Construction of Genomic Libraries of Mycobacterial Origin: Identification of Recombinants Encoding Mycobacterial-specific Proteins[1]

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Recent studies have put in evidence species-specific proteins in mycobacteria ([1, 8, 17]) in addition to the glycolipids unique to some organisms ([2, 10, 19]). These can be important for discriminatory immunodiagnosis as well as for immunological studies pertaining to the suppression or stimulation of immune response. Their isolation from parent mycobacteria is an onerous undertaking. An alternative source would be their preparation by DNA recombinant techniques. Genetic engineering approaches are particularly important for obtaining proteins of *Mycobacterium leprae* which is noncultivable in test cell-free conditions and whose supply is scarce.

The present communication is a report on the preparation of genomic libraries of *M. leprae* and bacillus Calmette-Guérin (BCG). *M. vaccae* was used to establish the methodology.

MATERIALS AND METHODS

Bacterial strains and growth conditions

M. vaccae and BCG were obtained from the standard collection of the Trudeau Institute, Saranac Lake, New York, U.S.A., and grown in Middlebrook 7H9 broth at 37°C. *M. leprae* purified from armadillo-infected liver was kindly made available by Dr. D. Gwinn (Bacteriology and Virology Branch, National Institutes of Health, Bethesda, Maryland, U.S.A.).

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Enzymes

Restriction endonucleases and other cloning enzymes were obtained from the Bethesda Research Laboratory, Bethesda, Maryland, U.S.A.

Isolation of DNA

DNAs of cultivable mycobacteria were isolated according to the method described by Imaeda, *et al.* ([11]) with modifications. Cultivable mycobacteria in the logarithmic phase of growth were treated with 0.2 M glycine for 12 hr at room temperature. The cells were centrifuged and treated with 1 mg/ml of lysozyme in TES buffer (3 mM Tris–6.00 mM NaCl–1 mM EDTA, pH 8.0) followed by treatment with pronase (200 μg/ml). Cell lysates were extracted with equal volumes of phenol : isoamyl alcohol : chloroform (25:1:24), and DNA was precipitated from the aqueous phase by adding 2 × volumes of cold ethanol. The crude DNA preparation was then subjected to treatment with RNase (50 μg/ml) for 1 hr at 37°C as well as cetyltrimethyl ammonium bromide (1.5% w/v). DNA was suspended in TE (10 mM Tris–1.0 mM EDTA, pH 8.0) and re-extracted with the phenol–isoamyl alcohol–chloroform mixture. From the aqueous phase, the DNA fibers were spooled on a glass rod after the slow addition of ethanol, and then air dried and resuspended in TE. Purity of the DNA was assessed spectrophotometrically at A260/280 nm.

Plasmid DNA isolation. *Escherichia coli* cells grown up to 0.8 optical density (OD) at 610 nm were added to chloramphenicol (200 μg/ml) and the culture continued for 16 hr. Amplified plasmid DNA was isolated by CsCl gradient centrifugation ([4]).

Construction of genomic libraries

Mycobacterial DNAs were digested to completion with the restriction endonu-

clease Bam HI. DNA fragments were ligated into the Bam HI site of *E. coli* plasmid pBR322 using T4 DNA ligase ([6]), and the ligated DNA was then used to transform *E. coli* strain RRI according to the procedure described by Mandel and Higa ([14]). Recombinant clones, scored on the basis of ampicillin resistance and tetracycline sensitivity, were pooled into groups of 20–25 and used for further analysis.

Nick translation

Mycobacterial DNA (2 μg) was labeled with ^{32}P dCTP by nick translation ([16]).

Colony hybridization

Colonies grown on nitrocellulose membrane filter for 18 hr at 37°C were lysed by treatment with 0.5 N NaOH, and the denatured DNA was neutralized and fixed by baking the filter at 80°C. Hybridization was carried out at 68°C in sixfold concentrated saline sodium citrate buffer (6 × SSC) (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2). The filters were washed 3 times with 200 ml of 2 × SSC, followed by 3 more times with 200 ml of 0.5 × SSC at 60°C, air dried, and exposed to Kodak X-R omat X-ray film at −70°C ([9, 15]).

Immunoenzymic detection of mycobacterial antigens

Preparation of *E. coli* extract. Recombinants pooled into groups were grown up to 1.2 OD (610 nm) in Luria broth in the presence of 50 μg/ml of ampicillin. The cells were harvested, resuspended in phosphate buffer (0.01 M phosphate buffered saline, pH 7.0), and then sonicated for 10 min (at 20 μm) using a MSE Soniprep 150 sonicator.

Protein estimation. Protein estimation was done according to Lowry, *et al.* ([13]).

Antiserum to *M. tuberculosis* (H37Rv) and *M. leprae.* Rabbits were immunized with a 15,000 g supernatant of *M. tuberculosis* (H37Rv) sonicate. Primary immunization was carried out with incomplete Freund's adjuvant (IFA). Rabbits were bled after two boosters with Leira's basic adjuvant (LBA; Leira's Pharmaceuticals, Finland), and serum was stored at 4°C with merthiolate. Anti-*M. leprae* serum was raised in a similar manner with irradiated *M. leprae.*

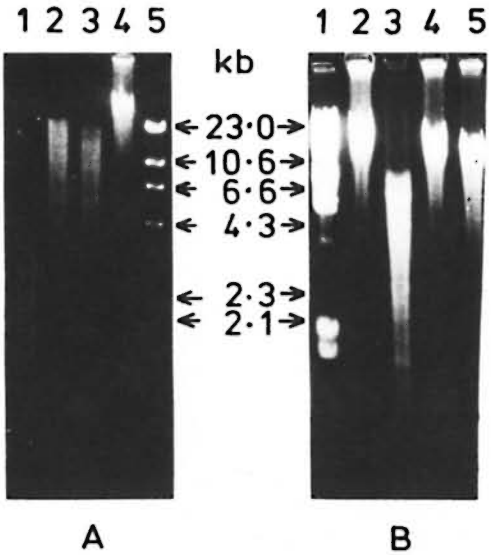

FIG. 1. Restriction analysis of mycobacterial DNAs. DNA from BCG and *M. vaccae* was digested for 16 hr with restriction endonucleases. DNA fragments were separated on 1% agarose gel electrophoresis and visualized by ethidium bromide staining. **A.** Columns 1 to 5 represent BCG DNA digested with BAM Hi, Hind III, EcoRI, undigested BCG DNA and Hind III digest of λ DNA, respectively. **B.** Columns 1 to 5 represent Hind III digest of λ DNA, undigested *M. vaccae* DNA, *M. vaccae* DNA digested with Bam HI, Hind III, and EcoRI, respectively.

Protein A horseradish peroxidase (HRPO) conjugate. Staphylococcal protein A (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was coupled at HRPO (Type VI RZ 3.0) by the periodate oxidation method of Wilson and Nakane ([18]).

ELISA. *E. coli* sonicates carrying cloned genes were coated on Nunc Micro ELISA plates and the immunoreactivity determined with respective polyvalent anti-H37Rv, anti-*M. leprae* sera, or LL and TB patient serum by conventional enzyme immunoassay in which the antibody bound was detected with protein A-HRPO ([7]). Each assay was done in duplicate, repeated at least five times, and the mean values were plotted.

RESULTS

Analysis of mycobacterial DNAs by restriction endonucleases. In view of the limited availability of *M. leprae,* initial exploratory studies were carried out with DNA from BCG and *M. vaccae,* which is reported

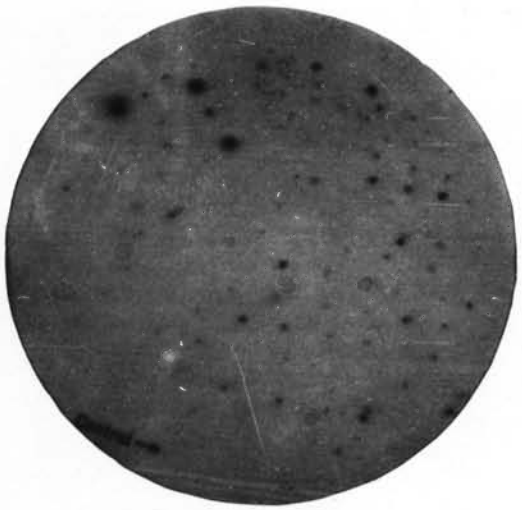

FIG. 2. Identification of recombinants by colony hybridization. Representative recombinant colonies of BCG origin were grown on nitrocellulose membrane filters. Colonies were lysed, and the DNA fixed to the membrane filter was hybridized with ^{32}P-labeled, nick translated BCG DNA (see Materials and Methods).

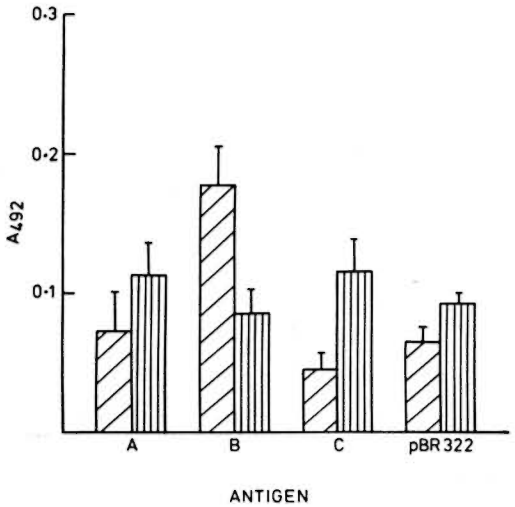

FIG. 3. Expression of cloned BCG DNA in *E. Coli.* Recombinant clones grown in Luria broth were sonicated, and sonicate supernates (100 μg protein/well) were coated on Nunc Micro ELISA plates and their reactivity to anti-H37Rv and anti-*M. leprae* sera were tested (see Materials and Methods). A, B, C = recombinant pools representing 20–25 independent clones; ▨ = reactivity against anti-H37Rv; ▥ = reactivity against anti-*M. leprae.*

to have several metabolic resemblances to *M. leprae* ([5]). Mycobacterial DNAs have high G:C contents ([11]) and, thus, some restriction nucleases may not be appropriate for the purpose of cloning. When DNA from *M. vaccae* was digested with restriction enzymes Bam HI, Hind III, and EcoRI (Fig. 1B, columns 3, 4, 5), complete digestion was observed only with Bam HI, while partial digestion was obtained with EcoRI and Hind III. Controls included in the assay were lambda and pBR322 DNA digested under identical conditions. Similar results were obtained with BCG (Fig. 1A, compare column 1 with columns 2 and 3). Figure 1 shows the presence of only a few restriction sites for Hind III in DNA but several restriction sites for Bam HI. Thus, Bam HI was used for the shot-gun cloning of mycobacterial DNAs.

Construction of genomic libraries. DNAs from *M. vaccae,* BCG, and *M. leprae* were digested to completion with restriction endonuclease Bam HI, and cloned into the Bam HI site of *E. coli* plasmid pBR322 essentially by the shot-gun approach as described above. The total number of recombinants accumulated for *M. vaccae* were 2785, while for *M. leprae* and BCG the number was 300 and 1750, respectively.

Evidence for insertion of mycobacterial DNA in plasmid pBR322. It was ascertained by more than one criteria that the recombinants are truly of mycobacterial origin. Representative colonies of BCG origin were grown on membrane filter. The colonies were lysed, and DNA fixed on the membrane was hybridized with ^{32}P-labeled, nick translated total BCG DNA. It is evident from the results presented in Figure 2 that a hybridization signal was detected for almost all of the clones, although the extent of hybridization was not the same in the case of the control (pBR322); even after 5 days' exposure no signal was observed. The same probe also hybridized partially with *M. leprae*-derived clones (data not presented). Insertion of mycobacterial DNA into pBR322 was further judged by isolating DNA from randomly picked recombinant colonies and analyzing them on 0.7% agarose gel electrophoresis. These experiments indicated the successful cloning of DNA of mycobacterial origin in *E. coli.*

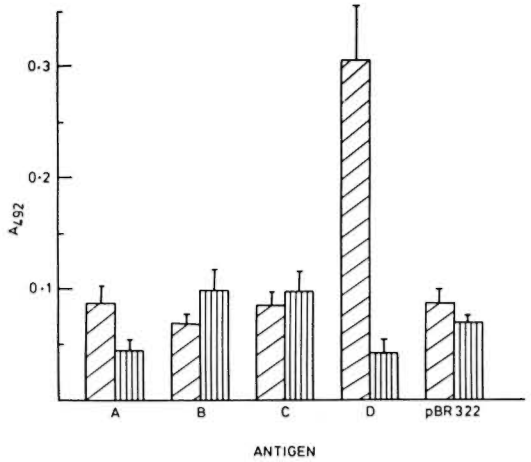

FIG. 4. Expression of cloned *M. leprae* DNA in *E. coli.* Recombinant clones pooled into groups of 20–25 were sonicated, and the sonicate supernates were tested for their reactivity against anti-*M. leprae* and anti-H37Rv sera (see Materials and Methods). A, B, C, D = recombinant pools representing 20–25 independent clones; ▨ = reactivity against anti-*M. leprae*; ▩ = reactivity against anti-H37Rv.

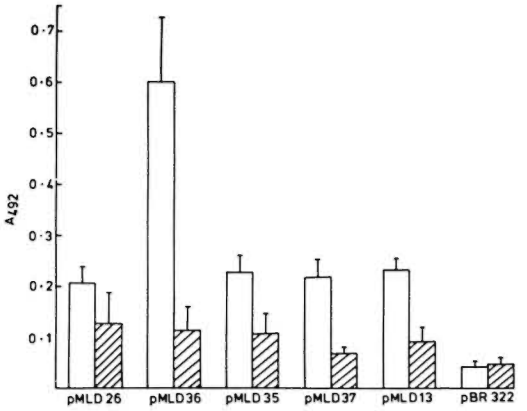

FIG. 5. Immunoreactivity of recombinants with sera from LL patients. Individual recombinants from *M. leprae* pool D were grown as described in Materials and Methods, and their sonicate supernates (100 ng protein/well) were tested for reactivity against pooled sera (10 individuals) of LL patients and TB patients. □ = reactivity against LL sera; ▨ = reactivity against TB sera.

Search for mycobacterial-specific clones. The eventual goal of the present investigation was to obtain, by DNA recombinant methodology, proteins unique to *M. tuberculosis* and *M. leprae.* Although the present system may not optimally express the cloned mycobacterial DNA, probing experiments revealed the immunoreactivity of cloned *E. coli* sonicates with anti-H37Rv and *M. leprae* polyclonal sera. Recombinants pooled into groups of 20–25 were grown as described above, and their 8000 g sonicate supernates were treated with two types of antibodies in the ELISA assay. It is evident from the results presented in Figures 3 and 4 that cloned DNA of BCG and *M. leprae* origin is expressed to some extent in *E. coli.* For BCG recombinant pools (Fig. 3), pool B demonstrated more immunoreactivity with anti-*M. tuberculosis* serum as compared to pools A and C. The same sonicates were tested with sera raised against *M. leprae.* The sonicate of pool B gave low immunoreactivity with these antibodies. Similar experiments were carried out with sonicates of pooled recombinants of *M. leprae* origin. Pool D had high immunoreactivity with anti-*M. leprae* serum; whereas its crossreactivity with anti-H37Rv was low and comparable to background levels (Fig. 4).

The results clearly indicate that cloned DNA sequences present in BCG pool B and *M. leprae* pool D are expressed as species-specific antigen(s) in *E. coli.* In order to identify clones that encode for antigens, individual colonies from B and D pools were grown and their sonicate supernates were analyzed by their reactivity to sera raised against *M. leprae* and *M. tuberculosis* (H37Rv). From *M. leprae* pool D, five clones were selected which were then tested for reactivity to sera of LL and TB patients. The results presented in Figure 5 clearly demonstrate that sonicates from pMLD36 show maximum reactivity to LL sera compared with TB sera.

Analysis of pMLD36 DNA on agarose gel. Plasmid pMLD36 DNA purified by CsCl density gradient centrifugation was subjected to digestion with the restriction endonuclease Bam HI. Analysis of the restriction fragments after Bam HI digestion revealed that pMLD36 has an insert of 5.1 kb (Fig. 6A). A partial restriction map was constructed for pMLD36 with respect to EcoRI, Hind III, PstI, Sal I, Pvu II, and Bgl II. Except for the restriction endonuclease Bgl II, all others were found to have sites on the pMLD36 insert (Fig. 6B).

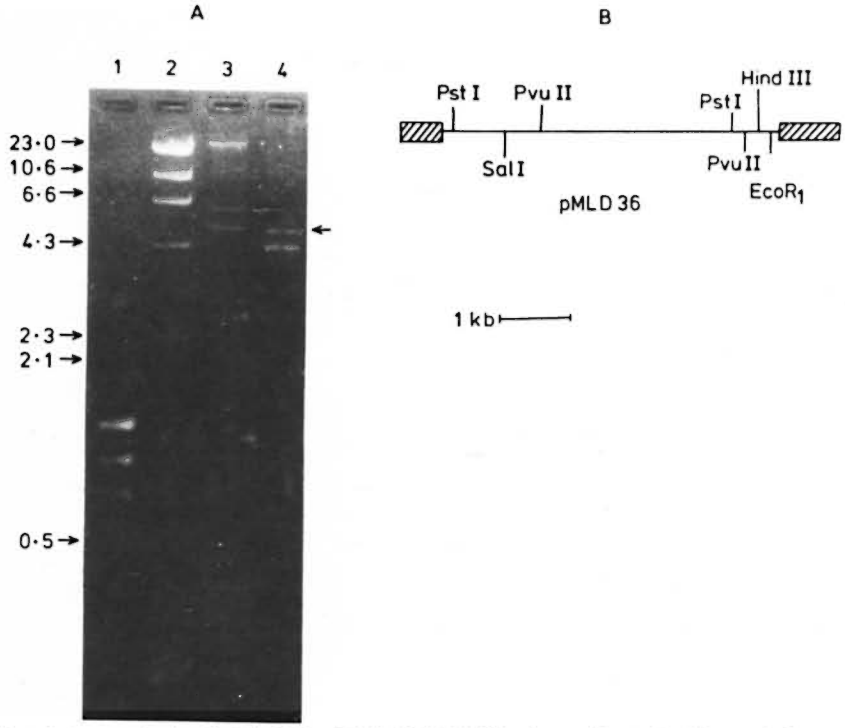

FIG. 6. Restriction analysis of pMLD36. pMLD36 DNA (2 μg) was digested with restriction endonuclease Bam HI, and the DNA fragments separated on 1% agarose gel electrophoresis were visualized after staining with ethidium bromide. **A.** Ethidium bromide stained pattern of pMLD36. Columns 1–4 represent Hae III digest of φX 174 DNA, λ DNA digested with Hind III, undigested pMLD36 DNA, and Bam HI digest of pMLD36 DNA, respectively. **B.** Restriction map of pMLD36.

DISCUSSION

Cloning of mycobacterial proteins by the DNA recombinant approach is attractive from two points of view: a) It offers an alternate possibility of obtaining proteins of *M. leprae,* which is not cultivable *in vitro* and whose supply is scarce. b) The approach also avoids cumbersome purification procedures to obtain mycobacterial-unique proteins in view of the fact that a large number of constituents are crossreactive in mycobacteria.

The preliminary studies reported here describe the preparation of partial genomic libraries of the mycobacteria *M. leprae* and BCG. Before these were prepared a complete genomic library of *M. vaccae* was made to establish the methodology. The total number of recombinants accumulated for *M. vaccae* was 2785, while the number was 300 and 1750 for *M. leprae* and BCG, respectively. According to Imaeda, *et al.* ([11]), the genome size for mycobacteria is 1–3 × 10^9D. The average sizes of the inserts in recombinants as analyzed on agarose gel electrophoresis were found to be between 3–7 kb, suggesting that the number of clones obtained in the case of *M. leprae* and BCG and the number of recombinants prepared so far represent about 30%–40% of the total genome. Construction of genomic libraries of *M. leprae* DNA has been reported by Clark-Curtiss ([3]), who used pBR322 as well as bacteriophage lambda derived cloning vectors.

Although the present number of recombinants do not claim cloning of the entire *M. leprae* and *M. tuberculosis* genes, the presence among them of inserts coding for proteins giving discriminatory serological reaction with anti-tuberculosis and *M. leprae* serum is of interest (Fig. 5). The successful identification of a recombinant pMLD36 from the *M. leprae* pool further supports the validity of the approach used in the screening procedure. Among the unique immunoreactive constituents are glycolipids ([2, 10, 19]) with terminal sugars ac-

counting for immunological reactivity and specificity. The immunodeterminants indicated in the present studies are not expected to be carbohydrates or glycolipids. Follow-up studies will provide definition of their nature.

The signals, although limited, obtained for expression of cloned DNA inserts in *E. coli* raise the question of whether mycobacterial DNA is expressed in *E. coli* from its own promotor or from a plasmid promotor. Studies on the expression of the tox 228 gene in *Corynebacterium diphtheriae* in *E. coli* is indicative of the functioning of a promotor of cloned DNA origin in such cases ([12]).

SUMMARY

A complete genomic library from *Mycobacterium vaccae* (2785 recombinants) and a partial genomic library of *M. leprae* and BCG (300 and 1750 clones, respectively) were constructed in the plasmid pBR322. Bam HI was selected as the restriction endonuclease for obtaining DNA cleavage products. Evidence was obtained for limited expression of the cloned mycobacterial DNA inserts in *Escherichia coli*. A recombinant has been identified which codes for antigen immunoreactive with rabbit anti-*M. leprae* antibody but not with anti-H37Rv antibody.

RESUMEN

Usando el plásmido pBR322 se construyó una biblioteca genómica completa del *Mycobacterium vaccae* (2785 recombinantes) y bibliotecas genómicas parciales del *M. leprae* y del BCG (300 clones y 1750 clones, respectivamente). Para los productos del rompimiento del DNA se seleccionó la endonucleasa de restricción Bam HI. Se lograron expresiones limitadas del DNA clonal micobacteriano insertado en *Escherichia coli*. Se identificó una recombinante que codifica para la síntesis de antígenos que reaccionan con anticuerpos de conejo anti-*M. leprae* pero no con anticuerpos anti-H37Rv.

RÉSUMÉ

Une bibliothèque complète du génome de *Mycobacterium vaccae* (2785 recombinants) a été établie chez le plasmide pBR322, de même qu'une bibliothèque partielle du génome de *M. leprae* et du BCG (respectivement 300 et 1750 clones). L'enzyme Bam HI a été choisi comme endonucléase de restriction pour obtenir les produits de clivage de l'ADN. On a observé des manifestations d'expression modérée des fragments d'ADN mycobactérien cloné chez *Escherichia coli*. On a identifié un recombinant qui code pour un antigène immunoréactif à l'égard de l'anticorps anti-*M. leprae* du lapin, mais non avec l'anticorps anti-H37Rv.

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Note added in proof: After this paper was communicated, Young, *et al.* published a paper on the cloning of genes for the major protein antigens of leprosy parasite *M. leprae* ([20]).

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