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Gene Replacement and Expression of Foreign DNA in Mycobacteria

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A system that permits molecular genetic manipulation of mycobacteria was developed on the basis of the yeast paradigm of gene replacement by homologous recombination. A shuttle vector that can replicate autonomously at a high copy number in *Escherichia coli* but must integrate into homologous DNA for survival in *Mycobacterium smegmatis* was constructed. The vector contains a ColE1 origin of replication, antibiotic resistance markers for ampicillin and kanamycin, a nutritional marker (*pyrF*) that allows both positive and negative selection in *E. coli* and *M. smegmatis*, and unique restriction sites that permit insertion of foreign DNA. Transformation of mycobacteria with this vector results in integration of its DNA into the genomic *pyrF* locus by either a single or a double homologous recombination event. With this system, the 65-kilodalton *Mycobacterium leprae* stress protein antigen was inserted into the *M. smegmatis* genome and expressed. This gene replacement technology, together with a uniquely useful *pyrF* marker, should be valuable for investigating mycobacterial pathobiology, for the development of candidate mycobacterial vaccine vehicles, and as a model for the development of molecular genetic systems in other pathogenic microorganisms.

The availability of molecular genetic tools for pathogenic microorganisms is useful for investigating the biology of these pathogens. The ease with which DNA manipulated in vitro can be introduced into the *Saccharomyces cerevisiae* genome has made *S. cerevisiae* one of the preferred systems for investigating a wide range of biological problems (6). The ability to replace genomic DNA sequences with homologous DNA manipulated in vitro has provided a particularly sophisticated tool for researchers who study the molecular genetics of *S. cerevisiae*. The development of similar gene replacement technology for pathogenic microorganisms would facilitate the study of these organisms and their pathobiology.

Mycobacterial diseases are among the oldest infectious diseases described, and they remain important causes of human morbidity and mortality worldwide. The most important mycobacterial diseases are leprosy, caused by *Mycobacterium leprae*; tuberculosis, caused by *M. tuberculosis*; and disseminated infection with *M. avium* in individuals with acquired immune deficiency syndrome (4, 8, 12, 17). Despite the importance of these pathogens, the mechanisms of mycobacterial pathogenesis are poorly understood.

M. bovis bacillus Calmette-Guérin (BCG) is an avirulent strain of *M. bovis*, attenuated by 230 serial passages in vitro over 13 years (7), and has been widely used as a vaccine for the prevention of tuberculosis. It has been estimated that 1.9 billion people were vaccinated with BCG between 1948 and 1974 (13). While its protective efficacy against tuberculosis varies in different settings (14), it is clear that BCG and other mycobacteria have potent nonspecific immunostimulatory properties, and preparations that contain mycobacterial components are widely used as adjuvants.

Mycobacteria have been proposed for use as live vaccine vehicles (18). Genes encoding antigens from a variety of pathogens could be expressed in recombinant mycobacteria such that the adjuvant properties of the mycobacteria would stimulate an immune response to the foreign antigens which, in turn, might provide protective immunity against these pathogens. Mycobacteria offer several advantages as vaccine vehicles, including a long record of use in humans with a very low incidence of complications, stability in the field, and single-dose usage. The ability to manipulate the mycobacterial genome is essential to developing this candidate vaccine vehicle.

The ideal molecular genetic system includes a transformation method, vectors that can be manipulated in vitro, selectable nutritional and drug markers, and an ability to replace specific genomic DNA sequences with exogenous sequences. Mycobacteria can be transformed through spheroplasting or by electroporation (11, 18). Bacteriophage and plasmid shuttle vectors that can be propagated in *Escherichia coli* and mycobacteria, confer kanamycin resistance, and permit phage lysogeny or autonomous plasmid replication in mycobacteria have been constructed (11, 18). We describe here the means to introduce DNA manipulated in vitro into the mycobacterial genome by homologous recombination and the use of this system to stably integrate foreign DNA and express foreign antigens in the rapidly growing, nonpathogenic mycobacterium *M. smegmatis*.

MATERIALS AND METHODS

Bacterial strains. *E. coli* Y1103 [*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rps* (Sm^r) *xyl-5 mtl-1 supE44 pyrF*::Tn*5*] (alias DB6507 [1]) was obtained from D. Botstein. *E. coli* DH5α was obtained from Bethesda Research Laboratories. The *M. smegmatis* prototrophic strain Y5001 (alias mc^2-6), a single-colony isolate of ATCC 607, was obtained from W. Jacobs (11).

***M. smegmatis* genomic DNA library.** *M. smegmatis* genomic DNA was obtained from strain Y5001, which was grown in tryptic soy broth supplemented with 1% glucose and 0.05% Tween 80. Cultures were grown at 37°C to an optical density at 600 nm of 0.5 to 0.7, glycine was added to 0.5%, and the cells were permitted to grow to an optical density at 600 nm of 1 to 2. The cells were harvested by centrifugation, washed and suspended in 50 mM Tris (pH

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8.0)–10 mM EDTA–10% sucrose–0.5 mg of lysozyme per ml, and incubated at 37°C for 1 h. EDTA was added to 50 mM, sodium dodecyl sulfate was added to 1%, and the mixture was incubated at room temperature for 15 min. Three phenol-chloroform extractions were performed and were followed by isopropanol precipitation, RNase treatment, phenol-chloroform extraction, chloroform extraction, and ethanol precipitation. The DNA was suspended in TE (10 mM Tris [pH 7.5], 1 mM EDTA).

To construct the genomic DNA library, *M. smegmatis* genomic DNA was partially digested with *Sau*3A, DNA fragments (2 to 6 kilobases [kb]) were isolated by agarose gel electrophoresis and electroelution, and the DNA was ethanol precipitated and ligated into *Bam*HI-digested, calf intestinal phosphatase-treated pUC19 (19) DNA. Transformation-competent *E. coli* DH5α cells were prepared by the method of Hanahan (10) and were transformed with the pUC19 *M. smegmatis* library and plated onto Luria Bertani agar (15) containing 50 μg of ampicillin per ml. Plasmid DNA was obtained from a pool of transformed colonies by an alkaline lysis method (15).

Isolation and characterization of *M. smegmatis pyrF*. *E. coli* uracil auxotrophs DB6507 and DB6656 were transformed with the recombinant *M. smegmatis* DNA library, and transformants capable of growing in the absence of uracil were isolated. Of approximately 2×10^5 transformants capable of ampicillin resistance, 31 produced colonies on plates lacking uracil. Plasmid DNA was isolated by alkaline lysis from 10 single Ura^+ colonies grown in liquid medium. The recombinant plasmid DNAs were mapped with restriction endonucleases by standard methods (15). The plasmid pY6001 (Fig. 1B) was selected for further study.

Construction of recombinant shuttle vectors. The 1.3-kb *Bam*HI fragment of Tn*903* encoding aminoglycoside phosphotransferase (*aph*) was isolated from pUC4KSac (2) and ligated into the unique *Bam*HI site of pY6001. The resulting construct was designated pY6002 (Fig. 1B). The 3.6-kb *Eco*RI insert DNA fragment of the λgt11 clone Y3178, which contains the gene encoding the 65-kilodalton (kDa) antigen of *M. leprae* (16, 20), was isolated, blunt-ended, and ligated into the unique *Eco*RV and *Xho*I sites of pY6002 to produce pY6003 and pY6004, respectively. The 3.6-kb fragment was ligated into pY6002 either directly (in the *Eco*RV site) or by using synthetic DNA linkers (in the *Xho*I site). In both cases, the transcriptional orientation of the *aph* and 65-kDa genes is the same as the transcriptional orientation of the mycobacterial *pyrF* gene.

Transformation and manipulation of mycobacteria. Electroporation of mycobacteria was carried out in a manner similar to the procedure recently described (18). *M. smegmatis* cells were grown in Middlebrook 7H9 broth (Difco Laboratories) supplemented with 10% albumin-dextrose complex (2% glucose, 5% bovine serum albumin, 0.85% NaCl) (ADC) and 0.05% Tween 80 (M-ADC-TW medium) to an optical density at 600 nm of approximately 0.5. Fifty milliliters of cells was pelleted, washed in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.0), and suspended in 2.5 ml of 10 mM HEPES (pH 7.0)–10% glycerol. DNA (1 to 10 μg) was added to 0.8 ml of cell suspension, and the cells were subjected to a single pulse of 6.25 V/cm at 25 μF with a Gene Pulser (Bio-Rad Laboratories). The cell-DNA mixture was then added to 3 to 5 volumes of M-ADC-TW medium and incubated at 37°C for 2 to 3 h. The cells were pelleted and resuspended in 0.5 to 1.0 ml of M-ADC-TW medium, and 0.1 ml of cells per plate was plated onto TSGUT medium (tryptic soy agar supplemented with 1% glucose, 0.2 mM uracil, and 0.05% Tween 80) containing 10 μg of kanamycin per ml. When appropriate, transformants were subsequently screened on plates containing TSGUT medium supplemented with 1 mg of 5-fluoroorotic acid (5-FOA) per ml.

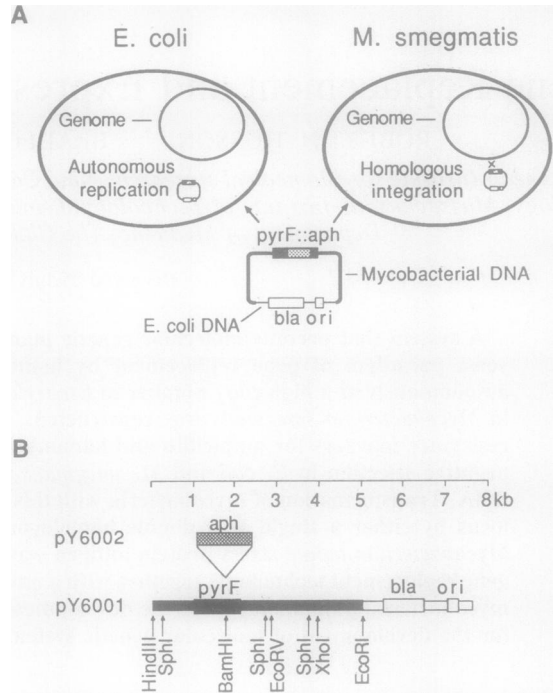

FIG. 1. Strategy for molecular genetic manipulation of mycobacteria. (A) Diagram of gene replacement strategy. A vector which permits autonomous replication in *E. coli* and integration by homologous recombination in *M. smegmatis* was constructed. The vector contains the ColE1 origin of replication (ori), which functions only in *E. coli*; the β-lactamase gene (bla), which confers ampicillin resistance; and mycobacterial DNA including the *pyrF* gene, which is disrupted by the aminoglycoside phosphotransferase gene (*aph*), which confers kanamycin resistance. (B) Diagram of the vectors constructed for genetic manipulation. Symbols: ▭, *E. coli* DNA; ▬, *M. smegmatis* DNA; ▩, the *aph* gene, which is inserted into the *Bam*HI site of pY6001 to make pY6002. The pY6001 vector is represented as linearized by *Hin*dIII.

Southern blot analysis. Genomic DNAs from *M. smegmatis* Y5001 and a variety of transformants were isolated as described above, digested with restriction enzymes, subjected to agarose gel electrophoresis in the presence of ethidium bromide, transferred to nitrocellulose, and probed with DNA labeled with ^{32}P by nick translation, all by standard procedures (15).

Stability of class I transformants in *M. smegmatis*. The stability of class I transformants, which contain an entire copy of the incoming plasmid integrated into homologous sequences in the genome, was investigated by determining the frequency at which the functional *pyrF* gene was lost from pY6002 class I transformants. This frequency should reflect the rate at which the plasmid loops out of the genome via homologous recombination. To determine the approximate frequency of loss of plasmid sequences in the absence of any selection, Y5004 cells from plates containing kanamycin and lacking uracil (selective medium) were streaked for single colonies on medium lacking kanamycin and containing uracil (nonselective medium). Each of 10 colonies (each colony contains approximately 10^6 cells) was sus-

pended in 0.2 ml of TSGUT medium, and the number of cells able to form colonies on nonselective medium was compared with the number of cells able to form colonies on TSGUT medium supplemented with 10 μg of kanamycin per ml and 1 mg of 5-FOA per ml. In this experiment, the fraction of cells that were able to grow in the presence of 5-FOA ranged from 1 in 1,000 to 1 in 20,000, with an average of approximately 1 in 5,000. Thus, the loss of functional *pyrF* sequences occurs at a frequency of about 2×10^{-4}. Assuming that this represents half of the loop-out events, the frequency of plasmid loss from the genome is approximately 4×10^{-4}. The frequency of spontaneous 5-FOA resistance in Y5001 wild-type cells was approximately 10^{-5}.

Western blot (immunoblot) analysis. Lysates of *M. smegmatis* and *E. coli* transformants were prepared by pelleting 1 ml of cells from a late-log-phase culture grown in tryptic soy broth, suspending the pellet in 0.2 ml of gel loading buffer (63 mM Tris [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 0.004% bromophenol blue, 5% β-mercaptoethanol), heating to 100°C for 5 min, and clearing the lysate by microcentrifugation. The cleared lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% minigels, transferred to nitrocellulose, and probed with the *M. leprae*-specific anti-65-kDa monoclonal antibody IIIE9 (9, 16) at a dilution of approximately 1:1,000. The Protoblot alkaline phosphatase detection kit (Promega Biotec) was used to detect binding of the antibody according to the instructions of the manufacturer.

RESULTS

Construction of an *E. coli-M. smegmatis* integrating shuttle vector. A shuttle vector capable of autonomous replication in *E. coli* and genomic integration in *M. smegmatis* (Fig. 1) was constructed. This vector contains pUC19 DNA, which has an origin of replication that permits high-copy-number, autonomous propagation in *E. coli* but not in *M. smegmatis*, and a gene for ampicillin resistance (Ap^r) (19). The vector also contains the *M. smegmatis pyrF* gene, which permits integration into homologous *M. smegmatis* genomic DNA and provides a nutritional marker that allows both positive and negative genetic selections. A functional *pyrF* gene, which encodes orotidine monophosphate decarboxylase, allows cells to grow in medium lacking uracil yet is lethal to cells grown in the presence of 5-FOA (5). Finally, unique *Bam*HI, *Xho*I, and *Eco*RV sites in the *M. smegmatis* DNA permit insertion of foreign DNA into the shuttle vector.

The shuttle vector was constructed by creating an *M. smegmatis* recombinant DNA library in pUC19 and isolating clones that complement the uracil auxotrophy of *E. coli pyrF* mutants. The pUC19 *M. smegmatis* DNA library contained 3×10^4 recombinants with insert sizes of 2 to 6 kb. The *pyrF* mutant DB6507 was transformed with the plasmid library, and transformants were selected on minimal media lacking uracil. Plasmid DNA was isolated from 10 colonies and was mapped with restriction endonucleases. All of the plasmids contained insert DNAs with overlapping restriction maps, and all of the inserts were oriented in the same direction in the vector. A representative clone, pY6001 (Fig. 1), was selected for further experiments.

Introduction of foreign DNA into the *M. smegmatis* genome. Because *M. smegmatis* cells are sensitive to the aminoglycoside kanamycin, an aminoglycoside phosphotransferase (*aph*) gene was selected as a drug-resistance marker. The *aph* gene of Tn*903*, which confers resistance to kanamycin in *E. coli* (3), was inserted into the unique *Bam*HI site of pY6001 to create pY6002 (Fig. 1B). *E. coli* transformed with this plasmid is resistant to kanamycin at concentrations in excess of 40 μg/ml. The uracil auxotrophy of *pyrF E. coli* mutants is not complemented by pY6002. Limited sequence analysis of the *M. smegmatis* DNA in pY6002 indicates that the lack of complementation is due to disruption of the *pyrF* coding sequence (not shown).

M. smegmatis Y5001 cells were transformed with pY6002 by electroporation and plated on TSGUT medium containing 10 μg of kanamycin per ml. In 10 independent experiments, the transformation frequency ranged from 10 to 500 transformants per μg of pY6002 DNA and colonies appeared 2 to 3 days after plating. The *M. smegmatis* colonies were resistant to at least 60 μg of kanamycin per ml in TSGUT plates. Four *M. smegmatis* transformants (Y5003, Y5004, Y5009, and Y5010) were colony purified, and DNA was prepared from each. DNAs from the wild-type strain and the four transformants were digested with the restriction endonuclease *Sph*I, subjected to agarose gel electrophoresis, transferred to nitrocellulose, and probed with labeled *M. smegmatis pyrF* DNA, *aph* DNA, and pUC19 DNA (Fig. 2).

The Southern blots revealed that two classes of transformants were obtained. In transformants Y5004 and Y5009 (class I transformants), the entire transforming DNA (including pUC19 DNA, mycobacterial sequences, and the *aph* gene) was integrated into homologous genomic DNA, apparently via a single homologous recombination event. In transformants Y5003 and Y5010 (class II transformants), the mycobacterial sequences containing the *aph* gene replaced the homologous wild-type genomic DNA sequences, apparently by a double crossover. DNA from the class II transformants contained only the *aph*-disrupted *pyrF* gene and did not contain pUC19 DNA. Episomal pY6002 was not detected in DNA from either class of transformants, as determined by Southern analysis of DNA digested with *Eco*RI (not shown). We infer that pY6002 DNA is stable in mycobacteria only if integrated in genomic DNA and that integration occurs via homologous recombination.

As expected, class I transformants, which contain one functional and one disrupted *pyrF* gene, are uracil prototrophs and are sensitive to 5-FOA. Class II transformants, with a single disrupted *pyrF* gene, are uracil auxotrophs and are resistant to 5-FOA. Thus, the two classes of transformants can be distinguished by differences in their requirements for uracil and their sensitivities to 5-FOA.

It is likely that the gene replacement observed in class II transformants is either a consequence of a double recombination event or a result of integration of the entire transforming plasmid DNA followed by removal of duplicated sequences. Class I and class II transformants occurred at similar frequencies in this and other experiments; analysis of 47 transformants revealed that 60% were class I and 40% were class II. However, the rate at which duplicated sequences in class I transformants loop out by recombination, producing cells identical to class II transformants, was found to be less than 10^{-3} (see Materials and Methods). We conclude that gene replacement in *M. smegmatis* transformants is predominantly a consequence of a double recombination event and is only infrequently the result of loss of the duplicated sequences in class I transformants.

To confirm and extend these results, the *aph*-disrupted *pyrF* gene in a class II transformant was replaced with a wild-type *pyrF* gene by electroporating the class II cells with pY6001 DNA and selecting for uracil prototrophs on minimal medium plates lacking uracil. Both classes of transformants were again obtained. Southern analysis of genomic DNA

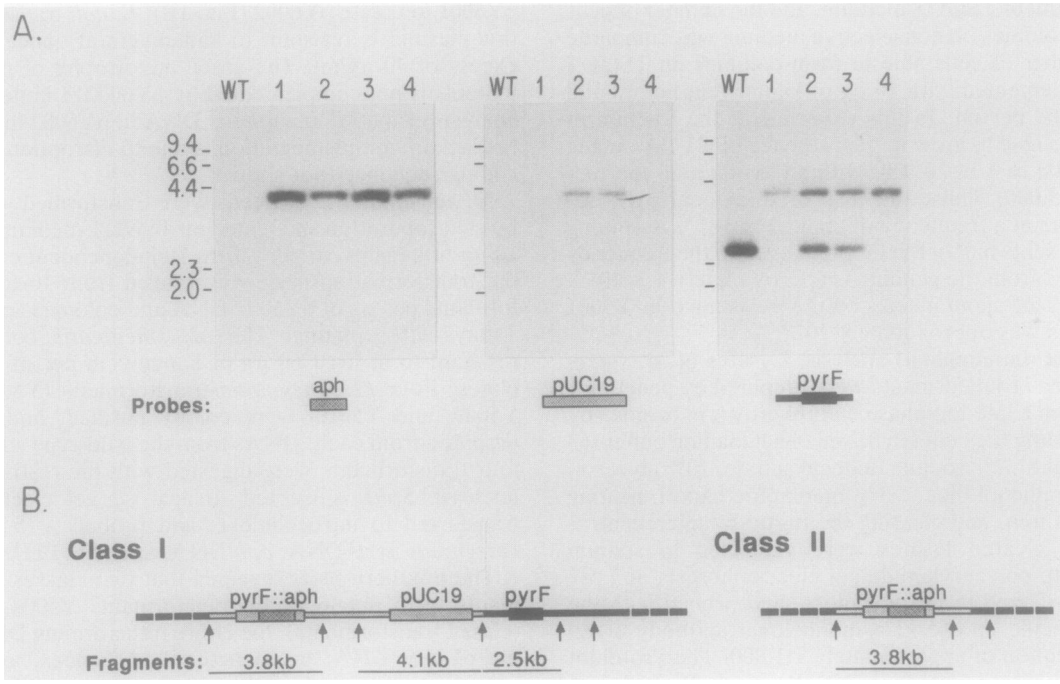

FIG. 2. Integration by homologous recombination. (A) Southern blot analysis of *M. smegmatis* wild-type and transformed cells. DNAs from wild-type cells and transformants Y5003 (lane 1), Y5004 (lane 2), Y5009 (lane 3), and Y5010 (lane 4) were digested with the restriction enzyme *Sph*I, subjected to agarose gel electrophoresis, transferred to nitrocellulose, and probed with labeled restriction fragments containing *aph*, pUC19, or *M. smegmatis pyrF* DNA. (B) Schematic interpretation of data shown in panel A. Shaded boxes represent exogenous DNA, and closed boxes represent wild-type *M. smegmatis* genomic DNA.

from the uracil prototrophs revealed that cells sensitive to both 5-FOA and kanamycin contained a single wild-type *pyrF* gene, while cells sensitive to 5-FOA but resistant to kanamycin contained the original *aph*-disrupted *pyrF* gene plus integrated pY6001 DNA.

Expression of a pathogen antigen in *M. smegmatis*. A gene encoding an antigen from a human pathogen was introduced into the *M. smegmatis* genome to examine the ability of *M. smegmatis* to express foreign DNA. The gene encoding the 65-kDa antigen of *M. leprae* was chosen because it is a well-characterized target of the immune response in persons with leprosy (20), because the *M. leprae* gene expression signals might be recognized by *M. smegmatis* and might not require additional genetic engineering for expression, and because of the availability of the monoclonal antibody IIIE9 (9), which detects an epitope on the *M. leprae* 65-kDa antigen that is not shared by the homologous antigen in other mycobacteria (H. D. Engers et al., Letter, Infect. Immun. **48**:603–605, 1985). The 3.6-kb *Eco*RI insert DNA of λgt11 clone Y3178, which contains the gene encoding the *M. leprae* 65-kDa antigen, was inserted into the unique *Eco*RV and *Xho*I sites of pY6002 to create pY6003 and pY6004, respectively (Fig. 3A). *M. smegmatis* Y5001 was transformed with plasmids pY6003 and pY6004, and cells were plated on medium containing 10 μg of kanamycin per ml. The frequency of transformation was comparable to that obtained with pY6002 DNA. A pY6003 transformant (Y5006) and a pY6004 transformant (Y5005) were colony purified. *M. smegmatis* Y5005 and Y5006 were both resistant to kanamycin but not to 5-FOA, indicating that both strains are class I transformants.

Lysates of Y5005 and Y5006 were prepared, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with the *M. leprae*-specific monoclonal antibody IIIE9. The results (Fig. 3) show that the *M. leprae* 65-kDa antigen is expressed in both Y5005 and Y5006. The pattern of bands, with a major band at 65 kDa and multiple minor breakdown products below, is characteristic of this antigen in lysates of *M. leprae* (Engers et al., Letter) and recombinant *E. coli* (Fig. 3).

DISCUSSION

We have developed a system that permits the molecular genetic manipulation of mycobacterial genomic DNA. A plasmid shuttle vector that allows insertion of foreign DNA at unique restriction sites in vitro, autonomous high-copy propagation in *E. coli*, and stable integration in the mycobacterial genome by homologous recombination has been constructed. This shuttle vector has been used to introduce and express foreign DNA at the *M. smegmatis pyrF* gene locus.

The ability to select for integration of transforming DNA in the mycobacterial genome is a consequence of the lack of a plasmid origin of replication that functions in mycobacteria and the presence of the selectable markers *pyrF* and *aph*. Integration of the transforming DNA occurs either by a single homologous recombination event, in which the entire recombinant vector is integrated into genomic DNA, or by a double homologous recombination event, in which sequences at the *pyrF* locus are replaced by homologous sequences into which foreign DNA has been inserted. The two types of integrants can be distinguished by virtue of their requirements for uracil and their sensitivities to the drug 5-FOA. Class I transformants, which contain the entire plasmid, have both a functional and a disrupted copy of the *pyrF* gene; the functional *pyrF* gene product makes these cells uracil prototrophs and renders them sensitive to 5-

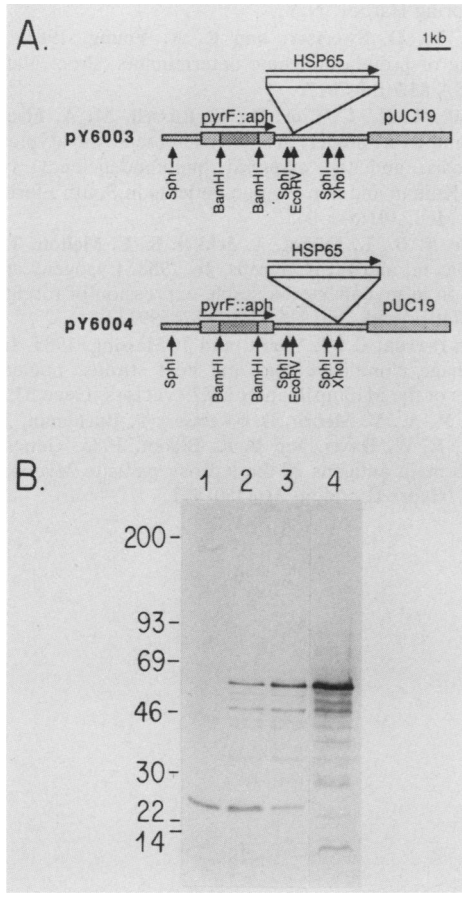

FIG. 3. Introduction and expression of the *M. leprae* gene encoding the 65-kDa stress protein (HSP65) in *M. smegmatis*. (A) Diagram of the plasmids used to transform *M. smegmatis* cells. The shaded region represents pY6002, and the *M. leprae* DNA is represented by an open box. (B) Western blot of protein lysates probed with the *M. leprae*-specific monoclonal antibody IIIE9. Lysates were from *M. smegmatis* wild-type cells Y5001 (lane 1), *M. smegmatis* transformants Y5005 (lane 2) and Y5006 (lane 3), and *E. coli* Y1089 containing the λgt11 clone Y3178, which expresses the intact *M. leprae* 65-kDa protein (lane 4). Transformants Y5005 and Y5006 contain DNA from plasmids pY6004 and pY6003, respectively. In addition to recognizing *M. leprae* HSP65, the monoclonal antibody IIIE9 binds to a 24-kDa *M. smegmatis* protein that is unrelated to HSP65 and that is observed in each of the lanes containing *M. smegmatis* extracts.

FOA. In contrast, class II transformants contain only a disrupted *pyrF* gene, are uracil auxotrophs, and are resistant to 5-FOA. Thus, cells that have undergone gene replacement (class II transformants) can be directly selected by plating transformants on medium containing both kanamycin and 5-FOA.

The *pyrF* gene is a particularly useful genetic tool because it permits positive and negative selections and because it does not suffer from two problems associated with drug resistance markers. Spontaneous resistance to many drugs occurs at relatively high frequencies in bacteria because of the large number of different mutations that can affect the ability of the cell to be permeated by drugs. In *S. cerevisiae*, disruption of the gene that encodes orotidine monophosphate decarboxylase (*URA3*) produces a uracil auxotroph that does not revert at measurable frequencies. The second potential problem with a drug resistance marker is that it is not desirable in potentially pathogenic organisms or in candidate live vaccines. Using the technology described here, one could replace the *aph*-disrupted *pyrF* gene in class II transformants with the wild-type *pyrF* gene without losing the adjacent foreign gene. Alternatively, one could begin with a *pyrF* deletion in the mycobacterial target cell which could be replaced with a wild-type *pyrF* gene plus adjacent foreign DNA.

The gene encoding the 65-kDa stress protein antigen of *M. leprae* was introduced into the *M. smegmatis pyrF* locus and was expressed at detectable levels. The gene expression signals utilized by *M. smegmatis* to produce this gene product, as well as that of the *aph* gene, remain undefined. The identification and incorporation of mycobacterial gene expression signals in the gene replacement system is necessary to obtain efficient and regulated expression of foreign genes in mycobacteria. The system described here should permit such an investigation of mycobacterial gene expression signals.

We and our colleagues (11, 18) have developed methods for molecular genetic manipulation of mycobacteria because of our interest in its biology and because of its potential use as a vaccine vehicle. Molecular genetic systems for most pathogenic bacteria and parasites remain to be developed. The use of electroporation to transform cells, shuttle vectors that do not rely on autonomous replication but can integrate via homologous DNA, and powerful selectable markers such as *pyrF* should be a useful strategy for the development of molecular genetic systems in many of these organisms.

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