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**Gene expression profiling and antigen mining of the tuberculin production strain
Mycobacterium bovis AN5**

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1 **Abstract**

2 Control of bovine tuberculosis (bTB) relies on regular testing of cattle with a crude preparation
3 of mycobacterial antigens termed PPD (Purified Protein Derivative). Worldwide production of
4 bovine PPD uses the *Mycobacterium bovis* AN5, a strain that was originally isolated circa
5 1948 in Great Britain (GB). Despite its worldwide use, the AN5 strain is poorly characterised.
6 AN5 was adapted to grow on glycerol in a process similar to that used for the derivation of the
7 BCG vaccine strains; during this process, it is known that BCG deleted the genes for some
8 potent antigens. Our previous analysis of the genome of *M. bovis* AN5 showed that it had not
9 suffered extensive gene deletion events during *in vitro* adaptation. However, glycerol
10 adaptation of AN5 strain may have caused differences in its global gene expression profile
11 that could affect antigen expression. To assess this, we determined the transcriptome profile
12 of AN5 and compared it to expression data for two endemic GB strains of *M. bovis* that
13 account for ~61% of all GB bTB cases. Genes expressed at lower levels in AN5 compared to
14 *M. bovis* field isolates were then screened for antigenicity in naturally infected animals. Using
15 this approach a number of genes were found to be expressed at lower levels in AN5,
16 including those for known antigens. Our results show that field strains of *M. bovis* show some
17 significant differences in gene expression to AN5, and that this differential gene expression
18 may impact on the antigen profiles expressed by AN5 during *in vitro* culture.

1 **Introduction:**

2 Bovine tuberculosis (bTB) is one of the most difficult animal health problems that the farming
3 industry in Great Britain (GB) faces today. The number of cattle infected with bTB has been
4 increasing year on year, leading to serious losses for affected farms. The reasons for this
5 inexorable rise are not obvious, yet many interacting factors contribute such as cattle
6 movement (Gilbert et al., 2005; Johnston et al., 2005), and a wildlife reservoir (Donnelly et al.,
7 2006; Griffin et al., 2005). Worldwide, bTB continues to be place a considerable drain on
8 agriculture, with annual losses predicted at >\$3 billion.

9
10 A cornerstone of the bTB control strategy is regular testing of cattle with a crude preparation
11 of mycobacterial antigens termed “purified protein derivative” or PPD, a.k.a tuberculin. PPD
12 production is based on the *Mycobacterium bovis* AN5 strain which was originally isolated in
13 England circa 1948 and which is now used worldwide (Paterson, 1948). The acceptance of *M.*
14 *bovis* AN5 as a standard for tuberculin production was principally based on its high yield of
15 cell mass on glycerinated-media, a phenotype that was selected by repeated subculture of
16 the bacillus on laboratory media. This selection for a desirable phenotype by passage through
17 glycerinated media has parallels with the method used by Calmette and Guérin to attenuate a
18 strain of *M. bovis* to generate BCG (Calmette and Guérin, 1909). It is known that during this
19 extended *in vitro* passage, BCG lost the genes for a number of potent antigens, and
20 accumulated a range of point mutations that effect gene expression and virulence (Behr et al.,
21 1999; Brosch et al., 2007).

22
23 We have previously shown that *M. bovis* AN5 suffered no extensive gene deletions during *in*
24 *vitro* culture (Inwald et al., 2003). However, the possibility exists that the AN5 strain may have
25 accumulated Single Nucleotide Polymorphisms (SNPs) during its derivation, and these
26 mutations may affect its global gene expression profile. To assess this, we determined the
27 transcriptome profile of AN5 and compared it to transcriptome data for two endemic GB
28 strains of *M. bovis* that account for ~61% of all bTB cases in the UK (Smith et al., 2003; Smith
29 et al., 2006). We identified genes expressed at lower levels in the AN5 strain, and then
30 checked whether these genes coded for antigens. Through this approach we have defined

1 the global gene expression profile of *M. bovis* AN5 and shown that its antigen expression
2 profile is similar to that of extant strains. This work therefore underpins continued use of AN5
3 to produce PPD for use in eradication and control programs.

4

5 **Methods**

6 **Bacterial strains and culture**

7 *Mycobacterium bovis* AN5 was provided by the Tuberculin Production Unit, VLA Weybridge
8 (Paterson, 1948). The spoligotype of AN5 is SB1417 as defined by the *M. bovis* international
9 spoligotype database (www.mbovis.org). The field isolates chosen were *M. bovis* 2122/97
10 (VLA designation spoligotype “9”; international designation spoligotype SB0140) and *M. bovis*
11 1121/01 (VLA spoligotype “17”; international designation SB0263); these two strains
12 represent the two dominant spoligotypes in GB, representing 34% and 29% of all isolates.
13 Mycobacterial strains were grown in ADC-supplemented Middlebrook 7H9 medium with
14 0.05% Tween80. *M. bovis* AN5 was also grown in glycerol-rich Bureau of Animal Industry
15 (BAI) medium which is used for PPD production (14 g of L-asparagine, 1.5 g of dipotassium
16 hydrogen phosphate, 0.74 g of sodium citrate, 1.5 g of magnesium sulfate, 0.3 g of ferric
17 sulfate, 0.08 g of zinc sulfate, 0.008 g of manganese chloride, 0.00138 g of cobaltous
18 chloride, 10 g of glucose, 100 g of glycerol/liter); as strains 2122/97 and 1121/01 are not
19 glycerol adapted, they will not grow in BAI medium (Keating et al., 2005). Strain stocks were
20 prepared from mid-log cultures and stored as 1 mL aliquots in 15% glycerol. One stock aliquot
21 was used to inoculate 10 mL medium and grown standing at 37°C until an OD₆₀₀ of
22 approximately 0.6 was reached. These seed cultures were used to set up roller cultures by
23 inoculating 2 mL of the starter culture into 200 mL of medium in 850 cm² roller bottles. Cells
24 were harvested in the mid-log phase of growth choosing a point in the growth curves where
25 the population doubling time was, approximately, the same for the three strains (0.4-0.5).

26

27 **Transcriptome analysis**

28 For transcriptome analysis, strains were grown in ADC-supplemented 7H9 medium with
29 0.05% Tween80. Total RNA from each strain was extracted, purified, reverse-transcribed, and
30 labeled with Cy5-dCTP as previously described (Golby et al., 2007). Cy3-labeled DNA

1 (AF2122/97) was used as a control. Probes were hybridised to whole-genome *M. bovis* / *M.*
2 *tuberculosis* composite microarrays (The array design is available in BμG@Sbase, accession
3 no. A-BUGS-31 (<http://bugs.sgul.ac.uk/A-BUGS-31>) and also ArrayExpress, accession no. A-
4 BUGS-31) and the arrays were scanned with an Affymetrix 428 scanner. Image processing
5 and data normalisation were performed with Bluefuse 3.2 software (BlueGnome, Cambridge,
6 UK). Data were further analysed with Genespring 7.0 software (Agilent Technologies).
7 Significance values were calculated by using 1-way ANOVA and final P-values were obtained
8 by Benjamini and Hochberg correction for multiple testing with a false discovery rate of 5%.
9 Results for selected genes were confirmed by quantitative RT-PCR analysis (qRT-PCR), as
10 previously described (Golby et al., 2007), and expression levels were normalised by using the
11 *sigA* gene as an internal reference.

12

13 **Antigen screening**

14 Peptides spanning the lengths of the selected coding sequences were purchased from
15 Mimitopes Ltd (UK). The peptides were 20 residues in length with a 12-residue overlap across
16 each neighbouring peptide and they were formulated into pools of approximately 8-12
17 peptides. The IFN- γ immunoassay methodology was described previously (Cockle et al.,
18 2002). For screening purposes, blood samples were taken from 20 *M. bovis* reactor cattle
19 identified through routine screening. The IFN- γ concentration was determined using the
20 BOVIGAM ELISA kit (Prionics, Schlieren, Switzerland). An antigen was defined as giving a
21 positive response when the OD₄₅₀ with antigen minus OD₄₅₀ without antigens were ≥ 0.1 .
22 Optical density readings were converted to concentration of IFN- γ (pg/ml) using the following
23 equation: $(OD_{450} \times 30.9) + 0.5658$.

24

25 **Accession numbers**

26 Fully annotated microarray data have been deposited in BμG@Sbase (accession no. E-
27 BUGS-64; <http://bugs.sgul.ac.uk/E-BUGS-64>) and also ArrayExpress
28 (<http://www.ebi.ac.uk/microarray-as/ae/>; accession number: E-BUGS-64).

1

2 **Results**

3 **Growth curves**

4 To ensure that differences in gene expression were not simply down to variation in growth
5 characteristics of the strains, growth curves of *M. bovis* AN5, *M. bovis* 2122/97 and *M. bovis*
6 1121/01 in ADC-supplemented 7H9 medium with 0.05% Tween 80 were determined in
7 triplicate. From the growth curves it was apparent that AN5 grew to a higher OD (~1.0)
8 compared to 2122 or 1121 (final OD ~ 0.8). However, during exponential growth their
9 doubling times were similar. Hence for transcriptome analysis cultures were harvested in the
10 mid-log phase of growth where the population doubling time was the same.

11

12 **Transcriptome analysis**

13 Comparison of global gene expression across *M. bovis* AN5, *M. bovis* 2122/97 and *M. bovis*
14 1121/01 in Middlebrook 7H9 medium revealed 66 genes with variable expression across the
15 strains. Thirty-one genes were found to be downregulated in AN5 compared to the 2122 and
16 1121 strains (Table 1). These included genes encoding the antigens Mb3646c, Mpb83,
17 Mpb70 and LpqH (19 KDa antigen); genes involved in the synthesis of molybdenum
18 cofactors; and genes encoding proteins of unknown function (Table 1).

19

20 The key focus of our study was the identification of genes expressed at lower levels in AN5
21 compared to field strains. However, we also identified thirty-five genes that were upregulated
22 in AN5 compared to the 2122 and 1121 strains. These included genes encoding a number of
23 ribosomal proteins; this may be indicative of an increased capacity for protein synthesis in
24 AN5 that could be linked to increased *in vitro* growth.

25

26 **Quantitative Real Time PCR (qRT-PCR)**

27 From Table 1 it was apparent that some genes were expressed at lower levels in AN5
28 compared to *M. bovis* field isolates. However, this was in Middlebrook 7H9 growth medium,
29 which supports growth of all *M. bovis* strains. For tuberculin production, AN5 is grown in
30 glycerol-based BAI, a medium that does not support the growth of field isolates as they can

1 not use glycerol as a sole carbon source (Keating et al., 2005). Therefore, we needed to
2 validate whether the genes that we saw as expressed at low levels in AN5 grown in 7H9
3 medium were also expressed at low levels when AN5 was grown in BAI medium. We
4 therefore used qRT-PCR to examine gene expression levels of selected genes (chosen on
5 the basis of fold change and putative function) across growth media (Figure 1). From this
6 comparison it was evident that while absolute level of expression varied across the strains
7 and growth media, genes expressed at low levels in 7H9 were also expressed at low levels in
8 BAI.

9

10 **Antigen screening**

11 From the expression data it was clear that some known *M. bovis* antigens were expressed at
12 lower levels in AN5 than in field isolates. MPB70, MPB83, 19 KD, and Mb3646c have
13 previously been shown to contain T-cell epitopes (Mustafa et al., 2006; Pollock et al., 1994;
14 Vordermeier et al., 1999). However, many of the other genes identified had not been
15 described as *M. bovis* antigens. We selected a subset of 6 genes based on their putative
16 function and level of down-regulation (*iniB*, *Mb3644c*, *Mb3645c*, *Mb3657*, *MT2941*, *MT3762*)
17 that were down regulated in AN5 for further analysis. The putative products of these genes
18 were synthesised as peptides and screened the ability of the peptides to induce *ex vivo* IFN- γ
19 in blood from *M. bovis* infected cattle.

20

21 *IniB*, *Mb3644c*, *Mb3657*, *MT2941*, *MT3762* were all found to be nonantigenic (data not
22 shown). However, protein *Mb3645c* was antigenic, coming up as positive in 9 out of 20
23 reactors. This protein had also been identified as antigenic through a parallel antigen mining
24 approach (Sidders *et al*, 2008), and it is now being taken forward for further analysis in a
25 larger number of reactors and negative cattle.

26

27 **Discussion**

28 *Mycobacterium bovis* AN5 is a cornerstone of bTB eradication policies as it is the source of
29 the PPD diagnostic reagent. However, there is a paucity of data on this strain and its
30 biological characteristics. With the upward trend in bTB incidence in GB and the worldwide

1 economic losses due to bTB we need to have a clear understanding of our disease control
2 tools to determine whether improvements are needed.

3

4 We previously performed comparative genomic analyses of AN5 using DNA microarrays, and
5 found that AN5 had not suffered any significant loss of genetic information compared to
6 recent field isolates. However, the global analysis we performed could not have identified
7 SNPs in the AN5 genome that may effect the expression of antigen-encoding genes. We
8 therefore determined the transcriptome of *M. bovis* AN5 grown in laboratory media and
9 compared it to that of the field strains *M. bovis* 2122/97 and *M. bovis* 1121/01. Of the 3,952
10 *M. bovis* genes, 66 showed a minimum 3-fold difference in expression when comparing AN5
11 against the two field strains (Tables 1 and 2).

12

13 Variation in gene expression between AN5 and field strains may also be indicative of
14 metabolic remodelling by AN5 during its adaptation to growth on glycerol. As we have
15 previously shown, *M. bovis* strains have a mutation in the *pykA* gene that prevents their
16 growth on glycerol as a sole carbon source; during glycerol adaptation of AN5 a “corrected”
17 *pykA* was selected to allow growth on glycerol. In a study looking at glycerol adaptation of *E.*
18 *coli*, Palsson and colleagues showed that point mutations in the gene encoding glycerol
19 kinase, *glpK*, were selected when they increased the efficiency of this key enzyme for growth
20 on glycerol. We sequenced *glpK* from AN5, but found it to have the same sequence to *M.*
21 *bovis* and *M. tuberculosis* (data not shown), suggesting that a variant allele has not been
22 selected. Similarly, we found no evidence for gene duplication of the *glpD2* gene (data not
23 shown), encoding glycerol dehydrogenase, whose amplification in the BCG genome has been
24 suggested to be driven by the need to adapt to growth on glycerol. Two genes for fatty acid
25 desaturases, *desA1* and *desA2*, were highly expressed in AN5 relative to the field strains.
26 Interestingly, transcriptome analysis of BCG, another glycerol adapted strain, showed
27 decreased expression of the *desA3* desaturase compared to the 2122 and 1121 *M. bovis*
28 strains (Brosch et al., 2007). This suggests that glycerol-adaptation may not necessarily
29 select for the same metabolic adaptations across all strains.

30

1 As stated above, a key limitation of our study is the fact that the comparative transcriptome
2 analysis could not be performed in the BAI medium that is used for tuberculin production,
3 since *M. bovis* field isolates cannot grow in this medium. We were therefore forced to use
4 Middlebrook 7H9 medium, and to capture the transcriptomes during the log-phase of bacterial
5 growth. It is possible that this approach led us to miss genes that are expressed at low levels
6 by AN5 during the extended culture (12 weeks) on BAI medium. Comparative proteome
7 analyses of PPD would provide a more direct approach to defining AN5's protein expression.

8
9 Decreased expression of genes encoding potent antigens may have implications for the
10 diagnostic value of the PPD reagent. The genes for some known antigens were expressed at
11 lower levels; for example, genes encoding MPB83, MPB70, 19 KDa and Mb3646 were
12 expressed at lower levels by AN5. To determine if any other of the genes with low expression
13 coded for antigens, we selected a subset and tested them for antigenicity using the
14 BOVIGAM assay. Of the peptides tested, only these derived from Mb3645 induced significant
15 levels of IFN- γ in *ex vivo* assays. However, it should be noted that in the final tuberculin
16 product, MPB83 and MPB70 are among the major protein constituents (A. Whelan, pers.
17 comm.). Hence, even though we identified antigen-encoding genes that were expressed at
18 lower levels in AN5, the final protein products are still present in significant quantities. The
19 quantitative gene expression differences seen here would therefore be expected to have
20 marginal effects on the diagnostic potential of tuberculin produced from AN5. This work
21 therefore underpins the use of *M. bovis* AN5 to produce PPD for use in eradication and
22 control programs.

23

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References

- Behr, M.A., Wilson, M.A., Gill, W.P., Salamon, H., Schoolnik, G.K., Rane, S., Small, P.M., 1999, Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 284, 1520-1523.
- Brosch, R., Gordon, S.V., Garnier, T., Eiglmeier, K., Frigui, W., Valenti, P., Dos Santos, S., Duthoy, S., Lacroix, C., Garcia-Pelayo, C., Inwald, J.K., Golby, P., Garcia, J.N., Hewinson, R.G., Behr, M.A., Quail, M.A., Churcher, C., Barrell, B.G., Parkhill, J., Cole, S.T., 2007, Genome plasticity of BCG and impact on vaccine efficacy. *Proc Natl Acad Sci U S A* 104, 5596-5601.
- Calmette, A., Guérin, C., 1909, Sur quelques propriétés du bacille tuberculeux d'origine, cultivé sur la bile de boeuf glycinée. *C.R. Acad. Sci. Paris* 149, 716-718.
- Cockle, P.J., Gordon, S.V., Lalvani, A., Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., 2002, Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infect Immun* 70, 6996-7003.
- Donnelly, C.A., Woodroffe, R., Cox, D.R., Bourne, F.J., Cheeseman, C.L., Clifton-Hadley, R.S., Wei, G., Gettinby, G., Gilks, P., Jenkins, H., Johnston, W.T., Le Fevre, A.M., McInerney, J.P., Morrison, W.I., 2006, Positive and negative effects of widespread badger culling on tuberculosis in cattle. *Nature* 439, 843-846.
- Gilbert, M., Mitchell, A., Bourn, D., Mawdsley, J., Clifton-Hadley, R., Wint, W., 2005, Cattle movements and bovine tuberculosis in Great Britain. *Nature* 435, 491-496.
- Golby, P., Hatch, K.A., Bacon, J., Cooney, R., Riley, P., Allnut, J., Hinds, J., Nunez, J., Marsh, P.D., Hewinson, R.G., Gordon, S.V., 2007, Comparative transcriptomics reveals key gene expression differences between the human and bovine pathogens of the *Mycobacterium tuberculosis* complex. *Microbiology* 153, 3323-3336.
- Griffin, J.M., Williams, D.H., Kelly, G.E., Clegg, T.A., O'Boyle, I., Collins, J.D., More, S.J., 2005, The impact of badger removal on the control of tuberculosis in cattle herds in Ireland. *Preventive veterinary medicine* 67, 237-266.
- Inwald, J., Hinds, J., Palmer, S., Dale, J., Butcher, P.D., Hewinson, R.G., Gordon, S.V., 2003, Genomic analysis of *Mycobacterium tuberculosis* complex strains used for production of purified protein derivative. *J Clin Microbiol* 41, 3929-3932.
- Johnston, W.T., Gettinby, G., Cox, D.R., Donnelly, C.A., Bourne, J., Clifton-Hadley, R., Le Fevre, A.M., McInerney, J.P., Mitchell, A., Morrison, W.I., Woodroffe, R., 2005, Herd-level risk factors associated with tuberculosis breakdowns among cattle herds in England before the 2001 foot-and-mouth disease epidemic. *Biology letters* 1, 53-56.
- Keating, L.A., Wheeler, P.R., Mansoor, H., Inwald, J.K., Dale, J., Hewinson, R.G., Gordon, S.V., 2005, The pyruvate requirement of some members of the *Mycobacterium tuberculosis* complex is due to an inactive pyruvate kinase: implications for in vivo growth. *Mol Microbiol* 56, 163-174.
- Mustafa, A.S., Skeiky, Y.A., Al-Attayah, R., Alderson, M.R., Hewinson, R.G., Vordermeier, H.M., 2006, Immunogenicity of *Mycobacterium tuberculosis* antigens in *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle. *Infect Immun* 74, 4566-4572.
- Paterson, A.B., 1948, The production of bovine tuberculo-protein. *J Comp Pathol Ther* 58, 302-313.
- Pollock, J.M., Douglas, A.J., Mackie, D.P., Neill, S.D., 1994, Identification of bovine T-cell epitopes for three *Mycobacterium bovis* antigens: MPB70, 19,000 MW and MPB57. *Immunology* 82, 9-15.
- Sidders, B., Pirson, C., Hogarth, P.J., Hewinson, R.G., Stoker, N.G., Vordermeier, H.M., Ewer, K. 2008 Screening highly expressed mycobacterial genes identifies Rv3615c as a useful differential diagnostic antigen for the *Mycobacterium tuberculosis* complex. *Infect Immun* June 2, e-pub ahead of print.
- Smith, N.H., Dale, J., Inwald, J., Palmer, S., Gordon, S.V., Hewinson, R.G., Smith, J.M., 2003, The population structure of *Mycobacterium bovis* in Great Britain: clonal expansion. *Proc Natl Acad Sci U S A* 100, 15271-15275.
- Smith, N.H., Gordon, S.V., de la Rúa-Domenech, R., Clifton-Hadley, R.S., Hewinson, R.G., 2006, Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. *Nat Rev Microbiol* 4, 670-681.

Vordermeier, H.M., Cockle, P.C., Whelan, A., Rhodes, S., Palmer, N., Bakker, D., Hewinson, R.G., 1999, Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle. Clin Diagn Lab Immunol 6, 675-682.

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Figure legends:**Figure 1: Gene expression differences for selected genes**

Selected genes were predicted by array analysis to be expressed at lower levels in AN5 compared to the field isolates 2122 and 1121. To validate these predictions qRT-PCR was used to measure gene fold-changes in expression between AN5, 2122, and 1121 grown in 7H9 medium (AN5/2122, AN5/1121), and between AN5 grown in BAI medium and 2122, and 1121 grown in 7H9 medium (AN5-BAI/2122, AN5-BAI/1121). Genes expressed at lower levels in AN5 in 7H9 medium were also lowly expressed in BAI medium, although fold changes varied

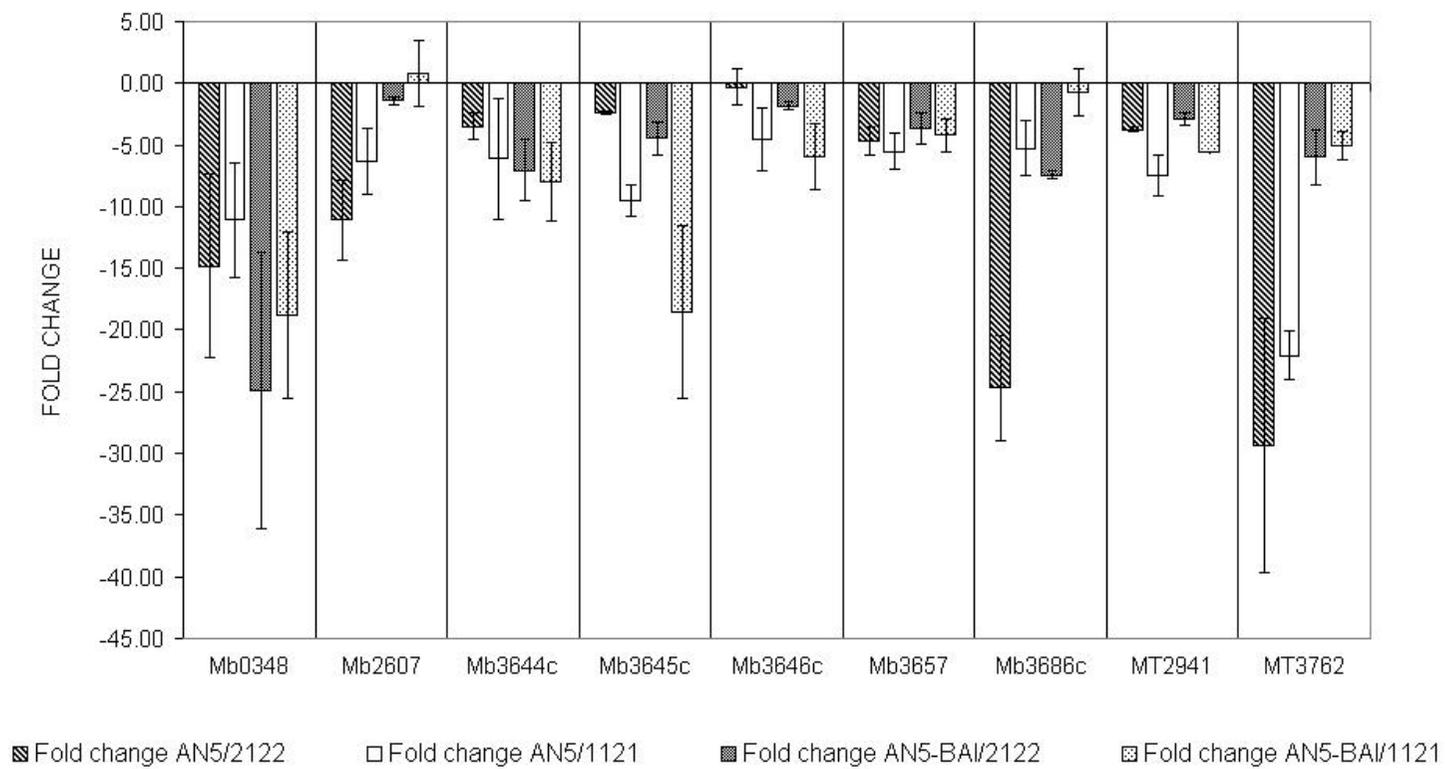


Figure 1

Table 1: Genes with minimum 3-fold reduced expression in *M. bovis* AN5 compared to *M. bovis* field isolates

| Gene | Relative Expression Values | | | Product |
|----------------|----------------------------|-------------------------|-------------------------|--------------------------|
| | <i>M. bovis</i> AN5 | <i>M. bovis</i> 2122/97 | <i>M. bovis</i> 1121/01 | |
| <i>MT3762</i> | 27.89 | 241.6 | 245.6 | Hypothetical Protein |
| <i>mpb70</i> | 16.68 | 50.56 | 41.2 | MPB70 antigen |
| <i>Mb2901</i> | 16.65 | 45.47 | 37.44 | Transmembrane protein |
| <i>Mb3645c</i> | 11.39 | 44.08 | 60.35 | Hypothetical Protein |
| <i>Mb2902c</i> | 9.078 | 26.58 | 20.78 | Membrane protein |
| <i>mpb83</i> | 5.994 | 34.71 | 41.53 | MPB83 antigen |
| <i>Mb3644c</i> | 5.826 | 25.46 | 31.4 | Hypothetical Protein |
| <i>Mb3646c</i> | 5.298 | 15.19 | 19.92 | Hypothetical Protein |
| <i>MT2941</i> | 3.833 | 15.03 | 14.79 | Hypothetical Protein |
| <i>Mb3643c</i> | 3.345 | 8.522 | 8.476 | Hypothetical Protein |
| <i>lpqH</i> | 2.936 | 6.157 | 6.516 | 19 KD Antigen |
| <i>Mb1939</i> | 1.841 | 3.977 | 3.798 | Hypothetical Protein |
| <i>Mb3657</i> | 1.483 | 4.199 | 5.298 | Hypothetical Protein |
| <i>dipZ</i> | 1.383 | 4.247 | 3.823 | Cytochrome biogenesis |
| <i>Mb2325c</i> | 1.063 | 2.146 | 2.65 | Antibiotic resistance |
| <i>moaA3</i> | 1.015 | 3.702 | 3.169 | Molybdpterin synthesis |
| <i>Mb2587</i> | 0.988 | 2.184 | 3.64 | Hypothetical Protein |
| <i>Mb3761c</i> | 0.98 | 2.306 | 2.713 | Hypothetical Protein |
| <i>iniB</i> | 0.911 | 8.162 | 2.734 | Isoniazid inducible gene |
| <i>Mb3356</i> | 0.898 | 3.554 | 4.088 | Hypothetical Protein |
| <i>moaB3</i> | 0.86 | 2.566 | 2.61 | Pterin Dehydratase |
| <i>Mb3686c</i> | 0.804 | 3.97 | 1.796 | Hypothetical Protein |
| <i>moaC3</i> | 0.69 | 1.557 | 1.976 | Molybdpterin synthesis |
| <i>MT1602</i> | 0.674 | 1.596 | 1.558 | Hypothetical Protein |
| <i>Mb2607</i> | 0.638 | 7.492 | 6.43 | Hypothetical Protein |

| | | | | |
|----------------|-------|-------|-------|-----------------------|
| <i>Mb2365c</i> | 0.626 | 1.257 | 1.301 | Hypothetical Protein |
| <i>Mb3760c</i> | 0.61 | 1.227 | 1.22 | Hypothetical Protein |
| <i>Mb3625</i> | 0.474 | 1.042 | 1.402 | Hypothetical Protein |
| <i>lipQ</i> | 0.45 | 1.717 | 2.361 | Carboxylesterase |
| <i>Mb0715</i> | 0.438 | 1.207 | 1.491 | Sugar transferase |
| <i>fadD2</i> | 0.238 | 2.346 | 2.033 | Fatty acid CoA ligase |

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Table 2: Genes with minimum 3-fold increased expression in *M. bovis* AN5 compared to *M. bovis* field isolates

| Gene | Relative Expression Values | | | Product |
|-----------------|----------------------------|-------------------------|-------------------------|-------------------------------|
| | <i>M. bovis</i> AN5 | <i>M. bovis</i> 2122/97 | <i>M. bovis</i> 1121/01 | |
| <i>desA1</i> | 20.80 | 6.80 | 2.93 | Acyl-desaturase |
| <i>bfrB</i> | 20.65 | 9.93 | 4.69 | Bacterioferritin |
| <i>rpmJ</i> | 19.68 | 8.14 | 5.91 | 50S ribosomal protein |
| <i>dnaK</i> | 15.42 | 7.06 | 7.53 | Chaperone |
| <i>rplM</i> | 14.18 | 6.20 | 4.38 | 50S ribosomal protein |
| <i>infA</i> | 13.62 | 6.80 | 5.31 | Translation initiation factor |
| <i>rpsK</i> | 10.09 | 4.84 | 4.53 | 30S ribosomal protein |
| <i>PPE41</i> | 8.60 | 4.09 | 1.78 | PPE family |
| <i>rpsM</i> | 8.51 | 4.01 | 2.99 | 30S ribosomal protein |
| <i>hspX</i> | 7.97 | 0.76 | 0.82 | 14 KDa antigen |
| <i>MT3220.2</i> | 7.95 | 1.65 | 1.64 | PE family |
| <i>rpsF</i> | 7.29 | 2.78 | 1.76 | 30S ribosomal protein |
| <i>fadD26</i> | 6.90 | 3.27 | 1.48 | Fatty Acid CoA Ligase |
| <i>rpfA</i> | 6.82 | 2.15 | 1.49 | Resuscitation factor |
| <i>MT3080.1</i> | 6.69 | 2.84 | 2.15 | Hypothetical Protein |
| <i>rpsB</i> | 6.67 | 3.05 | 2.04 | 30S ribosomal protein |
| <i>sigC</i> | 6.24 | 2.65 | 2.95 | Sigma Factor |
| <i>fdxA</i> | 6.00 | 0.46 | 0.61 | Ferredoxin |
| <i>glpQ1</i> | 5.58 | 2.10 | 1.75 | Phosphodiesterase |
| <i>lprQ</i> | 5.48 | 2.54 | 1.73 | Lipoprotein |
| <i>Rv2271</i> | 5.36 | 2.66 | 2.61 | Hypothetical Protein |
| <i>desA2</i> | 5.14 | 2.40 | 1.39 | Acyl-desaturase |
| <i>echA21</i> | 4.84 | 1.49 | 1.91 | Enoyl-CoA hydratase |
| <i>Mb3013</i> | 4.45 | 1.25 | 0.78 | Transcriptional regulator |

| | | | | |
|----------------|------|------|------|----------------------------------|
| <i>rplJ</i> | 4.41 | 1.87 | 1.14 | 50S ribosomal protein |
| <i>ilvB1</i> | 4.08 | 1.41 | 1.21 | Acetolactate synthase |
| <i>rplL</i> | 3.87 | 1.61 | 1.13 | 50S ribosomal protein |
| <i>ilvN</i> | 3.75 | 1.30 | 0.86 | Acetolactate synthase |
| <i>Mb3554</i> | 3.74 | 1.25 | 1.09 | Membrane protein |
| <i>Mb2659c</i> | 3.62 | 0.50 | 0.51 | Hypothetical Protein |
| <i>leuC</i> | 3.46 | 1.18 | 1.16 | 3-isopropylmalate dehydratase |
| <i>nadA</i> | 3.35 | 1.15 | 1.08 | Quinolinate synthase |
| <i>Mb3913c</i> | 3.33 | 1.31 | 1.47 | Secreted protease |
| <i>Mb3299c</i> | 3.15 | 1.31 | 1.49 | Membrane protein |
| <i>leuD</i> | 3.11 | 1.38 | 1.02 | Isopropylmalate dehydratase |