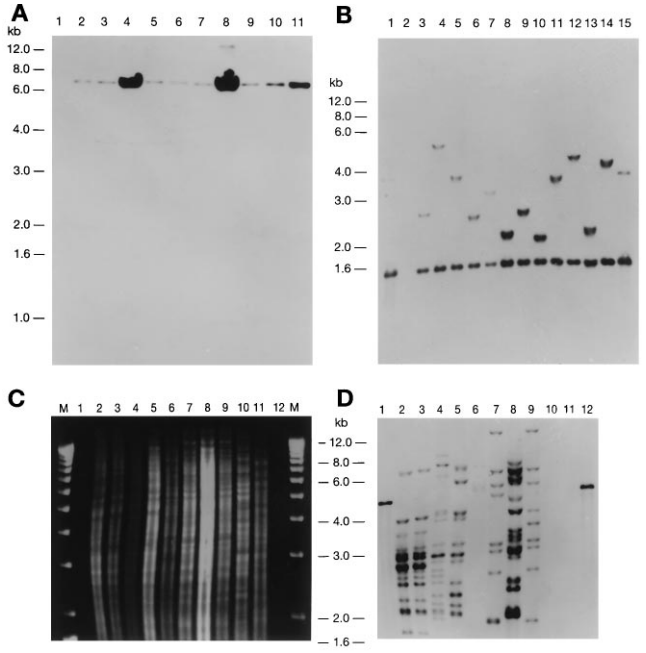

FIG. 2. Southern blot analysis of transposon mutants. (*A*) kan^r *M. smegmatis* mc^2 155 mutagenized with mini-Tn*10*(kan) using phAE78 as a delivery phage. Chromosomal DNAs were isolated from 11 independent mutants, digested with *Pst*I (which does not cleave in the transposon), and hybridized with a DNA fragment containing the gene that confers kan^r. (*B*) kan^r *M. phlei* cells mutagenized with Tn*5367* using phAE77 as a delivery phage. Chromosomal DNA digested with *Pst*I and *Apa*LI and hybridized with the *aph* gene. Both restriction sites are located within the transposon, so a common band is generated as well as the random fragment generated from the insertion of Tn*5367* into the *M. phlei* chromosome. Lane 1 contains pYUB553 digested with *Pst*I and *Apa*LI. (*C* and *D*) Southern blot analysis of IS*1096* in fast-growing mycobacteria. Ethidium bromide-stained agarose gel of chromosomal DNAs of various *M. smegmatis* strains (lanes 2–10); *M. phlei* (lane 11) and *M. vaccae* (lane 12) were digested with *Apa*LI (*C*) and hybridized with *tnpA* of IS*1096* (*D*).

Shuttle phasmids from D29 were first constructed, and one such phasmid, phAE60, which had a maximal deletion within the D29 phage, was subjected to hydroxylamine mutagenesis. From the initial screen of 2000 mutants only phages unable to replicate at 42°C were observed. One such mutant, phAE65, was subjected to further mutagenesis, and a set of independent mutants was identified that failed to form plaques at 38.5°C (Table 2). The mutations conferring thermosensitive growth reverted at frequencies of 10^{-4} at 38.5°C and $<10^{-7}$ at 42°C. Six mutants were tested for their inability to kill *M. smegmatis* when mixed at MOI of 10 and incubated at the nonpermissive temperature. One such mutant, phAE70, showing the lowest reversion frequency and inability to kill *M. smegmatis* when incubated at the nonpermissive temperature (38.5°C), was chosen as the D29-based delivery phage.

For the construction of the TM4 shuttle phasmid transposon delivery vector, we first mutagenized the wild-type TM4 phage and isolated the temperature-sensitive mutant PH101. This mutant failed to form plaques at 37°C, had a reversion frequency $<10^{-7}$, and failed to kill *M. smegmatis* at 37°C at an MOI of 10. As in the case of D29-based phasmids, two independent rounds of mutagenesis were needed. A shuttle phasmid was engineered from this phage and was designated phAE87. Both phAE70 and phAE87 were used for the construction of transposon delivery vectors.

Delivery of Transposons in Fast-Growing Mycobacteria Using Conditionally Replicating Shuttle Phasmids. Transposon constructs could be readily introduced into either phAE70 or phAE87 by replacing the pYUB328 cosmid with a cosmid containing the transposon of interest. Initial attempts to introduce transposon constructs, engineered in pYUB328, into the D29 shuttle phasmids were unsuccessful, most likely because of the packaging constraints of this phage. To further maximize the cloning capacities, a cosmid vector, pYUB552, was engineered to contain 2.1 kb less DNA than pYUB328. Tn*5367* (12) or mini-Tn*10*(kan) (5) was cloned into this cosmid to generate pYUB553 and pYUB554, respectively. Replacement of the pYUB328 in phAE70 with pYUB554 generated phAE78. Infection of *M. smegmatis* mc^{2}155 cells with phAE78 at MOI of 10 yielded kan^r colonies at frequencies slightly $>10^{-6}$ in four independent experiments (Table 3). Southern blot analysis revealed that chromosomal DNA from the kan^r colonies contained no D29 phage sequences (data not shown) but did contain the kan^r cassette of the mini-Tn*10*(kan) (Fig. 2*A*). Unfortunately, mini-Tn*10*(kan) appeared to integrate in a site-specific fashion in *M. smegmatis* and, thus, would not be useful for generating libraries of random insertional mutants. Nevertheless, these results confirmed that the D29 conditionally replicating shuttle phasmids could be used efficiently to deliver transposons to mycobacterial cells.

Table 4. Effect of incubation time on transpositions in BCG and *M. tuberculosis*

Strain	Time of adsorption, λ	kan^r colonies/10^9 input cells	
		phAE77	phAE94
BCG	0.5	0	0
BCG	2.0	0.9×10^{-6}	0
BCG	4.0	1.4×10^{-6}	0
BCG	6.0	1.2×10^{-6}	2×10^{-8}
BCG	8.0	0.5×10^{-6}	1×10^{-8}
M. tuberculosis (Erdman)	6.0	5×10^{-8}	1.7×10^{-7}
M. tuberculosis (Erdman)	6.0	0	3.5×10^{-7}
M. tuberculosis (Erdman)	6.0	ND	8.0×10^{-7}

ND, not determined.

Tn*5367* represented an attractive transposon for mutagenesis of mycobacteria because we had shown previously that it transposed in BCG in a relatively random fashion by a nonreplicative mechanism (12). pYUB553 was used to replace pYUB328 into both the D29 and TM4 conditionally replicating shuttle phasmids to generate phAE77 and phAE94, respectively. These vectors were used to deliver Tn*5367* into mc^2 155 cells at 38.5°C (phAE77) or 37°C (phAE94). Very few kan^r colonies were obtained. Southern analysis suggested that transposition had occurred because hybridization of the kan^r colonies with the *aph* gene yielded fragments of various sizes (data not shown). We hypothesized that the low frequency of transposition of Tn*5367* in mc^2 155 might be a result of a negative regulation resulting from the 11 copies of IS*1096*. Searching for an alternative fast-growing mycobacterial host, we screened various strains of *M. smegmatis*, *M. aurum*, *M. fortuitum*, *M. chelonei*, *M. phlei*, and *M. vaccae*. Transposition in three different *M. smegmatis* strains and one *M. phlei* [mc^2 19] strain were observed whereas no or very low frequency of transposition occurred in the others (Table 3). In subsequent experiments infecting mc^2 19 cells with phAE94 at a MOI of 1, we routinely obtained >2000 kan^r clones/10^8 input cells. Southern analysis revealed that the transposition occurred in a random fashion in *M. phlei* (Fig. 2*B*) and in the three *M. smegmatis* strains (data not shown). The inability to observe transpositions in some strains such as *M. fortuitum* or *M. chelonei* is likely a result of the inability of the phages to infect these strains. The ability of phAE77 and phAE94 to form plaques on the fast-growing strains at permissive temperature was shared by all of the strains that yielded transpositions but was not sufficient to predict the transposition efficiency. Nor was the number of copies of IS*1096* predictive of transposition efficiency because an *M. smegmatis* strain with 11 copies yielded as many transpositions as one strain with two and a *M. phlei* strain that had none (Fig. 2 *C* and *D*). Of interest, the presence of IS*1096*

Table 5. Mapping of Tn5367 insertion mutants in *M. tuberculosis* and BCG

Mutant	Gene interrupted	SC or MC cosmid	Sequence of the transposon duplication
M. tuberculosis	Erdman		
mc^{2}3002	Unknown	cY75.unf (SC)	CATTCATT
mc^{2}3003	Ferritin H (*rsgA*)	MTCY1A6 (SC)	AATAAACC
mc^{2}3004	Unknown	cY23H3 (SC)	CGTTATCG
mc^{2}3005	Unknown	cSCY03A11 (SC)	TGTTTGAC
mc^{2}3006	Transketolase	cY454 (SC)	GTGAAACC
mc^{2}3007	Unknown	MTCY31 (SC)	GCTTTTAC
mc^{2}3008	Acetyl–CoA synthetase	MSGY409 (MS)	GGTTTTGA
mc^{2}3011	Unknown	cY28.unf (SC)	GGTTTCCC
mc^{2}3014	Polyketide synthase	cSCY22G10 (SC)	CAGTAACG
mc^{2}3015	Thiosulfate sulfur transferase	cY164 (SC)	GGTGATCC
mc^{2}3017	Imidazole glycerol phosphate dehydratase	Not found	GTGATACA
mc^{2}3019	Polyketide synthase	cY275,unf (SC)	CGGTTTGC
mc^{2}3020	Alcohol dehydrogenase (C terminus)	MTCY190 (SC)	TGGAAACC
BCG			
mc^{2}1500	*leuD* (insertion in the 6th codon)	Not found	GTGAAAGG
mc^{2}1501	DR	cSCY16B7 (SC)	CCAAAACC
mc^{2}1502	DR	cSCY16B7 (SC)	CAAAACCC
mc^{2}1503	Unknown	Not found	CAAAACCC
mc^{2}1504	Unknown	cY28.unf (SC)	TTCAAACT
mc^{2}1505	Translation elongation factor G (*efg*)	Not found	GTTTTTCG
mc^{2}1506	8-Amino-7-oxononaate synthase (*bioF*)	MTCY10H4 (SC)	ACATTTGT

Homology to predicted ORF or analogous functions by alignment (BLAST) to National Center for Biotechnology Information database. DR, direct repeat; SC, Sanger Center, U.K.; MC, Genomic Therapeutics Corporation (Waltham, MA).

appears to be uniquely diagnostic for *M. smegmatis* strains; all eight strains that had the insertion sequence (Fig. 2 *C* and *D*) yielded a common restriction fragment length polymorphism after PCR amplification of the *hsp60* gene product (ref. 24 and data not shown).

Delivery of Transposons in Slow-Growing Mycobacteria Using Conditionally Replicating Shuttle Phasmids. For slow-growing mycobacteria, the expression time required for optimal transposition after phage infection was unknown. Therefore, we tested the effect of time of incubation with the phage before plating on kanamycin-containing media. The maximal number of kan^r colonies was obtained after 4 h of incubation of BCG with phAE77 or *M. tuberculosis* using phAE94 (Table 4). Surprisingly, phAE94 yielded no or relatively few transposon mutants in BCG. Using *M. tuberculosis,* in three independent experiments, phAE94 yielded 1700, 3500, and 8000 transposon mutants, respectively. In contrast, phAE77 yielded relatively few transposon mutants in *M. tuberculosis* whereas in BCG the number of the kan^r colonies was consistently higher than 10^4. Southern analyses of chromosomal DNA from 20 randomly picked kan^r colonies of either BCG or *M. tuberculosis* revealed a random distribution of the transposon insertions (data not shown). To further characterize the distribution of the Tn*5367* insertions, the DNA sequences adjacent to the transposon insertions were determined for 11 kan^r clones of BCG and 13 clones of *M. tuberculosis* (Table 5). All transposon insertions analyzed were accompanied by an 8-bp target duplication, as was described for nine other Tn*5367* insertions (12). The total of 33 insertions is within different target sites, demonstrating that Tn*5367* transposes with little or no sequence specificity. The chromosomal DNA sequence into which Tn*5367* had inserted could be readily identified because they revealed 100% identity on a DNA level with reported cosmid sequences (28). The inactivated genes could be predicted after a BLAST analysis compared with the National Center for Biotechnology Information database (Table 5). Notably, the *leuD* auxotroph mc^{2}1500 isolated in BCG resulted from Tn*5367* insertion in the sixth codon of the *leuD* gene. This insertion site is different from mc^{2}797 *leuD* and mc^{2}798 *leuD* transposon mutants reported previously (12). In two *M. tuberculosis* mutants, mc^{2}3014 and mc^{2}3019, two independent Tn*5367* insertions at widely separated regions of a large gene that has homology to polyketide biosynthesis gene were identified. The insertions in BCG and *M. tuberculosis* have been positioned on the *M. tuberculosis* H37Rv physical map (28), and we show that the insertions are scattered within every quadrant of the genome (Fig. 3).

DISCUSSION

To facilitate transposon-mediated insertional mutagenesis in pathogenic mycobacteria, we have developed conditionally replicating shuttle phasmids that efficiently deliver transposons to both fast-growing *M. smegmatis* and *M. phlei* species and the slow-growing species BCG and *M. tuberculosis*. Although, in the fast-growing mycobacteria, transposon mutagenesis has been achieved using nonreplicating plasmids (7–9, 12), phage delivery offers significant advantages for transposon mutagenesis of BCG and *M.*

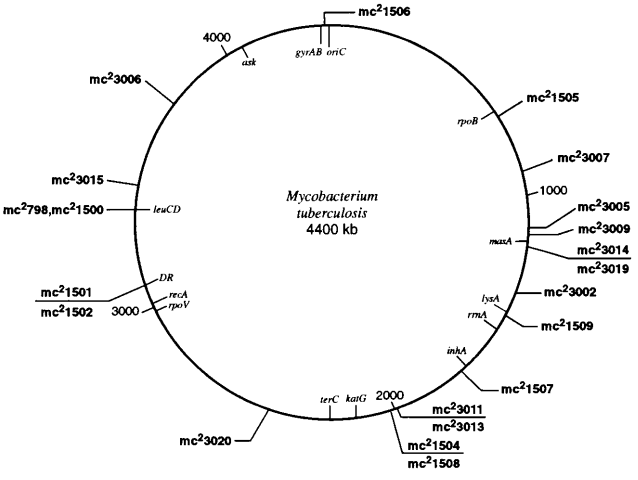

FIG. 3. Distribution of Tn5367 insertions on the genome map of *M. tuberculosis*. The location of the Tn*5367* insertions in mutants generated from *M. tuberculosis* Erdman [mc^2 3000s] or BCG [mc^2 1500s] were deduced from the determination of the sequence adjacent to the insertion and comparison to the *M. tuberculosis* genomic database and the *M. tuberculosis* physical map (28).

tuberculosis. The nonreplicating plasmids are of limited use in slow-growing mycobacteria because of the low transformation frequencies obtained with electroporation. The disadvantage of the conditionally replicating plasmid system is that, unless the transposition event is regulated, there is no means to select against propagation of transposon mutants during the outgrowth stage, with the consequence that many of the mutants selected will be siblings of a few mutated cells. In contrast, with a conditionally replicating phage system in which essentially every cell is infected, the selection can be executed shortly after infection, and the mutations are likely to represent independent events. Analysis of the BCG- and *M. tuberculosis*-independent mutants obtained in this study suggests that this is the case.

The transposon Tn*5367* that we have used for insertional mutagenesis in this study has several particularly desirable features. As shown previously (12) and confirmed in this study, Tn*5367* transposes in a relatively random fashion and therefore should allow for insertions in virtually every gene of a mycobacterial genome. Transposition frequencies could be improved by fusing the transposase to more active promoters or expression signals and additionally provide transposon elements (mini-Tn5367) that can no longer transpose once dissociated from their transposase. Transposon-containing, promoter-less reporter genes could be constructed that would allow for the generation of libraries that permit the screening of regulated promoters, as has been done in other genera (5).

In addition to delivering useful transposon constructs, the conditionally replicating shuttle phasmids represent potentially versatile vectors for genetic transfer. At present, we know that an additional 4 kb of DNA can be introduced into the pYUB552 backbone and still maintain the ability of the shuttle phasmids to yield functional mycobacteriophage. In addition to delivering transposons and creating marked insertional mutations, these vectors could be used to deliver any gene(s) to *M. tuberculosis* in a transient fashion. Infection of *M. tuberculosis* with conditionally replicating TM4 shuttle phasmid containing the firefly luciferase gene has already increased the sensitivity of the luciferase phage reporter assay (25). Infection at the nonpermissive temperature has been found both to increase the cumulative light emission and extend the time available for the assay because the phage is unable to proceed to lytic growth (25). Transient delivery of genes encoding γδ-resolvase or Cre recombinase to *M. tuberculosis* offers unique possibilities for precise gene deletions and removal of antibiotic selective markers that will be valuable in the creation of nonreverting, attenuated vaccine strains acceptable for use in humans. It is our hope that these phages may allow the development of specialized transducing phages that will efficiently mediate allelic exchange in *M. tuberculosis.*

The ability to generate libraries of transposon mutants in *M. tuberculosis* together with the complete genomic sequence shortly to become available provides an unprecedented opportunity to make rapid and substantial progress in the understanding of pathogenesis and development of novel therapeutics for tuberculosis. The rapid determination of sequences adjacent to the transposon insertion allows for an immediate classification of a gene as being nonessential for the multiplication of the tubercle bacillus. The set of essential genes would represent attractive candidates for analysis as practical drug targets. Many nonessential genes may represent important virulence determinants, as well. Indeed, among the mutants analyzed, it will be of immediate interest to examine the insertions in the mycocerosic acid biosynthetic gene (26) and the polyketide-biosynthetic gene [which shares significant homology to 6 of the 14 modules of the immunosuppressant rapamycin biosynthetic gene (27)] for their possible roles in virulence of *M. tuberculosis*. It is our view that mutational analyses represent the most powerful approach available to dissect the steps and functions governing the pathogenic process of *M. tuberculosis* and to support our hope that the conditionally replicating shuttle phasmids will be a valuable tool in facilitating that analysis.

We thank Marty Pavelka, Keith Derbyshire, and Jeffery Cox for their critical reading of this manuscript and Vladimir Pelicic, Christoph Guilhot, Mary Jackson, Jean-Mark Reyrat and Brigitte Gicquel for sharing their results before publication. This work was supported by grants from the National Institutes of Health (AI26170, AI27235, and AI23545) and the Burroughs Wellcome Fund.

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