INFECTION AND IMMUNITY, Dec. 1985, p. 800–806
0019-9567/85/120800-07$02.00/0

Vol. 50, No. 3

Cloning of *Mycobacterium bovis* BCG DNA and Expression of Antigens in *Escherichia coli*

JELLE E. R. THOLE, HANS G. DAUWERSE, PRANAB K. DAS, DICK G. GROOTHUIS, LEO M. SCHOULS, AND JAN D. A. VAN EMBDEN*

Rijksinstituut voor Volksgezondheid en Milieuhygiëne, 3720 BA Bilthoven, The Netherlands

Received 17 June 1985/Accepted 3 September 1985

A gene bank of *Mycobacterium bovis* BCG DNA in *Escherichia coli* was constructed by cloning *Sau*3A-cleaved mycobacterium DNA fragments into the lambda vector EMBL3. The expression of mycobacterial antigens was analyzed by Western blotting with hyperimmune rabbit sera. Among 770 clones tested, several were found that produced various mycobacterial antigens in low amounts, with concentrations generally close to the detection limit. One particular clone was chosen for further investigation. This clone produced a 64-kilodalton (kDa) antigen. By placing the lambda promoter P_L in front of the structural gene of this antigen, an overproducing *E. coli* strain was obtained. Rocket-line immunoelectrophoresis experiments showed that antigens cross-reacting with the 64-kDa protein are present in a wide variety of mycobacteria and also in so-called purified protein derivatives which are routinely used for skin tests. Preliminary experiments indicate the presence of antibodies against the 64-kDa antigen in sera from tuberculosis patients.

Pathogenic mycobacteria contain a complex mixture of antigens with varied chemical compositions (9, 10, 21). With regard to their specificity, Stanford and Grange distinguished four groups of mycobacterial antigens as follows: (i) antigens common to all mycobacteria and often also found in *Listeria*, *Corynebacterium*, and *Nocardia* spp.; (ii) common antigens present only in slowly growing mycobacteria; (iii) common antigens shared by fast growers and only present in these bacteria; and (iv) species-specific antigens not found in other species (22). Most studies on the specificity of soluble mycobacterial antigens are based on experimental techniques with immunoprecipitation in agarose and complex mixtures of antigens derived from either ultrasonicated bacteria or culture fluids.

Many attempts to isolate purified protein antigens from these complex mixtures to produce specific and readily standardizable antigens in sufficient amounts were unsuccessful (9, 10, 21). The unavailability of purified antigens has hampered a detailed study of the role of the individual antigens in the immunological and pathological events during mycobacterial infections. As a consequence, no satisfactory rapid diagnostic test for the serodiagnosis of tuberculosis has been developed, although many such attempts have been made. The relatively crude and nonstandardizable antigens employed in these studies resulted in nonreproducible tests, a great deal of nonspecificity, or both (10).

A different approach to obtaining large amounts of well-characterized, readily standardizable mycobacterial protein antigens is the molecular cloning and expression of mycobacterial DNA in a suitable host vector system. Clark-Curtiss et al. (6) reported the establishment of gene libraries of *Mycobacterium vaccae*, "*Mycobacterium lufu*," and *Mycobacterium leprae* in *Escherichia coli* with a cosmid as a vector. No expression of mycobacterial DNA was observed in *E. coli*, unless the vector was provided with a strong promoter. No antigen-expressing clones were found in a limited number of *M. leprae* recombinants provided with a transcription signal. More recently, Young et al. (27) reported the cloning and expression of *Mycobacterium tuberculosis* DNA in *E. coli*. By using the vector lambda gt11, which is provided with a transcription and translation signal, three different antigens were expressed as determined by their reaction with three monoclonal antibodies.

In this paper, we report the cloning and expression of *Mycobacterium bovis* BCG DNA in *E. coli* with the bacteriophage lambda vector EMBL3. In addition, the characterization of one immunogenic protein, expressed by *E. coli*, is described.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The bacterial strains used in this study are listed in Table 1. Strain Q359 and the lambda vector EMBL3 (13) were kindly provided by R. Koes. The plasmids pPLc236 (19) and pCI857 (20) were kindly given by E. Remaut. Strain M1070 was used as a host for latter plasmids or their derivatives.

Media and reagents. Mycobacteria were grown on Middlebrook 7H9 broth (DIFCO Laboratories, Detroit, Mich.) with some minor modifications as follows. Four-tenths of a gram of 0.4 g sodium citrate instead of 0.1 g, 1.5 g of Na_2HPO_4 instead of 2.5 g, and 1.5 g of NaH_2PO_4 instead of 1.0 g was added per liter. NZYM medium or NZYM agar (16) was used for growing *E. coli* K-12 cells. Antibiotic supplements were previously described (25). All enzymes used were from Boehringer GmbH, Mannheim, Federal Republic of Germany, New England BioLabs, Beverly, Mass., and P-L Biochemicals, St. Goar, Federal Republic of Germany, and they were used according to the recommendations of the manufacturer.

Anti-human and anti-rabbit immunoglobulins labeled with horseradish peroxidase were kindly provided by A. M. Hagenaars (Ryksinstitut voor Volksgezondheid en Milieuhygiëne [RVM], Bilthoven, The Netherlands). Various preparations of purified protein derivate (PPD) were kindly provided by E. C. Beuvery (RVM, Bilthoven, The Netherlands). Goat anti-mouse total immunoglobulin peroxidase-labeled conjugate was obtained from Nordic (Tilburg, The Netherlands). The antisera against *M. bovis* BCG, 1739 and 1438, were raised by repeated intravenous immunization of rabbits with disrupted cells of *M. bovis* BCG strain P3.

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Species	Relevant properties	Reference or origin
P3	*M. bovis* BCG	BCG strain used as vaccine strain in The Netherlands	R. H. Tiesjema
7114	*M. tuberculosis*	Clinical isolate	This laboratory
H37Rv	*M. tuberculosis*	Reference strain	This laboratory
4514	*M. tuberculosis*	Clinical isolate	This laboratory
3442	*M. scrofulaceum*	Clinical isolate	This laboratory
ATCC 14468	*M. smegmatis*	Reference strain	ATCC[a]
ATCC 25220	*M. gastri*	Reference strain	ATCC
1012	*M. kansasii*	Clinical isolate	This laboratory
3875	*M. avium*	Clinical isolate	This laboratory
ATCC 6841	*M. fortuitum*	Reference strain	ATCC
5544	*M. africanum*	Clinical isolate	This laboratory
8637	*ADM*	Armadillo-derived mycobacterium	F. Portaels (18)
LE392	*E. coli* K-12	$hsr_k^- hsm_k^-$	P. Leder (16)
Q359	*E. coli* K-12	hsr_k hsm_k P2 lysogenic	R. Koes (13)
1046	*E. coli* K-12	$recA^-$ hsr_k^- hsm_k	L. van de Ploeg (4)
M1070	*E. coli* K-12	1046, carrying plasmid pCI857	This laboratory

[a] ATCC, American Type Culture Collection.

Another anti-BCG antiserum was obtained from DAKO Laboratories, Copenhagen, Denmark. Murine monoclonal antibodies from clone F47-10 were kindly provided by A. H. J. Kolk (Royal Tropical Institute, Amsterdam, The Netherlands).

DNA technology. DNA of *M. bovis* BCG was obtained as follows. *M. bovis* BCG strain P3 was incubated in Middlebrook 7H9 broth at 37°C. To make the mycobacterial cells sensitive to lyzozyme, glycine was added in the late exponential phase (24) to a final concentration of 1%, and incubation was continued for 18 h. Cells from a 400-ml culture were harvested, washed in saline, and suspended in 25% sucrose–0.05 M Tris–0.05 M EDTA (pH 8.0). Lysozyme was added to a final concentration of 1 mg/ml, and the cells were incubated for 90 min at 37°C. Proteinase K was added to 200 μg/ml, and the incubation at 37°C was continued for 15 min. The cells were lysed by the addition of sodium dodecyl sulfate to a final concentration of 5%. After a 15-min incubation at 37°C, NaCl was added to a concentration of 1 M, and DNA was extracted twice with phenol, precipitated by ethanol, and further purified by CsCl density gradient centrifugation as previously described (25).

Standard procedures were used for the preparation of phage lambda and plasmid DNA, cleavage, ligation, transformation, and packaging of DNA (16). For cloning in phage EMBL3, the vector DNA was cleaved with *Bam*HI and *Eco*RI. The small *Eco*RI-*Bam*HI linker fragments were removed by isopropanol precipitation (13). This procedure prevented the stuffer fragment with *Bam*HI ends to ligate with the vector arms.

Immunological techniques. The expression of mycobacterial antigens in *E. coli* K-12 was determined by the binding of antibodies to proteins on nitrocellulose fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This so-called "Western blotting" was basically done as described by Burnette (2), with some modifications as described previously (25). For the detection of antigens in lambda-infected cells, 20 μl of the lysates was applied into lanes of polyacrylamide gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done on 13% acrylamide-containing gels as described by Laemmli (15). Antisera used for Western blotting were absorbed with *E. coli* K-12.

Soluble proteins of *E. coli* and mycobacteria were prepared by breaking the cells with a French press, followed by centrifugation for 5 min at 10,000 × *g*. Immunoelectrophoresis techniques were performed as described by Axelsen (1).

RESULTS

Construction of a gene bank of *M. bovis* BCG. The bacteriophage lambda vector EMBL3 was used to construct a gene bank of *M. bovis* BCG. Due to the lambda packaging requirements, this vector allows the cloning of DNA fragments with sizes of 9 to 22 kilobases (kb) (13). *M. bovis* BCG DNA was partially cleaved with the restriction endonuclease *Sau*3A, and fragments having sizes between 10 and 20 kb were pooled. These fragments were ligated with *Bam*HI-cleaved EMBL3 DNA, and after in vitro packaging and transduction into *E. coli* K-12 strain LE392, about 6,000 PFU of *M. bovis* DNA per μg were obtained. The same efficiency was found by using the P2-lysogenic *E. coli* strain Q359, indicating that virtually all plaque observed were from recombinant DNA phages, as only recombinants are able to grow on P2 lysogens (13). DNA from 12 randomly picked plaques was analyzed by cleavage with the restriction enzyme *Sal*I. All 12 restriction fragment patterns were different, showing that all of these clones contained various mycobacterium *Sau*3A fragments.

Screening of recombinant DNA clones on the production of mycobacterium antigens. Initially, an attempt was made to select mycobacterial antigen-producing clones by an in situ enzyme-linked immunoassay on plaques replicated onto nitrocellulose filters (23, 26). More than 5,000 plaques were screened by this method, and none of the recombinants showed a significant positive signal with either the anti-*M. bovis* BCG antiserum 1739 or the DAKO serum. This number of recombinants should contain about 10 to 20 mycobacterium genome equivalents, as the maximal genome size of *M. bovis* was estimated to be about 4,500 kb (3, 12). These results indicate that the expression of *M. bovis* DNA in *E. coli* K-12 is poor; therefore, a more sensitive assay was used to detect antigens in the recombinant clones. In a previous study (25), we found that Western blotting is quite sensitive, with a lower detection limit of about 50 protein molecules per *E. coli* cell. Therefore, Western blotting was used to screen individual clones.

We screened 135 lysates by using the anti-*M. bovis*

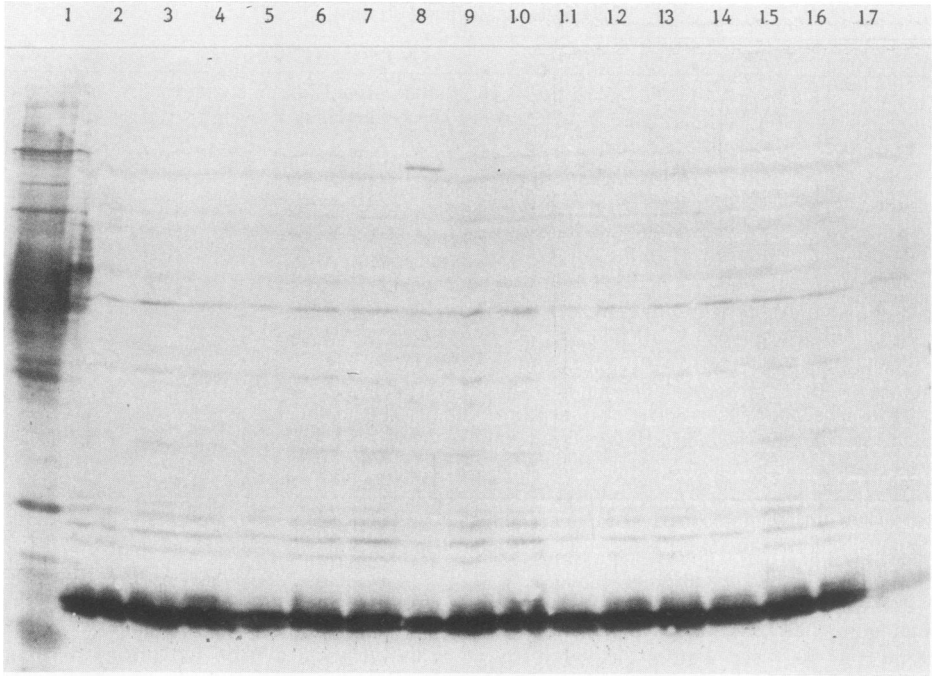

FIG. 1. Western blot of the primary screening of 16 recombinants of the *M. bovis* gene bank. Lanes 2 to 17 were loaded with 20 μl of *E. coli* K-12 lysates containing approximately 2.5 μg of protein. Lane 1 was loaded with a lysate of *M. bovis* BCG containing about 7 μg of protein. The blot was developed with anti-*M. bovis* BCG rabbit antiserum 1739. Lane 8, A representative of phenotype A, showing an antibody-binding protein antigen with an apparent molecular mass of 64 kDa.

antiserum 1739 as well as the DAKO anti-*M. bovis* antiserum to develop the nitrocellulose filters. As both antisera gave identical results, the other lysates, 635 in number, were screened only with serum 1739. Figure 1 is an example of the screening procedure by immunoblotting. It shows the results of the analysis of 16 individual recombinant clones, including the one that produced a 64-kilodalton (kDa) protein that reacted with antibodies in serum 1739.

In 21 of the 770 lysates tested, a band appeared, suggesting the presence of the mycobacterial protein antigen. According to their mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 6 different antigenic phenotypes were distinguished among these 21 clones (Table 2). Except for phenotype A, the bands stained weakly and the antigen concentration in these lysates was probably close to the detection limit, suggesting a poor expression in *E. coli*. All suspected positive clones were purified by replating, and lysates from these clones were tested again on the production of mycobacterial antigens. Only clones of the phenotypes A, B, and C showed a reproducibly positive picture on immunoblots. One clone, λRIB23, with phenotype A was chosen for further investigation.

TABLE 2. Molecular sizes of *M. bovis* BCG proteins expressed by EMBL3 recombinants in *E. coli*

Phenotype	No. of clones	Molecular size (kDa)	Designation of representative clone
A	10	64	λRIB23
B	1	70	λRIB555
C	1	95	λRIB415
D	3	>100	λRIB33
E	5	90	λRIB89
F	1	30	λRIB211

Subcloning of phenotype A clone λRIB23. The phenotype A clone λRIB23 expresses a 64-kDa protein antigen designated as antigen A. By cleavage of the DNA of λRIB23 with *Sal*I, the mycobacterium DNA insert in λRIB23 was estimated to be about 19 kb. To construct an antigen A-overproducing clone, λRIB23 was digested with *Eco*RI and subcloned into the *Eco*RI site of the expression vector pPLc236. This vector carries the left promoter, P_L, of bacteriophage lambda. The promoter can be fully repressed at 28°C when the thermolabile repressor product of the lambda CI857 gene is functional, whereas strong transcription takes place at 42°C (19). Two subclones, pRIB1000 and pRIB1001, were obtained that expressed the A antigen in the host at 28°C. Both plasmids carried a 4.9-kb *Eco*RI fragment; however, the orientation of this fragment in these two plasmids differed. Consistently, cells containing only one of these plasmids, pRIB1000, overproduced the antigen at 42°C (Fig. 2). To further localize the DNA regions of pRIB1000 involved in the expression of antigen A, deletion derivates of pRIB1000 were made by endonuclease digestion, followed by self-ligation at low DNA concentrations (1 to 5 μg/ml) and reintroduction into strain M1070. The physical map of pRIB1000 and its various deletion derivatives are shown in Fig. 3. The expression of antigen A by the various deletion mutants was analyzed by Western blotting, and the results (Fig. 3) indicate that the structural gene of antigen A is located within a 2.3-kb DNA segment on the pRIB1000 map (Fig. 2) between the 1.5- and 3.8-kb coordinates. One particular deletion mutant, pRIB1016, expressed an antigen of only 38 kDa in size, suggesting the production of a truncated protein.

Serological cross-reactivity of antigen A with other mycobacterial species. Immunoprecipitation by rocket-line immunoelectrophoresis is a powerful technique to detect the presence of cross-reacting antigens in complex protein mix-

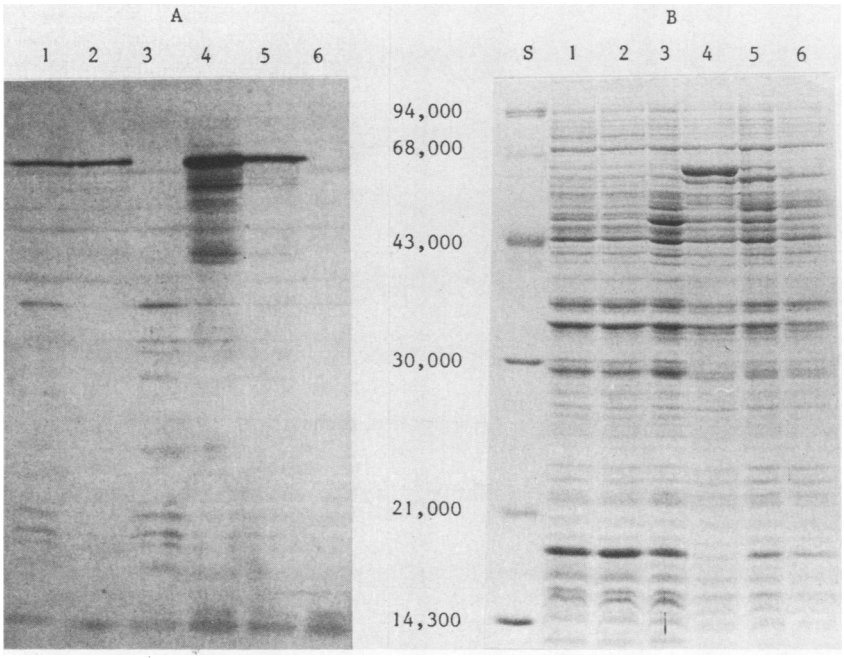

FIG. 2. Expression of the A antigen by *E. coli* at 28°C (lanes 1 to 3) and 42°C (lanes 4 to 6). (A) Western blots of cell lysates of M1070 (lanes 3 and 6), M1070 carrying pRIB1000 (lanes 1 and 4), and M1070 carrying pRIB1001 (lanes 2 and 5). The blots were developed with anti-*M. bovis* BCG antiserum 1739. (B) Coomassie blue-stained polyacrylamide gels loaded with the same lysates as shown in panel A. S, Molecular weight standards (Bio-Rad) as follows: lysozyme (14.3 kDa), soybean trypsin inhibitor (21.0 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (68.0 kDa), and phosphorylase B (94.0 kDa).

tures (1). Therefore, we checked whether antigen A precipitated in agarose by immunoelectrophoresis. The soluble protein fraction of induced cultures of strain M1070 carrying pRIB1000 gave rise to a number of precipitation lines in rocket-line immunoelectrophoresis with serum 1438. One of these lines could be identified as a precipitate of mycobacterium antigen, because only this line fused with a precipitation line derived from a *M. bovis* BCG lysate. Rocket-line immunoelectrophoresis was then used to determine whether an antigen cross-reacting with antigen A was present in the soluble protein fraction of the following mycobacterium species: *M. tuberculosis* (three strains), *M. scrofulaceum*, *M. smegmatis*, *M. gastri*, *M. kansasii*, *M. avium*, *M. fortuitum*, *M. africanum*, and armadillo-derived mycobacterium. In all of these bacteria, antigens cross-reacting with the A antigen produced by *E. coli* were detected. Furthermore, we analyzed the presence of cross-reacting antigens in PPDs from *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, *M. avium* and *M. marinum*. Rocket-line immunoelectrophoresis of all of these PPDs showed the presence of cross-reacting anti-

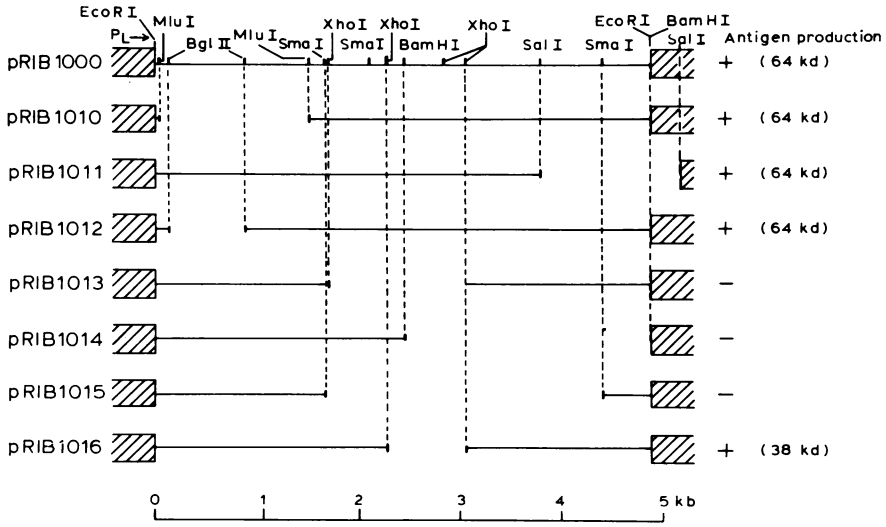

FIG. 3. Physical map and expression of the mycobacterial 4.9-kb DNA segment in pRIB1000. The crosshatched parts represent the vector pPLc236 DNA. The molecular masses (in kilodaltons) of the expressed proteins that bind antimycobacterium antibodies as detected by Western blots is indicated on the right. The lambda promoter P_L drives transcription from left to right on this map.

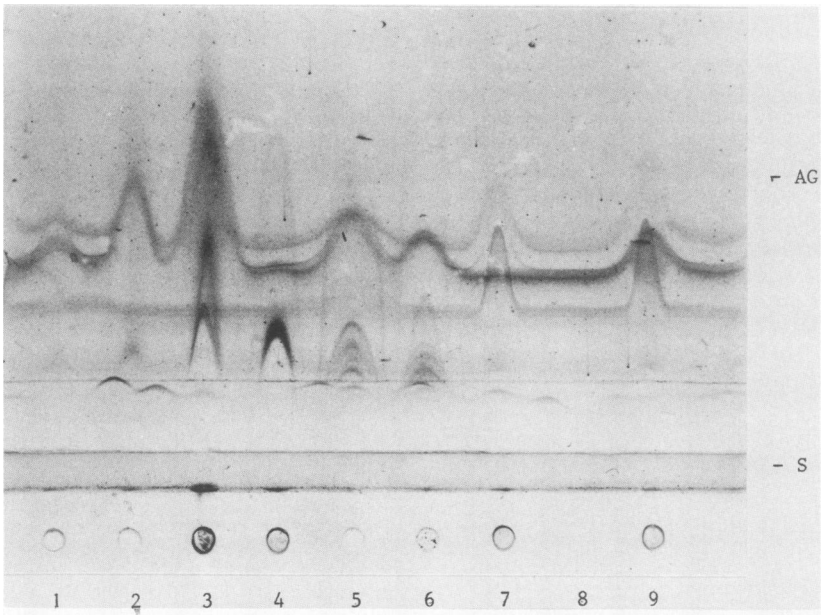

FIG. 4. Rocket-line immunoelectrophoresis showing the cross-reacting components in various mycobacteria and PPDs with antigen A. The sample gel S contained 400 μg of total soluble protein derived from induced cells of *E. coli* M1070(pRIB1000). The antiserum containing gel AG contained immunoglobulin IG (final concentration, 1.2 mg/ml) from anti-*M. bovis* BCG serum 1739. Wells 1 and 2 each contained 5 μg of PPD derived from *M. fortuitum* and *M. tuberulosis*, respectively. Also shown are wells containing total soluble protein derived from *M. smegmatis* strain (30 μg [lane 3]), *M. avium* strain (4 μg [lane4]), *M. tuberculosis* strain H37Rv (5 μg [lane 5]), *M. bovis* BCG (1 μg [lane 6]), *E. coli* M10170 (7 μg [lane 7]), and induced cells of *E. coli* M10170(pRIB1000) (5 μg [lane 9]). As a control, 10 μl of distilled water was applied to well 8. Note that the antigen A precipitate constitutes a continuous line that is deflected when cross-reacting antigenic material is present in a well.

gens in each of them. An example of such a rocket-line immunoelectrophoresis experiment is shown in Fig. 4. These results indicate that epitopes of antigen A of *M. bovis* BCG are present in many different species and that they are a constituent of various PPD preparations used for skin testing.

Recently, A. H. J. Kolk selected a hybridoma culture that produced a monoclonal antibody reacting with protein components in a variety of mycobacterial species. The molecular mass of these proteins was approximately 65 kDa. Therefore, we tested the reaction of these monoclonal antibodies with *E. coli* K-12 antigen A by Western blotting. These monoclonal antibodies do react with the A antigen (Fig. 5).

Presence of antibodies in sera from tuberculous patients. As a preliminary investigation to test the value of the A antigen for use in the serodiagnosis of tuberculosis, 16 sera from tuberculosis patients were investigated for the presence of antibodies against antigen A by Western blotting, with protein obtained from an induced culture of M1070 that carries pRIB1000. All of these sera reacted with the 64-kDa component. No reaction was observed with any of four control sera from humans with no history of a mycobacterium infection. These humans were negative in the skin test with *M. tuberculosis*-derived PPD.

DISCUSSION

A gene bank of *M. bovis* BCG in *E. coli* was constructed by insertion of partially *Sau*3A-cleaved *M. bovis* BCG DNA into the bacteriophage vector EMBL3. As the mean size of the inserts was ca. 15 kb, one might expect that the expression of cloned mycobacterial DNA depends almost entirely on transcription and translation expression signals of the mycobacterium DNA. Presently, no published information is available about the nature of these signals in mycobacterium. The most likely explanation for the relatively few antigen-expressing clones found among the EMBL3 recombinants seems to be a difference between expression signals in *M. bovis* and *E. coli*. Presumably the *E. coli* machinery for protein synthesis does not recognize well the expression signals of the distantly related bacterium *M. bovis* BCG. This explanation is consistent with the recent findings of Clark-Curtiss et al. on the expression of mycobacterium DNA in *E. coli* (6). No complementation of various genetic defects in *E. coli* was observed after screening for complementation by DNA from either *M. vaccae*, "*Mycobacterium lufu*," or *M. leprae*. However, the expression of a limited number of genes was observed after linkage of *M. leprae* DNA to a strong promoter (6).

In this study, 6 different phenotypes were observed among the 21 antigen-producing *M. bovis* BCG recombinant clones. The inability to reproducibly detect antigen production in the clones with the phenotypes D, E, and F might be related to the unstable nature of particular mycobacterial DNA segments in *E. coli*. Young et al. (27) reported the expression of three *M. tuberculosis* antigens, reacting with monoclonal antibodies against *M. tuberculosis* antigens, having molecular masses of 33, 18, and 16 kDa, respectively. The molecular weights of all expressed *M. bovis* BCG antigens, except phenotype F, observed in our study are higher than those expressed by the *M. tuberculosis*-derived clones of Young et al. Therefore, it is highly unlikely that any of the mycobacterium antigens obtained in this study are related to those

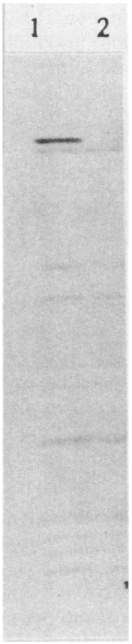

FIG. 5. Reaction of monoclonal antibodies of hybridoma F47-10 with the A antigen produced by *E. coli* K-12. Lanes 1 and 2, Western blot containing lysates derived from M1070 carrying pRIB1000 and from M1070, respectively.

described by Young et al. Among the antigen-producing clones obtained in this study, 10 were of one particular phenotype, phenotype A. All of these produced a 64-kDa protein antigen, designated antigen A. The structural gene of antigen A was found to be located on a 4.9-kb *Eco*RI fragment, and it was subcloned into the expression vector pPLc236. In both orientations of this fragment in the vector, an equally low level of production of the A antigen was observed under noninducing conditions. Therefore, the expression of this antigen is controlled by a mycobacterial promoter. This is the first example of a mycobacterial promoter that functions in *E. coli*. One of the recombinant plasmids, pRIB1000, expressed high levels of antigen A at 42°C, due to a strong transcription of the lambda P_L promoter. This overproducing strain enabled us to analyze the specificity of antigen A. Antigen A was found to be present in a variety of mycobacterial species; therefore, this common antigen is classified as a group i antigen according to the classification of Stanford (22). This result is consistent with the observation that antigen A reacts with monoclonal antibodies derived from the hybridoma clone F47-10. These antibodies react with protein antigens from several different mycobacterial species, including *M. leprae*. The hybridoma F47-10 was obtained from a mouse that had been immunized with *M. leprae* antigens (A. Kolk, personal communication).

Crossed immunoelectrophoresis studies of Closs et al. (7) have shown that virtually all antigens found in *M. bovis* BCG are also found in *M. tuberculosis*. Immunoblotting experiments with polyvalent anti-*M. tuberculosis* and anti-*M. bovis* antisera, absorbed with cell extracts of either *M. bovis* and *M. tuberculosis*, respectively, fully support the conclusions of these authors (J. Thole, unpublished data). Therefore, it will be difficult to clone genes expressing antigens that are specific for either one of these organisms and which are not found in other mycobacteria. However, such antigens might be of great value as skin test reagents and in the serodiagnosis of tuberculosis. Recently, Nagai et al. (17) purified a protein, designated MPB70, that seems to be present almost exclusively in certain strains of *M. bovis* BCG and only to a small extent in *M. tuberculosis* (11). As discussed by Grange (10), most other studies with partially purified mycobacterial protein antigens have been frustrated by their lack of specificity or by irreproducible results when tested by others. Recent studies with monoclonal antibodies, however, strongly suggest the existence of antigenic determinants that are species specific (8, 14). In this preliminary study, we screened the recombinant DNA clones with a polyvalent anti-*M. bovis* BCG antiserum which was not made specific by absorption with cell extracts of other mycobacteria. Therefore, it is not surprising that a clone was selected that produced a common mycobacterial protein antigen. Future studies will be directed to select clones that produce species-specific antigens or fusion proteins with specific antigenic determinants, with monoclonal antibodies as a probe. Furthermore, the use of expression vectors that provide the recombinant DNA with transcriptional or translational signals or both seem to be a prerequisite for the success of such studies.

It is well known that cell-mediated immunity plays a major role in the immunity of humans against mycobacterial diseases (5). Therefore, it is interesting to note that Emmrich and Kaufmann have identified a number of human T-cell clones that are stimulated by the A antigen produced by *E. coli*. These T-cell clones were derived from a patient with tuberculoid leprosy (F. Emmrich, J. E. R. Thole, J. D. A. van Embden, and S. H. E. Kaufmann, submitted for publication). It is likely that *M. leprae* contains an immunogenic protein that cross-reacts with antigen A and that causes a cell-mediated immune response. Studies are now in progress to purify antigen A and thus elucidate the possible role of this antigen in the immunity of mycobacterial infections.

ACKNOWLEDGMENTS

We thank A. H. J. Kolk for providing us with monoclonal antibodies and unpublished material, P. R. Klatser for providing us with soluble protein fractions of various mycobacteria, L. G. Berwald and J. G. Baas for excellent technical support, and Tineke Olij for perfect secretarial assistance.

This investigation received financial support from the World Health Organization Programme for Vaccine Development.

LITERATURE CITED

1. **Axelsen, N. H.** 1983. Handbook of immunoprecipitation-in-gel techniques. Scan. J. Immunol. Suppl. **17**:165–169.
2. **Burnette, W. N.** 1981. "Western Blotting": electrophoretic transfer of proteins for sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
3. **Bradley, S. G.** 1973. Relationships among mycobacteria and nocardiae based upon deoxyribonucleic acid reassociation. J. Bacteriol. **113**:645–651.
4. **Cami, B., and P. Kourilsky.** 1978. Screening of cloned recombinant DNA in bacteria by *in situ* colony hybridization. Nucleic Acids Res. **5**:2381–2390.
5. **Chaparas, S. D.** 1982. The immunology of mycobacterial infections. Crit. Rev. Microbiol. **9**:139–197.
6. **Clark-Curtiss, J. E., W. R. Jacobs, M. A. Docherty, L. R. Ritchie, and R. Curtiss III.** 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. J. Bacteriol. **161**:1093–1102.
7. **Closs, O., M. Harboe, N. H. Axelsen, K. Bunch-Christensen, and**

M. Magnusson. 1980. The antigens of *Mycobacterium bovis*, strain BCG, studied by crossed immunoelectrophoresis: a reference system. Scand. J. Immunol. **12**:249–263.

8. **Coates, A. R. M., B. W. Allen, J. Hewitt, J. Ivanyi, and D. A. Mitchison.** 1981. Antigenic diversity of *Mycobacterium tuberculosis* and *Mycobacterium bovis* detected by means of monoclonal antibodies. Lancet **ii**:167-169.
9. **Daniel, T. M., and B. W. Janicki.** 1978. Mycobacterial antigens: a review of their isolation, chemistry, and immunological properties. Microbiol. Rev. **42**:84–113.
10. **Grange, J. M.** 1984. The humoral immune response in tuberculosis: its nature, biological role and diagnostic usefulness. Adv. Tuberc. Res. **21**:1–78.
11. **Harboe, M., and S. Nagai.** 1984. MPB70, a unique antigen of *Mycobacterium bovis* BCG. Am. Rev. Respir. Dis. **129**:444–452.
12. **Imaeda, T., L. Barksdale, and W. F. Kircheimer.** 1982. Deoxyribonucleic acid of *Mycobacterium lepraemurium*: its genome size, base ratio, and homology with those of other mycobacteria. Int. J. Syst. Bacteriol. **32**:456–458.
13. **Karn, J., S. Brenner, and L. Barnett.** 1983. New bacteriophage lambda vectors with positive selection for cloned inserts. Methods Enzymol. **101**:3–19.
14. **Kolk, A. H. J., M. L. Ho, P. R. Klatser, T. A. Eggelte, S. Kuijper, S. de Jonge, and J. van Leeuwen.** 1984. Production and characterization of monoclonal antibodies to *Mycobacterium tuberculosis*, *M. bovis* (BCG) and *M. leprae*. Clin. Exp. Immunol. **58**:511–521.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Nagai, S., J. Matsumoto, and T. Nagasuga.** 1981. Specific skin-reactive protein from culture filtrate of *Mycobacterium bovis* BCG. Infect. Immun. **31**:1152–1160.
18. **Portaels, F., and S. R. Pattyn.** 1982. Isolation of fastidiously growing mycobacteria from armadillo livers infected with *Mycobacterium leprae*. Int. J. Lepr. **50**:370–374.
19. **Remaut, E., P. Stanssen, and W. Fiers.** 1981. Plasmid vectors for high-efficiency expression controlled by the P_L promoter of coliphage lambda. Gene **15**:81–93.
20. **Remaut, E., H. Tsao, and F. Fiers.** 1983. Improved plasmid vectors with a thermoinducible expression and temperature-regulated runaway replication. Gene **22**:103-113.
21. **Stanford, J. L.** 1983. Immunologically important constituents of mycobacteria: antigens, p. 85–127. *In* C. Ratledge and J. Stanford (ed.), The biology of the mycobacteria, vol. 2. Immunological and environmental aspects. Academic Press, Inc., London.
22. **Stanford, J. L., and J. M. Grange.** 1974. The meaning and structure of species as applied to mycobacteria. Tubercle **55**:143–152.
23. **Stanley, K. K., and J. P. Luzio.** 1984. Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO J. **3**:1429–1434.
24. **Udou, T., M. Ogawa, and Y. Mizuguchi.** 1982. Spheroplast formation of *Mycobacterium smegmatis* and morphological aspects of their reversion to the bacillary form. J. Bacteriol. **151**:1035–1039.
25. **van Embden, J. D. A., H. J. van der Donk, R. V. van Eijk, H. G. van der Heide, J. A. de Jong, M. F. van Olderen, A. D. Osterhaus, and L. M. Schouls.** 1983. Molecular cloning and expression of *Treponema pallidum* DNA in *Escherichia coli* K-12. Infect. Immun. **42**:187–196.
26. **Walfield, A. M., P. A. Hanff, and M. A. Lovett.** 1982. Expression of *Treponema pallidum* antigens in *Escherichia coli*. Science **216**:522-523.
27. **Young, R. A., B. R. Bloom, C. M. Grosskinsky, J. Ivanyi, D. Thomas, and R. W. Davis.** 1985. Dissection of *Mycobacterium tuberculosis* antigens using recombinant DNA. Proc. Natl. Acad. Sci. USA **82**:2583–2587.