

Heterologous Expression of the 65-Kilodalton Antigen of *Mycobacterium leprae* and Murine T-Cell Responses to the Gene Product

F. I. LAMB, ANN E. KINGSTON, IRIS ESTRADA-G, AND M. J. COLSTON*

Laboratory for Leprosy and Mycobacterial Research, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom

Received 9 November 1987/Accepted 5 February 1988

The gene encoding the immunodominant 65-kilodalton antigen of *Mycobacterium leprae* was subcloned from a lambda gt11 clone into the high-copy-number plasmid pUC8. *Escherichia coli* containing these recombinants produced large amounts of the antigen, which was purified by polyacrylamide gel electrophoresis in the presence of urea. The ability of *E. coli* to recognize the mycobacterial promoter was confirmed by constructing additional clones in which the gene is flanked by transcriptional terminators from phage fd. A similar approach was used to demonstrate the expression of this gene in *Streptomyces lividans*. Mice immunized with killed *M. leprae* showed cell-mediated immune reactivity to the purified 65-kilodalton protein which stimulated both in vitro lymphoproliferative and in vivo delayed-type hypersensitivity responses.

The failure to grow *Mycobacterium leprae* in vitro has severely restricted studies on the basic biology and immunology of this organism, which causes human leprosy. In recent years, the potential of recombinant DNA technology to provide proteins of *M. leprae* has begun to be exploited with the cloning of mycobacterial DNA into *Escherichia coli* (20, 26). Screening of these libraries, predominantly with monoclonal antibodies, has resulted in the identification of partial or complete clones for the genes of five major antigens (with molecular masses of 12, 18, 28, 36, and 65 kilodaltons [kDa]); some of these have been shown to be involved in T-cell-mediated responses (6, 8, 14-16). Since T-cell responses are known to be of vital importance in protection against leprosy, there is great interest in studying T-cell recognition of these recombinant polypeptides. The 65-kDa protein is highly conserved among mycobacterial species (11, 19, 24) and is an immunodominant antigen during the course of infection with *M. leprae* and *Mycobacterium tuberculosis* and after vaccination with *Mycobacterium bovis* BCG (2, 6, 14, 15). The suggested relatedness of this protein to a heat shock protein (D. B. Young, personal communication) also generates an interest in its basic molecular biology. Despite the relatively poor ability of *E. coli* to express mycobacterial genes (7), the promoters of the 65-kDa antigen genes from *M. leprae*, *M. tuberculosis*, and *M. bovis* all function in *E. coli* (18, 21; this work). In this study, we confirm the functioning of the mycobacterial promoter in *E. coli* and show that it also functions in *Streptomyces lividans*. We also report the purification of the antigen from an *E. coli* system providing high-level expression and describe T-cell responses to the antigen in mice immunized with *M. leprae*.

MATERIALS AND METHODS

Subcloning of the *M. leprae* 65-kDa antigen gene. DNA manipulations and general cloning procedures were performed as described by Maniatis et al. (12). *E. coli* Y1090 was lysogenized with phage Y3178, which contains the entire 65-kDa antigen gene (13). Phage were prepared from a

thermally induced culture, and DNA was extracted by dissolving the phage in a solution containing 6 M guanidinium chloride (1); this was extracted with chloroform, and DNA was precipitated with ethanol. The agarose gel-purified insert was recloned into the *EcoRI* site of pUC8 (23) to produce plasmids pIL161 and pIL164, which contain the insert in opposite orientations. In addition, after end filling with the Klenow fragment of DNA polymerase I, the insert of Y3178 was recloned into the end-filled *BamHI* site of plasmid pIJ666 (7) to produce plasmids pIL301 and pIL302, again with the insert in opposite orientations.

Expression in *S. lividans* was investigated by cloning the Y3178 insert into the *EcoRI* site of plasmid pIJ697 (T. Keiser and R. E. Melton, Gene, in press); only one orientation was studied, in plasmid pIL251. Manipulation of *S. lividans* was done as previously described (5).

Expression of the 65-kDa antigen gene in heterologous systems. *E. coli* strains were cultured overnight in LB broth (containing ampicillin or kanamycin, both at 50 µg/ml, for pUC8 and pIJ666 subclones, respectively) with IPTG (isopropyl-β-D-thiogalactopyranoside) added to 2.5 mM, as appropriate. *Streptomyces* clones were grown for 48 h in YEME (5) containing 5 mM MgCl₂ and 5 µg of thiostrepton per ml; expression was studied at 30 and 37°C. Samples (30 µl) were centrifuged, and bacteria were suspended in 50 µl of 100 mM Tris hydrochloride (pH 8.4)-250 mM sucrose-1 mM EDTA-0.005% bromophenol blue-2% sodium dodecyl sulfate (SDS)-5 mM dithiothreitol, heated to 95°C for 10 min, and analyzed on 12% polyacrylamide gels (9). Separated proteins were electrophoretically transferred to nitrocellulose for Western blotting (immunoblotting; 22), with the murine monoclonal antibody IIC8 (3) used at a dilution of 1:3,000. A goat horseradish peroxidase-conjugated anti-mouse second antibody was obtained from Bio-Rad Laboratories (Richmond, Calif.) and used at a dilution of 1:3,000, and blots were developed with 4-chloro-1-naphthol. Pre-stained molecular mass markers (Bethesda Research Laboratories, Gaithersburg, Md.) were used for size estimation of immunoreactive bands.

Purification of recombinant 65-kDa antigen. Cultures (5 liters) of *E. coli* TG2 containing pIL161 were grown over-

* Corresponding author.

night at 37°C in LB broth containing ampicillin, harvested by centrifugation ($2,500 \times g$ for 10 min), suspended in 50 ml of CE buffer (30 mM sodium citrate, 10 mM EDTA [pH 6.0 to 6.2]), and sonicated with three 1-min bursts at 100 W. The lysate was centrifuged at $10,000 \times g$ for 20 min, and the resulting pellet was washed three times with 100 ml of CE buffer. The washed pellet was resuspended in 50 ml of UPE buffer (6 M urea, 50 mM phosphate buffer [pH 7], 20 mM EDTA) by vortexing for 2 to 3 min, and the suspension was shaken gently at room temperature for 15 min. After removal of insoluble material by centrifugation at $10,000 \times g$ for 20 min, a 0.9 to 1.2 M ammonium sulfate fraction was prepared by slow addition of 3.6 M stock solution, incubation on ice for 30 min, and centrifugation at $10,000 \times g$ for 10 min. This fraction was dissolved in phosphate-buffered saline, extensively dialyzed against phosphate-buffered saline, and filtered (filter pore size, 0.45 μm) to produce a crude fraction.

Further purification was achieved by loading the UPE buffer-dissolved and centrifuged material without further preparation onto 6-mm-thick, 14-by-14-cm polyacrylamide gels (3% stacking gel and 7% resolving gel) by using the buffer system of Laemmli, except that SDS was replaced with 6 M urea. Gels were run at a constant power of 5 W for 17 to 24 h; after the band was located by staining a strip with Coomassie blue, the protein-containing gel was excised and homogenized (Sorvall Omnimixer; speed 4 for 20 s) with an equal volume of UPE buffer, and the protein was eluted by shaking overnight at 4°C. After filtration through Whatman no. 54 paper under negative pressure, the protein was precipitated by the addition of ammonium sulfate, to 2 M. The protein was then dissolved in phosphate-buffered saline, dialyzed, and filtered (filter pore size, 0.45 μm).

Mouse immunization and antigen preparations. Female BALB/c mice (6 to 8 weeks old) bred at the National Institute for Medical Research were immunized intradermally in both flanks with 10^7 to 10^8 gamma-irradiation-killed *M. leprae* (ML γ). The mice were sacrificed 28 days after receiving the immunization. Preparations of ML γ and a soluble, sonicated extract of *M. leprae* (MLS) were done as previously described (8).

Lymphocyte preparation and culture. Lymph node cell suspensions were prepared from the draining inguinal lymph nodes of immunized mice (five per group), pooled, and suspended in RPMI medium containing 10% fetal calf serum, 2-mercaptoethanol (5×10^{-5} M), HEPES (10 mM; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), L-glutamine (1 mM), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g/ml}$). Samples (200 μl) containing 3×10^5 cells were added to the wells of round-bottomed microtiter plates (Nunc, Roskilde, Denmark), and 20 μl of the various antigens was added over a range of concentrations.

Cultures were incubated at 37°C in a 5% CO₂-humidified incubator for 5 days, pulsed with 1.0 μCi of [³H]thymidine (specific activity, 5 Ci/mmol; Amersham International, Amersham, United Kingdom), and harvested after 18 h. [³H]thymidine incorporation was measured by standard liquid scintillation counting.

Delayed-type hypersensitivity testing. Groups of mice (six immune mice or nonimmune controls per group) were injected in the right hind footpad with 5 μg of MLS or 5 μg of purified antigen. Increases in footpad size were measured at 24, 48, and 72 h after antigen challenge by using a screw gauge micrometer (Moore and Wright, Sheffield, United Kingdom). Results are expressed as the mean percentage increase in footpad thickness relative to the unchallenged

footpad; diluent alone did not induce any footpad swelling over the period studied.

RESULTS

Expression in heterologous systems. Our results confirm the ability of the promoter of the *M. leprae* 65-kDa antigen gene to function in *E. coli*. The lambda gt11 lysogen (Fig. 1, lane C) and the pUC8 subclones (lanes D to G) all expressed the protein in the absence of IPTG, although in the lysogen, the antigen was hardly detectable even on the Western blot, except at very high loadings. The addition of IPTG did not enhance expression in the lysogen (data not shown) or in the pUC8 subclones (lanes D and E are subclones with the insert in the same orientation as the *lacZ* promoter; lane D is without and lane E is with IPTG).

That expression is under control of the cloned mycobacterial sequence is indicated by the independence of expression on orientation. Figure 1 (lane F) shows the clone in which transcription of the insert is in the direction opposite to that of the *lacZ* promoter; the addition of IPTG had no effect in this clone (lane G). Furthermore, subclones in plasmid pIJ666 (Fig. 1, lanes H and I) also expressed the antigen; the lower level of expression is attributable to the lower copy number of this plasmid. Since this vector contains transcriptional terminator sequences from phage fd on each side of the cloning site (Keiser and Melton, in press), expression in this system must be directed by a promoter in the cloned DNA.

The *M. leprae* 65-kDa antigen gene was also able to express its product in *S. lividans*. The cloning site of the *Streptomyces* vector used, pIJ697, is also flanked by phage fd terminators, again demonstrating initiation of transcription and translation in response to cloned sequences. Cultivation of subclones in *E. coli* and *S. lividans* at 30°C had no detectable effect on expression, although growth of the latter at 37°C was poor. Moreover, the levels of expression were similar throughout the growth cycle in *E. coli* (results not shown).

Isolation of the expressed antigen. In crude sonicates, the 65-kDa antigen expressed in *E. coli* was insoluble as defined by low-speed centrifugation ($10,000 \times g$, 20 min). Figure 2 shows the total sonicate (lane B) and supernatant (lane C) and pellet (lane D) fractions therefrom. The antigen was refractory to solubilization with a number of agents, including salt solutions and detergents (including deoxycholate and Triton X-100); although SDS (above 0.1%) was an effective solubilizing agent, we preferred to avoid its use because of the difficulty of its complete removal and its toxicity to cultured lymphocytes. Much of the host cell protein was removed by washing the pellet with citrate-EDTA buffer (Fig. 2, lanes E and F), and lanes G and H (Fig. 2) demonstrate the solubility of the antigen in buffer containing 6 M urea. Further purification of the antigen on urea-containing polyacrylamide gels yielded material containing predominantly the intact 65-kDa antigen as determined by SDS-polyacrylamide gel electrophoresis; although a number of lower-molecular-mass species were frequently observed (Fig. 2, lane J). These increased in quantity upon prolonged storage, particularly in the crude urea-dissolved fraction, with the appearance of a characteristic set of components that retained immunoreactivity on Western blotting (results not shown).

Murine T-cell responses to the recombinant 65-kDa antigen. Mice which had been vaccinated intradermally with whole irradiated *M. leprae* were used, since such mice are known

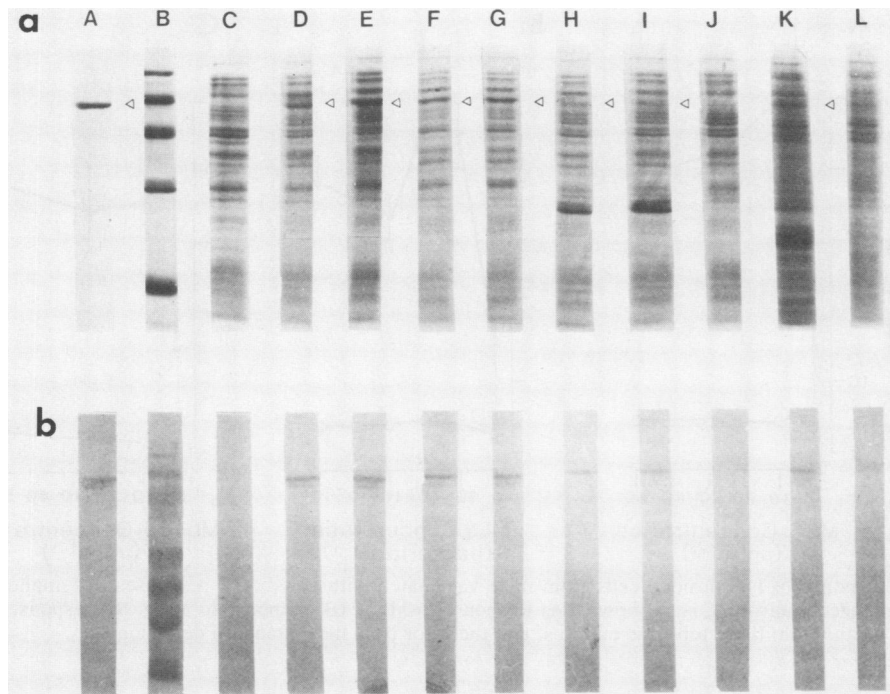


FIG. 1. Expression of the *M. leprae* 65-kDa antigen in *E. coli* and *S. lividans*. (a) SDS-polyacrylamide gels stained with Coomassie blue; (b) Western blots probed with monoclonal antibody IIC8. Lanes: A, 65-kDa antigen purified from *E. coli* TG2 containing pIL161; B, molecular mass markers (see below); C, total proteins from *E. coli* Y1090 lysogenized with phage Y3178; D to G, total proteins from *E. coli* strains containing pUC8 derivatives (in D and E, the direction of transcription of the insert is the same as that of *lacZ* [i.e., pIL161], while in F and G, transcription is in the opposite direction [pIL164]; E and G show cultures induced with IPTG); H and I, lysates of *E. coli* in which the inserts are flanked by transcriptional terminators of pIJ666; J, *E. coli* containing the vector pUC8; K, lysate of *S. lividans* containing the 65-kDa gene in pIJ697; L, *S. lividans* containing nonrecombinant pIJ697. Molecular mass markers (a) are, from the top, beta-galactosidase (116 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Prestained markers (b) are myosin H chain (apparent molecular mass, 214 kDa), phosphorylase *b* (111 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), α -chymotrypsinogen (24 kDa), β -lactoglobulin (18 kDa), and lysozyme (15 kDa). The arrowheads indicate the *M. leprae* 65-kDa antigen.

to be protected against *M. leprae* infection (17). The results of a representative lymphocyte proliferation assay using inguinal lymph node lymphocytes from such mice are shown (Fig. 3). It can be seen that strong proliferation responses were observed with MLS, ML γ , and the 65-kDa antigen, indicating that the latter is an important T-cell determinant in vaccinated mice. This was confirmed by the results of delayed-type hypersensitivity testing (Fig. 4); again, the 65-kDa antigen induced a response comparable with that of MLS. However, the kinetics of the response differed in that the peak response with the former occurred at 24 h and then declined, whereas the response to MLS was sustained for 48 h and only declined slightly over 72 h.

DISCUSSION

Considerable progress has been made recently in the expression of mycobacterial antigen genes in *E. coli*, particularly in the expression vector lambda gt11. Although this vector is designed for the expression of cloned DNA sequences as fusion proteins, it has been observed that three of the *M. tuberculosis* genes thus far identified with monoclonal antibodies can indeed be expressed under the control of their own regulatory sequences in lambda gt11 clones (25). We have concentrated on the gene encoding the 65-kDa antigen of *M. leprae*.

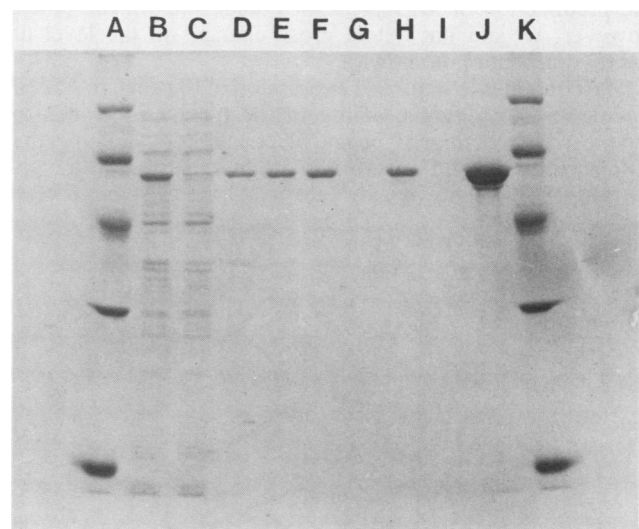


FIG. 2. Isolation of the *M. leprae* 65-kDa antigen from *E. coli* clones. Lanes: A and K, molecular mass markers (see the legend to Fig. 1); B, total sonicate; C, supernatant produced by low-speed centrifugation; D, pellet fractions produced by low-speed centrifugation; E and F, pellets from the first two washes; G and H, pellet and supernatant fractions after dissolution in UPE buffer and centrifugation at $10,000 \times g$ for 15 min; I and J, similar pellet and supernatant fractions after gel purification and dissolution in phosphate-buffered saline.

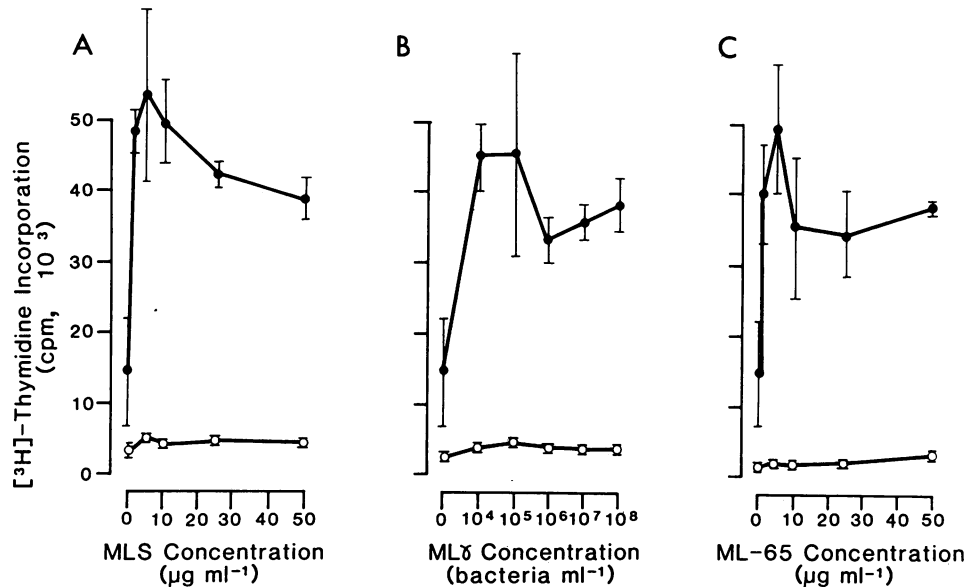


FIG. 3. Proliferative response of lymph node cells from mice vaccinated with ML γ . The responses of lymphocytes from immunized animals (●) and nonimmunized controls (○) are shown. (A) Response to MLS; (B) response to ML γ ; (C) response to the purified 65-kDa antigen. Results are the means from three replicate cultures, and the error bars show standard deviations.

Our initial aim was to investigate cloning systems able to synthesize much larger amounts of the antigen than lambda gt11 can to facilitate purification. In the course of this work, we have confirmed that the gene is indeed expressed from its own promoter in *E. coli* and that it also functions in *S. lividans*. The increase in the level of expression obtained by increasing the gene dosage is shown by comparison of the amounts of proteins produced by the lambda gt11 and pUC8 subclones (Fig. 1, lanes C and D, respectively). Cultivation of clones at different temperatures was studied because of the predilection of *M. leprae* for cooler sites in the body; however, we did not detect gross changes in the level of expression (data not shown).

The constitutive nature of expression of the gene in *E. coli* is demonstrated by the ability of the pIJ666 subclones to synthesize the antigen; phage fd terminators flanking the cloning site prevent transcription originating from promoter-

active sequences on the plasmid (Keiser and Melton, in press). Our interest in expression of the 65-kDa antigen gene in *S. lividans* arose from suggestions that this host is able to utilize mycobacterial regulatory sequences more frequently and more efficiently than *E. coli* can (7, 10). Although we have demonstrated that the gene does function in *S. lividans*, its level of expression was much lower than in *E. coli*. The validity of this gene as a general model of mycobacterial gene expression in heterologous systems must be considered dubious because of its proposed relatedness to heat shock proteins, which are widely conserved throughout the bacterial population. Significant sequence conservation has indeed been noted between the mycobacterial 65-kDa antigen genes and a gene from *E. coli* which complements the *ams* mutation (24), reducing the mRNA lifetime. The 65-kDa antigen gene varies little among the mycobacteria; Lu et al. (11) have shown identical restriction maps for the gene in *M. tuberculosis*, *Mycobacterium africanum*, and *M. bovis* BCG, whereas the *M. leprae* and *M. tuberculosis* gene sequences are 98% homologous (19). To better evaluate *S. lividans* as an expression system for mycobacterial genes, it might be more appropriate to study less conserved genes whose promoters are less likely to resemble those of *E. coli*.

It was apparent that the pUC8 clones expressed the *M. leprae* 65-kDa antigen in an essentially insoluble form; this is in contrast to a report of the expression of the analogous gene of *M. bovis* BCG in *E. coli* (21). Although in *M. leprae* the antigen may be associated with the cell wall (4), we believe that the insolubility in our recombinant *E. coli* strains is a consequence of the high level of overproduction, since the antigen synthesized by the lambda lysogen appears in the soluble fraction.

The 65-kDa proteins of *M. tuberculosis*, *M. bovis* BCG, and *M. leprae* have been shown to be recognized by T-cell clones derived from tuberculosis or leprosy patients (2, 14, 15), and Shinnick (18) has speculated that it might be a suitable antigen to include in a recombinant vaccine. Moreover, Kaufmann and colleagues have estimated that approx-

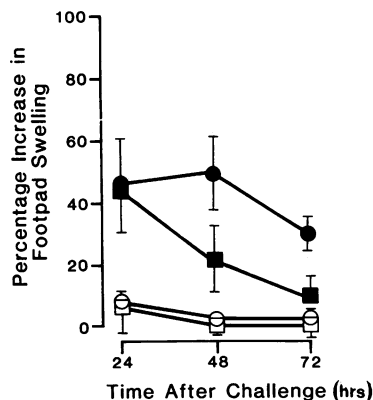


FIG. 4. Delayed-type hypersensitivity responses of mice immunized with ML γ . Responses of immunized mice to MLS (●) and the 65-kDa antigen (■) are compared with those of control mice (○ and □). Results are the means from six mice, and the error bars show standard deviations.

imately one in five *M. tuberculosis*-reactive T cells from *M. tuberculosis*-immune mice recognize determinants on this protein (6). Since mice vaccinated intradermally with killed *M. leprae* are immune to subsequent infection with *M. leprae* (17), we investigated whether T-cell responses to the antigen could be detected in such mice. The results indicate that the 65-kDa antigen is a major T-cell determinant in *M. leprae*-immunized mice. Experiments are now in progress to investigate whether the antigen itself can confer protection against *M. leprae* infection in mice.

ACKNOWLEDGMENTS

I. Estrada-G was supported by the British Leprosy Relief Association and the British Council.

We thank T. M. Buchanan for the kind gift of monoclonal antibody IIC8 and D. A. Hopwood and T. Kieser (John Innes Institutes, Norwich, United Kingdom) for much advice, as well as the *Streptomyces* strains and plasmids.

LITERATURE CITED

- Cox, R. A., and V. M. Katoch. 1986. Evidence for genetic divergence in ribosomal RNA genes in mycobacteria. *FEBS Lett.* **195**:194-197.
- Emmrich, F., J. Thole, J. van Embden, and S. H. E. Kaufmann. 1986. A recombinant 64 kilodalton protein of *Mycobacterium bovis* Bacillus Calmette-Guerin specifically stimulates human T4 clones reactive to mycobacterial antigens. *J. Exp. Med.* **163**: 1024-1029.
- Gillis, T. P., and T. M. Buchanan. 1982. Production and partial characterization of monoclonal antibodies to *Mycobacterium leprae*. *Infect. Immun.* **37**:172-178.
- Gillis, T. P., R. A. Miller, D. B. Young, S. R. Khanolkar, and T. M. Buchanan. 1985. Immunochemical characterization of a protein associated with *Mycobacterium leprae* cell wall. *Infect. Immun.* **49**:371-377.
- Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf. 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. John Innes Foundation, Norwich, United Kingdom.
- Kaufmann, S. H. E., U. Vath, J. E. R. Thole, J. D. A. van Embden, and F. Emmrich. 1987. Enumeration of T cells reactive with *Mycobacterium tuberculosis* organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur. J. Immun.* **17**: 351-357.
- Kieser, T., M. T. Moss, J. W. Dale, and D. A. Hopwood. 1986. Cloning and expression of *Mycobacterium bovis* BCG DNA in "*Streptomyces lividans*." *J. Bacteriol.* **168**:72-80.
- Kingston, A. E., P. R. Salgame, N. A. Mitchison, and M. J. Colston. 1987. Immunological activity of a 14-kilodalton recombinant protein of *Mycobacterium tuberculosis* H37Rv. *Infect. Immun.* **55**:3149-3154.
- Läemmlli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Lamb, F. I., and M. J. Colston. 1986. Cloning of *Mycobacterium leprae* genes in streptomyces. *Leprosy Rev.* **57**(Suppl.):225-229.
- Lu, M. C., M. H. Lien, R. E. Becker, H. C. Heine, A. M. Buggs, D. Lipovsek, R. Gupta, P. W. Robbins, C. M. Grosskinsky, S. C. Hubbard, and R. A. Young. 1987. Genes for immunodominant protein antigens are highly homologous in *Mycobacterium tuberculosis*, *Mycobacterium africanum*, and the vaccine strain *Mycobacterium bovis* BCG. *Infect. Immun.* **55**:2378-2382.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mehra, V., D. Sweetser, and R. A. Young. 1986. Efficient mapping of protein antigenic determinants. *Proc. Natl. Acad. Sci. USA* **83**:7013-7017.
- Mustafa, A. S., H. K. Gill, A. Nerland, W. J. Britton, V. Mehra, B. R. Bloom, R. A. Young, and T. Godal. 1986. Human T-cell clones recognise a major *M. leprae* protein antigen expressed in *E. coli*. *Nature (London)* **319**:63-66.
- Oftung, F., A. S. Mustafa, R. N. Husson, R. A. Young, and T. Godal. 1987. Human T cell clones recognise two abundant *M. tuberculosis* protein antigens expressed in *E. coli*. *J. Immunol.* **138**:927-931.
- Ottenhoff, T. H. M., P. R. Klatser, J. Ivanyi, D. G. Elferink, M. Y. L. de Wit, and R. R. P. de Vries. 1986. *Mycobacterium leprae*-specific protein antigens defined by cloned human helper T cells. *Nature (London)* **319**:66-68.
- Shepard, C. C., R. Van Ledingham, and L. L. Walker. 1980. Searches among mycobacterial cultures for antileprosy vaccines. *Infect. Immun.* **29**:1034-1039.
- Shinnick, T. M. 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. *J. Bacteriol.* **169**:1080-1088.
- Shinnick, T. M., D. Sweetser, J. Thole, J. van Embden, and R. A. Young. 1987. The etiologic agents of leprosy and tuberculosis share an immunoreactive protein antigen with the vaccine strain *Mycobacterium bovis* BCG. *Infect. Immun.* **55**:1932-1935.
- Thole, J. E. R., H. G. Dauwerse, P. K. Das, D. G. Groothuis, L. M. Schouls, and J. D. A. van Embden. 1985. Cloning of *Mycobacterium bovis* BCG DNA and expression of antigens in *Escherichia coli*. *Infect. Immun.* **50**:800-806.
- Thole, J. E. R., W. J. Keulen, A. H. J. Kolk, D. G. Groothuis, L. G. Berwald, R. H. Tiesjema, and J. D. A. van Embden. 1987. Characterization, sequence determination and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. *Infect. Immun.* **55**:1466-1475.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
- Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259-268.
- Young, D. B., J. Ivanyi, J. H. Cox, and J. R. Lamb. 1987. The 65 kDa antigen of mycobacteria—a common bacterial protein? *Immunol. Today* **8**:215-219.
- Young, D. B., L. Kent, and R. A. Young. 1987. Screening of a recombinant mycobacterial library with polyclonal antiserum and molecular weight analysis of expressed antigens. *Infect. Immun.* **55**:1421-1425.
- Young, R. A., V. Mehra, D. Sweetser, T. Buchanan, J. Clark-Curtiss, R. W. Davis, and B. R. Bloom. 1985. Genes for the major protein antigens of the leprosy parasite *Mycobacterium leprae*. *Nature (London)* **316**:450-452.