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Lysogeny and transformation in mycobacteria: Stable expression of foreign genes

(*Mycobacterium tuberculosis typus bovinus* var. Bacille-Calmette–Guérin/shuttle phasmid/electroporation/shuttle plasmid/vaccines)

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ABSTRACT **Requisite to a detailed understanding of the molecular basis of bacterial pathogenesis is a genetic system that allows for the transfer, mutation, and expression of specific genes. Because of the continuing importance of tuberculosis and leprosy worldwide, we initiated studies to develop a genetic system in mycobacteria and here report the use of two complementary strategies to introduce and express selectable genetic markers. First, an *Escherichia coli* cosmid was inserted into the temperate mycobacteriophage L1, generating shuttle phasmids replicating as plasmids in *E. coli* and phage capable of lysogenizing the mycobacterial host. These temperate shuttle phasmids form turbid plaques on *Mycobacterium smegmatis* and, upon lysogenization, confer resistance to superinfection and integrate within the mycobacterial chromosome. When an L1 shuttle phasmid containing a cloned gene conferring kanamycin resistance in *E. coli* was introduced into *M. smegmatis*, stable kanamycin-resistant colonies—i.e., lysogens—were obtained. Second, to develop a plasmid transformation system in mycobacteria, *M. fortuitum/E. coli* hybrid plasmids containing mycobacterial and *E. coli* replicons and a kanamycin-resistance gene were constructed. When introduced into *M. smegmatis* or BCG (*Mycobacterium tuberculosis typus bovinus* var. Bacille-Calmette–Guérin) by electroporation, these shuttle plasmids conferred stable kanamycin resistance upon transformants. These systems should facilitate genetic analyses of mycobacterial pathogenesis and the development of recombinant mycobacterial vaccines.**

Tuberculosis remains a devastating disease resulting in 3 million deaths per year, with greater than 10 million new cases annually predominantly found in the Third World (1). The incidence of tuberculosis in the United States is now increasing after a decline for the past 32 years (2), largely due to the increase in acquired immunodeficiency syndrome (AIDS). *Mycobacterium avium* infection is responsible for a significant proportion of fatalities in AIDS patients, because it is refractory to all standard antibiotics (3). Leprosy afflicts 10.5 million people, causing deformity in a third of its sufferers (4). On the other hand, BCG (*Mycobacterium tuberculosis typus bovinus* var. Bacille-Calmette–Guérin) is one of the most widely used vaccines in the world but has had variable effectiveness against tuberculosis and leprosy in different parts of the world (5, 6). Clearly, greater understanding of the mechanisms of pathogenesis of tuberculosis and leprosy and strategies to develop improved vaccines and drugs effective against mycobacterial diseases are urgently needed.

Mycobacteria remain virtually uncharacterized genetically, and our goal was to develop a system in which genes could be transferred between *Escherichia coli*, an organism that is easy to manipulate genetically, and cultivable mycobacteria. Because of the paucity of mycobacterial plasmids and absence of selectable markers for mycobacteria, we initially developed a phage-based strategy by creating a shuttle phasmid vector. An *E. coli* cosmid, pHC79, was inserted (7) into a nonessential region of the mycobacteriophage TM4 genome, generating a phasmid that allows one to shuttle replicons among *E. coli*, *Mycobacterium smegmatis*, and BCG substrains. The problem of pseudolysogeny has confounded much work with mycobacteriophages (8). Although TM4 was reported to lysogenize *M. avium* (9), we were unable to obtain a stable lysogenic state with this phage in *M. smegmatis* or BCG. Consequently, we have developed a methodology for expression of selectable markers by lysogeny and plasmid transformation in *Mycobacterium*, including BCG substrains.

METHODS AND MATERIALS

Bacterial Strains, Phages, and Cultures. The mycobacterial strains used were the *M. smegmatis* strain mc^{2}6 (7), a single-cell isolate of American Type Culture Collection (ATCC) 607; the unspeciated *Mycobacterium* ATCC 27199, lysogenized with mycobacteriophage L1 (10); and the BCG-Pasteur substrain, seed lot 1173P2, obtained from the BCG Laboratory of the Institut Pasteur (Paris). The *E. coli* strains used were χ2338 (11) and W5445 (12) and transformation protocols for these strains have been described. Mycobacterial cultures were grown in Middlebrook 7H9 broth (Difco) supplemented with ADC (albumin–dextrose complex) (Difco) and 0.05% Tween 80 (M-ADC-TW broth) with shaking at 37°C. Phage L1 was obtained by plating the culture supernatant fluid from the *Mycobacterium* ATCC 27199 on mc^{2}6 cells with Dubos agar medium without Tween (GIBCO) containing 2 mM $CaCl_2$. After plaque purification, high-titered plate lysates of L1, L1 shuttle phasmids, and the virulent mycobacteriophage D29 (13) were prepared, and the phage particles were purified by CsCl equilibrium density centrifugation as described (7).

Preparation of DNA and Hybridization Analyses. Chromosomal DNA was isolated by using a Braun homogenizer (packed cells were mixed with an equal volume of acid-washed 0.45-mm glass beads and homogenized for 2–6 min) and then phenol/chloroform extractions (C. Grosskinsky, J. E. Clark-Curtiss, W.R.J., and B.R.B., unpublished results). Phage and shuttle phasmid DNAs were isolated from phage particles after proteinase K digestion, phenol/chloroform extraction and extensive dialysis. Southern analyses

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Abbreviations: *aph*, aminoglycoside phosphotransferase; BCG, *Mycobacterium tuberculosis typus bovinus* var. Bacille-Calmette–Guérin; ADC, albumin–dextrose complex.

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were performed by using Biotrans (ICN) nylon membranes as described by the manufacturer. L1 DNA was radiolabeled by using a nick-translation kit (Bethesda Research Laboratories) and [α-^{32}P]dCTP (Amersham).

Construction of L1 Shuttle Phasmids. L1 shuttle phasmids were constructed in a manner similar to those constructed for the TM4 shuttle phasmids (7). Briefly, the 50-kilobase (kb) mycobacteriophage L1 DNA was ligated to form concatemers and partially digested with *Sau*3A to generate fragments 30–50 kb long. These fragments were ligated to the cosmid pHC79 (14) that had been cleaved with *Bam*HI. This DNA was packaged into bacteriophage λ heads by using Gigapack packaging extract (Stratagene, San Diego, CA), and the resulting packaged cosmid molecules were transduced into χ2338 cells that were then plated on L agar (GIBCO) containing ampicillin (30 μg/ml). Each resulting ampicillin-resistant clone contained a large piece of the L1 phage genome with pHC79 inserted within it. Closed circular plasmid DNA extracted from a pool of 40,000 ampicillin-resistant colonies therefore represented a library of pHC79 insertions in the L1 genome. Phasmid DNA from this library was transfected into *M. smegmatis* protoplasts as described (7). Five independent shuttle phasmids were obtained. All five formed turbid plaques on *M. smegmatis* and DNA isolated from these recombinant phage replicated as plasmids conferring ampicillin resistance in *E. coli*, thus confirming that these recombinant molecules were shuttle phasmids. Restriction analyses of these five L1 shuttle phasmids showed differences (S.B.S., B.R.B., and W.R.J., unpublished results). For this work, we used the L1 shuttle phasmid designated phAE15.

Cloning and Expression of the *aph* (Aminoglycoside Phosphotransferase) Gene into phAE15. The *aph* gene from Tn*903* (15) was inserted into the L1 shuttle phasmid, phAE15, by ligating phAE15 DNA concatemers cleaved at the unique *Eco*RI site to the TN*903* *Eco*RI *aph* cassette (Pharmacia). The bacteriophage λ cohesive end (*cos*) sequence present within the shuttle phasmid DNA permitted *in vitro* packaging of this ligated DNA into λ phage heads. After *in vitro* packaging, the resulting λ-packaged shuttle phasmids were transduced into the *E. coli* strain χ2338 and cells were selected for both ampicillin and kanamycin resistance. Plasmid DNA was isolated from an *E. coli* transductant and transfected into mc^{2}6 protoplasts, and the resulting mycobacteriophage was designated phAE19. Lysogens were purified from turbid plaques arising after spotting phAE19 on agar containing mc^{2}6 cells. Putative lysogens were tested for release of phages and resistance to superinfection by L1.

Expression of the *aph* gene in mycobacteria was demonstrated by the growth of *M. smegmatis* cells lysogenized with phAE19 on medium containing kanamycin. Dubos top agar was mixed with 5×10^7 cells and poured onto a Dubos agar plate containing kanamycin (15 μg/ml). Lysates of the L1 shuttle phasmids phAE15 and phAE19 were passed through a 0.45-μm filter and diluted to 5×10^6 plaque-forming units/ml with MP buffer (7). Serial 1:10 dilutions (10 μl) were spotted in designated areas and the plates were incubated for 5 days at 37°C and then photographed.

Construction of *E. coli–Mycobacterium* Shuttle Plasmids. Plasmid pAL5000 DNA, isolated from *Mycobacterium fortuitum* as described (16), was partially digested with *Mbo* I and linear fragments of 5 kb were isolated from an agarose gel after electrophoresis. These fragments were ligated to the positive selection vector pIJ666 (17) that had been cleaved with *Bam*HI and *Eco*RV. This ligated DNA was transformed into *E. coli* W5445 and plated on L agar containing chloramphenicol (25 μg/ml). pIJ666 contains the *neo* (neomycin/kanamycin phosphotransferase II) gene originating from Tn*5* (18), the P15A origin of replication, and the chloramphenicol acetyltransferase gene from pACYC184 (19). This plasmid has been described as a positive selection vector because the inverted terminator sequences present within pIJ666 must be separated by spacer DNA to allow for replication (17). Thus, chloramphenicol-resistant transformants were expected to contain pAL5000 DNA. Approximately 200 chloramphenicol-resistant transformants were pooled and grown in mixed culture, from which plasmids were isolated and purified as described (20).

Electroporation of Mycobacteria. Electroporation of mycobacterial cells was performed by using the Gene Pulser electroporator (Bio-Rad) and a modification of the protocol of Chassy and Flickinger (21). Cultures of mc^{2}6 or BCG-Pasteur were grown in M-ADC-TW broth with shaking at 37°C. The mc^{2}6 cultures were harvested when they reached an A_{600} of 1.7 and BCG cultures were harvested when they reached 4.5×10^7 colony-forming units/ml. After centrifugation, the cells were washed in electroporation buffer (7 mM sodium phosphate/272 mM sucrose, pH 7.2) and resuspended in 10% of the original volume. D29 phage DNA or plasmid DNA (1 μg) was added to an electroporation cuvette containing 0.8 ml of mycobacterial cells. After a 10-min incubation on ice, the cells were subjected to a single pulse of electroporation (25 μF at 6250 V/cm). Phage transfections were assayed after an additional 10-min incubation on ice, by mixing the electroporated cells with sensitive cells and plating for plaque-forming units. Cells electroporated with plasmid DNA were also incubated on ice for 10 min and then mixed with an equal volume of M-ADC-TW broth. After a 2-hr incubation at 37°C, these cells were spread on 7H10 agar (Difco) plates supplemented with ADC containing kanamycin (10 μg/ml). Plates used for BCG experiments also contained cycloheximide (100 μg/ml) to prevent mold contamination. Plates with *M. smegmatis* cells or BCG cells were incubated at 37°C for 7 days and 20–30 days, respectively. The kanamycin-resistant transformants were subcultured in M-ADC-TW broth con-

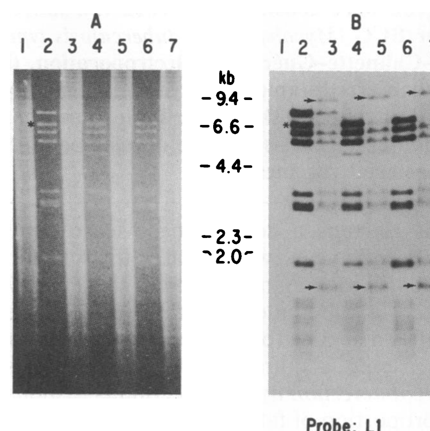


FIG. 1. Integration of mycobacteriophage L1 and L1 shuttle phasmid DNA into the *M. smegmatis* chromosome. DNAs from phage L1 and L1 shuttle phasmids and chromosomal DNAs from corresponding lysogens were digested with *Bam*HI and electrophoresed in 0.8% agarose. (*A*) Ethidium bromide-stained gel. (*B*) Autoradiograph of the Southern analysis of this gel probed with ^{32}P-labeled phage L1 DNA. Phage DNAs from the parent phage L1 (lane 2), shuttle phasmid phAE15 (lane 4), and shuttle phasmid phAE19 containing the *aph* gene (lane 6) and bacterial chromosomal DNAs from the parent *M. smegmatis* strain (lane 1), the parental strain lysogenized with L1 (lane 3), the parental strain lysogenized with phAE15 (lane 5), and the parental strain lysogenized with phAE19 (lane 7) are shown. L1, phAE15, and phAE19 have undergone site-specific integration within the chromosome of their respective lysogens (*B*, lanes 3, 5, and 7) as shown by the predominant loss of a single 6.7-kb band present in each phage (note asterisk in L1, lane 2) and the appearance of two new bands, 9.0 kb and 1.7 kb, in each lysogen (arrows).

taining kanamycin (10 μg/ml). The *M. smegmatis* transformants (in agar) were also found to be resistant to chloramphenicol at >100 μg/ml.

Isolation and Characterization of Plasmid DNA from Mycobacteria. Plasmid DNA was isolated from 1-ml samples of cells harvested at early stationary phase by a modification of the procedure of Birnboim and Doly (20). Sequential incubations in lysozyme, alkaline/$NaDodSO_4$, and high salt were each extended for 16 hr, and then the remainder of the protocol was followed. Plasmid DNA isolated from both *M. smegmatis* and BCG transformants would transform χ2338 cells and confer both kanamycin and chloramphenicol resistance.

RESULTS AND DISCUSSION

Development of Shuttle Phasmids Capable of Lysogenizing Mycobacteria. The introduction of foreign DNA into mycobacteria by means of phages initially prompted us to screen a group of mycobacteriophages to identify those that might be temperate and stably lysogenize the rapidly growing *M. smegmatis*. One such phage, L1, had been reported (10, 22) to produce turbid plaques on *M. smegmatis*; putative lysogens were resistant to superinfection and could be induced to produce phage. We confirmed these observations and have been able to demonstrate by Southern analysis that a prophage is integrated in the *M. smegmatis* chromosome (Fig. 1*B*, lanes 2 and 3). Analysis of multiple, independent lysogens revealed identical patterns of unique bands resulting from the phage integration, suggesting that L1 phage integration is site specific (Fig. 1).

Having established that L1 stably lysogenizes *M. smegmatis*, we proceeded to construct L1 shuttle phasmids by inserting the *E. coli* cosmid, pHC79, containing a ColE1 origin of replication and an ampicillin-resistance gene for selection in *E. coli* into a nonessential region of the L1 genome. The L1 shuttle phasmid phAE15 that was constructed replicates in *E. coli* as a plasmid and in *Mycobacterium* as a phage and, like the parent phage, integrates into the *M. smegmatis* chromosome (Fig. 1, lanes 4 and 5). Since the L1 phage is devoid of *Eco*RI sites, the introduction of pHC79 provided a unique *Eco*RI site for the phAE15 shuttle vector to allow the cloning of genes of interest. These genes can be introduced and stably maintained within mycobacteria

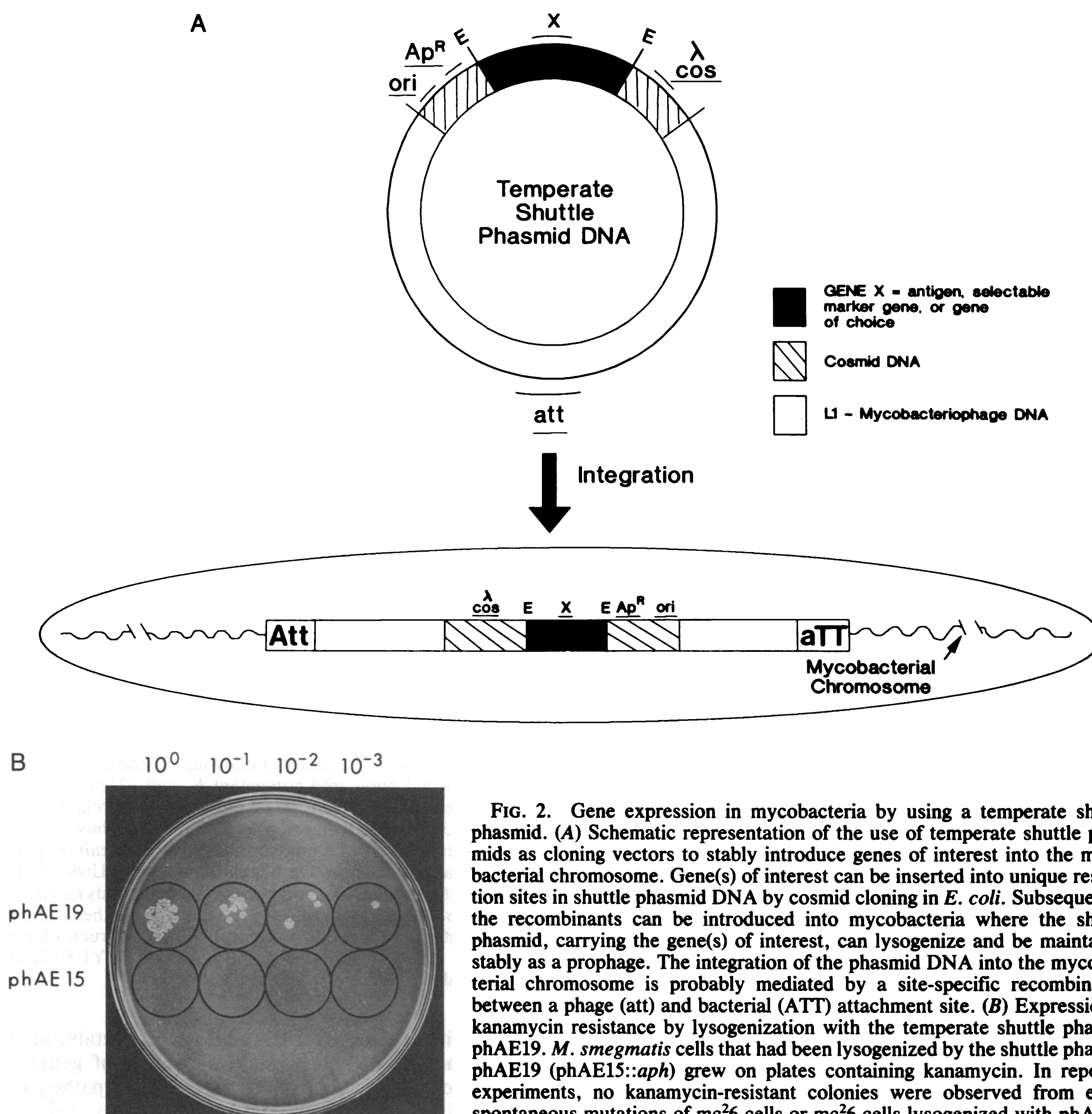
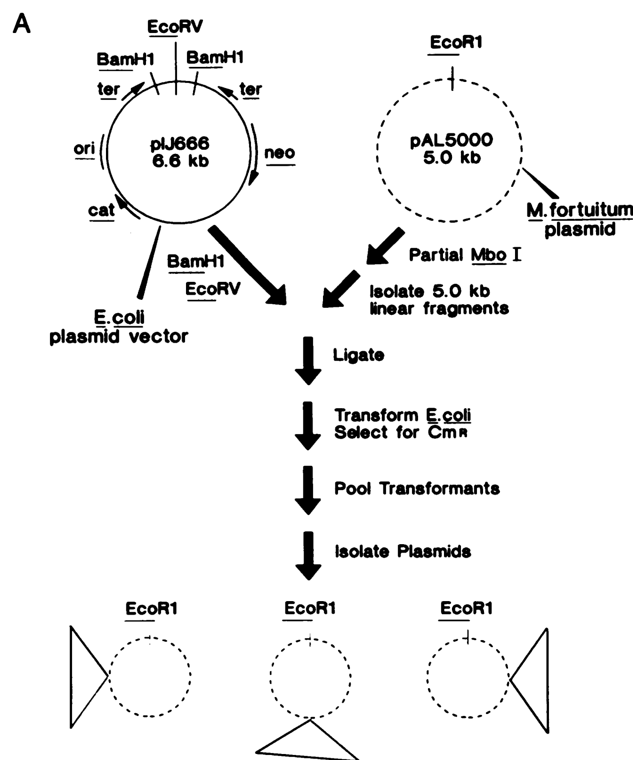


FIG. 2. Gene expression in mycobacteria by using a temperate shuttle phasmid. (*A*) Schematic representation of the use of temperate shuttle phasmids as cloning vectors to stably introduce genes of interest into the mycobacterial chromosome. Gene(s) of interest can be inserted into unique restriction sites in shuttle phasmid DNA by cosmid cloning in *E. coli*. Subsequently, the recombinants can be introduced into mycobacteria where the shuttle phasmid, carrying the gene(s) of interest, can lysogenize and be maintained stably as a prophage. The integration of the phasmid DNA into the mycobacterial chromosome is probably mediated by a site-specific recombination between a phage (att) and bacterial (ATT) attachment site. (*B*) Expression of kanamycin resistance by lysogenization with the temperate shuttle phasmid phAE19. *M. smegmatis* cells that had been lysogenized by the shuttle phasmid phAE19 (phAE15::*aph*) grew on plates containing kanamycin. In repeated experiments, no kanamycin-resistant colonies were observed from either spontaneous mutations of mc^26 cells or mc^26 cells lysogenized with phAE15.

upon lysogenization with the shuttle phasmid vector (Fig. 2A). A 1.6-kb DNA fragment containing the *aph* gene from Tn*903*, which confers kanamycin resistance in *E. coli*, was inserted into phAE15 by a cosmid cloning strategy. The *aph* gene with *Eco*RI ends was ligated to linear phAE15 DNA and packaged *in vitro* into bacteriophage λ heads, and the resulting recombinant molecules were transduced into *E. coli*. Closed circular phasmid DNA was isolated from an *E. coli* clone that was resistant to both ampicillin and kanamycin and was transfected into *M. smegmatis* protoplasts, yielding mycobacterial phage particles that had packaged phasmid DNA. This phasmid, designated phAE19, had the ability to lysogenize *M. smegmatis* cells and generate kanamycin-resistant colonies (Fig. 2B). In repeated experiments, kanamycin-resistant colonies did not arise from either spontaneous mutations of $mc^2 6$ cells or $mc^2 6$ cells lysogenized with phAE15. Mycobacteriophages spontaneously released from $mc^2 6$ (phAE19) lysogens simultaneously cotransduced resistance to L1 superinfection and kanamycin to recipient *M. smegmatis* cells. This demonstrates that the resistance to kanamycin results from expression of the cloned *aph* gene. The shuttle phasmid phAE19 also integrates into the *M. smegmatis* chromosome upon lysogenization (Fig. 1, lanes 6 and 7). Preliminary data indicate that phAE19 can also lysogenize and confer kanamycin resistance upon BCG (data not shown). Kanamycin resistance thus represents a useful selectable marker for the mycobacteria. Overall, these results establish lysogeny as one means to introduce and express foreign genes in mycobacteria.

Plasmid Transformation of Mycobacteria. Plasmid-based genetic systems extend the capabilities of phages by offering increased cloning capacity, ease of DNA manipulation, and increased copy number. Because plasmids from *M. smegmatis* had not been described and genetic manipulation in mycobacteria is difficult, we sought to construct a shuttle plasmid vector that expressed kanamycin resistance and was capable of replication and of expressing genes in *E. coli* and *Mycobacterium*. To ensure a functional replicon for mycobacteria, we randomly inserted an *E. coli* plasmid, pIJ666 (17), containing the *neo* gene from Tn*5* (17, 18), the P15A origin of replication, and chloramphenicol acetyltransferase gene from pACYC184 (19), into a plasmid, pAL5000 (16), that replicates in *M. fortuitum*. Fig. 3A outlines the construction of the pIJ666::pAL5000 library. Successful transformation of this library into *M. smegmatis* spheroplasts by standard techniques has been problematic, possibly due to the difficulty in regenerating viable cells from spheroplasts. DNA was therefore introduced directly into intact *M. smegmatis* cells by electroporation. Initially conditions were developed for electroporation with lytic D29 phage DNA to monitor uptake; it yielded $>5 \times 10^3$ plaque-forming units/μg. By using optimal conditions, electroporation of the pIJ666:: pAL5000 DNA library into *M. smegmatis* yielded 1–10 kanamycin-resistant transformants per μg of library DNA in six independent experiments. These transformants were also found to be resistant to chloramphenicol, thus demonstrating the expression of the chloramphenicol acetyltransferase gene in *M. smegmatis*. Plasmid DNA isolated from pools of *M. smegmatis* transformants from three experiments yielded kanamycin-resistant *E. coli* and *M. smegmatis* cells when retransformed. Although pIJ666 was inserted at different sites within pAL5000 in many of the isolated *E. coli* transformants (data not shown), at least three shuttle plasmids were stable in both bacterial genera (Fig. 3B). Finally, these methods have made it possible to transform BCG substrains with the pIJ666::pAL5000 recombinant library, with stable expression of kanamycin resistance (Fig. 4). Whether a pAL5000 origin of replication in the hybrid plasmids is functional in *M. smegmatis* or BCG remains to be critically examined.



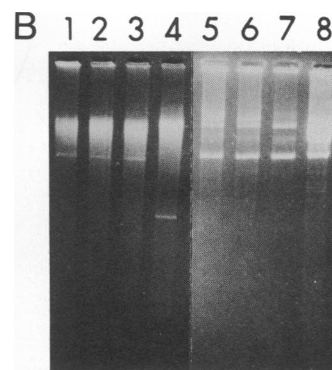


FIG. 3. Construction and analysis of *E. coli–Mycobacterium* shuttle plasmids. (*A*) Schematic of the overall strategy used to generate a library of hybrid plasmid molecules consisting of an *E. coli* plasmid pIJ666 that contains marker genes conferring resistance to neomycin/kanamycin (*neo*) and chloramphenicol (*cat*), inserted at random sites around the pAL5000 genome. (*B*) Agarose gel electrophoretic analysis of DNA from pIJ666::pAL5000 recombinant shuttle plasmids isolated from three independent pools of *M. smegmatis* transformants (lanes 1, 2, and 3). Following separate transfer of each of these plasmid pools into competent *E. coli* χ2338 cells, unique plasmids were isolated from single purified transformants and designated pYUB12, pYUB16, and pYUB17; they are shown in lanes 5, 6, and 7, respectively. Lane 4 contains the *M. fortuitum* plasmid, pAL5000, and lane 8 contains the library of pIJ666::pAL5000 recombinant plasmids. The size of the shuttle plasmids isolated from either *M. smegmatis* or *E. coli* is identical to the size of the recombinant library indicating stability of the construct. (A restriction map of the pIJ666::pAL5000 shuttle plasmid, pYUB12, will be made available to any investigator upon request.)

The ability to express foreign genes and selectable markers in mycobacteria makes possible a number of genetic approaches to understanding questions of disease pathogenesis. By using either phage or plasmid vector systems, it should be

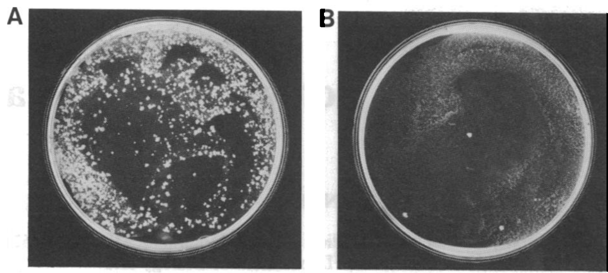


FIG. 4. Transformation of BCG with shuttle plasmid DNA. Kanamycin-resistant colonies of BCG that arose after electroporation of BCG with shuttle plasmid DNA isolated from a pool of BCG transformants (*A*) and mock transformed cells (*B*).

possible to insertionally inactivate and mark specific genes of pathogenic *M. tuberculosis*, either by homologous recombination or by transposon-mediated insertion, with the aim of identifying specific genetic determinants required for virulence and pathogenesis. By specifically deleting or replacing those genes, it may be possible to develop a more specific and effective attenuated vaccine against tuberculosis than the BCG vaccine. Alternatively, as specific protective antigens for tuberculosis and leprosy are identified by studying antigens recognized by T cells from resistant individuals, it will now be possible to incorporate and express them in existing BCG vaccines. With the genetic approaches described here, we have established that the *neo* gene encoding kanamycin resistance can be stably expressed in certain BCG substrains. Since BCG is currently one of the most widely used vaccines in the world—and one of the safest—it is our hope that the genetic systems described here will permit the development of BCG into a multivaccine vehicle, capable of simultaneously expressing genes encoding protective antigens of a variety of bacterial, viral, and parasitic pathogens.

In this collaboration, the temperate shuttle phasmids were developed by S. Snapper, the hybrid shuttle plasmid was constructed by A. Jekkel, R. E. Melton, and T. Kieser, and transformation of BCG was accomplished by L. Lugosi. We express our appreciation to Drs. Ron Davis, Richard Young, Bruce Chassy, Frank Bayliss, David Hopwood, Julius Marmur, and Stanley Falkow for helpful discussion and suggestions; and to Margareta Tuckman for important technical assistance. This work was supported by grants and the Medical Scientist Training Program (S.B.S) from the National Institutes of Health, United Nations Development Programme/World Bank/ World Health Organization Special Programme for Research and Training in Tropical Diseases, World Health Organization Special Programme for Vaccine Development, the Rockefeller Foundation, the Irvington House Institute for Medical Research, and the Weinstock Chair (B.R.B.).

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