

Sequence and Immunological Characterization of a Serine-Rich Antigen from *Mycobacterium leprae*

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Sera from lepromatous leprosy patients were used to screen a *Mycobacterium leprae* λ gt11 library. Three positive plaques were picked, and lysogens were constructed. Immunoblot analysis showed that all of the lysogens expressed an apparently identical β -galactosidase fusion protein which reacted strongly with the sera. The 1.7-kbp insert from one clone was subcloned into the *lacZ* gene in pUR290; sequence analysis of the end fused to *lacZ* revealed an open reading frame with no significant homology to previously published sequences. The insert was used to screen an *M. leprae* cosmid library, and five clones were isolated. The insert was also found to hybridize to clones expressing the *M. leprae* antigen which had previously been designated class III and 25L. A 1.8-kbp *Hind*III fragment was subcloned from one of the cosmids and sequenced. The sequence revealed a 1,227-bp open reading frame, encoding a 408-amino-acid protein with a predicted molecular mass of 42,466 Da. The protein contains amino- and carboxy-terminal hydrophobic domains and a hydrophilic central domain; the amino-terminal domain shows some homology to a 51-kDa hypothetical antigen of *Mycobacterium tuberculosis*, while the hydrophilic region contains a high proportion of serine residues, and we have therefore designated the protein serine-rich antigen (Sra). Some repeated motifs are present in the protein, but their significance is unknown. Seventy-eight percent of serum samples from multibacillary leprosy patients and 68% of serum samples from paucibacillary leprosy patients recognized the fusion protein, showing that this is a major *M. leprae* antigen. In contrast, all serum samples from endemic controls were negative, while 26% of serum samples from tuberculosis patients were weakly positive.

It is hoped that the identification of the critical antigens of *Mycobacterium leprae* and their characterization will lead to a better understanding of the complex immune response which takes place in leprosy. The first genes encoding *M. leprae* protein antigens were isolated from a genomic library in λ gt11 in 1985 by using mouse monoclonal antibodies raised against *M. leprae* to screen the library (36). Since that time, other strategies have been employed, including the use of sera from patients with tuberculoid or lepromatous leprosy to identify genes encoding antigens recognized in humans (5, 21, 26, 27) or the use of T cells to screen the library (22). A number of the genes isolated in this way have been sequenced, and the protein products have been characterized immunologically. In addition, some proteins have had functions assigned to them (reviewed by Young et al. [35]).

This approach to understanding the immunological response in leprosy requires the analysis of many antigens. We have shown previously that sera from lepromatous leprosy patients recognize *M. leprae* antigens which have not yet been cloned or characterized. This project therefore aimed to identify and isolate novel immunoreactive recombinant

DNA clones from an *M. leprae* genomic library in the vector λ gt11. To screen the library, a pool of serum samples from lepromatous leprosy patients was prepared which contained immunoglobulin G antibodies to a number of previously uncloned antigens. We report the isolation and characterization of the gene encoding an immunoreactive serine-rich antigen with a predicted molecular mass of 42 kDa.

MATERIALS AND METHODS

Serum samples. Serum samples were from leprosy patients from Mexico ($n = 60$) and Pakistan ($n = 16$). Most of the patients from Mexico were under treatment, either with dapsone monotherapy or multidrug therapy, whereas the patients from Pakistan were newly diagnosed, untreated patients. Although it was difficult to establish the *Mycobacterium bovis* BCG status of the patients, it is likely that the majority had not been vaccinated. Patients from Mexico with active pulmonary tuberculosis undergoing standard multidrug therapy were included as controls. Healthy control subjects came from hyperendemic leprosy areas in Mexico and had not suffered from relevant clinical events before or at the time this study took place. About half of these had received BCG vaccination.

Chemicals and enzymes. Most chemicals were purchased from Sigma Chemical Co. (Poole, United Kingdom) or BDH-Merck (Lutterworth, United Kingdom). Buffer-equilibrated phenol was obtained from Rathburn Chemicals Ltd. (Walkerburn, United Kingdom). Enzymes were obtained from GIBCO-BRL (Paisley, United Kingdom) or Northumbrian Biological Laboratories (Cramlington, United King-

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dom). Luria (L) broth and fetal calf serum were purchased from GIBCO-BRL.

Oligonucleotides. Oligonucleotides were synthesized by using phosphoramidite chemistry in a Pharmacia Gene Assembler Plus and used without further purification. The two primers were O1 (5'-GCAGCTCGACGGCAAGTA) and O2 (5'-CACTATGACCAACTAGCA).

Plasmids, bacteriophages, and bacterial strains. The plasmids used in this study were pUR290 (24), pNGS21 (30), pUC9-2 (11), and Lawrist4 (7a, 13). Bacteriophage M13mp19 (33) was used in sequencing studies, and *Escherichia coli* K-12 strains BNN97, Y1088, Y1089, and 1046 have been described elsewhere (4, 15).

Monoclonal antibodies. The monoclonal antibodies used were L7 (70-kDa antigen) (10), L12 (65-kDa antigen) (3), F47-9-1 (36-kDa antigen) (20), SA1-B11H (28-kDa antigen) (34), L5 (18-kDa antigen) (2), and ML06 (12-kDa antigen) (16). These were obtained through the World Health Organization IMMLEP bank, except ML06, which was a kind gift from J. Ivanyi, Hammersmith Hospital, London, United Kingdom.

Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting procedures were as described previously (32), except that the secondary antibody was alkaline phosphatase-conjugated goat anti-human immunoglobulin G. For the detection step, the filters were incubated for 2 min in a substrate solution prepared by adding 33 μ l of nitroblue tetrazolium (75 mg/ml in 70% dimethyl formamide) and 25 μ l of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 50 mg/ml in 70% dimethyl formamide) to 7.5 ml of Tris-buffered saline (TBS) just prior to use.

Removal of anti-*E. coli* antibodies from sera. A culture of *E. coli* BNN97 was grown to stationary phase, and, after centrifugation, the pellet of cells was resuspended in TBS, sonicated, and diluted to 1.8 mg of protein per ml. Nitrocellulose membranes (BA85; 82-mm diameter; Schleicher & Schuell, Dassel, Germany) were incubated in the *E. coli* extract for 20 min, transferred to fresh extract for a further 20 min, and washed in TBS for 10 min. The filters were then allowed to dry and frozen at -70°C until used. Each filter was used to absorb 10 ml of sera at a 1:10 dilution in TBS for 30 min at room temperature. The serum sample was incubated with a second filter and then passed through a 0.2- μm -pore-size filter. Pooled patient serum samples were diluted to 1:50 in TBS containing 20% fetal calf serum and stored at -20°C prior to use.

Screening the λ gt11 library. Manipulations of the *M. leprae* λ gt11 library were generally as described by Huynh et al. (15). The library was amplified once by plating to confluence on *E. coli* Y1088. To screen the library, phages were plated at a density of 2×10^4 to 3×10^4 PFU per 90-mm plate. Filters were probed with a serum pool made by mixing 5-ml serum samples from six lepromatous leprosy cases and diluting them 1:10 in TBS (50 mM Tris-HCl [pH 8.0], 150 mM NaCl). Detection was achieved by using nitroblue tetrazolium-BCIP as described above.

Lysogen construction. Lysogens of recombinant phages were constructed by infecting a culture of *E. coli* Y1089 with the phages at a multiplicity of infection of 5 for 20 min at 32°C in L broth containing 10 mM MgCl_2 . The cells were plated onto L agar to single colonies and incubated at 32°C , and colonies were then tested for temperature sensitivity at 42°C . Approximately 50% of the colonies were found to be lysogenic. Induction of the lysogens was carried out as described elsewhere (15).

Recombinant DNA techniques. DNA manipulations were carried out as described elsewhere (13, 25).

Subcloning from λ gt11 into pNGS21. DNA from recombinant λ gt11 clones was prepared by harvesting phages from a confluent plate lysate with Qiagen columns (Hybaid) as described in the manufacturer's instructions. This produced approximately 4 μg of DNA. To subclone the insert into pNGS21, the plasmid vector was digested with *EcoRI*, treated with calf-intestinal alkaline phosphatase, and then ligated with *EcoRI*-digested phage DNA. The ligation was used to transform *E. coli* JM101, and isopropyl- β -D-thiogalactopyranoside (IPTG)-X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) color selection was used to identify recombinant clones. One of these, pFVL5, was used for subcloning into pUR290.

Construction of pFVL9. pFVL5 DNA was purified by CsCl-ethidium bromide density gradient centrifugation and digested with *EcoRI*, and the 1.7-kbp insert was purified by fractionation in low-melting-point agarose. The subcloning into pUR290 was the same as described for pNGS21.

Purification of *M. leprae* DNA. *M. leprae* bacilli were isolated from 10 g of infected armadillo liver, which had been stored at -70°C by using the method of Draper (32a). Microscopic examination confirmed that a suspension of acid-fast bacilli which contained very little armadillo tissue had been prepared.

DNA was prepared by using a modification of the method of Clark-Curtiss et al. (6). The *M. leprae* cell suspension was frozen on dry ice and ground in a pestle and mortar in the presence of glass beads. The resultant fine powder was transferred to a 50-ml polypropylene tube, and 10 ml of a solution containing 10 mM EDTA, 100 mM NaCl, 150 mM Tris-HCl (pH 8.0), 3.5% SDS, and 250 μg of proteinase K per ml was added. This mixture was incubated at 55°C for 2 h and then gently extracted sequentially with buffer-equilibrated phenol, phenol-chloroform mix, and chloroform-isoamyl alcohol (100:4). DNA was precipitated by the addition of 0.6 volume of propan-2-ol and centrifugation at 4,000 rpm for 20 min (Beckman JS4.2 rotor). The pellet was washed with 70% ethanol and dissolved in 1 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Field-inversion gel electrophoresis showed most of the DNA to be greater than 50 kbp in size, and the total yield was estimated at 70 μg .

Construction of an *M. leprae* cosmid library. A genomic library was constructed in the cosmid Lawrist4. The DNA was partially digested with *Sau3A*, suitable conditions being determined by serial dilution of the enzyme. High-molecular-weight partial digests were extracted with a phenol-chloroform mix and chloroform-isoamyl alcohol and precipitated with ethanol. The DNA was size selected by centrifugation through sucrose. Sucrose gradients (10 to 40%) in 1 M NaCl-20 mM Tris (pH 7.2)-20 mM EDTA were prepared by layering 0.5 ml each of 40, 35, 30, 25, 20, 15, and 10% sucrose solutions into 5-ml ultracentrifuge tubes. A 0.5-ml amount of DNA was layered on top, and the gradients were centrifuged at 25,000 rpm for 17 h at 10°C in a Beckman SW55Ti rotor. The gradient was collected in 250- μ l fractions, and the size of the DNA was checked by agarose gel electrophoresis. High-molecular-weight fractions were pooled, and the DNA was precipitated with ethanol, washed with 70% ethanol, and resuspended in 50 μ l of TE buffer. The DNA was cloned into Lawrist4 as described for pMSC1 (13), obtaining approximately 2,000 transformants. Cosmid DNA was purified from 12 colonies by using the miniprep method of Coulson and Sulston (7), digested with *Bam*HI, and analyzed by agarose gel electrophoresis. All 12 clones

contained large inserts, and all showed different restriction patterns. The library was picked onto nitrocellulose filters which had been placed on L agar containing 40% sucrose and 40 μ g of kanamycin per ml. After the colonies had grown, a second sterile filter was placed on top of the colonies, and the filter sandwich was stored at -70°C . Colonies were picked into Micronics tubes (Flow Laboratories, High Wycombe, United Kingdom) in a 96-tube array, each tube containing 0.6 ml of L broth and 40 μ g of kanamycin per ml, and shaken at 37°C overnight. The clones were then transferred to nitrocellulose filters by using a 96-pin device; four sets were transferred to a single filter, slightly offsetting each set, so that 384 clones could be screened on a single filter. A total of 576 colonies were picked in this way. The filters were incubated on L agar containing kanamycin, and the colonies were lysed and used for hybridizations by standard techniques. When positive clones were identified, the parent clone was cultured, and cosmid DNA was prepared for further analysis.

Construction of pMFG3. Cosmid C508 was digested with *Hind*III, and the 1.8-kbp fragment was purified and cloned into pUC9-2. Southern hybridization with the insert from pFVL9 was used to confirm that the correct fragment had been cloned.

DNA sequencing. Shotgun sequencing of the 1.8-kbp *Hind*III fragment in M13mp19 was accomplished by the method of Bankier and Barrell (1). The template for double-stranded sequencing was prepared by denaturing 3 μ g of DNA in 50 μ l of 0.4 M NaOH–0.2 mM EDTA at 37°C for 30 min. The DNA was precipitated with ethanol, resuspended in 7 μ l of TE buffer, and used in a standard sequencing reaction. Sequence assembly was accomplished by using Staden software at the Human Genome Mapping Project computing facility at the Clinical Research Centre, Northwick Park, United Kingdom. After 28 clones had been sequenced, two groups of overlapping sequences (contigs) of 1.5 and 0.26 kbp had been assembled. Double-stranded sequencing of the ends of the *Hind*III insert in pMFG3 oriented the contigs in relation to each other, and the contigs were joined by sequencing with two primers (O1 and O2) which annealed on either side of the gap. Regions which had not been sequenced on both strands were analyzed on the other strand by polymerase chain reaction amplification of inserts in M13 by using a biotinylated forward primer followed by capture on streptavidin-coated magnetic beads and direct sequencing using the reverse primer (17). In addition, regions containing compressions were sequenced by using dITP instead of dGTP and electrophoresed in parallel to a standard dGTP sequencing reaction. Note that although almost all of the *Hind*III fragment was sequenced, one end (at the 3' end of *sra*) was not quite reached. Sequence analysis was carried out with Genetics Computer Group (University of Wisconsin) software (8) at the Human Genome Mapping Project computing facility and also with PC-Gen software (Intelligenetics, El Camino, Calif.).

Nucleotide sequence accession number. These sequence data will appear in the EMBL, GenBank, and DDJB Nucleotide Sequence Data Libraries under the accession number X68431.

RESULTS

Screening λ gt11 library. Leprosy sera from six lepromatous leprosy patients were pooled and diluted 1:10 in TBS buffer. Cross-reacting antibodies were removed by absorption with *E. coli* extracts; the efficiency of this procedure

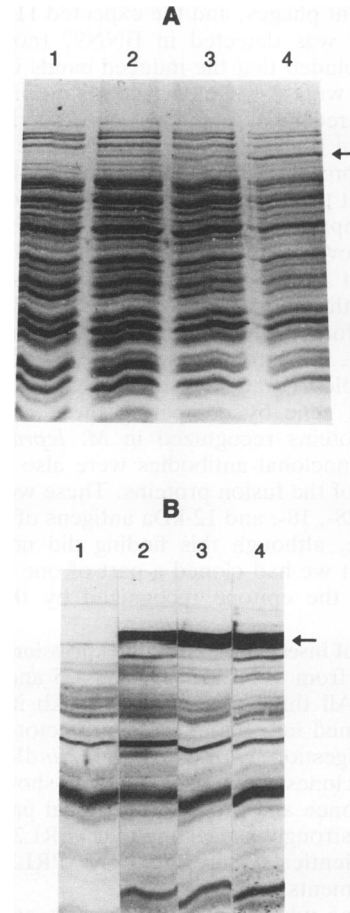


FIG. 1. Recognition of β -galactosidase fusion proteins by pooled leprosy sera. Ten micrograms from induced *E. coli* BNN97 (lane 1) and lysogens were fractionated by SDS-8% PAGE. (A) Coomassie blue-stained gel; (B) immunoblot with pooled leprosy patient sera. Lanes: 1, BNN97; 2, Y1089(λ PRL21); 3, Y1089(λ PRL23); 4, Y1089(λ PRL25). The arrows indicate the fusion protein induced in the recombinant clones.

was monitored by Western blot analysis. The serum pool was then used to screen a total of 2×10^5 plaques from an *M. leprae* λ gt11 library. Three positive plaques were identified and purified until all plaques were colorless when plated with IPTG and X-Gal, and all produced positive signals on filters after screening with the serum pool. These three clones were called λ PRL21, -23, and -25.

Preliminary immunological characterization of fusion protein. Lysogens of the recombinant phages were constructed in *E. coli* Y1089, and protein extracts were analyzed after induction with IPTG. Crude extracts were fractionated by SDS-PAGE, and total protein was visualized by Coomassie blue staining. A distinct band which was not present in the control λ gt11 lysogen, BNN97, was observed with the recombinant lysogens (Fig. 1A). The protein extracts were Western blotted, and the filters were incubated with the pooled patient sera. The induced band in the recombinant lysogens was strongly recognized by the leprosy sera, whereas no equivalent band was recognized in the BNN97 track (Fig. 1B). When the experiment was repeated, and the filter was probed with anti- β -galactosidase antiserum, the induced protein was again recognized in the tracks contain-

ing recombinant phages, and the expected 116-kDa β -galactosidase band was detected in BNN97 (not shown). We therefore concluded that the induced bands in the recombinant lysogens were β -galactosidase fusion proteins and the leprosy sera recognized an *M. leprae*-derived sequence rather than the β -galactosidase moiety. The three induced recombinant proteins were of the same molecular weight, suggesting that they might be from sibling clones.

In an attempt to relate the fusion proteins to proteins previously shown to have been recognized by individual leprosy serum samples (32), Western blots were probed separately with the six serum samples which had been combined to form the pooled sera. All six recognized the fusion protein, four strongly and two less so. However, it was not possible to draw any conclusions about the identity of the cloned gene by comparing these results with the pattern of proteins recognized in *M. leprae* extracts. A variety of monoclonal antibodies were also used to probe immunoblots of the fusion proteins. These were specific for 70-, 65-, 36-, 28-, 18-, and 12-kDa antigens of *M. leprae*. All were negative, although this finding did not exclude the possibility that we had cloned a part of one of these genes which lacked the epitope recognized by the monoclonal antibody.

Subcloning of inserts into plasmid expression vectors. DNA was prepared from λ PRL21, -23, and -25 and was digested with *EcoRI*. All three contained a 1.7-kb insert. Each of these was cloned into the expression vector pNGS21 (30). Restriction digestion with *PstI* and *HindIII* of selected recombinant clones from each ligation showed that both enzymes cut once and produced identical patterns with all three inserts, strongly suggesting that λ PRL21, -23, and -25 were in fact identical. Therefore, only λ PRL25 was used in further experiments.

To determine which end of the insert encoded the *M. leprae* antigen, clones containing the insert in opposite orientations were induced with IPTG, and Western blots were probed with the pooled patient sera. No recombinant protein was seen; this could be attributed to the instability of the product or perhaps the product was too small to resolve clearly (the leader peptide in the fusion protein would only be 2 kDa). The insert was therefore recloned from λ PRL25 into pUR290, to create the same β -galactosidase fusion as was present in the recombinant phage, and the immunoblotting experiment was repeated. This time, fusion proteins were observed which reacted with the sera and had a molecular mass identical to that produced by λ PRL25 were observed. One recombinant plasmid, pFVL9, which expressed the fusion protein, was used in later studies.

Restriction mapping and partial sequencing of the insert. The restriction map of the insert in pFVL9 was determined. The single restriction sites for *PstI* and *HindIII* were located approximately 90 and 500 bp, respectively, from the same end of the fragment. Double digestion with *EcoRV*, which lies within the *lacZ* gene, showed that the insert was oriented such that the end of the fragment closer to the *PstI* and *HindIII* sites was transcribed from the *lac* promoter and therefore was the end which encoded the *M. leprae* antigen (Fig. 2). Partial sequencing of this end was carried out, from double-stranded sequencing of both pFVL9 and an M13mp18 clone containing the 1.7-kb *EcoRI* fragment. This revealed an open reading frame (ORF), but neither the DNA sequence nor the predicted amino acid sequence showed significant homology to previously published sequences.

Cloning of the entire gene. To clone the entire gene, a cosmid library array was screened with the *M. leprae*

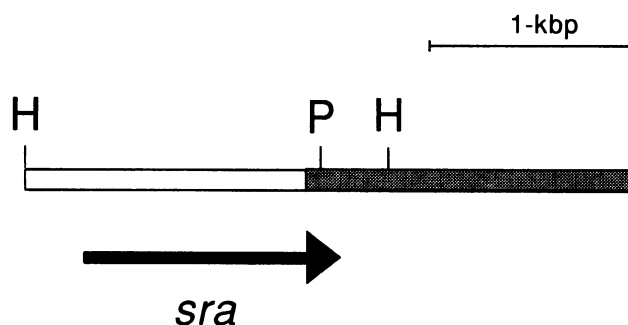


FIG. 2. Diagram showing position of *sra* gene. The shaded region represents the DNA insert in λ PRL25, which overlaps the *HindIII* fragment which was sequenced. The position and direction of transcription of *sra* are shown at the bottom. H, *HindIII*; P, *PstI*.

fragment. Five clones were isolated and shown by restriction digestion to be different but related, indicating that they overlapped. We noticed that the clones isolated were identical to those hybridizing to the plasmid pYA1090, which contains a 4.0-kbp insert from the λ gt11 clone L17 (5a, 26). This belonged to what had been designated hybridization group III antigen by these workers and given the code number 25L by Young et al. (35). It was therefore concluded that the gene we were analyzing was identical or adjacent to this gene.

Analysis of the cosmid DNA by Southern hybridization after digestion with *EcoRI*, *PstI*, and *ApaI* by using the 1.7-kbp insert in pFVL9 as a probe resulted in bands of the same sizes (7.0, 1.9, and 3.5 kbp, respectively) in four of the five cosmids (C508, C510, C517, and C545; Fig. 3). This was good evidence that these bands were genuine *M. leprae* bands and that there were no major rearrangements in the area surrounding this gene in these cosmid clones. The fifth cosmid, C205, produced bands with different sizes from those of the other four, suggesting that the sequence lay near the end of the insert in this clone. Digestion with *KpnI* produced different-sized bands in all clones, all larger than 9.0 kbp, suggesting that most or all of the cosmids contained inserts beginning within a large *KpnI* fragment. Our earlier results with the 1.7-kb insert in pFVL9 showed that a *HindIII* site lay 400 bp into the insert (Fig. 2) and indicated that *HindIII* might be a suitable enzyme for subcloning the entire gene. Southern analysis of *HindIII*-digested cosmids by using the 1.7-kbp insert as a probe showed two bands of 5 and 1.8 kbp (not shown). The 1.7-kbp fragment was then digested with *HindIII*, and the two resulting fragments were purified and used separately as probes. The results suggested that the gene encoding the antigen would lie within the 1.8-kbp *HindIII* fragment seen in the cosmid clones. Accordingly, the 1.8-kbp *HindIII* fragment was purified from cosmid C508 and cloned into pUC9-2, producing pMFG3.

Sequence analysis. The 1.8-kbp *HindIII* fragment in pMFG3 was sequenced by shotgun cloning into M13mp8 (Fig. 4). The DNA had a G+C content of 55.6%, similar to that calculated for the *M. leprae* genome (6), and was found to contain an ORF of 1,227 bp, which is in the same reading frame as the protein expressed, fused to β -galactosidase in pFVL9. Taking the first ATG as the putative start codon, the ORF encodes a polypeptide of 408 amino acids, with a predicted molecular mass of 42,466 Da and a predicted pI of 3.98. A canonical Shine-Dalgarno sequence was located upstream of the putative initiation codon, with a gap of 9 bp (Fig. 4). No clear promoter sequence could be located.

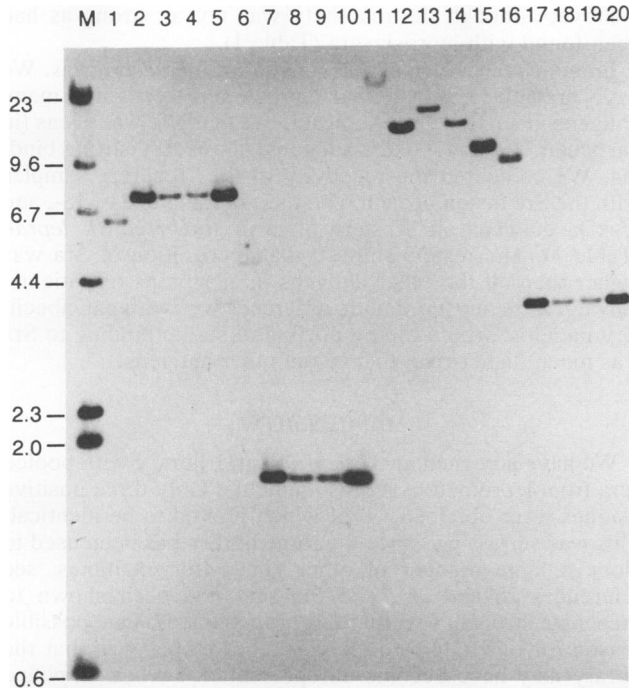


FIG. 3. Hybridization of the 1.7-kbp insert from pFVL9 to *M. leprae* cosmid clones. Five cosmid clones were digested with *EcoRI* (lanes 1 to 5), *PstI* (lanes 6 to 10), *KpnI* (lanes 11 to 15), and *ApaI* (lanes 16 to 20) and electrophoresed in an 0.8% agarose gel. After Southern transfer, the filter was hybridized with the ³²P-labeled 1.7-kbp insert from pFVL9 and autoradiographed. The cosmids used were C205 (lanes 1, 6, 11, and 16), C508 (lanes 2, 7, 12, and 17), C510 (lanes 3, 8, 13, and 18), C517 (lanes 4, 9, 14, 19), and C545 (lanes 5, 10, 15, and 20). M, ³²P-labeled λxHindIII markers.

1 AAGCTTACTCTCTGTA ACCACCAAGGTTGTC CCACCGCCGCCGAC CAAGTGTGCAAGCTA
 61 GTATCGCAGGTATTT AGTTCCTACGGGAAG CAGTACGAGGGTAT GCCGTCAGGGTGTGTA
 121 GACCAGAGTCGGGTTG TTTGTCCAGAGTCTG AAAAGATGCCGCAGTG ATTATATGGACAGTG
 181 ATCATATGTATCTAA ACACAGAGGATTAAG CCTCCTAAGTGTGG AGTGTTAAGCCTCAC
 241 TGTGGGGCATCGTAT TGTGTGAAGTTTCTG GCGGTATCGGAGTGG CTACCGCGGAGTAGG
 301 AAGATTATAGAGAAG ATATAGAGGTAACAA AATC ATG TTC GAC TTC ATG GTG
 Met Phe Asp Phe Met Val
 353 TAT TCG CCC GAG GTC AAT GCT TTT CTT ATG AGT CGT GGC CCG GGT TCT
 Tyr Ser Pro Glu Val Asn Ala Phe Leu Met Ser Arg Gly Pro Gly Ser
 401 ACT CCT CTA TGG GGC GCC GCT GAG GCA TGG ATT AGT CTG GCA GAG CAG
 Thr Pro Leu Trp Gly Ala Ala Glu Ala Trp Ile Ser Leu Ala Glu Gln
 449 TTA ATG GAG GCG GCG CAG GAA GTG TCG GAC ACA ATA GTC GTC GCG GTG
 Leu Met Glu Ala Ala Gln Glu Val Ser Asp Thr Ile Val Val Ala Val
 497 CCG GCG TCA TTT GCG GGC GAG ACG TCG GAT ATG TTA GCC AGC CGT GTC
 Pro Ala Ser Phe Ala Gly Glu Thr Ser Asp Met Leu Ala Ser Arg Val
 545 AGC ACT TTT GTG GCG TGG CTG GAT GGT AAC GCC GAG AAT GCC GGG CTG
 Ser Thr Phe Val Ala Trp Leu Asp Gly Asn Ala Glu Asn Ala Gly Leu
 593 ATT GCT CGC GTC CTC CAC GCA GTG GCA TAC GCC TTC GAG GAG GCG CGT
 Ile Ala Arg Val Leu His Ala Val Ala Tyr Ala Phe Glu Glu Ala Arg
 641 GCG GGC ATG GTG CCA CTG CTG ACG GTG CTC GGG AAC ATC ATA CAC ACC
 Ala Gly Met Val Pro Leu Leu Thr Val Leu Gly Asn Ile Ile His Thr
 689 ATG GCG CTG AAG GCA ATA AAC TGG TTC GGG CAA GTA TCT ACC ACG GTC
 Met Ala Leu Lys Ala Ile Asn Trp Phe Thr Gly Gln Val Ser Thr Thr Val
 737 GCG GCT TTG GAA GCC GAC TAC GAT CTG ATG TGG GTC CAA AAC TCG ACG
 Ala Ala Leu Glu Ala Asp Tyr Asp Leu Met Trp Val Gln Asn Ser Thr
 785 GCA ATG ACG ACC TAT CCG GAT ACC GTG CTC AGA GAA ACG GGA AAA ATG
 Ala Met Thr Thr Tyr Arg Asp Thr Val Leu Arg Glu Thr Gly Lys Met
 833 GAA AAT TTT GAA CCA GCA CCG CAG TTG GTC TCT AGA TAT TGC ATG GAT
 Glu Asn Phe Glu Pro Ala Pro Gln Leu Val Ser Arg Tyr Cys Met Asp
 881 CGG CCG GAT TCC GTT AAT TCG TTT CAT TCT TCG TCT TCG TCC GAT TCA
 Arg Arg Asp Ser Val Asn Ser Thr His Ser Ser Ser Ser Ser Asp Ser

A hydropathicity plot of the polypeptide showed that it possesses hydrophobic amino- and carboxy-terminal domains and a hydrophilic central domain (Fig. 5A). The central part of the protein is marked by a large number of serine residues (Fig. 5B). Serine makes up 14.9% of the total protein and 29.9% of a 137-residue central region which constitutes a third of the entire protein. The average serine composition found in published protein sequences is 6.76% ± 2.52% (derived from OWL protein data base version 9 [16a]), so the serine content for the whole protein is 3 standard deviations from the mean, while the serine content of the 137-residue region is 9 standard deviations from the mean. We have therefore named the protein serine-rich antigen (Sra).

The greatest homology found between Sra and published sequences was with a 51-kDa hypothetical antigen of *Mycobacterium tuberculosis* (28). This was a sequence deduced from an ORF found adjacent to the gene encoding the 65-kDa antigen of *M. tuberculosis*. The homology was mainly found in the amino-terminal part of the protein (Fig. 6); the identity in a 149-residue region close to the amino terminus was 34% (55% including conserved residues). Outside this region, the homology was much lower.

Immune recognition of fusion protein by sera from leprosy patients and endemic controls. To investigate the humoral response to Sra in leprosy patients, the fusion protein from *E. coli* (pFVL9) was induced, and the bacterial extracts were Western blotted. A total of 76 serum samples from leprosy patients were used to probe the blots. Seventy-five percent of all samples recognized the fusion protein (Fig. 7). Lack of recognition of β-galactosidase in induced cultures of *E. coli* BNN97 confirmed that this reactivity was due to the *M. leprae* sequence (not shown). Analysis of the antibody

929 CTT TAC GAA TCA ATC GAT AAT CTG TAT GAC TCG GTG GCT CAG TCC GAA
 Leu Tyr Glu Ser Ile Asp Asn Leu Tyr Asp Ser Val Ala Gln Ser Glu
 977 GAA CAC GGA TCG GAC AGT ATG TCC CAA TCG TAC AAT ACG TGT GGA TCG
 Glu His Gly Ser Asp Ser Met Ser Gln Ser Tyr Asn Thr Cys Gly Ser
 1025 GTC GCT CAG TCT GAA TTG TGC GAT TCG CCG TTT GGC ACC CCT TCT CAG
 Val Ala Gln Ser Glu Leu Cys Asp Ser Pro Phe Gly Thr Pro Ser Gln
 1073 AGC TCT CAG AGC AAC GAC CTT TCT GCT ACG CTT ACA CAG CAG TTG
 Ser Ser Gln Ser Asn Asp Leu Ser Ala Thr Ser Leu Thr Gln Gln Leu
 1121 GGA GGC CTG GAC AGT ATC ATA TCG AGT GCT AGT GCA AGT CTG CTT ACA
 Gly Gly Leu Asp Ser Ile Ile Ser Ser Ala Ser Ser Ile Met Pro Ile Val
 1169 ACG AAC AGC ATA AGC AGC TCG ACG GCA AGT AGT ATT ATG CCT ATA GTG
 Thr Asn Ser Ile Ser Ser Ser Thr Ala Ser Ser Ile Met Pro Ile Val
 1217 GCT TCT CAG GTT ACA GAA ACA CTG GGT CGA AGT CAA GTG GCT GTG GAG
 Ala Ser Gln Val Thr Glu Thr Leu Gly Arg Ser Gln Val Ala Val Glu
 1265 AAA ATG ATA CAG TCG ATT AGC AGC ACC GCG GTG AGC GTG GAT GTG GCG
 Lys Met Ile Gln Ser Ile Ser Ser Thr Ala Val Ser Val Asp Val Ala
 1313 GCC AGT AAA GTG GTT GCC GGC GTA GGT CAG GCT GTG TCG GTT GGT GCT
 Ala Ser Lys Val Val Ala Gly Val Gly Gln Ala Val Ser Val Gly Ala
 1361 TTG CGG GTG CCG GAA AAT TGG GCC ACG GCC AGT CAG CCG GTC ATG GCG
 Leu Arg Val Pro Glu Asn Trp Ala Thr Ala Ser Gln Pro Val Met Ala
 1409 ACG GCG CAC TCA GTG CCG GCA GGC TGC AGC GCG ATT ACC ACG GCA GTA
 Thr Ala His Ser Val Pro Ala Gly Cys Ser Ala Ile Thr Thr Ala Val
 1457 AGC GGG CCG CTG GAA GGG GTG ACT CAG CCT GCT GAG GAA GTT CTC ACG
 Ser Gly Pro Leu Glu Gly Val Thr Gln Pro Ala Glu Glu Val Leu Thr
 1505 GCT TCC GTT GCC GGC GGC AGT GGG ACT GGA GGT CCG GCC TTC AAC GAA
 Ala Ser Val Ala Gly Gly Ser Gly Thr Gly Pro Ala Phe Asn Glu
 1553 GCG GTT TGA CCGTGAAGTGGGTTG GTTGAACAATTATCG AGGTGCTAGTTGGTC
 Ala Val ***
 1607 ATAGTGTAGCTTTT TTGATACGTGCATAA GACCATCCCGTCACA CACCTCGTGAAGCA
 1667 GTTTCGTGATGTTTG TGAATGTTTGATTC GTCTGTTGATCGGGT TGTACGAAATGGCCT
 1727 GAGTGGTCTCTGCC TCCAAGTCACATTTG CTATGTTATGAGCAC GAAGCCCATCT

FIG. 4. Nucleotide sequence of *HindIII* fragment and the deduced amino acid sequence of Sra. The arrow marks the location of the fusion with β-galactosidase in pFVL9. SD, putative Shine-Dalgarno sequence.

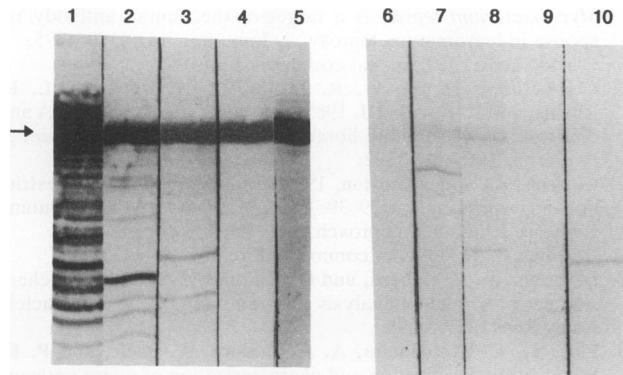


FIG. 7. Recognition of Sra- β -galactosidase fusion protein by sera from leprosy patients. Seven hundred fifty micrograms of crude lysate from an induced culture of JM101(pFVL9) was separated by SDS-8% PAGE. After transfer to nitrocellulose, individual strips were probed with *E. coli*-absorbed sera from leprosy patients at a concentration of 1:50. Examples of the strips are shown. Lanes: 1 to 5, positive strips; 6 to 10, negative strips. The arrow indicates the Sra- β -galactosidase fusion protein.

in cell-cell interaction during mating, contains a very serine-threonine-rich C-terminal domain, which is thought to be heavily glycosylated and the part of the protein that is anchored in the cell wall (23). There are precedents for glycosylation of proteins in mycobacteria. It has been reported that several antigens of *M. bovis* are glycosylated (9). In addition, we have shown that some proteins in *M. leprae* bind concanavalin A (not shown) but have not excluded the possibility that the carbohydrate moieties identified in this way are noncovalently associated. We know nothing about the cellular localization of Sra, but in view of its antigenicity it might be expected to be present in the cell wall. In addition, the hydrophobic nature of the amino- and carboxy-terminal domains suggests that the protein may lie in a hydrophobic environment, such as the cytoplasmic membrane or the lipid-rich cell wall. However, there was no indication of an amino-terminal signal sequence. An alternative role for serines has been postulated for some eukaryotic

TABLE 1. Recognition of different *M. leprae* antigens by sera from leprosy patients^a

Subjects	No. of serum samples	No. (%) of subjects with serum recognition of:			
		Sra- β -gal	<i>M. leprae</i> antigen		
			33 kDa	18 kDa	15 kDa
All leprosy patients	76	57 (75)	36 (47)	31 (41)	13 (17)
Multibacillary patients	54	42 (78)	34 (63)	26 (48)	11 (20)
Paucibacillary patients	22	15 (68)	2 (9)	5 (23)	2 (9)
Patients with history of ENL	17	16 (94)	14 (82)	7 (41)	5 (29)
Patients with no ENL	37	26 (70)	20 (54)	19 (51)	6 (16)
Tuberculosis patients	31	8 (26)	5 (16)	14 (45)	0 (0)
Endemic controls	26	0 (0)	0 (0)	0 (0)	1 (4)

^a Sera from leprosy patients, tuberculosis patients, and healthy endemic controls were used to probe immunoblots of JM101(pFVL9) and sonicated *M. leprae*. Recognition of Sra- β -galactosidase (Sra- β -gal) and of the 33-, 18-, and 15-kDa antigens of *M. leprae* was scored. Multibacillary patients were also divided into those who had been documented as having had an erythema nodosum leprosum (ENL) reaction and those for whom there was no history of ENL.

Repeat 1

209 S V A Q S E
230 S V A Q S E

Repeat 2

192 S S S S S
267 S I I S S
281 S I S S S
315 S I S S T

Repeat 3

286 T A S S I
294 V A S Q V
326 A A S K V
351 T A S Q P
390 T A S V A

FIG. 8. Repeated motifs found in Sra. The numbers indicate the locations of the first amino acid residues in the protein sequence.

transcription factors where serine-threonine-rich domains are thought to be sites for phosphorylation (18).

Within the protein, some repeated sequences can be discerned, mostly within the central domain (Fig. 8). The significance of these repeats is not known, but in Pra, the *M. leprae* proline-rich antigen, repeated sequences in the amino-terminal region have been shown to be highly immunogenic (31). The greatest homology to Sra that we found with a published sequence was with a hypothetical 51-kDa *M. tuberculosis* protein. This was deduced from an ORF adjacent to the gene encoding the *M. tuberculosis* 65-kDa antigen (28). The homology is only present in the amino-terminal parts of the proteins and does not extend to the serine-rich domain of Sra. Interestingly, however, the 51-kDa protein also contained many repeated sequences outside of this region of homology.

We have analyzed the immune response in leprosy patients to the carboxy-terminal part of Sra only, as this was all that was present in the β -galactosidase fusion protein, but there is a strong antibody response to this region. We showed that serum samples from 75% of leprosy patients contained antibodies to the fusion protein. This figure was 78% for multibacillary patients and 68% for paucibacillary patients. This compares with recognition of the 18-kDa antigen by 40% of multibacillary patients and 6% of paucibacillary patients by an enzyme-linked immunosorbent assay (ELISA) (14) and recognition of the LSR2 antigen by 55.8% of multibacillary patients and 21% of paucibacillary patients by a dot-ELISA (21). Although more-sensitive tests have been reported, on the basis of the *M. leprae*-specific phenolic glycolipid I (up to 100% of multibacillary and 75% of paucibacillary patients; reviewed by Smith [29]) or the Pra-specific monoclonal antibody F47-9 (100% of multibacillary and 91% of paucibacillary patients [19]), we conclude that Sra is a highly immunogenic protein and an important antigen. Serum samples from tuberculosis patients also bound the fusion protein, albeit more weakly than those from leprosy patients. This suggests that there is a homolog of Sra in *M. tuberculosis*. An extremely high percentage (94%) of multibacillary patients with a history of erythema nodosum leprosum reacted to Sra. This may indicate that Sra is involved in the immunological processes which take place during these reactions, but studies specifically addressing this issue would have to be carried out.

The antibody level that bound to the fusion protein was

compared indirectly with the levels of antibodies binding other major antigens by scoring bands detected in Western blots of *M. leprae* extracts. Sra was recognized by more serum samples than were the 33-, 18-, and 15-kDa proteins. However, this comparison is not ideal, since the levels of the 33-, 18-, and 15-kDa antigens in the *M. leprae* sonicate may have been low while the concentration of the Sra- β -galactosidase fusion protein was high. On the other hand, this is a minimum estimate for the proportion of patients recognizing Sra; by expressing the entire protein, or by using a more sensitive immunoassay, more antibodies might be detected. There was variation in the seroreactivity in the class III clones reported by Sathish et al. (26). All of the clones expressing the 45-kDa protein were recognized more weakly than those expressing a fusion protein. This might be due to the instability of the recombinant 45-kDa product, or epitopes might be hidden when the whole protein is present, perhaps because of anomalous folding in *E. coli*, and are exposed when only part of the protein is present. The high proportion of positive paucibacillary sera suggests that this protein may be of diagnostic value.

To further characterize the antigenicity of Sra, it will be necessary to use the whole protein. The observation that it can be expressed as a nonfusion protein is encouraging for this. We do not know at present if the *sra* promoter or just the translational signals function in *E. coli*. Access to recombinant Sra, preferably as a nonfusion protein, will allow the evaluation of the cellular immune response, which is thought to be the dominant arm of the immune response in protection to leprosy, to this protein.

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