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Expression of *Mycobacterium tuberculosis* genes in *Escherichia coli*

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Abstract. Two *Escherichia coli* clones expressing *Mycobacterium tuberculosis* antigens were isolated from a gene-bank in the plasmid vector pBR 325. 'Western blot' analysis revealed the presence of a unique protein band of molecular weight 68,000 and 38,000, respectively in cell-extracts from each clone. The 68,000 dalton antigen was found to be expressed on *Escherichia coli* outer surface. Plasmid DNA from a third clone could confer leucine independence on two different *leu B* mutants of *Escherichia coli* but not on mutants in other *leu* genes, pointing to the possibility ofgenetic complementation. Thus, *Mycobacterium tuberculosis* DNA is capable of expression in *Escherichia coli.*

Keywords. *Mycobacterium tuberculosis* gene-expression; western blot; cloned *M. tuberculosis* DNA.

Introduction

Tuberculosis remains a major health problem in the world, especially in Third World countries like India. It is believed that protective immunity against *Mycobacterium tuberculosis,* the causative organism, is generated *via* the cell-mediated immune response (Collins, 1982). The role of different subsets of T-cells and their fine specificities in tuberculosis is poorly understood. Such a study requires large quantities of relevant *M. tuberculosis* antigens in purified form—a need easily met if *M. tuberculosis* gene-products could be synthesised in *Escherichia coli.* As a first step towards this goal, we report here the expression of *M. tuberculosis* genes in *E. coli* to produce antigenically identifiable proteins.

Materials and methods

Bacterial strains and growth conditions

M. tuberculosis was grown in Middlebrook 7H9 medium containing 1 % glucose. *E. coli* strains were grown in Luria broth or in M9 medium. All cells were grown with aeration at 37°C.

Abbreviations used: PBS, Phosphate buffered saline; BSA, bovine serum albumin; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis; $^{M}r'$ molecular weight.

Cloning of M. tuberculosis DNA

High molecular weight DNA from *M. tuberculosis* $H_{37}R_a$ was extracted, partially digested with *EcoRI,* inserted into the *cam* site of plasmid pBR325 and cloned in *E. coli* HB101 as described before (Bhattacharya *et al.,* 1984).

Rabbit anti M. tuberculosis antiserum

M. tuberculosis $H_{37}R_V$ cells in normal saline (60 mg wet wt/ml) were sonicated for 12 min at 150 W and emulsified with incomplete Freund's adjuvant (1:1, v/v). 1·0 ml of emulsion was injected subcutaneously at multiple sites in each rabbit both for primary and subsequent boosters at two-weeks intervals. Last booster of 0·5 ml sonicate was given intravenously at the end of 8th week. Rabbits were bled every 3 weeks.

Purification of immunoglobulins and radioiodination

Immunoglobulins were purified from sera by a two-step method involving ammonium sulphate precipitation and ion-exchange chromatography as described (Mishell and Shiigi, 1980). Purified anti-bodies (10-20 μg) were radioiodinated with 200 μCi of carrier-free $Na^{125}I$ (Bhabha Atomic Research Centre, Bombay) in the presence of iodogen as previously described (Bhattacharya *et al.,* 1981).

Absorption of anti M. tuberculosis serum

1×10^6–5×10^6 cpm of [^{125}I]-labelled immunoglobulins, diluted to 2·0 ml with phosphate buffered saline (PBS; 10 mM phosphate, 0·14 M sodium chloride, pH 7·4) containing 1 % bovine serum albumin (BSA) were mixed with washed pellet of *E. coli* cells (100 ml cells grown to mid-log). The absorption was carried out at 4°C overnight. Unabsorbed antibodies were collected after centrifugation at 6000 *g* for 10 min at 4°C. Anti *M. tuberculosis* serum was also absorbed with sonicated *E. coli* extracts for enzyme immunoassays essentially as described (Stahl *et al.,* 1984).

Whole-cell binding assay

E. coli cells (5 ml) grown to mid-log were washed and resuspended in PBS, 1 % BSA. [^{125}I]-Labelled, absorbed *M. tuberculosis* antibodies (3×10^6 cpm) were added to the cells. After 2 h, incubation at room temperature with shaking, cells were washed 4 times with PBS, 0·1 % BSA. Bound radioactivity was eluted with 200 μl of 0·1 M glycine-HCl, pH 2·5. The radioactivity in the supernatant was determined using an ECIL gamma counter.

Western blotting

1-2 ml of recombinant clones grown in L-broth were centrifuged, washed with 10 mM Tris, pH 6·8, resuspended in sodium dodecyl sulphate (SDS)-sample buffer (100 μl), incubated at 100° C for 5 min and separated on 10 % Polyacrylamide gels as described (Laemmli, 1970). Gels were stained with Coomassie blue and transferred electrophoretically onto a nitrocellulose sheet as described (Towbin *et al.,* 1979). Nonspecific binding sites on nitrocellulose filters were blocked with 3 % BSA in PBS

containing 2 % normal goat serum and the antigens were detected by absorbed rabbit anti *M. tuberculosis* antiserum (dil. 1:100) followed by goat anti-rabbit immunoglobulin G-horse radish peroxidase.

Results and discussion

Clones selected by antibody screening

E. coli clones from the *M. tuberculosis* gene-bank were screened using anti *M. tuberculosis* antibodies by two methods, one for clones expressing antigens on the cell-surface (screening of whole cells) and the other for intracellular antigens (screening of lysed cells). One clone each was identified by both methods and further analysed.

The clone detected by screening of whole cells was designated M-86 (cloned DNA insert size 13·8 kb). To further demonstrate the presence of *M. tuberculosis* antigens on M-86 cell-surface, a whole-cell binding assay was performed with M-86 cells and control cells *(E. coli* HB101 containing pBR325), using [^{125}I]-labelled rabbit anti *M. tuberculosis* antibodies. The results presented in table 1 show that M-86 bound significantly higher amounts of antibodies (6825 cpm) compared to control cells (392 cpm) suggesting that *M. tuberculosis* antigens are, indeed, expressed on the cell-surface of M-86 cells.

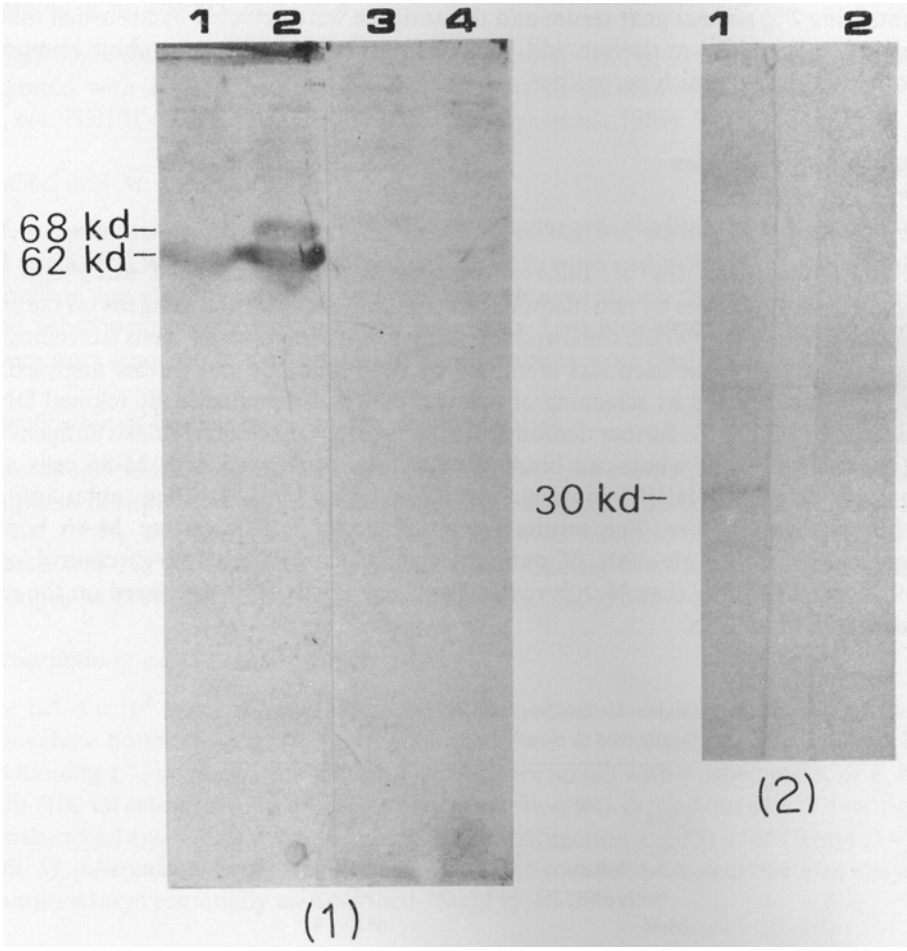
Table 1. Binding of [^{125}I]-anti *M. tuberculosis* antibodies to clone M-86 cells.

Cells	[^{125}I]-Antibody bound (cpm)
E. coli HB101 (with pBR325)	392 ± 90
M-86	6825 ± 82

The binding of [^{125}-I]-anti *M. tuberculosis* antibodies to whole cells was determined by the whole cell binding assay described in 'materials and methods'.

Further information on the nature of gene-products of M-86 responsible for the antigenic reactivity was obtained by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting of total cell-extracts from M-86 and *E. coli* containing pBR325. The results presented in figure 1 indicate the presence of a unique peptide of molecular weight (M_r) 68,000 in M-86 (lane 2). Another peptide of M_r 62,000 showed up on the western blot both in M-86 and in *E. coli* controls (lanes 1,2). The appearance of this peptide despite exhaustive preabsorption of the rabbit antibodies with *E. coli* whole cells suggests that it is highly immunogenic and may be located intracellularly or in the inner membrane of *E. coli.*

The clone detected by screening of lysed cells was designated C-45 (DNA insert size 15 kb). On further analysis by western blotting, cell extracts from C-45 showed a major unique band of M_r 30,000 which was absent in the *E. coli* control (figure 2). These results



Figures 1 and 2. 1. Western blot analysis of proteins from M-86. Proteins from the clone M-86 (lanes 2,4) and *E. coli* containing pBR325 (lanes 1,3) were analysed by Western blotting as described in 'materials and methods'. Lanes 1,2—blots treated with rabbit antiserum against *M. tuberculosis.* Lanes 3, 4—blots treated with preimmune rabbit serum. 2. Western blot analysis of proteins from C-45. Proteins from the clone C-45 (lane 1) and *E. coli* containing pBR325 (lane 2) were analysed by western blotting as described in 'materials and methods'. Blots were treated with rabbit antiserum against *M. tuberculosis.*

demonstrate the feasibility of expression of *M. tuberculosis* antigens from cloned DNA fragments in *E. coli.*

Clone selected by genetic complementation

E. coli HB101 carries the *leu B*6 mutation which renders it auxotrophic for leucine. In order to select for clones which might complement this leucine requirement, clones growing on nutrient agar plates were replica-plated on minimal agar plates lacking leucine. One clone which on repeated sub-culturing continued to grow independent of

leucine was further studied. It was designated as L-3. It had a DNA insert size of 2·7 kb.

Leucine independence could be generated by mechanisms other than genetic complementation. To test this, plasmid DNA from L-3 (after passaging through *E. coli* DH1 to modify the DNA) was used to transform other *E. coli leu* mutants (*leuA*371, *leuB*401, *leuC*171, *leuD*101). Tiny transformant colonies came up on leu-plates for all the 4 *leu* mutants tested. When these colonies were re-tested for growth on *leu*- medium, it was found that only the colonies from *leuB*401 continued to grow while the others failed to grow on *leu* $^{-}$ medium. Since two different *leuB* mutants were rendered leucine independent by plasmid DNA from L-3, it seems likely that this result may be due to genetic complementation. The small amount of initial growth on *leu*$^{-}$ plates observed for all *leu* mutants may have been due to trace amounts of leucine provided by the Luria broth in which transformants were allowed to grow to enable the expression of antibiotic-resistance markers.

The expression of *M. tuberculosis* and *M. leprae* antigens in *E. coli* has recently been reported (Young *et al.,* 1985a, b) using the λ expression vector, λ gt 11. When cloning of *M. leprae* DNA was done in a cosmid vector (pHC79), no expression of mycobacterial antigens was observed (Clark-Curtiss *et al.,* 1985). However, when DNA from this library was sub-cloned into an expression vector, pYA626, polypeptides encoded by *M. leprae* DNA could be detected in minicells. Thus, all mycobacterial promoters may not be recognised by the *E. coli* transcription system. Whether they may be recognised by a gram-positive host, such as *Bacillus subtilis* remains to be investigated.

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