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Molecular Analysis of DNA and Construction of Genomic Libraries of *Mycobacterium leprae*

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Molecular analysis of DNA from *Mycobacterium leprae*, "*Mycobacterium lufu*," and *Mycobacterium vaccae* has demonstrated that the G+C (guanine plus cytosine) contents of the DNAs are 56, 61, and 65%, respectively, and that the genome sizes are 2.2×10^9, 3.1×10^9, and 3.1×10^9 daltons, respectively. Because of the significant differences in both G+C content and genome size among *M. leprae*, "*M. lufu*," and *M. vaccae* DNAs, these species are not related, although hybridization experiments under nonstringent conditions, with two separate cloned *M. leprae* DNA inserts as probes, indicate that there are some conserved sequences among the DNAs. The G+C content of *Dasypus novemcinctus* (armadillo, the animal of choice for cultivating *M. leprae*) DNA was determined to be 36%. Genomic libraries potentially representing more than 99.99% of each genome were prepared by cloning into the cosmid vector, pHC79, in *Escherichia coli* K-12. A genomic library representing approximately 95% of the genome of *M. vaccae* was prepared in pBR322. *M. leprae* DNA was subcloned from the pHC79::*M. leprae* library into an expression vector, pYA626. This vector is a 3.8-kilobase derivative of pBR322 in which the promoter region of the *asd* (aspartate semialdehyde dehydrogenase) gene from *Streptococcus mutans* has been inserted in place of the *Eco*RI-to-*Pst*I fragment of pBR322. Several (44% of those tested) pYA626::*M. leprae* recombinants and one pBR322::*M. vaccae* recombinant synthesized new polypeptides in minicells of *E. coli*, indicating that mycobacterial DNA can be expressed in *E. coli* K-12, although expression is probably dependent upon use of nonmycobacterial promoters recognized by the *E. coli* transcription-translation apparatus.

Although *Mycobacterium leprae* was one of the first microorganisms to be implicated as the causative agent of a disease (leprosy), inability to grow this organism in the laboratory has greatly hampered studies on its genetics, physiology, and mechanism(s) of pathogenicity. In 1971, Kirchheimer and Storrs (21, 38) reported the successful experimental infection of armadillos (*Dasypus novemcinctus* Linnaeus), and since that time, experimentally infected armadillos have been a source of *M. leprae* for investigators throughout the world. Because available supplies of *M. leprae* are limited, investigators have attempted to identify other mycobacterial species that share antigens or antigenic determinants with *M. leprae* (e.g., *Mycobacterium vaccae*, *Mycobacterium bovis* BCG, and *Mycobacterium lepraemurium*) and thus might be used for development of a vaccine against leprosy (16, 37). Other investigators have used other mycobacterial species (e.g., "*Mycobacterium lufu*") with drug sensitivity profiles similar to those of *M. leprae* as model systems for determining the mode of action of diaminodiphenyl sulfone (dapsone), the drug of choice for treating leprosy, and for testing the efficacy of new drugs in the treatment of this disease (30, 34).

Recombinant DNA technology offers an obvious advantage for studying the genetics and physiology of *M. leprae*, provided that *M. leprae* genes are expressed in the host bacterial strain. In preparation for cloning *M. leprae* DNA, the mole percent G+C (guanine plus cytosine) content and genome sizes of *M. leprae*, *M. vaccae*, and "*M. lufu*" DNAs were determined. In addition, it was important to determine the G+C content of armadillo DNA to ensure separation of *M. leprae* DNA from any residual armadillo DNA before cloning. Moreover, the percent G+C of this DNA has not been reported.

Recombinant banks or libraries of *M. vaccae*, "*M. lufu*," and *M. leprae* DNAs were prepared with the cosmid cloning vector, pHC79 (18). In this paper we report the initial molecular analysis of these three mycobacterial DNAs, the construction of the cosmid libraries, and evidence for expression of mycobacterial DNA in *Escherichia coli* K-12.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. *E. coli* K-12 χ925, χ2001, and χ2819 were used as host strains for preparing the recombinant cosmid and plasmid libraries. All other bacterial strains were used as sources of DNA.

Armadillo tissue. An uninfected armadillo was obtained from the Singleton Trapping Company, Riverview, Fla., since armadillos with naturally occurring leprosylike infections have not been observed in Florida. This animal was sacrificed, and its liver was aseptically removed and used as a source of uninfected armadillo DNA.

Media and reagents. The cultivable bacteria were grown in the following media: *E. coli* and *Pseudomonas aeruginosa*, L broth (24) or minimal salts broth or agar supplemented with amino acids and glucose (9); *M. vaccae*, TB broth (Difco Laboratories, Detroit, Mich.); and "*M. lufu*," modified Dubos-Davis broth (Difco); the last two media were supplemented with 0.25% (wt/vol) bovine serum albumin fraction V (Difco).

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TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
E. coli K-12 χ289	F⁻ *tte-1* prototroph	11
E. coli K-12 χ925	F⁻ *thr-1 ara-13 leu-6 azi-8 tonA2 lacY1 minA1 glnV44 gal-6* λ⁻ *minB2 rpsL35 malA1 xyl-7 mtl-2 thi-1*	Single colony isolate of P678-54; 1
E. coli K-12 χ1849	F⁻ *tonA53 dapD8 minA1 purE41 glnV42* Δ*(gal-uvrB)40* λ⁻ *minB2 his-53 gyrA25 metC65 oms-1 tte-1* Δ*(bioH-asd)29 ilv-277 cycB2 cycA1 hsdR2*	17
E. coli K-12 χ2001	F⁻ Δ*araC766 tonA53 dapD8 proA370* Δ*lacZ39 minA1* Δ*(gal-chlD)69* λ⁻ *tyrT58* Δ*galU183* Δ*trpE5 minB2 rfb-2 recA56 relA1* Δ*thyA57 endA1 oms-1* Δ*asd-4 rpoB402 cycB2 cycA1 hsdR2*	7
E. coli K-12 χ2819	F⁻ *lacY1 glnV44 galK2 galT22* (λ *cI857 b2 red3 S7*) *recA56* Δ*thyA57 metB1 hsdR2*	This paper
M. leprae	Wild type	Armadillo liver #29 infected with *M. leprae* pooled from seven patients; received from C. Shepard, Centers for Disease Control, Atlanta, Ga.
"*M. lufu*"	Wild type	J. K. Seydel, Institut Borstel, West Germany
M. vaccae	Wild type	C. Shepard, Centers for Disease Control
Micrococcus luteus	Wild type	J. Lebowitz, University of Alabama in Birmingham
P. aeruginosa PAO-2	Wild type	J. B. Hansen, University of Alabama in Birmingham

Reagents for separation of *M. leprae* cells from armadillo tissue were those recommended by Draper (14). Buffer A contained 0.15 M NaCl, 0.015 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfon acid) and 1 mM $MgSO_4$, and buffer B contained 0.15 M NaCl, 0.2 M Tris buffer, and 1 mM $MgSO_4$. Both buffers were adjusted to pH 7.2. Buffered saline with Tween (BST) contained 0.15 M NaCl, 2.2 mM KH_2PO_4, and 4.2 mM Na_2HPO_4 (pH 7.0). Each of these three buffers contained 1 mM benzamidine as an inhibitor of armadillo cellular proteases (at the suggestion of T. Buchanan) and 0.2% (wt/vol) Tween 80. Percoll (Pharmacia Fine Chemicals, Piscatoway, N.J.), a colloidal suspension of silica particles coated with polyvinylpyrrolidone, was used to form density gradients. Polyethylene glycol (PEG)-palmitate–dextran was a phase mixture in which 7.0 g of dextran T500, 4.9 g of PEG 6000, and 0.1 g of PEG-palmitate (a generous gift of P. Brennan) were added to 63 ml of sterile 0.01 M potassium phosphate buffer (pH 6.9)–0.01 M NaCl and allowed to dissolve overnight.

Reagents for DNA isolation were described by Marmur (28). Saline-EDTA was 0.15 M NaCl–0.01 M EDTA (pH 8.0). Reagents used for deproteinization were 5 M $NaClO_4$ and Sevag solution (24 parts chloroform to 1 part isoamyl alcohol). Standard saline-citrate (SSC) was 0.15 M NaCl–0.015 M sodium citrate (pH 7).

All restriction endonucleases and T4 ligase were obtained from Bethesda Research Laboratories, Bethesda, Md., or New England Biolabs, Boston, Mass. All other enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions of proteinase K (protease XI; 10 mg/ml) and ribonuclease A (2 mg/ml) were divided into 1-ml portions and stored frozen at −20°C. Before freezing, the RNase stock solution was boiled for 10 min and then slowly cooled to inactivate DNases.

Separation of *M. leprae* cells from armadillo liver tissue. The method for quantitative recovery of *M. leprae* cells from infected armadillo liver was adapted from a method developed by Draper (14) and Shepard et al. (36).

Tissue homogenization was accomplished by cutting a 15-g sample of infected liver into pieces of approximately 1 cm^3 which were placed into a sterile 200-ml stainless steel Omnimixer cup (Sorvall Instruments, Norwalk, Conn.) with 60 ml of ice-cold buffer A. While the cup was immersed in ice, the tissue was homogenized for a total of 5 min (1 min of homogenization alternated with a 1-min rest) at top speed and then allowed to rest for 20 min. This homogenate was then transferred to sterile 30-ml Oak Ridge polycarbonate centrifuge tubes and spun at 12,000 rpm in a Sorvall SS34 rotor in an RC-5B centrifuge for 10 min at 4°C. (All subsequent sedimentations throughout the separation procedure were done in 30-ml polycarbonate Oak Ridge tubes in a Sorvall SS34 rotor.) As much of the supernatant fractions as possible was carefully removed (and discarded) without disturbing the pellets. The pellets were mixed with residual supernatant fluid with the piston of a Pyrex tissue grinder (13 by 100 mm) and then suspended in 10 ml of buffer A (total) by pipetting up and down with a 10-ml pipette until clumps were suspended. All suspended cells were transferred to the Omnimixer cup, and the centrifuge tubes were rinsed with buffer A until a final volume of 60 ml was obtained. All washes were added to the Omnimixer cup. This mixture was homogenized for an additional 3 min, allowed to rest, and centrifuged as described above. As much of the supernatant fractions as possible was again carefully removed (and discarded), and the pellets were completely suspended in the residual supernatant fluid plus 10 ml of fresh buffer A as described above. This mixture was passed through a sterilized wire mesh tea strainer (diameter, 3 in.) (to remove tissue strands) into a sterile 250-ml beaker. The contents of the beaker were then transferred to a sterile 250-ml screw-capped flask. Sufficient buffer A to make a final volume of 60 ml was used to thoroughly wash the centrifuge tubes,

strainer, and beaker; all washes were added to the flask containing the homogenized mixture.

Enzymatic digestion of armadillo collagen and DNA was accomplished by adding 0.2 ml of 1 M $CaCl_2$ and 6 mg of collagenase to the flask containing the homogenized mixture. The flask was incubated at 30°C with gentle aeration for 1 h. During a second hour of incubation, 0.6 mg of DNase was added to the mixture to digest armadillo DNA. The mixture was then transferred to sterile tubes and centrifuged at 12,000 rpm for 10 min. The supernatant fractions were carefully removed, and each pellet was suspended by vortexing in 5 ml of buffer B. The mixture can be stored overnight at 4°C at this point. In the morning, 10 ml of buffer B was added to each tube, and the tubes were centrifuged at 12,000 rpm for 10 min. The supernatant fractions were removed, the pellets were mixed with the tissue grinder piston and suspended in a total of 5 ml of BST, and the mixture was divided equally among six centrifuge tubes.

Density separation of *M. leprae* cells from armadillo tissue residue was accomplished by Percoll gradient separation with two different densities of Percoll. BST was added to each tube to bring the total volume to 9.6 ml per tube. Percoll (10.4 ml) was added to each tube over a period of approximately 2 min per tube (to avoid osmotic shock to the *M. leprae* cells) to give a final concentration of 52%. The six tubes were centrifuged at 15,000 rpm for 15 min at 4°C. The gradient formed has a number of bands (all measured in distance below the meniscus): (i) a white milky band that extends from the meniscus to approximately 3 mm, (ii) a brown liver cell band from 3 to 10 mm, (iii) an area of light-brown turbidity from 10 to 14 mm, (iv) a relatively clear area from 14 to 27 mm, (v) a band containing *M. leprae* cells (which starts out slightly turbid and then increases to a heavier band) from 27 to 46 mm, and (vi) a brown band from 46 to 49 mm. The top three bands and some of the clear portion were carefully removed from each tube with a Pasteur pipette; BST was added slowly to each tube until the total volume per tube was 15 ml. The tubes were mixed gently by inversion and then centrifuged at 12,000 rpm for 10 min at 4°C. As much supernatant fraction as possible was removed from each tube, the pellets were gently suspended in BST, and the mixture was divided evenly into four centrifuge tubes. The volume of BST in each tube was adjusted to 15 ml, and the tubes were centrifuged at 12,000 rpm for 10 min. At this point, the supernatant fractions were completely removed, and each pellet was suspended in BST. The density of the next Percoll gradient was dependent upon the extent of separation between liver cells and *M. leprae* cells achieved in the first gradient. If band iv was turbid, the four pellets each were suspended in 9.6 ml of BST and a second 52% Percoll gradient was run, as described above. When band iv was clear and bands v and vi overlapped, separation was on an 80% Percoll gradient. The pellets recovered from the 52% gradient were each suspended in 4 ml of BST. To each tube, 16 ml of Percoll was slowly added (to give a final concentration of 80% Percoll), and the four tubes were centrifuged at 15,000 rpm for 15 min. The gradient formed this time had the following bands (all measured in distance below the meniscus): (i) a brown layer at the meniscus; (ii) a white or beige layer of *M. leprae* cells from 2 to 5 mm (heavy band) plus a more diffuse band from 5 to 10 mm, (iii) a clear area from 10 to 35 mm, (iv) a diffuse brown band (containing liver tissue) from 35 to 48 mm, and (v) a pellet of brown and white material. The top two bands (containing *M. leprae* cells) were removed with a Pasteur pipette, diluted with BST, mixed by inversion, and centrifuged as they were after the first Percoll gradient. The pellets were washed with BST and centrifuged as described above; after the centrifugation, the pellets were suspended and combined in a total of 20 ml of BST.

The final purification of the *M. leprae* cells was achieved by phase separation. The cell suspension in BST was added to 80 ml of PEG-palmitate–dextran in a 250-ml separatory funnel, which was then inverted 100 times. The contents of the funnel were allowed to separate for approximately 30 min. More *M. leprae* cells can be recovered if they are collected when the liver band (which is in the lower half of the separatory funnel) is approximately 2 cm wide than when the liver band forms more tightly. The PEG-palmitate layer (cloudy white) was removed from the top of the funnel with a Pasteur pipette, added to two sterile centrifuge tubes, and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant fraction was removed and added to the dextran in the separatory funnel, and the phase separation was repeated two to four times (as long as good pellets of *M. leprae* cells were recovered after centrifugation of the PEG-palmitate layer). After the final phase separation, all cell pellets were combined and suspended in 15 ml of BST–1.5 ml of dimethyl sulfoxide in a polycarbonate centrifuge tube. The cells were frozen slowly at −70°C until used for DNA isolation. A 20-μl sample of the cells was removed before the addition of dimethyl sulfoxide for determining the titer of acid-fast bacilli.

The liver bands from the two Percoll gradients also were collected and washed with BST until good pellets were formed. All pellets were combined and suspended in a total of 15 ml of BST. A sample was removed for acid-fast staining to determine whether a significant number of *M. leprae* cells remained associated with the liver residues. If a significant titer of *M. leprae* cells was still present, the suspension was put through the PEG-palmitate–dextran phase separation to recover the *M. leprae* cells.

Isolation of DNA. *E. coli*, *P. aeruginosa*, and armadillo DNA were isolated by the Marmur procedure (28). To extract DNA from *M. leprae*, frozen cells from three separation procedures (1×10^{11} to 2×10^{11} cells) were thawed, centrifuged at 10,000 rpm for 10 min at 4°C, washed with saline-EDTA, and sedimented by centrifugation with complete removal of the supernatant fluid. The centrifuge tube was placed on dry ice, and the frozen pellet of cells was removed from the tube. *M. vaccae* and "*M. lufu*" cells were frozen at −20°C after they were harvested from their respective growth media. DNA was isolated from the mycobacteria as follows. Frozen cells were placed in a mortar which contained dry ice and glass beads (0.1 g of 20-μm-diameter beads; 3M Co., Minneapolis, Minn.). The cells were triturated for approximately 15 min (until the cells were ground to a fine powderlike consistency and the dry ice had sublimed) and were then transferred to a sterile screw-capped 125-ml flask. The mortar was allowed to warm to room temperature, and then 10 ml of saline-EDTA was used to wash any residual cells out of the mortar into the screw-capped flask. Egg-white lysozyme was added to a final concentration of 200 μg/ml, and the flask was incubated at 37°C with moderate aeration (e.g., 100 to 150 rpm on a platform shaker; Lab-Line Instruments, Inc., Melrose Park, Ill.) for 1 h. Proteinase K was added to the flask to a final concentration of 250 μg/ml, and the flask was incubated at 60 to 65°C for 15 min with occasional gentle swirling. Sodium dodecyl sulfate (SDS) was added to a final concentration of approximately 3.5% (vol/vol), and the flask was incubated at 60 to 65°C for an additional 15 min with occasional gentle

swirling. The flask was quickly cooled to room temperature by running cold water over it, and $NaClO_4$ then was added to a final concentration of 1 M. The flask was shaken gently (e.g., 75 rpm on a Lab-Line platform shaker) for 5 min at room temperature or 37°C. A volume of Sevag solution equal to the contents of the flask was added, and gentle shaking was continued for 15 min (at either room temperature or 37°C). The contents of the flask were transferred to chloroform-resistant 50-ml Oak Ridge centrifuge tubes and centrifuged at 10,000 rpm in a Sorvall SS34 rotor for 15 min at 4°C. The top aqueous layer was removed from each tube and placed in silicanized 30-ml Corex centrifuge tubes. The nucleic acids were recovered from these tubes by ethanol precipitation, suspended in 4.5 ml of 0.015 M NaCl–0.0015 M sodium citrate (when the nucleic acids were in solution, the salt concentration was adjusted to 0.15 M NaCl–0.015 M sodium citrate), and transferred to a 50-ml screw-capped flask. RNase was added to a final concentration of 50 μg/ml, and the flask was incubated at 37°C with gentle shaking for 1 h. Proteinase K was added to a final concentration of 100 μg/ml, and the flask was incubated at 60 to 65°C for 15 min with occasional gentle swirling. The flask was cooled to room temperature, and 0.98 g of cesium chloride (Kaweki Berylco Industries, Inc., Reading, Pa.) per ml of DNA solution was added. The solution was transferred to Beckman VTi65 Quik-seal tubes (Beckman Instruments, Inc., Palo Alto, Calif.), and 2.5 mg of ethidium bromide was added to each tube. The tubes then were centrifuged in a Beckman VTi65 rotor in an ultracentrifuge for 20 h at 45,000 rpm at 14°C. After centrifugation, the DNA bands were visually located with a short-wavelength UV light and recovered from the gradient. The bands were combined and run on a second cesium chloride-ethidium bromide gradient as described above. After collecting the bands from the second gradient, the ethidium bromide was removed with NaCl-saturated isopropanol (27), and the DNA was then dialyzed at 4°C against 10 mM Tris buffer (pH 8): three changes of 250 to 300 ml of buffer over an 18-h period. The DNA was removed from the dialysis tubing and placed in a screw-capped tube. EDTA (pH 8) was added to a final concentration of 1 mM, and the DNA was stored at 4°C.

Determination of G+C content. The G+C content of each DNA was determined from the midpoint of the melting curve measured optically at 260 nm (T_m) and from the isopycnic bouyant density of the DNA (32). The T_ms of the DNAs were determined in 0.015 M NaCl–0.0015 M sodium citrate by the method of Marmur and Doty (29) with a Gilford model 250 spectrophotometer equipped with a model 2257 Thermoprogrammer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). *E. coli* K-12 χ289 DNA was used as a reference standard. The G+C content of *E. coli* K-12 χ289 was calculated from the equation % G+C = 100 [(T_m/50.2) − 0.990], and the G+C contents of the other DNAs were calculated from the equation % G+C of unknown = 100 [G+C of *E. coli* + 0.0199 (T_m of unknown − T_m of *E. coli*)] (26).

The bouyant densities were determined by isopycnic ultracentrifugation in a Beckman model E ultracentrifuge, with *Micrococcus luteus* DNA (a gift of J. Lebowitz) as a reference standard. These densities were determined by Cheryl Goguen, Department of Microbiology, University of Alabama in Birmingham. The G+C contents of the DNA samples were determined from their bouyant densities by the equation % G+C = 100 [(ρ − 1.660)/0.098] (32).

Determination of genome sizes. Genome sizes of the mycobacterial DNAs were determined from the rates of reassociation of denatured DNA (C_0t analysis) as measured optically in the Gilford spectrophotometer (5, 6, 15). Each DNA sample was sheared by sonication of 10-s bursts alternating with 10-s rests for a total of 1 min. Sonication was done with the small probe of a Braun Sonic model 1510 (B. Braun Melsungen AG) at the highest speed (100 W). The DNA samples were suspended in 1.2 M NaCl–0.12 M sodium citrate, and the tubes containing the DNA were submerged in ice throughout the sonication. The DNA was diluted to 40 μg/ml, and formamide was added to a final solvent concentration of 25% (vol/vol) in 0.9 M NaCl–0.09 M sodium citrate (5). Denaturation and reassociation of the DNA samples were performed as described by Bradley (5). Absorbance of the DNA samples at 270 nm was monitored until the denatured DNA was at least 65% reassociated. *E. coli* χ289 DNA was the reference standard for the reassociation experiments. Genome sizes were determined from the equation $C_0t_{1/2}$ of *E. coli*/$C_0t_{1/2}$ of unknown = (2.5×10^9)/molecular weight of unknown DNA (15). A factor derived by Seidler and Mandel (33) was used to correct for the differences in the G+C content between the mycobacterial DNAs and *E. coli* DNA.

The DNA from the total pHC79::*M. leprae* recombinant library was sheared, denatured, and reassociated in the same way as the chromosomal DNAs to estimate the fraction of the *M. leprae* genome that is represented in the recombinant library. Sheared DNA from pHC79 was simultaneously denatured and reassociated as a control.

Construction of genomic libraries. To prepare DNA for cosmid cloning, mycobacterial and pHC79 DNAs were digested with restriction endonucleases by previously published procedures (27). "*M. lufu*" DNA was completely digested (in separate reactions) with *Hin*dIII and *Eco*RI, and *M. leprae* and *M. vaccae* DNAs were partially digested with *Pst*I. The vector DNA was completely digested with the appropriate restriction enzyme and then treated with alkaline phosphatase to inhibit self-ligation (27).

The chromosomal DNA fragments were fractionated on linear sucrose gradients. Sucrose solutions of 5 and 25% (wt/vol) were prepared in 10 mM Tris–1 mM EDTA (pH 8.0) (TE) buffer and used to form 4.0-ml linear gradients in Beckman SW56 nitrocellulose centrifuge tubes. The digested DNAs (at concentrations of 20 μg in 200 μl) were layered on top of separate gradients. The tubes were centrifuged at 34,000 rpm in a Beckman SW56 rotor for 10.5 h at 10°C. The gradients were fractionated by puncturing the bottom of the tubes with an 18-gauge needle and collecting 2- to 3-drop fractions. The DNA was recovered by ethanol precipitation, with 20 μg of yeast carrier RNA per fraction. Since the RNA did not appear to inhibit ligation, no effort was made to eliminate it. The sizes of the digested DNA fragments were estimated from 0.4% agarose (type 1; Sigma) gels run at 2 V/cm. Molecular weight standards included in the gels were DNA from bacteriophages T4, T5, T7, and λ and λ DNA digested with the restriction endonuclease *Sal*I.

For *M. leprae* DNA, two types of ligations were done: (i) the 15-kilobase (kb) fragments plus the 30-kb fragments were mixed with pHC79 and (ii) the 20-kb fragments were mixed with pHC79. For *M. vaccae* DNA, only the 20-kb fragments were mixed with pHC79. "*M. lufu*" *Hin*dIII-restricted or *Eco*RI-restricted DNA fragments were not separated by size on sucrose gradients; a sample of each DNA digestion mixture was ligated to pHC79 DNA that had been digested with the same restriction endonuclease. In all ligation mixtures the ratio of chromosomal DNA to vector DNA was 2 to 1 (mole:mole) and approximately 0.5 U of bacteriophage T4

TABLE 2. Base composition and genome sizes of DNA samples

DNA	Mol% G + C[a] by:		Genome size[a] (10^9)
	T_m	Buoyant density	
D. novemcinctus	35.7 ± 1 (2)	36.2	ND
E. coli K-12 χ289	51.2 ± 0.6 (6)	49.0	2.5[c]
Micrococcus luteus	71.5 ± 1 (2)	72.6	ND
M. leprae	56.2 ± 1 (3)	57.6	2.2 ± 0.3
"*M. lufu*"	61.3 ± 0.9 (3)	ND	3.1 ± 0.5
M. vaccae	65.3 ± 1 (3)	64.7	3.1 ± 0.8
P. aeruginosa PAO-2	65.7 ± 1 (3)	66.1	ND

[a] Values within parentheses denote the number of determinations.
[b] Calculated from equations given in the text; values represent the mean ± the standard deviation. ND, Not determined.
[c] Value from reference 5.

ligase was added to each 20-μl reaction mixture (containing a total of 2.5 to 10 μg of DNA). All ligation reactions were done at 16°C for 16 h.

The recombinant cosmids were packaged in vitro (8) and used to transduce strain χ2001 or χ2819. After the in vitro-packaged cosmids had adsorbed to the cells, the cultures were incubated at 30°C for 30 min, and the transductants were selected for ability to grow on L agar containing (i) 25 μg of ampicillin per ml (final concentration) when *Hin*dIII- or *Eco*RI-digested "*M. lufu*" DNAs were used or (ii) 5.0 μg of tetracycline per ml (final concentration) when *Pst*I-digested *M. leprae* or *M. vaccae* DNAs were involved in the transduction. The Ap^r colonies obtained after transduction with the *Hin*dIII-digested DNA were tested for sensitivity to 12.5 μg of tetracycline per ml, and the Tc^r colonies obtained after transduction with the *Pst*I-digested DNA were tested for sensitivity to 12.5 μg of ampicillin per ml. Although a portion of the Ap^r Tc^r colonies obtained from the *Hin*dIII-digested reaction mixture could have had *M. leprae* DNA inserts, analysis of the recombinant molecules was confined to those which were Ap^r Tc^s. Colonies that were Ap^r Tc^s from the *Hin*dIII-digested mixture were grown in L broth containing 12.5 μg of ampicillin per ml, and colonies that were Tc^r Ap^s from the *Pst*I-digested mixtures were grown in L broth containing 5.0 μg of tetracycline per ml; cosmid DNA was extracted from both types of recombinants by the method of Birnboim and Doly (3) or Birnboim (2). Both undigested and endonuclease-digested DNA samples were analyzed by electrophoresis on 0.7% agarose gels.

Recombinant cosmid libraries prepared in strain χ2001 were stored frozen at −70°C in 1% peptone broth plus 5% glycerol as plasmid-containing cells. When the recombinant cosmid libraries were introduced into strain χ2819, the transductants were grown to high titers by taking advantage of the presence of the λ prophage in this host strain. The mutations in the prophage (i) reduce the ability of the prophage to integrate into and be excised from the chromosome of the host cell (*b2*), (ii) preclude recombination between the prophage and the recombinant cosmid (*red3*), (iii) preclude lysis in a suppressor-free host cell (*S7*), and (iv) allow for thermal induction of phage protein synthesis (*cI857*). Thus, when a transductant culture is thermally induced, the only DNA available for packaging is that of the recombinant cosmids. Since strain χ2819 is $tyrT^+$, cell lysis by the prophage is prevented, so the cells become elongated and filled with in vivo-packaged recombinant cosmids until they are lysed externally (W. R. Jacobs, J. E. Clark-Curtiss, L. R. Ritchie, and R. Curtiss III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H147, p. 130). Lysates containing 10^{10} packaged recombinant cosmids per ml were prepared by concentrating the transduced χ2819 cells by centrifugation at 4,000 × *g* for 10 min at 4°C, gently suspending the pellets in 1/10 to 1/50 the original volume, and lysing the cells by adding 0.01 volume of chloroform and vigorously shaking the culture at 37°C for 10 min. In some cases, these lysates were further concentrated to titers of 10^{11} Tc or Ap resistance-conferring transducing phage particles per ml by centrifugation through CsCl. Purified lysates were stored at 4°C.

To enhance the possibility of expression of cloned *M. leprae* DNA in *E. coli*, *M. leprae* DNA was subcloned from the pHC79::*M. leprae* libraries into an expression vector, pYA626 (a 3.8-kb plasmid containing the promoter region from the *Streptococcus mutans asd* gene [13, 20] cloned in between the *Pst*I and *Eco*RI sites of pBR322, constructed by Guy Cardineau and Roy Curtiss).

Recombinant cosmids containing *M. leprae* DNA were isolated as plasmid DNA (2, 3) from a pool of Tc^r Ap^s colonies and were completely digested with *Pst*I. These fragments were mixed with *Pst*I-digested pYA626 DNA and T4 ligase. The resulting pYA626::*M. leprae* recombinant plasmids were transformed (27) into *E. coli* K-12 χ925, and transformants were selected on L agar containing 5.0 μg of tetracycline per ml. These transformants again were tested for sensitivity to 12.5 μg of ampicillin per ml, and the plasmid DNA of the Tc^r Ap^s transformants was analyzed by agarose gel electrophoresis as described above.

A recombinant library of *M. vaccae* chromosomal DNA was prepared in the plasmid cloning vector, pBR322 (4). *M. vaccae* and pBR322 DNAs were digested to completion with the restriction endonuclease *Bam*HI and ligated as described above, and the recombinant molecules were transformed (10, 12) into *E. coli* χ1849. Transformant colonies were selected for growth on L agar containing 25 μg of ampicillin per ml; these were tested for sensitivity to tetracycline as described above. Recombinant DNA was extracted from Ap^r Tc^s transformants and analyzed by agarose gel electrophoresis as described above.

Hybridization of probe DNA to chromosomal DNA. Two different pYA626::*M. leprae* recombinant molecules (pYA1026 and pYA1031) were completely digested with *Pst*I, and the fragments were separated by electrophoresis in 0.7% agarose (type 1, Sigma). The *M. leprae* insert DNA fragments from each probe were recovered by removing the appropriate slices from the gel and electroeluting the DNA as described by Maniatis et al. (27). The DNA fragments then were concentrated by passage through a NACS Prepac (Bethesda Research Laboratories), ethanol precipitated, and nick translated, with [α-^{32}P]dATP (27).

Chromosomal DNAs from *E. coli*, armadillo, *M. leprae*, "*M. lufu*," and *M. vaccae* each were completely digested with *Pst*I and separated by electrophoresis on a 0.7% agarose gel (2 V/cm). The digested DNAs were transferred from the agarose gel to a filter of GeneScreen*Plus* (New England Nuclear Corp., Boston, Mass.) by capillary action, with 10× SSC as the transfer buffer, as described by Maniatis et al. (27).

The labeled probe DNAs were denatured by boiling for 10 min, followed by immersion in ice. The chromosomal DNAs fixed to the GeneScreen*Plus* filters were prehybridized for 20 h at 65°C and sealed in plastic bags with 10 ml of a solution containing 1% SDS, 1 M NaCl, and 10% dextran sulfate per filter. The denatured radioactive probes were added at a concentration of 10 ng/ml (final concentration in the reaction mixture) together with denatured salmon sperm DNA (final concentration, 100 μg/ml) to separate filters. The

plastic bags were resealed and incubated at 65°C with continuous agitation for 24 h. After washing the filters twice with 2× SSC at room temperature for 5 min each, twice with 2× SSC plus 1% SDS at 65°C for 30 min each, and twice with 0.1× SSC at room temperature for 30 min each, the filters were air dried briefly and then autoradiographed with Kodak XAR-2 film (Eastman Kodak Co., Rochester, N.Y.) for 18 h.

Complementation analysis of recombinant molecules. Recombinant molecules were tested to determine whether the cloned DNA contained genes that could complement any of the genetic defects present in the host *E. coli* strains. This was done by plating 10^7 to 10^8 transformed or transduced *E. coli* cells on minimal salts agar either lacking one required growth supplement or containing one nonmetabolizable carbon source. All media contained either 12.5 μg of ampicillin per ml or 5.0 μg of tetracycline per ml.

Minicell analysis of recombinant plasmids. *E. coli* K-12 χ925 cells containing the pYA626::*M. leprae* recombinants or χ1849 cells containing pBR322::*M. vaccae* recombinants were grown to late-log phase in minimal salts broth supplemented with 0.5% glucose, thiamine, and all amino acids except methionine (minimal salts broth growth medium). The minicells (1) from the cultures were separated from the parental cells by differential centrifugation, followed by sedimentation through one or two sterile linear gradients of 5 to 20% sucrose in buffered saline plus gelatin (7, 31). The final minicell preparations had less than one contaminating parental cell per 10^7 minicells after two sucrose gradients. The minicells were suspended in 1 ml of fresh minimal salts broth growth medium in a sterile Eppendorf tube, incubated for 10 min at 37°C, and then labeled with 10 μCi of [^{35}S]methionine (1,000 Ci/mmol) for 2 min. The reaction was stopped by immersing the minicell preparation in ice water for 30 min. The minicells were sedimented by a 2-min centrifugation in a Beckman Microfuge, and the minicell pellet was suspended in 50 μl of Laemmli buffer (23). This suspension was boiled at 100°C for 2 min to lyse the minicells. A 5-μl sample was removed from each suspension, spotted on a Whatman 3MM filter, and assayed for incorporation of the [^{35}S]methionine into hot trichloroacetic acid-insoluble material. The remaining 45 μl of the lysed minicell preparations were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (23).

RESULTS

Separation of *M. leprae* cells from armadillo liver tissue. By using the method for purifying *M. leprae* cells from armadillo liver tissue described in this paper, 92 to 100% of the cells in the tissue were recovered, based on microscopic counts of the acid-fast bacteria in the tissue versus those in the purified preparation.

Isolation of *M. leprae* DNA. Approximately 1×10^{11} to 2×10^{11} purified *M. leprae* cells were used for the DNA isolation procedure, from which we were able to recover 60% of the DNA present in those cells, assuming that each cell had only a single chromosome of 2.2×10^9. Using the viability stain developed by Kvach and Veras (22), we determined that 60 to 65% of the acid-fast bacteria recovered from the armadillo tissue were viable (i.e., had intact cytoplasmic membranes). Thus, the amount of DNA recovered from these cell preparations may be the maximal amount recoverable, since nucleases may have degraded the DNA in the nonviable cells. Analysis of the *M. leprae* DNA by electrophoresis on 0.3% agarose gels, with bacteriophage T4 (178-kb), T5 (110-kb), and lambda (48.6-kb) DNAs as molecular weight standards, indicated that the *M. leprae* DNA fragments are 100 to 200 kb in length (data not shown).

Determination of G+C content and genome sizes of mycobacterial DNA. The G+C content of the DNAs, determined by thermal denaturation and by isopycnic bouyant density centrifugation, are given in Table 2. Thermal denaturations were conducted at least twice and as many as six times, whereas the bouyant densities were usually determined only once. *E. coli* K-12 χ289 DNA served as the reference standard for the T_m determinations, and *Micrococcus luteus* DNA served as the reference standard for the bouyant density determinations. Each of these DNAs was analyzed by the alternative method as well. *P. aeruginosa* DNA was included as an additional control, since the published values for its G+C content are similar to those reported for mycobacterial species (39), although the two genera are unrelated.

In most cases, there is very good agreement between the values determined by the two methods. Armadillo DNA has a G+C content slightly lower than those reported for other mammals (39 to 44% [35]), which is significantly different from that of *M. leprae*. *M. vaccae* DNA has a G+C content similar to the reported values for other mycobacterial DNAs, which fall into two groups: one with G+C contents of 64 to 66.4% and the other with G+C contents of 67 to 70% (39). In contrast, *M. leprae* DNA has a G+C content significantly different from those of other mycobacteria, as has been reported by Imaeda et al. (19). "*M. lufu*" DNA also has a G+C content that is lower than those of other mycobacterial DNAs.

The molecular weights of "*M. lufu*" and *M. vaccae* genomes are similar to those of a variety of mycobacterial species reported by Bradley (5). The *M. leprae* genome is slightly smaller than other mycobacteria.

The molecular weight of the pHC79::*M. leprae* recombinant library estimated from the $C_0t_{1/2}$ value was 1.9×10^9 by using the correction factor for the difference in G+C content between *M. leprae* and *E. coli* chromosomal DNAs. The pHC79 DNA alone reassociated very quickly; this DNA was more than 50% reassociated by the time the temperature within the cuvette chamber had dropped to and stabilized at the reassociation temperature for the chromosomal DNA (2.5 to 3 min).

Preparation of genomic libraries. Concurrently with the molecular analysis, we prepared recombinant libraries, initially with *M. vaccae* and "*M. lufu*" DNAs, since these DNAs were more plentiful, and then with *M. leprae* DNA. Recombinant cosmid libraries prepared from these DNAs each consist of approximately 1,000 recombinant molecules with inserts of mycobacterial DNA of 40 to 45 kb. Thus, each library theoretically represents more than 99.99% of the respective mycobacterial genome. In addition, a pBR322::*M. vaccae* library was prepared which consists of 1,500 recombinant molecules with inserts of 0.8 to 19.2 kb of *M. vaccae* DNA and which potentially represents 95% of the *M. vaccae* genome.

Each library was screened for complementation of the following genetic defects in the host *E. coli* K-12 strains: *proA*, *trpE*, *thyA*, *asd*, *araC*, and *lacZ*. No complementation of any of these defects by mycobacterial DNA has been detected.

Expression of mycobacterial DNA in *E. coli*. The *M. leprae* DNA therefore was subcloned into the *Pst*I site of the plasmid expression vector pYA626. This vector contains the promoter region from the *S. mutans asd* gene; when the entire *S. mutans* gene is present in the plasmid (pYA575),

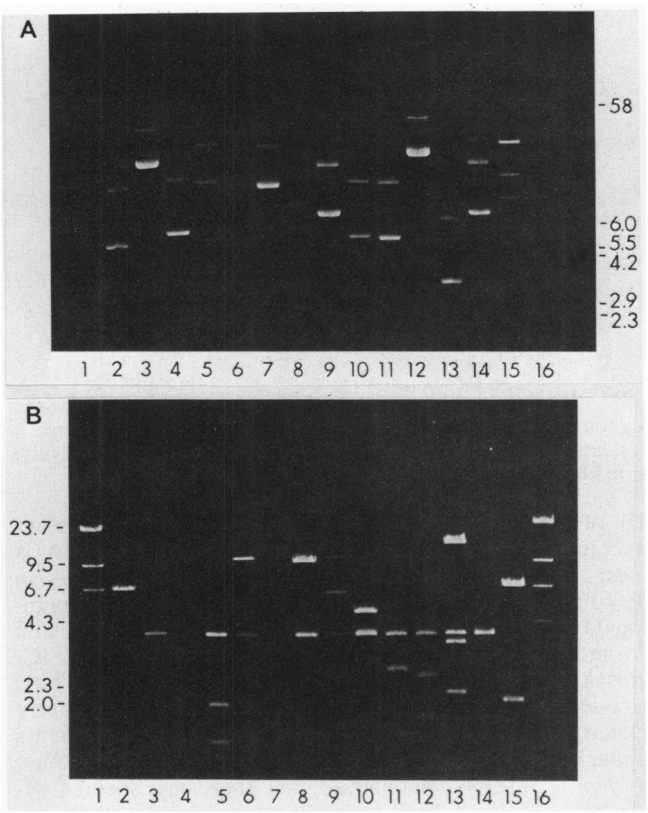

FIG. 1. Agarose gel electrophoresis of DNA from pYA626::*M. leprae* recombinant molecules before (A) and after (B) digestion with *Pst*I restriction endonuclease. (A) Lanes 1 through 14 are, respectively, pYA626, pHC79, pYA1025, pYA1026, pYA1027, blank, pYA1028, pYA1029, pYA1030, pYA1031, pYA1032, pYA1033, pYA1034, and pYA1035; lane 15 is λ DNA digested with *Hin*dIII, and lane 16 is plasmid DNA from *E. coli* V517 (25), the sizes of which (in kb) are given to the right of the gel. (B) Lanes 1 and 16 are λ DNA digested with *Hin*dIII (the sizes of the fragments are given, in kb, to the left of the gel); lanes 2 through 15 are, respectively, pHC79, pYA626, pYA1025, pYA1026, pYA1027, blank, pYA1028, pYA1029, pYA1030, pYA1031, pYA1032, pYA1033, pYA1034, and pYA1035.

the *asd* gene product comprises 7% of the total protein produced by whole *E. coli* cells (13). Moreover, the *asd* promoter apparently has a greater affinity for *E. coli* RNA polymerase than does the β-lactamase (*bla*) promoter from the pBR322 portion of the plasmid in that the synthesis of β-lactamase is almost totally shut off in minicells (which have a limited amount of RNA polymerase) harboring pYA575 (13, 20). Therefore, the *asd* promoter is an extremely strong promoter, and we anticipated that subcloning *M. leprae* DNA into such an expression vector would enhance expression of *M. leprae* genes in *E. coli*. The recombinant molecules were transformed into *E. coli* K-12 χ925, and tetracycline-resistant transformants were recovered at a frequency of 5×10^3 per μg of DNA. Six hundred transformants were screened for sensitivity to ampicillin: approximately 50% were sensitive. Since the subcloned DNA was from the *Pst*I-digested pHC79::*M. leprae* library, several types of recombinant molecules could be formed: (i) religated pHC79, (ii) pHC79::*M. leprae* DNA, (iii) pHC79::pYA626, and (iv) pYA626::*M. leprae* DNA. The transformants that were Tc^r Ap^r were cells that inherited the religated pHC79 molecules. Of the recombinant molecules analyzed, 80% contained inserts of sizes different than 6 kb (the size of pHC79). Figures 1A and B are photographs of 0.7% agarose gels on which plasmid DNAs from 11 of these transformants have been analyzed: Figure 1A depicts the intact recombinant molecules, and Fig. 1B depicts a gel on which *Pst*I-digested DNA from the same sources was analyzed. The inserted DNA present in most of these recombinant molecules ranges in size from approximately 1.4 to 10.5 kb except pYA1025, in which two bands of approximately 22 and 25 kb were faintly visible on the original photograph of the gel. It is evident (Fig. 1B) that none of the 11 recombinant cosmids contained an insert of the size of pHC79, with the possible exception of pYA1035 (lane 15) which gave a doublet band with fragments of approximately 6.7 and 6.9 kb in size.

Hybridization of the *M. leprae* 2.8-kb insert DNA fragment from pYA1031 (labeled with ^{32}P) to chromosomal DNA is shown in Fig. 2. Figure 2A is a photograph of a 0.7% agarose gel on which *Pst*I-digested chromosomal DNAs from *E. coli*, armadillo, *M. leprae*, "*M. lufu*," and *M. vaccae* were separated. Figure 2B depicts the autoradiograph showing that this insert DNA hybridized only with *M. leprae* chromosomal DNA. The larger (2.1-kb) fragment of pYA1026 and the 2.5-kb insert of pYA1036 each hybridize strongly to *M. leprae* chromosomal DNA, slightly to "*M. lufu*" chromosomal DNA and less well to *M. vaccae* chromosomal DNA, indicating that there are some conserved sequences present in the genomes of these mycobacterial DNAs (data not shown).

Minicells then were isolated from 9 of the 11 transformants, and the polypeptides produced by the recombinant molecules were labeled with [^{35}S]methionine and analyzed by SDS-PAGE. Figure 3A and B are autoradiographs of the gels, which show that at least four of the recombinant molecules (pYA1025, pYA1026, pYA1028, and pYA1031) specified polypeptides in addition to the 33-kilodalton (kDa) product of the tetracycline resistance gene of the vector, which is shown in lane 8 of each gel. Two of the recombinant molecules specified multiple polypeptides: pYA1025 specified polypeptides of 40 and 52 kDa and pYA1028 specified polypeptides of 20, 37, and 40 kDa.

Minicells also were isolated from *E. coli* χ1849 isolates containing two of the pBR322::*M. vaccae* molecules (pYA1001 and pYA1002). The polypeptides specified by these recombinant molecules were labeled and analyzed by SDS-PAGE. Figure 4 is an autoradiograph of this gel which demonstrates that pYA1001 specifies a new polypeptide of approximately 50 kDa.

DISCUSSION

The base composition and genome size determinations on *M. vaccae* and "*M. lufu*" DNAs were initiated because both of these organisms are cultivable (and thus provide easily accessible DNA in adequate amounts) and because each has been reported to show similarities to *M. leprae* (16, 30, 34, 37). One or both of these organisms could then be used to optimize procedures for preparing genomic libraries of mycobacterial DNA and for obtaining expression of mycobacterial DNA in *E. coli* K-12.

As is evident from the data in Table 2, the *M. leprae* genome is significantly different from the genomes of *M. vaccae* and "*M. lufu*", as well as other mycobacteria that have been studied, both in base composition and in molecular weight of the genome. Bradley (5) reported genome sizes of 2.8×10^9 to 4.5×10^9 daltons for 10 different mycobacterial species; only *Mycobacterium tuberculosis*

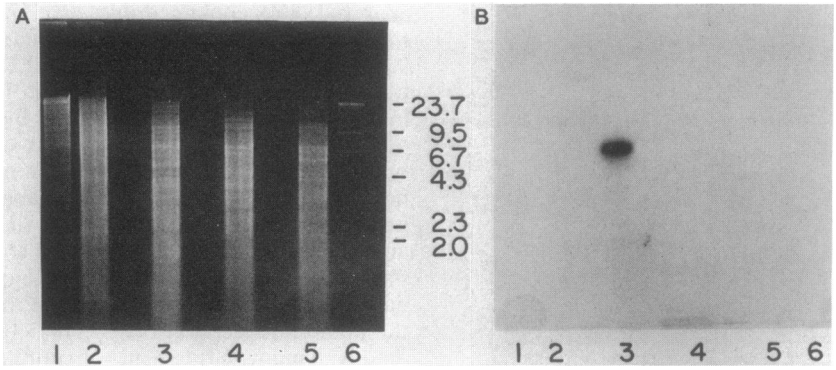

FIG. 2. Hybridization of ^{32}P-labeled *M. leprae* insert DNA from pYA1031 to *Pst*I-digested chromosomal DNAs. (A) Photograph of the 0.7% agarose gel on which the chromosomal DNAs were separated; (B) photograph of the autoradiograph of the hybridization. Lanes 1 through 5 contain completely digested chromosomal DNA from *E. coli* K-12, armadillo, *M. leprae*, "*M. lufu*," and *M. vaccae*, respectively. Lane 6 contains λ DNA digested with *Hin*dIII (the sizes of the fragments, in kb, are given to the right of the gel).

H37Ra, with a genome of 2.5×10^9 daltons, was similar to that of *M. leprae* determined in this study. (However, *M. tuberculosis* has a G+C content of 65% [5].)

M. vaccae DNA is similar to other mycobacterial DNAs in G+C content and molecular weight of its genome. The size of the "*M. lufu*" genome is similar to those of other mycobacteria, although the G+C content of its DNA is lower. Little homology (less than 15%) has been observed in filter hybridization experiments (under nonstringent conditions) between total chromosomal DNA from *M. leprae* and chromosomal DNA from either "*M. lufu*" or *M. vaccae* (C. Grosskinsky and B. Bloom, personal communication). Thus, we conclude that *M. vaccae* and "*M. lufu*" are not closely related to *M. leprae*, although hybridization experiments (under nonstringent conditions) with two independent cloned *M. leprae* inserts demonstrate that there are specific sequences that have been somewhat conserved among the three species.

The results on the G+C content of *M. leprae* DNA presented in Table 2 concur with those of Imaeda et al. (19). However, the molecular weights of the *M. leprae* and *M.*

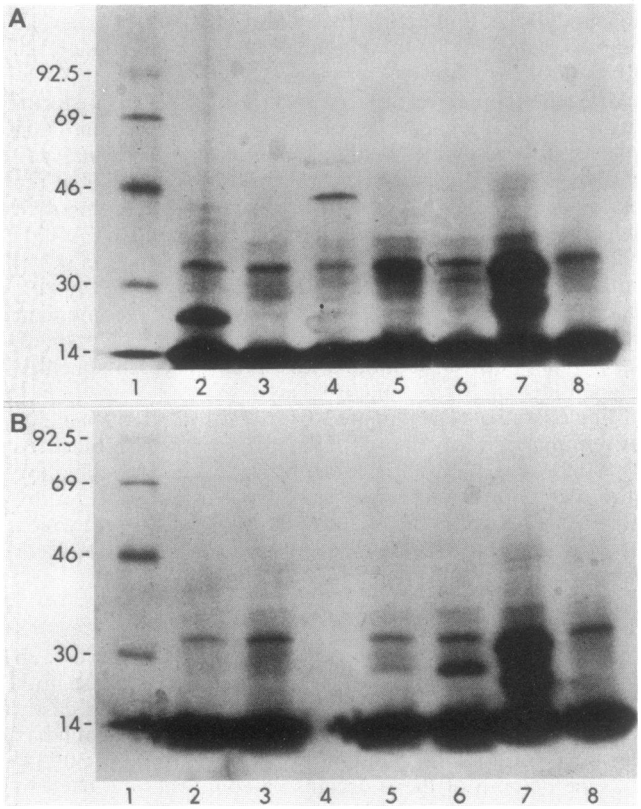

FIG. 3. SDS-PAGE analysis of ^{35}S-labeled polypeptides synthesized by minicells containing pYA626, pHC79 or pYA626::*M. leprae* clones. Lane 1 in A and B contains ^{14}C-labeled protein standards (the sizes of which are given in kDa to the left of the gels); lane 7 contains polypeptides synthesized by pHC79, and lane 8 contains polypeptides synthesized by pYA626. (A) Lanes 2 through 6 contain polypeptides synthesized by pYA1028, pYA1029, pYA1025, pYA1034, and pYA1026, respectively; (B) Lanes 2 through 6 contain polypeptides synthesized by pYA1027, pYA1032, no plasmid, pYA1030, and pYA1031, respectively.

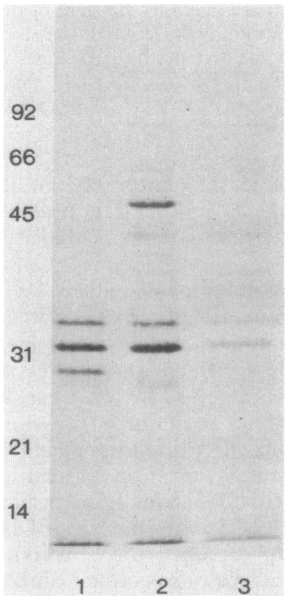

FIG. 4. SDS-PAGE analysis of ^{14}C-labeled polypeptides synthesized by minicells containing pBR322::*M. vaccae* recombinant molecules. Strain χ1849 containing pBR322 (lane 1), pYA1001 (lane 2), and pYA1002 (lane 3). The numbers to the left of the gel are the sizes (in kDa) of the ^{14}C-labeled protein standards separated by SDS-PAGE.

vaccae genomes which we determined differ from those published by Imaeda et al. There were several differences in the way in which the reassociation studies presented in this study were conducted: (i) all DNA samples were sheared by sonication to yield fragments of 300 to 800 base pairs (determined by agarose gel electrophoresis); (ii) the DNA samples were denatured by maintaining the temperature in the spectrophotometer at 99°C for 20 min after the maximal hyperchromicity was achieved, and (iii) the $C_0t_{1/2}$ values were determined after the DNA was 50 to 65% renatured. In contrast, Imaeda et al. sheared some of their DNA by passage through a 26-gauge needle, which yields fragments of approximately 10 kb (average size), and some DNA by passage through a cell fractionator, which presumably yields smaller fragments. However, there is no indication of which method was used to shear specific DNAs. Imaeda et al. held the DNA samples at 96°C for 2 min, after maximal hyperchromicity had been achieved, which may be insufficient to completely denature DNA with G+C contents over 60%. Finally, these investigators determined their $C_0t_{1/2}$ values from reaction rates observed during the first 15 min of the reaction. It has been pointed out by other investigators (33) that the reaction is not second order during that period, possibly leading to erroneous calculations of genome size.

If the genome size of *Corynebacterium* 2628 LB reported by Imaeda et al. (19) is correct, then the genome of *M. leprae* is significantly different from this organism. Because of the disparities in genome sizes, it is very surprising that the *Corynebacterium* DNA showed 68% homology to *M. leprae* DNA as reported by those investigators.

Recombinant libraries representing more than 99.99% of the genomes of *M. leprae*, *M. vaccae*, and "*M. lufu*" have been prepared by cloning into the cosmid vector, pHC79. The pHC79::*M. leprae* and pHC79::*M. vaccae* libraries were transduced into an in vivo packaging strain (*E. coli* χ2819), amplified, induced, and stored at 4°C as high-titer phage lysates as described above. The pHC79::"*M. lufu*" libraries were transduced into *E. coli* χ2001 and stored frozen at −70°C as plasmid-containing cells. Fragments of *M. leprae* DNA subcloned from the recombinant cosmids into pYA626 can result in synthesis of polypeptides in minicells of *E. coli* K-12. Since DNA hybridization experiments with radioactively labeled fragments of inserted *M. leprae* DNA have demonstrated that these probes hybridize only to *M. leprae* chromosomal DNA, the unique polypeptides that are produced by minicells containing recombinant molecules must be encoded by the inserted *M. leprae* DNA. Two of the pYA626::*M. leprae* recombinant molecules which were analyzed produced more than one polypeptide, which could indicate that some polypeptides may be expressed from an *M. leprae* promoter, a polycistronic mRNA may be produced from the *asd* promoter, or a newly synthesized polypeptide may be degraded by *E. coli* cellular proteases into one or several smaller stable polypeptides.

At present, there is no evidence that *M. leprae* promoters are functioning in *E. coli* K-12. We recently have obtained complementation of the *gltA* (citrate synthase) and *aroB* (dehydroquinate synthetase) mutations of *E. coli* by several different pYA626::*M. leprae* recombinant molecules, but the *M. leprae* enzyme polypeptides are only expressed when the encoding DNA sequences are linked to the *S. mutans asd* promoter in one of the two possible orientations (W. R. Jacobs, M. A. Docherty, J. E. Clark-Curtiss, and R. Curtiss III, manuscript in preparation).

Experiments are in progress to further optimize expression of *M. leprae* genes in *E. coli*. This is being done to obtain expression of *M. leprae* genes specifying proteins that are potential targets for development of new antileprosy drugs. In addition, efficient expression of *M. leprae* gene products will facilitate immunological screening to identify protein antigens that might yield new diagnostic reagents or potential components of vaccines effective in preventing *M. leprae* infections. The recombinant molecules that have been shown to specify polypeptides by minicell analysis have been tested by an enzyme-linked immunosorbant assay with sera from eight leprosy patients (four borderline tuberculoid or tuberculoid patients and four borderline lepromatous or lepromatous patients; the sera were a gift from R. Gelber, Seton Medical Center, San Francisco, Calif.), with monoclonal antibodies against specific *M. leprae* proteins (WHO-4, WHO-6, WHO-30, IIIE9, IIH9, E4/2, and IIC8; the monoclonal antibodies were gifts from J. Ivanyi, The Wellcome Foundation Ltd., Kent, England, and T. P. Gillis, National Hansen's Disease Center, Carville, La.), and with polyclonal anti-*M. leprae* serum prepared in rabbits (a gift from T. P. Gillis). To date, no positive enzyme-linked immunosorbent assay reaction has been observed.

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