Published in final edited form as:

Gene. 1997 April 29; 190(1): 37–44.

Construction of shuttle vectors for genetic manipulation and molecular analysis of *Mycobacteria*¹

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Abstract

Two novel shuttle vectors for mycobacteria are described which have been derived from the expression system pSD5 developed in our laboratory. Plasmid pSD5B is a promoter-selection vector containing a promoterless *lacZ* gene and allows the identification of mycobacterial promoters by the blue colour of the colonies on solid media containing XGal. Moreover, the chronological order of appearance of blue colonies and intensity of colour provide a qualitative index of transcriptional strengths of the cloned promoters. Plasmid pSD5C has been designed to construct mycobacterial genomic libraries and express the cloned DNA inserts as fusion proteins with maltose binding protein in mycobacteria. Libraries in pSD5C provide feasibility for their screening with either DNA probes or specific antisera for identifying the genes of interest and for isolation of specific genetic loci by complementation of *Escherichia coli* and mycobacterial mutants. These vectors combine the ease of working in *E. coli* with the advantage of directly propagating them in mycobacteria without further manipulations. Finally, we demonstrate that these vectors function efficiently both in fast growing *Mycobacterium smegmatis* and slow growing mycobacteria including *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG.

Keywords

Gene expression; Promoter; Genomic library; Fusion protein; Genetic complementation

1. Introduction

Tuberculosis (TB), the cause of the largest number of deaths from a single infectious agent worldwide, continues to present serious health concerns around the globe. More than 50 000 people die of tuberculosis every week and one third of the world's population is estimated to be infected with the tubercle bacilli asymptotically (Kochi, 1994). While 95% of all cases of tuberculosis occur in developing countries, the developed world too has seen its revival due to the spread of the human immunodeficiency virus. Infection with AIDS virus is associated with elevated susceptibility to tuberculosis and increased mortality and morbidity

¹Presented at the International Conference on 'Eukaryotic Expression Vector Systems: Biology and Applications', National Institute of Immunology, New Delhi, India, 4–8 February 1996.

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(Chaisson and Slutkin, 1989). The emergence of multi-drug resistant strains of *Mycobacterium tuberculosis* has further aggravated the problem (Frieden et al., 1993).

The present challenge for mycobacteriologists is to apply molecular genetic approaches to understand the biology of mycobacteria and utilize this knowledge for the development of more effective prophylactic, diagnostic and curative methods to combat mycobacterial infections. The study of molecular biology of mycobacteria in the past was rendered difficult due to reasons such as slow growth rate and high GC content of their genomes and the fact that they remained refractory to transformation with DNA due to their tough cell wall and lack of suitable plasmid vectors for gene transfer. The last decade, however, has witnessed considerable progress in this field and genetic tools for cloning and expression of mycobacterial genes have become available. Bacteriophage λ gt11 was the first to be used as a vehicle for the construction of mycobacterial genomic libraries that permitted the use of specific antisera to isolate immunoreactive antigens (Young et al., 1985, 1987). Subsequently these libraries were used to perform complementation of *Escherichia coli* mutants to identify mycobacterial genes (Garbe et al., 1990; Aldovini et al., 1993; Anderson and Hansen, 1993; Cirillo et al., 1994). Isolation of high-efficiency-transformation strains of the fast growing *M. smegmatis* (Snapper et al., 1990) and development of electroporation to transform mycobacteria (Snapper et al., 1988) were crucial achievements in our ability to express genes in mycobacteria with a variety of shuttle vectors that were based on the plasmid pAL5000 from *M. fortuitum* (Labidi et al., 1985; Snapper et al., 1988; Raney et al., 1990; Stover et al., 1991). Plasmids and integration proficient vectors based on sequences derived from mycobacteriophages have enabled stable introduction and expression of genes in mycobacteria (Jacobs et al., 1987; Lee et al., 1991; Stover et al., 1991).

We had designed an expression system pSD5 for mycobacteria which provides varied levels of expression of a gene by changing the mycobacterial promoter regulating its expression (S.K.D., S.J., D.K. and A.K.T., unpublished). We report here the construction of two vectors, pSD5B and pSD5C, which have been derived from pSD5 and can be used for the isolation of transcriptionally active DNA fragments from mycobacteria and the construction of genomic libraries of mycobacteria respectively. We describe the salient features and applications of these vectors that overcome some of the limitations of the mycobacterial vectors described previously.

2. Experimental and discussion

2.1. Isolation of mycobacterial promoters based on blue-white selection

The vector pSD5B was constructed to isolate promoter sequences from the mycobacterial genome (Fig. 1). Promoter cloning sites *Xba*I and *Sph*I are preceded by the transcriptional terminator *TER3* to prevent any readthrough transcription from the mycobacterial origin of replication (*oriM*). Translational terminators in all three reading frames between the promoter cloning sites and *IacZ* are present to uncouple transcriptional initiation from translational initiation. A transcriptional terminator, *TER4*, derived from fd phage is present downstream from *IacZ* to facilitate cloning of strong promoters (Gentz et al., 1981).

A partial promoter library using mycobacterial DNA was prepared in pSD5B (see legend to Fig. 2). The clones exhibiting promoter activities were identified by the appearance of blue coloured mycobacterial colonies on LB agar containing XGal, due to expression of β Gal in these bacteria. Using pSD5B, we were able to isolate mycobacterial promoters with a frequency of 10%. The plasmid DNA purified from promoter clones was subjected to restriction digestion with *Sph*I and it was observed that all the plasmids carried DNA inserts ranging from 0.1 to 0.5 kb in size (Fig. 2). The clones were assigned serial numbers in the order of their acquisition of blue colour. β Gal activities in the cell-free extracts prepared from individual clones indicated that the promoter clones 1–17 exhibited activities ranging between 2879 and 135 nmol/min/mg of protein, thus providing a 20-fold variation in promoter strengths (Fig. 3A). The extracts were also analysed by 0.1% SDS-7.5% PAGE and probed with anti- β Gal Ab (Fig. 3B). The expression profile correlated with β Gal activities from respective clones thereby confirming the range of promoter strengths. To assess the functioning of pSD5B in slow-growing species of mycobacteria, six promoter clones (1, 2, 5, 8, 10 and 14 in Fig. 3) were electroporated into *M. tuberculosis* H₃₇Ra and *M. bovis* BCG. Transformants of all these six promoters were blue in both species. Cell-free extracts from the promoter clones exhibited β Gal activities comparable with those observed in *M. smegmatis* (data not shown) thus substantiating that the vector pSD5B functions efficiently in slow-growing mycobacteria as well.

Selection of promoters based on *lacZ* (β Gal) expression by pSD5B offers an advantage in that cloning of a wide range of promoters is possible without incorporating any bias towards the promoters of a certain range as can occur in vectors using antibiotic resistance-encoding genes as a basis for promoter selection (DasGupta et al., 1993). Besides, the mycobacterial promoters isolated in pSD5B can be directly assessed for their transcriptional strength in *E. coli*. The presence of *ori* both for *E. coli* (P15A) and mycobacteria (*oriM*) and the gene for kanamycin resistance which functions in *E. coli* and mycobacteria provide the basis for propagation of this vector for isolation and analysis of promoters in these bacterial species. It has been observed that vectors maintaining a high copy number in *E. coli* often express the reporter gene in the absence of any promoter sequences due to a large accumulative read-through transcription thus rendering them unsuitable for use in *E. coli* (Timm et al., 1994). We have circumvented this problem in pSD5B by using the P15A *ori* which maintains a plasmid copy number of 15–20 in *E. coli* (Chang and Cohen, 1978) and does not produce any β Gal in the absence of promoter sequences (data not shown).

2.2. Construction of pSD5C

In designing the vector pSD5C for expression of genes in mycobacteria, we have taken advantage of the fact that the majority of *E. coli* promoters function in mycobacteria albeit with efficiencies different from those in *E. coli* (Bashyam et al., 1996). In pSD5C, gene expression is under the transcriptional control of the *P_{tac}* promoter. Thus, expression of cloned DNA fragments in an *E. coli* strain overexpressing *lacI* (encoding *lac* repressor) commences only upon the addition of IPTG which induces expression from *P_{tac}* promoter. This promoter, although not induced by addition of IPTG in mycobacteria, can function constitutively at a level much lower than that observed after induction in *E. coli*. As such, while the induced level of expression in *E. coli* is high enough to be seen on Coomassie blue

stained polyacrylamide gels, that in mycobacteria can be detected using Ab. pSD5C contains the *malE* gene (encoding maltose-binding protein, MBP) fused to the *lacZα* fragment downstream from *Ptac* promoter (Fig. 4). DNA fragments can be cloned in the unique *Pst*I site within *lacZα*. The transcriptional terminator from fd phage (*TER4*) present downstream from *lacZα* prevents any transcription originating from *Ptac* from interfering with plasmid functions. In addition, the presence of *oriM* and *Km^R* gene facilitates selection and expression from pSD5C or its recombinants in mycobacteria.

2.3. Application of pSD5C in expression of genes in *E. coli* and mycobacteria

In *E. coli* transformed with pSD5C, addition of IPTG induces the expression of the *malE-lacZα* genes, producing a 55-kDa fusion protein (Fig. 5A and B, lane 2). To examine that all necessary features of pSD5C function appropriately, a fragment encoding CAT (gene *cat*, containing its own ribosome-binding site and start codon without the promoter region) derived from pSD7 (DasGupta et al., 1993) was cloned in the *Pst*I site of pSD5C to obtain pSD5C-CAT and the expression of *cat* was analysed in *E. coli* and *M. smegmatis* by 0.1% SDS-10% PAGE and Western blotting. Since *cat* was cloned in a reading frame different from *malE*, it was not expressed as an MBP fusion protein. Instead, two proteins, corresponding to MBP (38 kDa) and CAT (26 kDa), were detected in induced cultures of *E. coli* (Fig. 5A, lane 4). Their authenticity was confirmed with anti-MBP Ab (Fig. 5B, lane 4) and anti-CAT Ab (Fig. 5C, lane 4).

Cell-free extracts prepared from *M. smegmatis* transformed with pSD5C or pSD5C-CAT were also analysed similarly. While Coomassie blue stained SDS-polyacryl-amide gels did not reveal any band due to MBP-LacZα, MBP or CAT (Fig. 5A, lanes 5 and 6), their expression was detected when Ab to MBP (Fig. 5B, lanes 5 and 6) and to CAT (Fig. 5C, lane 6) were used. *M. smegmatis* transformed with pSD5C-CAT formed colonies on LB agar plates containing 25 µg Km/ml and 20 µg Cm/ml thus confirming that enzymatically active CAT could be expressed in *M. smegmatis* using pSD5C. Based on the amounts of protein loaded for a comparable expression of MBP-LacZα fusion protein and CAT in *E. coli* and mycobacteria as detected using Ab (Fig. 5B and C) the constitutive level of expression from *Ptac* promoter in mycobacteria is approx. 1–2% of the expression in *E. coli* after induction with IPTG.

2.4. Construction of mycobacterial genomic library in pSD5C

A partial genomic library of *M. tuberculosis* H₃₇Ra was constructed in pSD5C and expression from some of the recombinants tested in *E. coli* and *M. smegmatis*. Upon induction with IPTG (Fig. 6A), MBP fusion proteins of various sizes were expressed in eight of the *E. coli* recombinants. The same recombinant proteins could be detected with anti-MBP Ab when expressed in *M. smegmatis* (Fig. 6B). The ability of this expression system to express genes both in mycobacteria and *E. coli* should provide ease of screening the libraries constructed in pSD5C either using nucleic acid probe or a specific Ab in *E. coli*. The identified clones can be directly electroporated into mycobacteria for expressing the proteins without further manipulations, an advantage not reported for any of the existing plasmid vectors for constructing mycobacterial genomic libraries. Such libraries may be

extremely useful in genetic complementation of both *E. coli* and mycobacterial mutants for isolation of mycobacterial genes that can be identified based on phenotypic selection.

2.5. Use of pSD5C for protein expression in *M. tuberculosis* and *M. bovis* BCG

Expression of *malE-lacZα* and *cat* in pSD5C was examined in the slow-growing mycobacterial species *M. tuberculosis* H₃₇Ra and *M. bovis* BCG. The 55-kDa MBP-LacZα fusion protein was detected in cell-free extracts of *M. smegmatis*, *M. tuberculosis* H₃₇Ra and *M. bovis* BCG transformants harbouring pSD5C (Fig. 7A, lanes 1, 3 and 5). pSD5C-CAT transformants of *M. smegmatis*, *M. tuberculosis* H₃₇Ra and *M. bovis* BCG, respectively, displayed a 38-kDa protein band due to MBP alone (as described above in Section 2.3) when probed with anti-MBP Ab (Fig. 7A, lanes 2, 4 and 6) and a 26-kDa band of CAT protein when probed with anti-CAT Ab (Fig. 7B, lanes 2, 4 and 6). The ability to express genes in the slow-growing mycobacteria using pSD5C is of extreme importance. Complementation of non-pathogenic mycobacterial species such as *M. tuberculosis* H₃₇Ra and *M. bovis* BCG can be carried out with the genomic libraries of their pathogenic counterparts such as *M. tuberculosis* H₃₇Rv and *M. bovis* for identifying the genetic determinants responsible for the disease causing ability of the latter. Such a strategy has been employed using the cosmid library of *M. tuberculosis* H₃₇Rv to complement *M. tuberculosis* H₃₇Ra and has led to the identification of DNA fragments that provide a growth advantage to the recombinant *M. tuberculosis* H₃₇Ra in mice (Pascopella et al., 1994).

Post-translational modifications such as glycosylation and acylation and secretion of proteins that occur specifically in mycobacteria (Garbe et al., 1993; Burlein et al., 1994; Matsuo et al., 1990) cannot be achieved if these genes are expressed in *E. coli*. pSD5C provides the possibility of expressing such genes in mycobacteria and easy purification of appropriately modified proteins directly from mycobacteria on affinity columns using amylose resins. Due to the presence of factor Xa cleavage site between MBP and the expressed protein, recombinant protein can be cleaved with factor Xa to release pure mycobacterial protein synthesized in its native environment.

3. Conclusions

1. Two shuttle vector systems pSD5B and pSD5C have been constructed for mycobacteria by modifying the expression system pSD5 and are suitable for analysis of gene expression in fast and slow-growing mycobacteria.
2. pSD5B is a promoter selection vector carrying the promoterless *lacZ* gene that can be used to identify transcriptionally active DNA fragments in mycobacteria based on the blue-white selection.
3. The vector pSD5C is designed for construction of genomic libraries of mycobacteria wherein gene fragments are expressed as MBP fusion roteins under the transcriptional control of the *E. coli* *Ptac* promoter. Protein expression in *E. coli* can be induced with IPTG, hence, it is possible to screen such genomic libraries in *E. coli* and the selected clones can be expressed constitutively in mycobacteria without any further molecular manipulations.

4. The presence of MBP as a fusion protein tag in pSD5C would aid in affinity purification of fusion proteins directly from mycobacteria which can be subsequently cleaved with factor Xa to release mycobacterial proteins in their native conformation and with proper posttranslational modifications.

Acknowledgement

This work was supported by a grant from the Department of Biotechnology, Govt. of India. *M. smegmatis* LR222 was a generous gift from Dr. J.T. Crawford, Centers for Disease Control and Prevention (Atlanta, GA, USA). We thank Dr. J.S. Tyagi, Department of Biotechnology, All India Institute of Medical Sciences, New Delhi for providing *M. tuberculosis* H37Ra and *M. bovis* BCG and for critically reading the manuscript. M.D. Bashyam is acknowledged for critically reading the manuscript and valuable help with the computer art work. S.J. and D.K. are recipients of fellowships from the University Grants Commission and the Council of Scientific and Industrial Research, India, respectively. We thank Manisha Jain and Birinder Kaur for excellent technical help and Rajiv Chawla for efficient preparation of this manuscript.

Abbreviations

A	absorbance
Ab	antibody (ies)
βGal	β-galactosidase
CAT	chloramphenicol acetyl transferase
Cm	chloramphenicol
IPTG	isopropyl-β-D-thiogalactopyranoside
Km	kanamycin
LB	Luria-Bertani (medium)
M.	<i>Mycobacterium</i>
MBP	maltose-binding protein
MBP-LacZα	fusion protein of maltose-binding protein with α-fragment of β-galactosidase
ONPG	o-nitrophenyl-α-D-galactopyranoside
ori	origin of DNA replication
PAGE	poly-acrylamide gel electrophoresis
R	resistance
SDS	sodium dodecyl sulfate
Tc	tetracycline
XGal	5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside
□	denotes plasmid-carrier state

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Fig. 1.

Strategy for construction of promoter selection vector pSD5B. The vector pMC1871 (Pharmacia Fine Chemicals, Uppsala, Sweden) was digested with *SmaI* + *ScaI* yielding 3.3-kb and 4.2-kb fragments. The 3.3-kb fragment containing the promoterless *lacZ* gene was purified and cloned into *ScaI*-digested, dephosphorylated pSD5 in the orientation as shown. *P15A ori* and *oriM* are the origins of DNA replication for propagation in *E. coli* and mycobacteria, respectively. Promoter cloning sites, *XbaI* and *SphI*, transcriptional terminator *TER3* to prevent read-through transcription from *oriM*, translational terminators in all three

reading frames between the promoter cloning sites and *lacZ*, and the transcriptional terminator *TER4* to facilitate cloning of strong promoters are all shown in the figure.

Fig. 2.

Restriction analysis of mycobacterial promoters isolated using pSD5B. For isolation of promoters in pSD5B, genomic DNA from *M. smegmatis* LR222 was digested with *Sph*I, and the fragments from 100 bp to 1 kb were purified. In a typical experiment, 60 ng of purified genomic DNA fragments were ligated to 100 ng of *Sph*I-cleaved, dephosphorylated pSD5B, and electroporated into *M. smegmatis* LR222. Transformants were selected on LB agar containing 25 µg Km/ml and 40 µg XGal/ml (Life Technologies, Gaithersburg, MD, USA) and assigned numbers in the serial order of appearance of blue colour. Individual transformants were grown in LB medium supplemented with 0.5% glycerol/0.2% Tween-80/25 µg Km/ml to an $A_{600\text{nm}}$ of 2.0. Plasmid DNA was isolated by a modified alkaline SDS method, subjected to restriction digestion with *Sph*I and analysed on a 6% polyacrylamide gel. Lanes: 1, pSD5B digested with *Sph*I; 2–9, plasmid DNA from eight of the promoter clones digested with *Sph*I; M1, phage λ DNA digested with *Hind*III M2,

pGEM-5Z f(+) DNA digested with *Hae*III. Sizes of marker bands in kb are indicated on the left for M1, and in bp on the right for M2.

Fig. 3.

Expression of *lacZ* in the promoter clones isolated using pSD5B. (A) Bar diagram depicting β Gal activities of pSD5B promoter clones in *M. smegmatis*. (B) Western blot analysis with anti- β Gal Ab (Sigma, St. Louis, MO, USA). The position of β Gal is indicated by an arrow.

Methods: Individual promoter clones were grown in LB medium supplemented with 0.5% glycerol/0.2% Tween-80/25 μ g Km/ml to an A_{600nm} of 2.0. Cells were harvested, resuspended in one sixth of the culture volume of 0.25 M Tris $\cdot$ HCl (pH 7.6), disrupted by sonication and centrifuged at $12\,000 \times g$ for 10 min at 4°C . Total protein and β Gal activities were estimated in cell-free extracts as described by Miller (1972) and expressed as nmol ONPG converted to *o*-nitrophenol per min per mg of protein. Protein samples for SDS-PAGE were prepared by addition of $4 \times$ loading buffer to a final concentration of $1 \times$ (50 mM Tris $\cdot$ HCl pH 6.8/1% SDS/1% β -mercaptoethanol/10% glycerol/0.1% bromophenol blue), boiled for 10 min, centrifuged at $12\,000 \times g$ for 10 min at room temperature, subjected to 0.1% SDS-7.5% PAGE and transferred to nitrocellulose membrane (Schleicher&Schuell, Keene, NH, USA) for probing with Ab.

Fig. 4.

Strategy for construction of the vector pSD5C. pSD5 DNA was digested with *Xba*I + *Mlu*I. The 6.2-kb DNA fragment containing *oriM* P15A *ori* and the Km^R gene was purified, dephosphorylated, end repaired and ligated to a 2.1-kb *Ssp*I fragment, containing the *malE* gene fused to *lacZ α* obtained from pMAL-C2 (New England Biolabs, Beverly, MA, USA). The clones were selected with the orientation of *malE-lacZ α* fragment as shown. For other designations, see legend to Fig. 1.

Fig. 5.

Expression in *E. coli* and *M. smegmatis* using pSD5C. (A) Coomassie blue stained SDS-polyacrylamide gel. (B) Western blot analysis with anti-MBP Ab (New England Biolabs, Beverly, MA, USA). (C) Western blot analysis with anti-CAT Ab. Lanes: A1, B1 and C1, 20 pg, 2 μ g and 2 μ g protein, respectively, from cell-free extract of *E. coli* XL1 Blue[pSD5C] uninduced; A2, B2 and C2, 20 μ g, 2 μ g and 2 μ g protein, respectively, from cell-free extract of *E. coli* XL1 Blue[pSD5C] induced with IPTG; A3, B3 and C3, 20 μ g, 2 μ g and 2 μ g protein, respectively, from cell-free extract of *E. coli* XL1 Blue[pSD5C-CAT]

uninduced; A4, B4 and C4, 20 µg, 2 µg and 2 µg protein, respectively, from cell-free extract of *E. coli* XL1 Blue[pSD5C-CAT] induced with IPTG; A5, B5 and C5, 20 µg, 100 µg and 100 µg protein, respectively, from cell-free extract of *M. smegmatis* LR222[pSD5C]; A6, B6 and C6, 20 µg, 100 µg and 100 µg protein, respectively, from cell-free extract of *M. smegmatis* LR222[pSD5C-CAT]. Molecular masses of marker proteins in A and expressed proteins in B and C are indicated in the left margin. **Methods:** *E. coli* XL1 Blue (Stratagene, La Jolla, CA, USA) was transformed with pSD5C-CAT and grown to an $A_{600\text{ nm}}$ of 0.6 in LB medium supplemented with 25 µg Km/ml and 10 µg Tc/ml. The culture was then divided into two equal portions. After addition of IPTG (to a final concentration of 50 µM) to one of the portions, both cultures were further incubated for 1 h with shaking at 37°C before harvesting. *E. coli* XL1 Blue[pSD5C] was subjected to identical treatments and served as a control. For expression in mycobacteria, *M. smegmatis* was transformed with pSD5C and pSD5C-CAT separately and grown as described for Fig. 3. Cell-free extract from each transformant was prepared as described in the legend to Fig. 3, subjected to 0.1% SDS-10% PAGE and stained with Coomassie brilliant blue R-250 (0.25% in 50% methanol and 10% acetic acid) or transferred to nitrocellulose membrane (Schleicher&Schuell, Keene, NH, USA) for probing with Ab.

Fig. 6.

Expression of MBP fusion proteins in pSD5C recombinant clones containing mycobacterial genomic DNA fragments. **(A)** Analysis of MBP fusion proteins on SDS-polyacrylamide gel stained with Coomassie Brilliant Blue. 20 μ g total protein from cell-free extracts was loaded in each lane. Lanes: 1, *E. coli* XL1 Blue[pSD5C] uninduced; 2, *E. coli* XL1 Blue[pSD5C] induced with IPTG; 3–10, *E. coli* XL1 Blue[pSD5C] recombinants containing *M. tuberculosis* H₃₇Ra genomic DNA fragments induced with IPTG. **(B)** Western blot analysis of MBP fusion proteins with anti-MBP Ab. 100 μ g of total protein from cell-free extracts

was loaded in each lane. Lanes: 1, *M. smegmatis* LR222[pSD5B] as control; 2, *M. smegmatis*[pSD5C]; 3–10, *M. smegmatis* [pSD5C] recombinants containing *M. tuberculosis* H₃₇Ra genomic fragments (loaded in the same order as in A). Molecular mass markers (in kDa) are indicated on the left of each panel. **Methods:** Genomic DNA isolated from *M. tuberculosis* H₃₇Ra was digested with *Pst*I and fragments ranging between 2 and 7 kb were purified. These were ligated to *Pst*I-digested, dephosphorylated pSD5C, and the ligation mixture was used to transform *E. coli* XL1 Blue. Transformants were selected on LB agar containing 25 µg Km/ml and 10 µg Tc/ml. For expression studies, individual transformants were grown in LB medium containing Km and Tc to A_{600nm} of 0.6, and induced with 50 µM IPTG for 1 h with shaking at 37°C. *M. smegmatis* transformants were grown and their cell-free extracts were prepared and analysed by 0.1% SDS-10% PAGE, as described in the legend to Fig. 3.

Fig. 7.

Expression of *cat* using pSD5C in different mycobacterial species. **(A)** Western blot analysis with anti-MBP Ab. **(B)** Western blot analysis with anti-CAT Ab. Lanes: A1 and B1, *M. smegmatis*[pSD5C]; A2 and B2, *M. smegmatis*[pSD5C-CAT]; A3 and B3, *M. tuberculosis* H₃₇Ra[pSD5C]; A4 and B4, *M. tuberculosis* H₃₇Ra[pSD5C-CAT]; A5 and B5, *M. bovis* BCG[pSD5C]; A6 and B6, *M. bovis* BCG[pSD5C-CAT]. **Methods:** *M. tuberculosis* H₃₇Ra and *M. bovis* BCG were separately transformed with pSD5C and pSD5C-CAT as described previously (Bashyam et al., 1996) and transformants were selected on Middlebrook 7H10

agar supplemented with OADC (Difco Laboratories, Detroit, MI, USA), 25 µg Km/ml and 20 µg Cm/ml. Single colonies were grown in Middlebrook 7H9 medium containing ADC (Difco Laboratories, Detroit, MI, USA) and Km to $A_{600\text{nm}}$ of 2.0 and cell-free extracts were prepared and analysed by 0.1% SDS-10% PAGE as described in the legend to Fig. 3. 100 µg total protein from cell-free extracts was loaded in each lane.