Cloning and characterization of the gene for the '19 kDa' antigen of *Mycobacterium bovis*

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Monoclonal antibody CMA134.1 reacted with a protein antigen of apparent molecular mass 22 kDa from *Mycobacterium bovis* and *Mycobacterium tuberculosis*, and with an apparently 24 kDa antigen of *Mycobacterium kansasii*, but not with other mycobacteria or related species. This antibody was used to screen a gene library of *M. bovis* in λ gt11 and identified a recombinant clone that expressed a protein with an apparent molecular mass of 19–20 kDa. Gene expression occurred from the *lac* promoter in λ gt11, but used an unidentified vector promoter, possibly that of the replication primer RNA, in the final plasmid construct. The sequence of an 840 bp fragment was determined and shown to code for a product of 15 kDa. This sequence is identical to that, independently determined, of a gene from *M. tuberculosis*, usually referred to as the 19 kDa antigen. The reasons for the apparent size discrepancies are discussed.

Introduction

Bovine tuberculosis persists at a significant level in certain areas of the south-west of England as a result of the reservoir of *Mycobacterium bovis* infection present in the badger population (Wilesmith & Williams, 1987). Disease caused by infection with *M. bovis* is also recognized as a potential problem in farmed deer (Stuart *et al.*, 1988).

Diagnosis of *M. bovis* infection in both cattle and deer relies on isolation of organisms from tissues of suspect animals. These animals must first be identified as potentially infected by screening skin-test reactions to the crude *M. bovis* antigen preparation known as PPD. However, PPD is a heterogeneous preparation and considerable cross-reactions occur with other non-pathogenic organisms (Thorns & Morris, 1986; Chaparas *et al.*, 1970). Improvement of immunodiagnosis-

tic screening for *M. bovis* infection relies on the identification and purification of antigens exhibiting a high degree of specificity.

The fractionation of mycobacterial antigens has proved difficult due to the complexity of the organisms and problems associated with identifying specific, biologically active components. Hybridoma technology has allowed the identification and characterization of appropriate antigens using monoclonal antibodies (mAbs) specific for individual species. The use of mAbs coupled with molecular biology techniques has enabled recombinant antigens bearing these specific epitopes to be produced and purified (Young *et al.*, 1985). This paper describes the isolation from *M. bovis* and characterization of a gene encoding an antigen which binds a mAb specific for *M. bovis*, *Mycobacterium tuberculosis* and *Mycobacterium kansasii*.

Independently of this work, Ashbridge *et al.* (1989) have determined the sequence of the *M. tuberculosis* gene coding for a 19 kDa antigen, which is identical to the sequence described in this paper.

Methods

Strains and vectors. The *Escherichia coli* host strains Y1088, Y1089 and Y1090 for the λ vector gt11 are described by Huynh *et al.* (1985); *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) was used as the host for

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Abbreviations: PPD, purified protein derivative; mAb, monoclonal antibody; ORF, open reading frame.

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pUC18 and M13 recombinants. All other strains used were from the collection at the Central Veterinary Laboratory.

Preparation of antigens. Ten isolates of *M. bovis* (five from cattle and five from badgers), plus isolates of *Mycobacterium phlei*, *Mycobacterium chelonae*, *Mycobacterium kansasii*, *Mycobacterium smegmatis* and *Mycobacterium fortuitum*, were grown in modified Middlebrook's 7H11 medium (Difco) + Tween 80. Other mycobacteria were grown on Lowenstein-Jensen medium. *Corynebacterium bovis*, *Corynebacterium pseudotuberculosis*, *Yersinia pseudotuberculosis* and two *Nocardia* isolates from bovine lung were grown on blood agar. Cells were harvested in saline; sonicated extracts were prepared as described by Morris *et al.* (1985) and protein concentrations estimated by the Lowry method.

Determination of mAb specificity. Microtitre plates (Nunc Maxisorp) were coated with 50 µl of antigen containing 10 µg protein ml⁻¹ in PBS (10 mM-sodium phosphate buffer, 0.85% saline, pH 7.2) overnight at room temperature. Non-specific sites were blocked with 1% (w/v) polyvinylpyrrolidone (Sigma) for 1 h at 37 °C. mAb CMA134.1 (provided by Coralabs Research) was added at optimal concentration (previously determined by titration with *M. bovis* antigen) and incubated for 1 h at 37 °C. After washing, the plates were incubated with affinity-purified sheep anti-mouse immunoglobulin biotinylated species-specific F(Ab')₂ fragment (Amersham) in 1% (w/v) BSA-PBST (PBS containing 0.05% Tween 20) for 90 min at 37 °C. The plates were washed and further incubated for 30 min at 37 °C in streptavidin biotinylated horseradish peroxidase complex (Amersham) diluted in PBST containing 1% (w/v) BSA. The biotinylated anti-immunoglobulin and streptavidin complex were titrated previously to determine the optimal dilutions. The substrate used was *o*-phenylenediamine hydrochloride. Four replicates of each antigen preparation were tested, and the mean values were compared with the value obtained for a standard *M. bovis* antigen which was included on each plate.

Characterization of antigen. Extracts of mycobacteria prepared by sonication were separated under reducing conditions (2-mercaptoethanol) in 12.5% SDS-PAGE gels using a discontinuous Tris/HCl buffer system (Laemmli, 1970). Antigens were transferred to nitrocellulose sheets by electroblotting using a Tris/glycine/methanol buffer (Towbin *et al.*, 1979). Non-specific binding sites were blocked by incubating the filter in PBST containing 3% (w/v) BSA for 1 h. mAbs were diluted in 1% (w/v) BSA-PBST to ten times the concentration giving maximum binding in the ELISA; the filters were incubated in this mixture for 1 h at room temperature with continuous gentle shaking. After washing, bound mAb was detected using the biotinylated anti-immunoglobulin and streptavidin-biotin horseradish peroxidase complex (as described above) with 0.05% chloronaphthol + 0.015% hydrogen peroxide as substrates.

Construction of gene libraries. The procedure was essentially that described by Huynh *et al.* (1985). DNA was extracted from *M. bovis* (field strain 381/79) as described by Zainuddin & Dale (1989) and fragmented by repeated passage through a syringe needle until the major part of the DNA was between 2 and 9 kb. The DNA fragments were end-filled using T4 DNA polymerase and internal *EcoRI* sites protected by methylation with *EcoRI* methylase before addition of *EcoRI* linkers and digestion with *EcoRI* restriction endonuclease.

The DNA was then size-fractionated on a 0.8% agarose gel, collecting fragments between 2.3 and 9 kb. About 250 ng of the fragmented *M. bovis* DNA was ligated with 1 µg of λgt11 arms (*EcoRI*-cut and dephosphorylated, from Vector Cloning Systems). The ligation mixture was divided into two aliquots for *in vitro* packaging, using a Gigapack kit (Vector Cloning Systems), yielding libraries designated MB7 and MB8. Samples of the libraries were assayed using Y1088 as the host, on L-agar + ampicillin, using IPTG/X-gal overlays. Samples of the libraries were subsequently amplified, using the host strain Y1090.

Antibody screening of gene libraries. The procedure was based on that described by Huynh *et al.* (1985). After infection of the host strain (Y1090) with the library, a plate of L-agar containing ampicillin (50 µg ml⁻¹) was overlaid and incubated for 3.5 h at 42 °C. A nitrocellulose filter, soaked in IPTG (10 mM) and allowed to dry, was laid on the agar surface and the plate was incubated for a further 3.5 h at 37 °C. After removal from the plate, the filter was washed in TBST (50 mM-Tris/HCl, 150 mM-NaCl, pH 7.5 + 0.05% Tween 20) and blocked overnight in TBST containing 3% (w/v) BSA. The blocking solution was then replaced by TBST + BSA + antibody. After incubation for 1 h, the filter was washed twice with TBST and then incubated for 2 h in TBST + BSA + biotinylated rabbit antimouse antibody. The filter was washed again in TBST and incubated for 30–60 min in TBST containing streptavidin-horseradish peroxidase complex (Amersham). After washing in TBS (without Tween) the filters were developed in 0.05% chloronaphthol + 0.015% hydrogen peroxide.

Lysogen construction and testing of cell extracts. Lysogens of λgt11 recombinants in Y1089 were constructed as described by Huynh *et al.* (1985), except that the phage:bacterium input ratio was increased to approximately 100:1. Single colonies able to grow at 30 °C but not at 42 °C were purified and tested for antigen production by SDS-PAGE and Western blotting as described above. Extracts of plasmid-containing cultures were examined in the same way.

Sequence analysis. DNA sequencing of M13 subclones was carried out using the dideoxy procedure (Sanger *et al.*, 1977), and the data were assembled and analysed with the 'Staden-Plus' software (Amersham). Searches of GenBank, EMBL and NBRF databanks were carried out using the SEQNET node at the SERC facility at Daresbury, by means of the UWGCG package and WordSearch program (Wilbur & Lipman, 1983; Devereux *et al.*, 1984), and the NAQ program from the Protein Identification Resource.

Other procedures. Restriction endonucleases and other enzymes were used according to the manufacturers' instructions. Other procedures were as described by Maniatis *et al.* (1982).

Results and Discussion

Specificity of the mAb

Extracts prepared by sonication of a range of mycobacteria and related species were tested by ELISA for binding of the mAb CMA134.1. A high degree of antibody binding to most isolates of *M. bovis* and *M. tuberculosis* and BCG was observed (Table 1). Two *M. tuberculosis* isolates showed no activity, suggesting that either the gene was not present or it was not expressed. All other species of mycobacteria and related organisms tested were negative, with the exception of the two strains of *M. kansasii*.

Western blotting (Fig. 1, lanes 1 and 2) showed that CMA134.1 bound to an antigen in sonicated extracts of *M. tuberculosis* and *M. bovis* with an apparent molecular mass of 22 kDa. The value of 22 kDa differs from that of 19 kDa obtained for the *M. tuberculosis* antigen by Ashbridge *et al.* (1989). This discrepancy is discussed later. The mAb detected a specific band in the *M. kansasii* extract with an apparent molecular mass of 24 kDa. This reaction confirms the ELISA results which showed cross-reaction of mAb CMA134.1 with *M.*

Table 1. Relative binding of mAb CMA134.1 to a range of antigens

Four replicates were tested, as described in Methods, for each antigen preparation; the results are expressed as a percentage of the value obtained for a standard *M. bovis* antigen which was included on each plate.

Species	Strain details	Percentage binding
<i>M. bovis</i> (bovine strains)	7/88	24
	2215/88	77
	264/88	86
	1664/88	91
	949/88	89
<i>M. bovis</i> (badger strains)	1697	108
	1395	73
	1291	34
	1974	69
	1318	69
	Bilthoven	46
	Dakar	35
BCG strains	Tokyo	72
	USSR	77
	Glaxo	28
	Paris	38
	Copenhagen	48
	Toronto	22
	Brazil	58
	Sweden	74
	Chicago	55
		1
		1
<i>M. phlei</i>		1
<i>M. chelonae</i>		1
<i>M. kansasii</i>	6595	34
	6411	69
<i>M. microti</i>	8710	10
<i>M. smegmatis</i>		1
<i>M. fortuitum</i>		1
<i>M. vaccae</i>		1
<i>M. paratuberculosis</i>	S250	1
	S100	1
<i>M. tuberculosis</i>	1156	33
	1130/79	56
	1132/79	84
	1139	1
	1143	33
	1145	51
	1146	64
	1147	48
	1151	1
	1156	32
	Serotype 2	0
<i>M. avium</i>	Serotype 3	2
<i>Corynebacterium bovis</i>		1
<i>Corynebacterium pseudotuberculosis</i>		1
<i>Nocardia</i> sp.	126/82	1
	207/82	1
<i>Yersinia pseudotuberculosis</i>		0

kansasii. An additional band at about 75 kDa in track 3 is probably a non-specific reaction due to the presence of a biotin-carrying protein (Collins *et al.*, 1987).

Construction and screening of gene libraries

Gene libraries MB7 and MB8 each contained approximately 10^5 phage particles, of which 90% were ap-

parently recombinant, i.e. they produced white plaques. Ten white plaques (five from each library) were picked at random and DNA samples subjected to *Eco*RI digestion. Three samples showed no apparent insert, while the average size of insert in the other seven samples was 2.7 kb.

Both libraries were amplified, each in two batches, using the host strain Y1090. The amplified stocks MB7.1, 7.2, 8.1 and 8.2 each contained approximately 10^{11} phage ml⁻¹. Each amplified library was screened by testing filters containing about 10^5 phage plaques for the presence of clones expressing antigens that reacted with a pool of mAbs including CMA134.1. One positive clone from the MB8.1 library (MB8.1.1) reacted specifically with CMA134.1 and was plaque purified. Parallel plaque lifts in the presence and absence of IPTG showed that expression was inducible, indicating that the cloned gene was transcribed from the *lac* promoter on the *gt*11 vector.

To confirm the nature of the expression product, lysogens were produced by infecting Y1089 and testing for the inability to form colonies at 42 °C. Western blotting of heat-treated and IPTG-induced lysogens showed the expression of an antigen of 19–20 kDa that was detected by CMA134.1 (results not shown). The β -galactosidase band was visible in gels stained for protein, at a very similar position to the authentic β -galactosidase in the marker track (116 kDa). These results indicate that although the cloned gene was transcribed from the *lac* promoter in the recombinant λ gt11 lysogens, it was translated independently of the β -galactosidase and not as a fusion protein.

Subcloning and characterization of the cloned gene

Attempts to recover the inserted fragment by *Eco*RI digestion of the λ gt11 recombinant were unsuccessful, indicating that one or both of the *Eco*RI sites had been lost in the cloning procedure. This was subsequently verified by DNA sequencing (see below). The major part of the inserted DNA was recovered, together with about 1 kb of λ gt11 DNA, using an internal *Kpn*I site and an *Sst*I site in the flanking vector sequence (see Fig. 2a). This fragment was cloned, using pUC18 as the vector, generating plasmid pUS30 (Fig. 2b). This strategy maintained the insert in the same orientation with respect to the *lac* promoter on the vector. This clone, pUS30, was also able to express the antigen, and expression was enhanced by induction with IPTG (Fig. 1, lanes 4 and 5).

The superfluous λ gt11 DNA was removed by *Bal*31 digestion of *Sst*I-cut pUS30, under conditions that reduced the DNA size by 1–2.5 kb. Twenty clones were selected and tested for antigen expression by Western

Fig. 1. Western blots of mycobacterial antigens and recombinant *E. coli* clones with mAb CMA134.1. Samples were subjected to SDS-PAGE, transferred to nitrocellulose sheets and reacted with CMA134.1 as described in Methods. Lanes: 1, *M. tuberculosis* 1145; 2, *M. bovis* 98; 3, *M. kansasii* 219; 4 and 5, *E. coli* clone containing the recombinant plasmid pUS30, uninduced and IPTG-induced respectively; 6 and 7, *E. coli* clone containing the recombinant plasmid pUS32, uninduced and induced; 8 and 9, non-recombinant *E. coli* control, uninduced and induced. 'Rainbow' protein markers (Amersham) were used as size standards. The upper (22 kDa) band in the *E. coli* tracks (4-9) is a non-specific reaction due to the *E. coli* biotin carrier protein which has a monomer size of 22 kDa (Fall & Vagelos, 1972; Collins *et al.*, 1987).

(a) MB8.1.1

(b) pUS30

(c) pUS31

(d) pUS32

Fig. 2. Structure of recombinant clones. Only the cloned fragment and relevant adjacent regions of the vector are shown. Stippling indicates *M. bovis* DNA; hatching indicates *lambda gt11* DNA. (a) Recombinant *lambda gt11* clone recovered from the library; (b) *KpnI*-*SstI* fragment cloned in pUC18; (c) *Bal31* deletion of pUS30; (d) removal of *PstI* fragment of pUS31. Restriction enzyme sites marked are: E, *EcoRI*; K, *KpnI*; N, *NdeI*; Pst, *PstI*; Pvu, *PvuII*; S, *SstI*. E* indicates the position of the *EcoRI* site (on the *lambda gt11* vector) lost in the cloning procedure.

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      10              30              50
GAATTTTCCGCCGCACCTAGACCTCGGGCCCTGCTAACGCGCATACTGCCGAAGCGGTCC

      70              90              110
TCAATGCCGATGGACCGCTACGACAGGCAAAGGAGCACAGGGTGAAGCGTGGACTGACGG
      -----          V* K R G L T V

      130             150             170
TCGCGGTAGCCGGAGCCGCCATTCTGGTCGCAGGTCTTTCCGGATGTTCAAGCAACAAGT
      A V A G A A I L V A G L S G C S S N K S

      190             210             230
CGACTACAGGAAGCGGTGAGACCACGACCGCGGCAGGCACGACGGCAAGCCCCGGCGCCG
      T T G S G E T T T A A G T T A S P G A A

      250             270             290
CCTCCGGGCGGAAGGTCGTCATCGACGGTAAGGACCAGAACGTCACCGGCTCCGTGGTGT
      S G P K V V I D G K D Q N V T G S V V C

      310             330             350
GCACAACCGCGGCCCGGCAATGTCAACATCGCGATCGGCGGGGCGGCGACCGGCATTGCCG
      T T A A G N V N I A I G G A A T G I A A

      370             390             410
CCGTGCTCACCGACGGCAACCCTCCGGAGGTGAAGTCCGTTGGGCTCGGTAACGTCAACG
      V L T D G N P P E V K S V G L G N V N G

      430             450             470
GCGTCACGCTGGGATACACGTCGGGCACCGGACAGGGTAACGCCTCGGCAACCAAGGACG
      V T L G Y T S G T G Q G N A S A T K D G

      490             510             530
GCAGCCACTACAAGATCACTGGGACCGCTACCGGGGTCGACATGGCCAACCCGATGTCAC
      S H Y K I T G T A T G V D M A N P M S P

      550             570             590
CGGTGAACAAGTCGTTTCAAATCGAGGTGACCTGTTTCCTAACCTAAAGCGTGTGATGCG
      V N K S F E I E V T C S *

      610             630             650
GGCTGTGAACAGCGCGTCCGAGCCGGGCAGTCAGGCCTAGCGCGGCGACGATTCGAGCGG
      -----

      670             690             710
TTGCCATCCGTCAAGTGGCAACCCGACCGCAAACCTCGGTATATCCGGGTGAGCTACTCAC
      ----->          <-----

      730             750             770
GGTGATCGTTCGGTTGTTTCGGCCTTGACCACAGCGGAGACGATCGCCAGGCCGAGCCCGG

      790             810             830
TGCTACCGCTTGGCCGGGACCGTGACGTATCGCCGCGGGCAACCGCTCGAAAACCTCGGAC

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Fig. 3. Nucleotide sequence of the *M. bovis* '19 kDa' antigen gene. The sequence shown starts at the *EcoRI** site at the 5' end of the cloned fragment. The underlined sequence (89–95) is the presumptive ribosome-binding site, and the arrows indicate a potential stem-loop structure. The deduced amino acid sequence is shown in one-letter code; V*, presumed *N*-formylMet initiation codon.

blotting. Two clones (pUS31 and pUS33) showed a positive reaction while fifteen were negative and three showed a weak reaction. The apparent size of the antigen specified by pUS31 and pUS33 was unchanged. Restriction fragment sizes indicated that in both cases about 1500 base pairs of pUS30 had been deleted, which is consistent with the observation that both *PvuII* sites in this region had been deleted, but not the *NdeI* site (see Fig. 2c). This deletion had therefore removed the *lac* promoter; the nature of expression from this plasmid is

discussed later. Removal of a *PstI* fragment to give plasmid pUS32 (Fig. 2d) did not affect the expression or size of the product (Fig. 1, tracks 6 and 7). As predicted from the deletion of the *lac* promoter, expression from pUS32 was not inducible by IPTG.

Sequence determination and analysis

For DNA sequence determination, the 900 bp *NdeI*–*PstI* fragment of pUS32 was end-filled and blunt-end

ligated with *Sma*I-cut M13mp18. Sequence data from these clones identified an internal *Stu*I site, and additional sequence data were obtained from a cloned *Kpn*I/*Stu*I fragment in M13mp19. These data were supplemented by using synthetic primers, so that the complete sequence was determined using overlapping fragments on both strands. The sequence (Fig. 3) starts with GAATTTTC rather than the expected *Eco*RI site. The sequence 5' to this position is that of the expected region of the λ gt11 vector (not shown). The absence of an *Eco*RI site at this position accounts for the failure of *Eco*RI to excise the insert.

Ashbridge *et al.* (1989) have independently published the sequence of a 19 kDa antigen from *M. tuberculosis*, which is identical in its coding sequence to the *M. bovis* sequence presented here. Their sequence (as deposited with the EMBL databank, accession number X07945) extends beyond the 5' end shown in Fig. 3 to a genuine *Eco*RI site, indicating that the GAATTTTC sequence is present in the mycobacterial DNA; in the construction of our library this site may have been cut by *Eco*RI prior to the ligation step, presumably due to *Eco*RI* activity.

The orientation of the gene was established by the inducibility of the λ gt11 lysogen and confirmed by Northern blot analysis using single-stranded probes (data not shown). There is only one open reading frame (ORF) of the required size, extending from position 21 to 578; this was identified as a probable coding region by the positional base preference procedure of Staden (1984). Ashbridge *et al.* (1989) identified the same ORF for the gene coding for the 19 kDa *M. tuberculosis* antigen. Although there is no AUG start codon within this ORF, there is a GUG codon at position 102 and a putative ribosome-binding site (AAAGGAG) lying six bases upstream. GUG sequences have been reported as start codons in several other mycobacterial genes (see Dale & Patki, 1990), which is consistent with the high G+C content of these organisms. The translation product initiated from this position consists of 159 amino acids, with a calculated molecular mass of 15115 Da. This is significantly less than the observed size of 22 kDa in *M. bovis* (and 19–20 kDa for the recombinant *E. coli* product), which could be due to different post-translational modifications and/or anomalous behaviour of the protein on SDS-PAGE gels.

In the original λ gt11 lysogen, expression was induced by IPTG and was therefore under the control of the *lac* promoter of the vector, although a fusion protein was not seen. Inspection of the sequence shows that translation of the transcript initiating from the *lac* promoter in the λ gt11 recombinant would terminate at the stop codon UGA (position 103) which overlaps with the suggested GUG start codon, which would therefore be available for re-initiation of translation. In the pUC recombinant

pUS30, the reading frame of the remaining portion of the λ gt11 *lacZ* gene within the insert is altered by the recombination and is terminated by stop codons within the *lacZ* region. Translation of the *M. bovis* insert in pUS30 therefore requires the ribosome-binding site adjacent to the start codon identified above.

With plasmids pUS31 and pUS32, it was deduced that the *lac* promoter had been deleted, and this is supported by the absence of IPTG induction (Fig. 1). It is unlikely that transcription in *E. coli* occurs from a promoter within the *M. bovis* insert since a detailed examination of the short region of DNA between the start of the insert and the translational start codon revealed no sequence resembling an *E. coli* consensus promoter. The size of the transcript from pUS32 was estimated by Northern blotting (results not shown) to be about 800 nucleotides, which would allow scope for a considerable leader region; transcription may therefore be occurring from a site within the *lacZ* region that does not normally act as a promoter, or by readthrough from a promoter within the vector. The sequences of pUC18 and the portion of the *lac* region contained in λ gt11 were examined for the presence of possible consensus *E. coli* promoters; the only candidate is the region, upstream from the origin of replication, from where synthesis of the replication primer RNA is initiated.

The base composition of this sequence (64.2% G+C) is typical of mycobacteria, and the codon usage (Table 2) also shows the high degree of codon bias normally seen in these organisms (see Dale & Patki, 1990). An analysis for structural motifs of the sequence revealed one possibly significant hairpin loop structure, formed by an 8 base inverted repeat starting at position 659 (see Fig. 3). This is about 80 bases beyond the putative stop codon, and could be involved in transcriptional termination. The inverted repeat is not accompanied by a following run of U residues; the high G+C content of mycobacteria may encourage the preferential use of terminators resembling those rho-dependent terminators of *E. coli* that do not have a sequence of U residues.

A search was carried out for homology with other nucleic acid and protein sequences in the GenBank, EMBL and NBRF databanks; no homologies considered to be significant were detected, other than that of the 19 kDa *M. tuberculosis* antigen (Ashbridge *et al.*, 1989). The sequences of the coding region of these genes from *M. tuberculosis* and *M. bovis* are identical. Together with the high degree of DNA homology (greater than 90%) between *M. bovis* and *M. tuberculosis* (Wayne, 1984), and the observations that the '65 kDa' and 10–12 kDa antigen genes of *M. tuberculosis* and BCG are completely or virtually identical in *M. tuberculosis* and BCG (Shinnick, 1987; Thole *et al.*, 1987; Baird *et al.*, 1989; Shinnick *et al.*, 1989; Yamaguchi *et al.*, 1988), our

Table 2. Codon usage of the *M. bovis* '19 kDa' antigen gene

Ala	GCU	1	Gly	GGU	5	Pro	CCU	1
	GCC	10		GGC	10		CCC	1
	GCA	4		GGA	6		CCA	0
	GCG	6		GGG	5		CCG	4
Arg	CGU	1	His	CAU	0	Ser	UCU	0
	CGC	0		CAC	1		UCC	5
	CGA	0					UCA	2
	CGG	0	Ile	AUU	2		UCG	4
	AGA	0		AUC	5		AGU	0
	AGG	0		AUA	0		AGC	4
Asn	AAU	1	Leu	UUA	0	Thr	ACU	2
	AAC	9		UUG	0		ACC	11
				CUU	1		ACA	2
Asp	GAU	0		CUC	2		ACG	6
	GAC	5		CUA	0			
				CUG	3	Trp	UGG	0
Cys	UGU	2						
	UGC	1	Lys	AAA	0	Tyr	UAU	0
				AAG	8		UAC	2
Gln	CAA	0						
	CAG	2	Met	AUG	2	Val	GUU	1
							GUC	9
Glu	GAA	1	Phe	UUU	0		GUA	1
	GAG	3		UUC	1		GUG	7*

* Including the presumptive *N*-formylMet initiation codon.

observations provide further support for the view that *M. tuberculosis*, *M. bovis* and BCG should be regarded as variants of the same species, although distinguishable by the use of probes specific for a polymorphic repetitive DNA element (Zainuddin & Dale, 1989). However, the 65 kDa and 10–12 kDa proteins are related to highly conserved stress ('heat-shock') proteins of other species (Young *et al.*, 1988; Shinnick *et al.*, 1989), which reduces the significance of their conserved nature among members of the *M. tuberculosis* complex. Searches of the databanks have revealed no evidence that the 19 kDa antigen is related to any known stress proteins, and the complete identity of the sequence of this gene in *M. bovis* and *M. tuberculosis* may therefore be a better indication of the close relationship of these organisms. In addition, the absence of silent nucleotide substitutions may indicate that such changes are constrained by the high degree of codon bias in the genomes of these organisms.

Since the '19 kDa' antigen gene is identical in both organisms, reagents derived from it will not be able to differentiate members of the *M. tuberculosis* complex. The mAb CMA134.1 shows some diagnostic potential where this distinction is not important. Elimination of cross-reaction with *M. kansasii* may be obtained by fractionating the antigen or by using specific parts of the gene as probes.

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