Functional analysis of a clonal deletion in an epidemic strain of *Mycobacterium bovis* reveals a role in lipid metabolism

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Previous work on the population structure of *Mycobacterium bovis* strains in Great Britain has identified highly successful clones which are expanding across the country. One such clone, designated *M. bovis* type 17, differs from all other members of the *Mycobacterium tuberculosis* complex in having a region of deletion, termed RDbovis(d) _0173, of seven genes between Mb1963c and Mb1971. Three of these genes have functions annotated in lipid metabolism. To explore the molecular basis for the success of this clone, we examined the impact of this deletion on lipid metabolism. While type 17 isolates had similar lipid composition to other *M. bovis* strains, their ability to incorporate propanoate into mycolic acids was remarkably low. When expressed as a reciprocal (the ratio of incorporation of label from acetate : propanoate into mycolic acids) the ratio was higher for all three type 17 field strains tested (mean: 18.90) than the values of 7.30 to 7.61 for other field strains (\(P<0.002\)) and values of 6.50 for all other strains in the *M. tuberculosis* complex tested. The label from propanoate was diverted to pyruvate, at significantly higher levels in *M. bovis* type 17 than all other strains (\(P<0.021\)). Complementation of *M. bovis* type 17 with an integrating cosmid, IE471, carrying the *M. tuberculosis* orthologues of Mb1963c–Mb1971 resulted in the ability of the recombinant strain to incorporate label from propanoate into mycolic acids in a manner similar to other strains. *M. bovis* type 17 : : IE471 labelled pyruvate from propanoate about four times more slowly than the parent strain. Thus, RDbovis(d) _0173 results in a profound effect on carbon metabolism, providing the ability to compensate for the inactivation of the ald and pykA genes, involved in pyruvate metabolism, that is seen in *M. bovis* (but not in *M. tuberculosis*). This shift in carbon metabolism may be a factor in the extraordinary clonal expansion reported for *M. bovis* type 17.

**INTRODUCTION**

Genomic technologies now allow us to scan pathogen genomes in remarkable depth. In the field of human tuberculosis, this has been exemplified by the work of Small and colleagues, who have used gene chip technology to identify unique event polymorphisms across a worldwide distribution of *Mycobacterium tuberculosis* isolates, providing unprecedented insight into the population structure of this pathogen (Gagneux et al., 2006). Furthermore, their work suggest that *M. tuberculosis* strains evolve to adapt to the local host population. The role of gene loss in virulence has been
examined from a functional point of view by Wilkinson and colleagues, who showed that a gene deletion from an epidemic strain of *M. tuberculosis* conferred an altered immunomodulatory phenotype that differed from that of strains where the gene was intact (Henao-Tamayo et al., 2007). Hence, mutational events can clearly alter the phenotype of *M. tuberculosis* isolates in ways that may affect their success as pathogens.

While *M. tuberculosis* causes death and disease in humans, leading to 2 million fatalities a year worldwide, *Mycobacterium bovis* (which is 99.95% similar at the nucleotide level) causes financial devastation, with losses of $3 billion a year to agriculture (Garnier et al., 2003). Molecular typing is used in tracing and surveillance of bovine tuberculosis in cattle and wildlife in the UK, and in trying to understand why the epidemic is increasing year on year – in contrast to continental Europe, where a similar test and slaughter control policy has reduced the incidence in cattle to a negligible level (Smith et al., 2006). The current means of bovine tuberculosis control in Great Britain (GB: England, Scotland and Wales) is the ‘test and slaughter’ strategy, whereby animals giving a positive skin reaction to a crude preparation of mycobacterial antigens are identified as infected and subsequently slaughtered. Cases of bovine tuberculosis are then confirmed by culturing the bacilli from post-mortem material. *M. bovis* isolates are analysed at VLA Weybridge by spacer-oligonucleotide typing (spoligotyping), which is based on a polymorphic region of the genome called the direct repeat (DR) locus that is composed of multiple, identical 36 bp DR repeats interspersed by unique sequences called spacers. Isolates of *M. bovis* differ in the presence or absence of spacers and adjacent DRs, allowing a ‘barcode’ to be generated for each molecular type (Kamerbeek et al., 1997). Spoligotype patterns are named at VLA on the basis of a sequential numbering system; e.g. spoligotype 9 was the ninth pattern to appear, spoligotype 17 the seventeenth, etc. One notable feature of the UK epidemic is that 60% of the affected cattle are infected by just two spoligotypes of *M. bovis*, type 9 and the essentially clonal type 17. Type 17 represents an emerging microepidemic as it has increased significantly compared with all other field strains of *M. bovis* (Smith et al., 2003). Furthermore, we have used Fourier-transform infrared spectroscopy (FT-IR) to measure the vibration characteristics of functional groups across replicate cultures of the major spoligotypes. Using this analysis one can measure quantitative differences in polysaccharides, proteins, lipids, etc., between samples. Strikingly, multivariate statistical analysis clustered the resultant spectra in agreement with the molecular phylogeny; for example, all type 17 strains clustered together (Winder et al., 2006). This global metabolic analysis therefore showed that strains of the same molecular type have distinct phenotypic traits.

Given that type 17 strains are a growing threat to cattle in the UK, and are increasing as a result of natural selection rather than genetic drift (Smith et al., 2003), we were interested in discovering the biochemistry that underlies the selectable phenotype of type 17 *M. bovis*. A major clue is that the phenotype is related to lipid biochemistry, given that type 17 has a small region of deletion, termed RDbovis(d)_0173 (Mostowy et al., 2005). This deletion affects seven genes between *Mb1963c* and *Mb1971* (*M. tuberculosis* H37Rv designation Rv1928c–Rv1936), three of which are annotated as being involved in fatty acid degradation (Table 1). The expected substrates for the three predicted enzymes are fatty acyl-CoA for the FadEs and 2-unsaturated enoyl-CoA for the EchA. However, with 21 echA genes and 36 fadE genes, each encoded enzyme may have a narrow specificity, rather than there being massive redundancy. Yet there is no clue what the substrates of these deleted genes might be, nor is it clear whether their deletion affects catabolism or anabolism. Although they are genes associated with the catabolic, β-oxidation pathway, their deletion could allow the accumulation of fatty acids and polyketides and might even influence remodelling of mycobacterial lipids with changed fatty acyl moieties. Therefore, the global metabolic labelling of lipids was analysed in strains with and without the RDbovis(d)_0173 genes.

### Table 1. RDbovis(d)_0173: genes deleted in *M. bovis* type 17

<table>
<thead>
<tr>
<th><em>M. bovis</em> gene</th>
<th><em>M. tuberculosis</em> orthologue</th>
<th>Predicted product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mb1963c</em></td>
<td>Rv1928c</td>
<td>Probable short-chain type dehydrogenase/reductase</td>
</tr>
<tr>
<td><em>Mb1964c</em></td>
<td>Rv1929c</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td><em>Mb1965c</em></td>
<td>Rv1930c</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td><em>Mb1966c</em></td>
<td>Rv1931c</td>
<td>Probable transcriptional regulatory protein</td>
</tr>
<tr>
<td><em>Mb1967</em></td>
<td><em>tpx</em></td>
<td>Probable thiol peroxidase <em>tpx</em></td>
</tr>
<tr>
<td><em>Mb1968c</em></td>
<td><em>fdeE18</em></td>
<td>Probable acyl-CoA dehydrogenase <em>fdeE18</em></td>
</tr>
<tr>
<td><em>Mb1969c</em></td>
<td><em>fdeE17</em></td>
<td>Probable acyl-CoA dehydrogenase <em>fdeE17</em></td>
</tr>
<tr>
<td><em>Mb1970c</em></td>
<td><em>EchA13</em></td>
<td>Possible enoyl-CoA hydratase <em>EchA13</em> (enoyl hydrase (unsaturated acyl-CoA hydratase) (crotonase)</td>
</tr>
<tr>
<td><em>Mb1971</em></td>
<td>Rv1936</td>
<td>Possible monoxygenase</td>
</tr>
</tbody>
</table>
The outcome of the global metabolic labelling experiments reported in this study was a striking difference between the labelling of mycolic acids with propanoate between type 17 *M. bovis* and every other strain examined. Therefore, we focussed on how propanoate might label mycolates and alternative products. Mycolic acids are present in all actinobacteria, whether they are saprophytes, pathogens, or even those used in biotechnology such as *Corynebacterium glutamicum* and *M. bovis* BCG, which is used for the production of the vaccine against tuberculosis (Goodfellow et al., 1976). Although the biotechnology strains have a much changed metabolism to adapt to the media used for their growth (Guerin, 1980) and for production requirements (Jetten & Sinskey, 1995), the mycolic acids remain essentially unchanged. This is not surprising since most of the mycolic acids are covalently bound, via a branched glycan, to the peptidoglycan (McNeil et al., 1990). As such, they form the inner leaf of an outer permeability membrane, complete with porins (Niederweis, 2003). While mycolates are universal to actinobacteria, loss of some classes of mycolate (Behr et al., 2000), and even more subtle structural differences in their cyclopropane ring structures, can lead to changes in the virulence of mycobacteria (Glickman et al., 2001). In the current work, no differences in structure or abundance of mycolic acid classes were detected between strains. However, it was established that mycolic acids are labelled much less efficiently with propanoate by *M. bovis* type 17 than any other *M. bovis* strain and all strains of *M. tuberculosis* tested. Thus, verification of this finding and investigation of the metabolic consequences of this differential efficiency of labelling with propanoate were the major areas we investigated after obtaining the global data.

**METHODS**

**Strains and their culture.** An objective before starting metabolic labelling experiments was to be able to grow all strains on the same medium, using the same carbon source, at the same rate. All the strains in Table 2 were grown in Sauton medium with pyruvate replacing glycerol as sole carbon source. The composition was: l-asparagine, 4 g l\(^{-1}\); citric acid, 2 g l\(^{-1}\); K\(_2\)HPO\(_4\), 0.5 g l\(^{-1}\); MgSO\(_4\).7H\(_2\)O, 0.5 g l\(^{-1}\); ammonium ferric citrate, 0.05 g l\(^{-1}\); sodium pyruvate, 4.16 g l\(^{-1}\). The pyruvate had to be added to get any growth at all. Deionized water was used dissolve the medium constituents and the pH was adjusted with KOH to pH 7.2. In experiments with alternative sole carbon sources, pyruvate was omitted and either sodium propanoate at 2 g l\(^{-1}\), or Tween 80 at 2 g l\(^{-1}\) with BSA at 5 g l\(^{-1}\) was included. For growth of recombinant strains that contained

<table>
<thead>
<tr>
<th>Cosmid or strain</th>
<th>SB no.*</th>
<th>Description</th>
<th>RDbovis (d)_0173</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cosmids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pYUB412</td>
<td></td>
<td>Integrating shuttle cosmid, carries HysR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IE471</td>
<td></td>
<td>pYUB412-based integrating cosmid containing a <em>M. tuberculosis</em> Erdman insert corresponding to genes Rv1918–Rv1963</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td></td>
<td>Type strain of the <em>M. tuberculosis</em> complex</td>
<td>+</td>
<td>VLA Weybridge stock</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Tb12</td>
<td></td>
<td>Bangladeshi clinical isolate</td>
<td>+</td>
<td>Banu et al. (2004)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Tb30</td>
<td></td>
<td>Bangladeshi clinical isolate</td>
<td>+</td>
<td>Banu et al. (2004)</td>
</tr>
<tr>
<td><em>M. canetti</em></td>
<td></td>
<td>Reference strain140010059</td>
<td>+</td>
<td>Institut Pasteur stock; Gutierrez et al. (2005)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 9</td>
<td>SB0140</td>
<td>Sequenced strain (Garnier et al., 2003)</td>
<td>+</td>
<td>VLA Weybridge stock, AF2122/97</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17</td>
<td>SB0263</td>
<td>Field isolate with different spoligotype from sequenced strain and with the deletion RDbovis(d)_0173</td>
<td>–</td>
<td>VLA Weybridge stock, 61/1121/01</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17</td>
<td>SB0263</td>
<td>–</td>
<td>VLA Weybridge stock, 61/1121/01</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> type 17</td>
<td>SB0263</td>
<td>–</td>
<td>VLA Weybridge stock, 61/3139/06</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 :: IE471</td>
<td></td>
<td>Type 17 strain transformed with cosmid IE471 (described above)</td>
<td>+</td>
<td>This paper</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 :: pYUB412</td>
<td></td>
<td>Type 17 strain transformed with empty cosmid control</td>
<td>–</td>
<td>This paper</td>
</tr>
<tr>
<td><em>M. bovis</em> type 35</td>
<td>SB0134</td>
<td>Field isolate with different spoligotype from sequenced strain</td>
<td>+</td>
<td>VLA Weybridge stock, 61/1307/01</td>
</tr>
</tbody>
</table>

*SB, Spoligotype Bovis.*

† +, Region present; –, region not present.

‡ The data obtained with *M. tuberculosis* Canetti ("M. canetti") are presented only in the supplementary tables.
the empty pYUB412 vector or the pYUB412-based cosmid IE471, hygromycin was added to the medium up to 100 μg ml⁻¹ as required. Cultures (100 ml) in 2 l capacity Greiner Bio-One bottles were grown at 37 °C and were aerated by rolling at 2 r.p.m. Growth rates, using pyruvate or Tween 80, typically of 0.033 h⁻¹ (range with pyruvate: 0.029 h⁻¹ to 0.040 h⁻¹, range with Tween 80: 0.030 h⁻¹ to 0.035 h⁻¹) were obtained with all the strains listed in Table 2 except M. bovis type 35, which grew at 0.023 h⁻¹ to 0.026 h⁻¹ with either carbon source. The variation was between groups of batches; within each group the growth rates were very similar, except for that of M. bovis type 35, which was notably slower. Thus the objective was largely achieved across a wide range of strains. Regardless of the medium that was used, M. bovis type 35 always grew more slowly than the other strains in Table 2. Both growth rates (<0.022 h⁻¹) and yields were much lower (<10 mg dry weight (100 ml culture)⁻¹) for all strains when propanoate was the sole carbon source.

The decision not to use detergent in the medium so that culture filtrate lipids could be analysed meant that the cultures were of a granular appearance and could not be monitored by their OD₆₀₀ values. However, samples taken at two or three intervals during growth, heat-killed, and dried revealed that their weights were similar to those in a duplicate culture including 0.025 % (w/v) of the detergent tyloxapol. Therefore, growth was monitored in the duplicate, parallel detergent-containing cultures. The tyloxapol did not support any growth. Yields of 36 mg (dry wt) per 100 ml culture could be obtained readily, except for M. bovis type 35, which gave 25 mg (dry wt) per 100 ml. However, such cultures were entering lag phase and all metabolic labelling was done at mid-exponential phase, when the OD₆₀₀ in parallel, detergent-containing cultures was 0.4 to 0.6. These OD₆₀₀ values equated to 12 to 18 mg (dry wt) per 100 ml for every strain in Table 2.

For construction of recombinant M. bovis type 17 knock-ins, the IE471 cosmid was selected from a M. tuberculosis Erdman library of pYUB412 integrating shuttle cosmids (Bange et al., 1999; Glickman et al., 2001) by hybridization with PCR-amplified probes from the Mb1963c–Mb1971 region and sequencing of the insert termini following a previously published strategy (Brosch et al., 1998). Highly concentrated cosmid DNA from clone IE471 containing a 42 kb insert of M. tuberculosis Erdman DNA that corresponds to genes Rv1918–Rv1963 was then electroporated into M. bovis type 17 electrocompetent cells, where it stably inserted into the attB site situated in the glyV-tRNA gene. The same procedure was used for the pYUB412 vector control. Hygromycin-resistant transformants appearing after 4 weeks on Middlebrook 7H11 medium (Difco) supplemented with oleic acid-albumin-dextrose-catalase (OADC, Difco) and 100 μg hygromycin ml⁻¹ were analysed for the presence of genes orthologous to Mb1963c–Mb1971 by specific amplification and sequencing. The resulting M. bovis type 17::IE471 and the M. bovis type 17::pYUB412 vector control strains were used for further biochemical analyses.

**Labelling with acetate or propanoate.** Cultures growing at the same rate (as near as possible), during exponential phase, were labelled with [1-¹⁴C]acetate or [1-¹⁴C]propanoate, to minimize any differences in metabolism due to growth rate or phase. The density of bacteria, 12 to 18 mg (dry wt) per 100 ml, was chosen to maximize the yield of exponential-phase bacteria. All labelling was done for 2 h with 50 μCi (1850 kBq) sodium salt, 50 to 60 Ci mol⁻¹, per 100 ml medium, adding the labelled compound directly to cultures and incubating in growth medium and conditions. Radioisotopically labelled substrates were from American Radiolabelled Chemicals (ARC 101A for acetate; ARC 203A for propanoate). The main conclusions of this work were drawn from labelling followed by analysis—‘pulse-only’ experiments. As part of our global analysis of lipid labelling, some pulse-chase experiments were done. For these, a 10 ml sample was analysed at the end of the 2 h pulse phase and the remainder of the suspension was washed again in medium and resuspended in 90 ml medium free of labelled compounds for a chase phase. After 4 h and 20 h chase phase, 45 ml samples were taken.

**Lipid analysis.** After 2 h, the bacteria were harvested by centrifugation and washed once in MilliQ water. When pulse-chase experiments were done, additionally, the samples after the 4 h and 20 h chase were also harvested by centrifugation and washed once in MilliQ water. In most of the experiments, superficial lipids were extracted with 0.05 % decylamine in hexane (Converse et al., 2003), the unbound lipids were extracted with successive mixtures of hexane and chloroform/methanol/water mixtures to give ‘non-polar’ and polar lipid fractions (Besra, 1998; Dobson et al., 1985), and the wall-bound mycolic acids were released from the remaining residue with 15 % (w/v) tetrabutylammonium hydroxide (TBAH) at 100 °C for 18 h. The TBAH salts thus produced were then methylated and analysed by TLC (Fig. 1c) (Wheeler, 2008).

Most of the label in the mycolates was in the wall-bound fraction. However, during the global analysis of metabolic labelling of lipids that was conducted in this study, minor amounts of mycolate were detected in the unbound lipids (see Supplementary Tables S1 and S2, available with the online version of this paper). Unbound lipids were analysed by successive 2D TLC (Besra, 1998; Dobson et al., 1985). The unbound mycolate-containing lipids are shown in Fig. 1(a, b) with the solvent systems used to develop them.

The amount of radioactivity in wall-bound mycolates was determined by sampling 10 % of the volume of the methyl esters dissolved in hexane and scintillation counting in Scintran NA (VWR). Samples containing 30 000 to 100 000 d.p.m. were applied to the concentrating zone of Silica Gel 60 TLC plates (Merck 13748) and the plates developed three times in hexane/ethyl acetate (95/5, v/v). The amount of radioactivity in each band on TLC plates (Fig. 1c) was quantified using a phosphorimager or by scraping off individual radioactive spots. These two methods gave values that agreed. The total amount in the wall-bound mycolates was then calculated by working out the proportion of radioactivity in the bands corresponding to mycolates (Fig. 1c) and multiplying by the value for all of the material dissolved in hexane.

A step-by-step protocol for the labelling and global lipid analysis performed in this work is available (Wheeler, 2008; Wheeler & Anderson, 1996).

**Lipid and pyruvate analysis after quenching metabolism: experiments 1150 and 1160.** In order to analyse the pyruvate formed from labelled substrates, metabolism had to be rapidly quenched. This was achieved by pipetting 20 ml samples of cultures, after 2 h labelling, into 20 ml methanol at –80 °C. The mixture was kept on dry ice in centrifuge buckets pre-cooled to –80 °C and transferred quickly into a pre-cooled Sigma 3K10 centrifuge set at –20 °C and centrifuged for 8 min at 4800 g. The pellet was recovered by removing all the supernatant while the buckets were kept on dry ice and stored at –80 °C. Labelled keto acids were extracted from the pellet by adding 1 ml methanol at –80 °C, freezing by plunging into liquid nitrogen and thawing on dry ice three times, collecting the pellet and supernatant by centrifuging in the pre-cooled centrifuge, repeating the extraction using 0.5 ml, and pelleting and combining the two supernatants. The supernatants were freeze-dried at –80 °C, then 330 μl 25 mM 1,2-diamino-4,5-dimethoxybenzene (DDB) in 1 M HCl was added directly to the dried material. DDB derivatives of the keto acids were recovered and their radioactivity analysed as described previously (Wheeler et al., 2005). The pellets were washed twice at 25 °C (room temperature: not critical) with 2 ml chloroform/methanol (2:1, v/v); their wall-bound mycolates were prepared as methyl esters (MAMes), and their radioactivity was quantified and analysed as described above.
Statistics and expression of data. Values of d.p.m. per mg dry weight of bacteria were calculated from the d.p.m. and dry weight data for each sample; numbers of determinations are shown in the tables. Examples of d.p.m. mg\(^{-1}\) values are given in Fig. 2(b); all values are in the supplementary tables. The ratio of d.p.m. per mg dry weight of bacteria in mycolates labelled with acetate : propanoate were calculated for each pair of samples done in parallel, i.e. in the same experiment, and their mean values ±SEM are presented. Fisher’s exact t-test was conducted on the ratio values to test for statistical differences of the ratios. The percentage of label in each lipid was calculated (i) relative to the d.p.m. mg\(^{-1}\) values after the pulse phase, with no chase, and (ii) relative to the label in each sample. Linked spreadsheets with each of these sets of percentage values are given in Supplementary Tables S1 and S2. Percentage values thus calculated were used in the Results section, with SEM values calculated on the percentage values.

RESULTS

Global metabolic labelling of lipids

Mycobacterial lipids were labelled and the distribution of label followed in pulse–chase experiments for all lipids. The full data for labelling of unbound, non-polar lipids are included in Supplementary Table S1. Those data failed to show any differences in the unbound lipids that could be
Distribution of the labelled mycolates

Most of the metabolic labelling of mycolates by acetate was into wall-bound mycolates (mean of 79% ± 1.3%, SEM in all 38 determinations across all strains and time points) with nearly all the remaining 21% (range: 4% to 37%) going into trehalose mycolates. Usually, only a trace was detected in triacylglycerols (TAGs) as di-non-hydroxyacyl, monomycolyl glycerol (DAGMM: mean of 1.85% ± 0.44%, SEM in all 14 determinations across all strains and time points).

In pulse–chase experiments, label was chased out of TMMs (Fig. 2b) while label was chased into TDMs (Fig. 2a). Label was also especially rapidly chased out of TAGs (Fig. 2a), though very little of this label is in mycolyl glycerol (DAGMM: mean of 1.85% ± 0.44%, SEM). The values for propanoate were calculated from values obtained with all the M. bovis spoligotypes used in this study as their incorporation of acetate into each of TMM, TDM and TAG was similar. All data from pulse–chase experiments are shown. In (a) wall-bound mycolates are represented in calculating mean values for acetate incorporation at each time point as there were no consistent differences in this activity when the cosmid was present. In (a), unbound lipids are shown: TMM (▼, ▽), TDM (▲, △), TAG (●, ◆). TAG is included in this graph as it represents the lipid from which most label was chased out over the 20 h chase. All values are percentage of label in each sample ± SEM.

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However, relative to the labelling with acetate, labelling with propanoate was significantly lower in *M. bovis* type 17. The relative efficiency of labelling is expressed as the ratio of labelling with acetate : propanoate (Table 3) so the relative efficiency of labelling with propanoate is the reciprocal of the values in Table 3. For the reasons stated above, wall-bound mycolates were used to calculate this ratio. In the wall-bound mycolic acids, the proportion of label in the three classes of mycolic acid was the same regardless of either the strain or whether the labelling was with acetate or propanoate (Fig. 1c). The values were 43–46 % of the label in \( \alpha \)-mycolates, 36–37 % in methoxymycolates and 17–20 % in ketomycolates. Even when the caveat about assigning d.p.m. mg\(^{-1}\) values to propanoate-labelled TMM was ignored and the ratio of propanoate : \( \alpha \)-mycolates was calculated for all mycolates, i.e. including the trehalose mycolates and DAGMMs, the ratio was higher in *M. bovis* type 17 strains, showing their less efficient labelling of total mycolates with propanoate (these ratio values are in Supplementary Table S2).

Especially compelling was the difference in the labelling of wall-bound mycolates between all type 17 field strains and the other strains of *M. bovis* when the data for type 9 and type 35 strains were combined (Table 3). These three spoligotypes are genetically extremely similar apart from type 35 strains were combined (Table 3). These three spoligotypes are genetically extremely similar apart from type 17 strains, showing their less efficient labelling of total mycolates with propanoate (these ratio values are in Supplementary Table S2).

To determine whether this effect was related to the function of genes *Mb1963c–Mb1971*, missing from type 17 *M. bovis* strains due to the RD*bovis(d)_0173* deletion, recombinant *M. bovis* type 17 variants were constructed that carried an integrating shuttle cosmid (IE471) containing the *M. tuberculosis* orthologues of *Mb1963c–Mb1971* and large portions of their flanking regions. The IE471 clone was selected from a *M. tuberculosis* Erdman pYUB412 cosmid library (Bange *et al.*, 1999; Glickman *et al.*, 2001) by hybridization and sequencing of the insert termini following a previously published strategy (Brosch *et al.*, 1998).

### Table 3. Acetate : propanoate labelling ratios of wall-bound mycolic acids

<table>
<thead>
<tr>
<th>Strain</th>
<th>All experiments(^a)</th>
<th>Quenched experiments only(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>6.50 ± 0.66 (14)</td>
<td>7.70 ± 0.58 (3)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Tb12</td>
<td>5.37 ± 0.98 (5)</td>
<td>7.14 ± 0.44 (2)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Tb30</td>
<td>2.88 ± 0.14 (3)</td>
<td>Not done(^c)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 9 AF2122/97</td>
<td>7.61 ± 0.76 (8)</td>
<td>7.95 ± 0.93 (6)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 61/1121/01</td>
<td>20.34 ± 3.77 (8)</td>
<td>19.68 ± 1.90 (3)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 61/3139/06</td>
<td>22.56 ± 1.54 (3)</td>
<td>22.56 ± 1.54 (3)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 21/7917/05</td>
<td>11.39 ± 1.44 (3)</td>
<td>11.39 ± 1.44 (3)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17::IE471</td>
<td>9.61 ± 1.36 (5)</td>
<td>7.43 ± 0.40 (3)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17::pYUB412</td>
<td>20.48 ± 2.99 (5)</td>
<td>18.53 ± 4.29 (3)</td>
</tr>
<tr>
<td><em>M. bovis</em> type 35 61/1307/01</td>
<td>7.30 ± 1.84 (5)</td>
<td>Not done</td>
</tr>
</tbody>
</table>

\(^a\)All time points were included in calculating these ratios as d.p.m. (mg bacteria\(^{-1}\)), as ratio values did not show any pattern of increase or decrease with time. The mean of the values ± SEM (number of ratios calculated from data) is shown.

\(^b\)Experiments in which labelling of intracellular pyruvate and wall-bound mycolates were determined in the same samples of bacteria; only a pulse phase was done in these experiments, followed by rapid quenching of metabolism to preserve intracellular metabolites. These are referred to as experiments 1150 and 1160 in Supplementary Table S2.

\(^c\)Ratio not significantly different between Tb12 and Tb30 in experiments where both strains were labelled (see Supplementary Table S2).
17::pYUB412 ($P=0.041$). The general pattern of restoration of more efficient propanoate labelling of mycolates was evident from the ratios obtained in every experiment in which the two constructs were tested (‘all experiments’ in Table 3), and also in experiments in which intracellular metabolites as well as wall-bound mycolates could be determined in the same sample (‘Quenched experiments only’ in Table 3). As propanoate is expected to be converted to either acetyl-CoA, which would label mycolates, or to pyruvate, as suggested by examination of likely metabolic pathways, the labelling of pyruvate was also determined in the quenched experiments.

**Propanoate is incorporated more efficiently into pyruvate in *M. bovis* type 17**

The metabolic labelling of pyruvate with propanoate was around four times higher in *M. bovis* type 17 than in *M. bovis* type 17::IE471 (Table 4). Labelling of pyruvate was very similar ($P=0.38$) in the type 17 field strain and type 17::pYUB412 (with the empty vector introduced), giving the option of combining these data as the ‘Type 17 aggregate’ for significance testing (Table 4). The *M. bovis* type 17 aggregate incorporated label from propanoate into pyruvate significantly more efficiently ($P$ values are in Table 4) than *M. bovis* type 17::IE471, the other *M. bovis* strains tested, and *M. tuberculosis*. When metabolic labelling of pyruvate with propanoate was compared with two other field strains of type 17, the labelling was indistinguishable between the type 17 strains but significantly different from the labelling in type 9 (Table 4). These data may explain the inefficient incorporation of label from propanoate into mycolate by *M. bovis* type 17 as a result of its diversion to pyruvate (Fig. 3), an effect that is reversed when the genes deleted in RDbovis(d)_0173 are restored. Note that d.p.m. mg$^{-1}$ values are given in this section because label from acetate was not incorporated into pyruvate and therefore no ratio could be calculated.

Given that label from propanoate was incorporated into pyruvate as an alternative to acetyl-CoA, it would have appeared to be preferable to grow the bacteria with an alternative carbon source to pyruvate. However, the only alternative carbon sources that all the strains in Table 2 can be grown on is a lipid, or propanoate itself. All the strains grew on Tween 80, a source of oleate, at similar rates to their growth on pyruvate. However, when they were pulsed with either labelled acetate or propanoate, incorporation of label into lipids was three to six times lower than when they were grown, in parallel, on pyruvate. As an example, data of all incorporation values obtained in this work are shown in Fig. 2(b) for all *M. tuberculosis* constructs and all the *M. bovis* type 17 constructs. Fatty acids such as oleate are good feedback inhibitors of *de novo* fatty acid biosynthesis (Bloch, 1977) and this effect was previously demonstrated when it was shown that lipids in culture media depressed acetate incorporation into lipids by up to 10-fold (Wheeler & Ratledge, 1988). Propanoate as a sole carbon source (in Sauton medium) supported the growth of all the strains in Table 2, although on subculturing from Sauton medium with pyruvate as sole carbon source, or Middlebrook 7H9 with ADC, Tween 80 and pyruvate, lag phases of 3 to 6 weeks occurred. On subculturing from Sauton with propanoate, lag phases were always less than 2 weeks but the maximum growth yield was 10 mg (dry wt) per 100 ml culture medium, and growth rates varied between the strains (fastest $\sim0.022$ h$^{-1}$). Together with the problem that labelled propanoate would have been diluted out with unlabelled propanoate, at $2$ g l$^{-1}$, to support growth, propanoate too proved to be unsuitable as a carbon source in these labelling experiments.

**Table 4. Incorporation of label from propanoate into pyruvate**

All values (means ± SEM) were from three determinations, except for *M. bovis* type 9 AF2122/97 (six) and the *M. bovis* type 17 aggregate results, which were calculated from data from the type 17 field strains and *M. bovis* type 17::pYUB412 combined. These sets of data for the different type 17 strains were not significantly different (for pairwise comparisons: $P>0.38$).

<table>
<thead>
<tr>
<th>Strain</th>
<th>D.p.m. (mg bacteria)$^{-1}$</th>
<th>$P$ vs <em>M. bovis</em> type 17 aggregate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>1983±258</td>
<td>0.021</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Tb12</td>
<td>1216±313</td>
<td>0.010</td>
</tr>
<tr>
<td><em>M. bovis</em> type 9 AF2122/97</td>
<td>2368±248</td>
<td>0.010</td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 61/1121/01</td>
<td>3522±471</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 61/3139/06</td>
<td>3454±548</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 21/7917/05</td>
<td>3575±45</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> type 17::IE471</td>
<td>835±146</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> type 17::pYUB412</td>
<td>2887±170*</td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> type 17 aggregate</td>
<td>3516±237</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from value for *M. bovis* type 17::IE471 ($P=0.002$).
Fig. 3. Metabolic fate of propanoate. Pathways leading to acetyl-CoA and methylmalonyl-CoA, needed for lipid biosynthesis, are in black. The methylcitrate cycle, for generating pyruvate, is shown in green. An alternative possible route for generating pyruvate is shown in blue, with shared reactions in turquoise: acetyl-CoA could be generated through either of these two routes, too, by decarboxylation of pyruvate. Acc, acetyl-CoA carboxylase; Max, mycocerosic acid synthase; Mcl, methylisocitrate lyase; Mcs, mycolic acid synthase; Mez, malic enzyme.

DISCUSSION

Bovine tuberculosis is one of the most difficult animal health problems that the British farming industry faces. The number of cattle infected with bovine tuberculosis has been increasing year on year by 18%, which leads to serious losses for affected farms due to the slaughter of infected animals and the imposition of cattle movement restrictions. One possibility is that new forms of M. bovis have evolved in GB that are able to circumvent the current restrictions. One possibility is provided by the presence of a range of different types of M. bovis circulating in GB that seem to be successful in spreading around the country from their original place of isolation. The hallmark of this clonal expansion is provided by the M. bovis type 17 clone, which accounts for more than 30% of all clinical isolates of M. bovis circulating in GB that seem to be successful in spreading around the country from their original place of isolation. The hallmark of this clonal expansion is provided by the M. bovis type 17 clone, which accounts for more than 30% of all clinical isolates of M. bovis. By determining the molecular basis of the success of this strain we hope to understand the selective forces that drive the emergence of new strains. As mycobacterial lipids have been shown to be key immunomodulators (Reed et al., 2004; Sinsimer et al., 2008), we were intrigued by the clonal deletion of genes involved in lipid metabolism from type 17 strains, and sought to determine the effect of this deletion on surface lipid profiles of type 17.

Mycobacterial lipids can be readily labelled during growth of the bacteria with radioactive acetate or propanoate. Usually one carbon source gives optimal labelling. When polyketide synthases are involved in the biosynthesis of a lipid, with the incorporation of methyl branches in its fatty acyl moieties, propanoate is the best labelled carbon source (Minnikin et al., 2002; see also Supplementary Table S1). However, when methyl branches are not present in the fatty acids, or if present are not derived from methylmalonyl-CoA, label from propanoate cannot be directly incorporated into the lipid, as in the case of mycolic acids. Mycolic acids are 1-alkyl 2-hydroxy fatty acids. Their biosynthetic pathways have been worked out: their acyl chains are built up solely from acetyl-CoA (Marrakchi et al., 2008) and any methyl groups are derived from S-adenosylmethionine using Mmas, Cmas and related methyltransferases (Marrakchi et al., 2008). Nevertheless, incorporation of label from propanoate into mycolates was observed in this study. Some strains were quite efficient at labelling with propanoate but M. bovis type 17 was remarkably inefficient and we wondered if this might be related to its loss of some fadE and echA genes (Table 1). Therefore, we analysed the relative incorporation of label from acetate and propanoate into mycolates further. Given that metabolic fingerprints of M. bovis cluster with molecular type (Winder et al., 2006) but the actual metabolites were not identified, we conducted some identification of metabolites labelled in this study. We targeted keto acids as they include pyruvate – a key metabolite – and tricarboxylic acid cycle intermediates, and are likely to be derived from propanoate (Fig. 3).

Our data can be rationalized from consideration of metabolic pathways and our knowledge, outlined above, of mycolate biosynthesis. Propanoate must be metabolized first to acetyl-CoA to initiate mycolate biosynthesis and for malonyl-CoA to be incorporated into the growing acyl chains of mycolates (Fig. 3). It is just possible that the carbon from propanoate is incorporated only into the methyl groups of mycolic acids, or via some other route than via acetyl-CoA, although any pathways or metabolic nodes would be highly tenuous. However, experimental support for propanoate being converted to acetyl-CoA was obtained in this work from the observation that it labelled the three classes of mycolates (with different degrees of methylation required to form each of them) in the same proportion as when acetate was used as the label (Fig. 1c). The ability to convert propanoate to acetyl-CoA and malonyl-CoA is indeed a prerequisite for growth on propanoate as a single carbon source, a growth property demonstrated both here and by McKinney’s group (Munoz-Elias et al., 2006; Savvi et al., 2008). It is probably also needed to remove excess propanoate generated by catabolism of odd-chain fatty acids from metabolic pools, as this acid is toxic (Savvi et al., 2008). The likely pathways are shown in Fig. 3; the route to pyruvate is the methylcitrate cycle as outlined previously (Munoz-Elias et al., 2006) and acetyl-CoA can be generated from the pyruvate. Alternatively, to generate acetyl-CoA or malonyl-CoA, a new pathway via malonate semialdehyde is proposed (Fig. 3). The overall reactions for both pathways are:

Propanoate + ATP + CoA + 2 NAD(P)⁺ + FAD = Malonyl-CoA + AMP + 2 P + + 2 NAD(P)H + FADH₂ + 2 H⁺

Acetyl-CoA is generated by the same pathway initially with
the same reduction and phosphorylation cost of coenzymes, with the additional generation of CO₂. Both pathways are feasible as they are less costly in energy than the generation of methylmalonyl-CoA, which has been demonstrated directly (Rainwater & Kolattukudy, 1982):

Propanoate + 2 ATP + CoA + CO₂ = Methylmalonyl-CoA + AMP + ADP + 3 P₃

The closeness of the relationship of the *M. bovis* strains suggested that it might be the major genetic difference between them, the loss of genes *Mb1963c-Mb1971* (Table 1), that gives rise to this phenotype, especially as three of the genes were predicted to be involved in lipid catabolism (Table 1). This was confirmed by adding back this genetic region to *M. bovis* type 17 and restoring the incorporation of label from propanoate to levels comparable with all the other strains surveyed that had the *Mb1963c-Mb1971* genes or their orthologues. If, in *M. bovis* type 17, very little propanoate was channelled to acetyl-CoA, a feasible alternative metabolic fate would be to pyruvate (Fig. 3).

As well as being a feasible pathway that is not contrived, this was an attractive scenario because all *M. bovis* field strains lack alanine dehydrogenase (Chen *et al.*, 2003) and pyruvate kinase (Keating *et al.*, 2005), with their respective *ald* and *pykA* genes being inactive (Garnier *et al.*, 2003). In these strains, pyruvate would have to be acquired from an exogenous source or by anaerobic reactions using the acetyl-CoA generated from β-oxidation of fatty acids via the glyoxylate cycle and malic enzyme (Mez). Therefore a genetic change that led to further compensation of the lesions in pyruvate production might be selectable in new conditions for *M. bovis*. When it was tested whether the loss of the *Mb1963c-Mb1971* genes or their orthologues led to the production of pyruvate, *M. bovis* type 17 and type 17::pYUB412 (the empty vector control) produced significantly more labelled pyruvate from labelled propanoate than the isogenic type 17::IE471 with the genes added back in, or any of the non-isogenic strains with the *Mb1963c-Mb1971* genes or their orthologous genes. The pathways in Fig. 3 show the methylcitrate cycle for generating pyruvate, a cycle that uses bifunctional isocitrate lyases for the Mcl (methylisocitrate lyase) step and that is needed for *M. tuberculosis* to grow on propanoate (Gould *et al.*, 2006; Munoz-Elias *et al.*, 2006). A feasible alternative that could possibly be used if methylmalonyl-CoA (Savvi *et al.*, 2008) is produced in excess is shown in blue in Fig. 3. This would also allow malate, a product of the glyoxylate cycle, to be used for pyruvate production. Both have the same overall reaction:

Propanoate + XTP + FAD + NAD⁺ = Pyruvate + XMP + 2 P₃ + FADH₂ + NADH + H⁺

Although the complementation experiment confirmed the role of the genes *Mb1963c-Mb1971* in propanoate metabolism, this work does not define the mechanism of their action. It is unlikely that any of these genes encode enzymes for the steps in the pathways in Fig. 3, although the genes for the two enzymes for generating, then oxidizing, malonate semialdehyde have not been annotated in the *M. tuberculosis* complex genomes and *Mb1964c* is a probable dehydrogenase (Table 2). However, the FadE and EchA enzymes might generate fatty acyl-CoAs or their derivatives that could affect the pathways as these are powerful regulatory compounds at both transcriptional and allosteric levels (Bloch, 1977; Flick & Bloch, 1975; Iram & Cronan, 2005; van Aalten *et al.*, 2001). Further, *Mb1966c* is predicted to be a transcriptional regulatory protein, and may play a role in regulating the pathways in Fig. 3.

Previous studies have linked subtle changes in mycolic acid structure to virulence. However, in this study differential labelling of mycolic acids with propanoate across strains of *M. bovis* and the *M. tuberculosis* complex revealed a difference in the central pathways of carbon metabolism that was related to the loss of RDbovis(d)_0173. This region, lost in *M. bovis* type 17, includes lipid catabolism genes and a possible transcriptional regulator. We suggest that the loss of the RDbovis(d)_0173 leads to very little propanoate being channelled into acetyl-CoA (for mycolate biosynthesis) but more being channelled into pyruvate, using the central pathways illustrated in Fig. 3. Given the need for *M. bovis* to compensate for natural mutations in *ald* and *pykA* to replenish its intracellular pool of pyruvate, this additional compensatory mechanism may confer an advantage to *M. bovis* type 17 and may contribute to its extraordinary expansion in a microepidemic of bovine tuberculosis.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Altered metabolism in an epidemic strain of bovine TB


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