Microbiology (2004), **150**, 3821–3829 DOI 10.1099/mic.0.27284-0

Comparative proteome analysis of *Mycobacterium tuberculosis* grown under aerobic and anaerobic conditions

Joakim Starck,[1] Gunilla Källenius,[1,2] Britt-Inger Marklund,[3] Dan I. Andersson[1,2] and Thomas Åkerlund[1]

Correspondence
Thomas Åkerlund
Thomas.Akerlund@smi.ki.se

[1]Department of Bacteriology, Swedish Institute for Infectious Disease Control, S-17182, Solna, Sweden

[2]Microbiology and Tumour Biology Centre, Karolinska Institute, S-17177, Stockholm, Sweden

[3]Department of Chemistry and Biomedical Sciences, University of Kalmar, SE-39182, Kalmar, Sweden

Data are presented from two-dimensional (2-D) PAGE analysis of *Mycobacterium tuberculosis* strain Harlingen grown during aerobic and anaerobic culture, according to a modified Wayne dormancy model. *M. tuberculosis* cultures were grown to the transition point between exponential growth and stationary phase in the presence of oxygen (7 days) and then part of the cultures was shifted to anaerobic conditions for 16 days. Growth declined similarly during aerobic and anaerobic conditions, whereas the ATP consumption rapidly decreased in the anaerobic cultures. 2-D PAGE revealed 50 protein spots that were either unique to, or more abundant during, anaerobic conditions and 16 of these were identified by MALDI-TOF. These proteins were the α-crystalline homologue (HspX), elongation factor Tu (Tuf), GroEL2, succinyl-CoA : 3-oxoacid-CoA transferase (ScoB), mycolic acid synthase (CmaA2), thioredoxin (TrxB2), β-ketoacyl-ACP synthase (KasB), L-alanine dehydrogenase (Ald), Rv2005c, Rv2629, Rv0560c, Rv2185c and Rv3866. Some protein spots were found to be proteolytic fragments, e.g. HspX and GroEL2. These data suggest that *M. tuberculosis* induces expression of about 1 % of its genes in response to dormancy.

Received 28 April 2004
Revised 16 July 2004
Accepted 17 July 2004

INTRODUCTION

Human tuberculosis (TB) is caused by an intracellular pathogen, *Mycobacterium tuberculosis*, which causes both latent and acute illness. Approximately eight million active cases are reported annually with 2 million deaths. In addition, about one-third of the global population is estimated to suffer from latent tuberculosis (Dye *et al.*, 1999), which can be reactivated even after several decades. The fact that the bacilli can shift into a dormant state is of therapeutic importance, since this dormant state induces resistance to the two most important TB-drugs, rifampicin and isoniazide (Wayne & Sramek, 1994). One of the most challenging problems in TB research is to reveal the mechanisms behind latency.

Latency is believed to involve a non-replicating persistence of mycobacteria or a very slow growth. One important condition believed to contribute to latency is reduced access to oxygen. *M. tuberculosis* is generally regarded as a strictly aerobic bacillus, although it can survive for a long time in a micro-aerophilic environment as long as the shift is not too abrupt (Wayne & Hayes, 1996). An *in vitro* model to study this persistent state is the so-called Wayne dormancy model, in which the bacteria downregulate their metabolism due to reduced access to oxygen (Wayne & Hayes, 1996). Another dormancy model utilizes nutrient starvation to induce a state where *M. tuberculosis* arrests growth and decreases its respiration rate (Betts *et al.*, 2002). Recently it was demonstrated that inhibition of respiration by nitric oxide might induce a dormant state in *M. tuberculosis* (Voskuil *et al.*, 2003). One should recognize, however, that the evidence linking *in vitro* dormant states of *M. tuberculosis* to human latent infection still remains circumstantial.

The vaccine strain *Mycobacterium bovis* BCG has been reported to occasionally persist in a latent state in humans, and in rare instances to cause disseminated disease, mainly in patients that are immunosuppressed (Talbot *et al.*, 1997). This is, however, an extremely rare event compared to the reactivation occurring in latent TB and could conceivably be caused by other mechanisms. The completed DNA sequences of *M. tuberculosis* and *M. bovis* BCG (Cole *et al.*, 1998) show that BCG lacks a number of genes present in *M. tuberculosis* (Brosch *et al.*, 1998; Mahairas *et al.*, 1996).

Abbreviations: 2-D, two-dimensional; USP, universal stress protein.

Furthermore, comparative proteome analysis of *M. tuberculosis* and *M. bovis* BCG has shown differences in the expression of several protein classes (Jungblut *et al.*, 1999). There are also differences in protein expression between different laboratory strains, such as *M. tuberculosis* Erdman and H37Rv (Jungblut *et al.*, 1999). To study latency it is therefore important to use a model with a relevant experimental *M. tuberculosis* strain.

Two-dimensional (2-D) PAGE is a potent tool to analyse the proteome (total protein expression) of a certain organism. The proteome represents not only the gene product, but also translational rate and post-translational modifications. Identification of proteins that are upregulated during latency is important in order to study the mechanisms of latency and to identify vaccine targets as well as targets for drug development. There are several reports on the proteomes of mycobacterial strains where cytosolic proteins, as well as cell wall proteins and culture filtrate proteins, have been identified (Boon *et al.*, 2001; Jungblut *et al.*, 1999; Mattow *et al.*, 2001; Monahan *et al.*, 2001; Rosenkrands *et al.*, 2000; Sonnenberg & Belisle, 1997; Wong *et al.*, 1999; Yuan *et al.*, 1996).

Here, we studied the cytosolic proteome of the virulent clinical isolate *M. tuberculosis* Harlingen strain grown under aerobic and anaerobic conditions. A modified Wayne dormancy model (Wayne & Hayes, 1996) was used where the bacteria were grown aerobically for 8–10 days and then shifted to anaerobic growth conditions.

METHODS

Bacterial strain. The clinical isolate *M. tuberculosis* strain S-02293 Harlingen was kindly provided by Dr J. van Embden, National Institute of Public Health and the Environment, The Netherlands. Strain Harlingen is a clinical isolate of *M. tuberculosis* that was isolated as a particularly transmissible and virulent strain in The Netherlands in 1993 (Kiers *et al.*, 1997).

Culture and growth conditions. The bacteria were thawed from a stock solution maintained in the laboratory at −70 °C and grown in Löwenstein–Jensen vials with pyruvate for 4 weeks. Approximately 1×10^6 bacteria of the *M. tuberculosis* isolate Harlingen were inoculated into BACTEC 13A vials (Becton & Dickinson) carrying 7H13 medium containing ^{14}C-labelled palmitate. The vials were incubated at 37 °C, and every other day the amount of $^{14}CO_2$ in the gas phase of the vial was measured in a BACTEC460 apparatus (Becton & Dickinson) and the removed gas was exchanged with an equal amount of oxygen (5 % CO_2, synthetic air). After 8–10 days half of the cultures were shifted to anaerobic growth conditions, i.e. the measurements were performed with the air valve of the BACTEC460 apparatus connected to a non-oxygen mixture (85 % N_2, 10 % H_2, 5 % CO_2). After 22–26 days the cultures were harvested by centrifugation and the bacterial pellets were washed once in PBS and protein extracts were prepared.

Adaptation of the cells to oxygen-free conditions was monitored by daily recordings of the growth index. The metabolic activity of the adapted bacteria was monitored by measuring intracellular ATP levels. The extraction and quantification of ATP were accomplished using a Bio-Orbit 1251 luminometer, following the improved method described by Nilsson *et al.* (1998).

Protein extraction. The bacterial pellets were washed in PBS 1–3 times, dissolved in 20 µl 0·3 % SDS, 200 mM DTT, 28 mM Tris base either before or after bead-beating and transferred to Eppendorf tubes containing 200–400 µl 0·1–0·5 mm zirconium beads (Techtum). The cells were disrupted in a Mini-BeadBeater (Biospec Products) for 3 min at 5000 Hz. The lysate was boiled for 2 min and cooled on ice. Four microlitres of a mix of 0·5 M Tris/HCl, 50 mM $MgCl_2$, 1 mg DNaseI ml^{-1}, 0·25 mg RNaseA ml^{-1} was added to the Harlingen extracts and the incubation on ice was continued for 10 min. Then, 160 µl of a mix of 9·9 M urea, 4 % NP-40, 2·2 % ampholytes, 100 mM DTT was added either before or after storage at −80 °C.

2-D PAGE. Between 10 and 40 µg protein was loaded on IPG strips pH 4–7, 18 cm (Amersham Biosciences). The gel strips were focused on a Multiphor electrophoresis unit and the second dimension was run on vertical 12 % acrylamide SDS-PAGE gels (Protean II; Bio-Rad). The gels were stained by silver, dried on Novex frames and scanned in a Hewlett Packard ScanJet 3c/T scanner. For preparative gels, protein was precipitated in 10 % TCA and washed three times in acetone at 4 °C. About 0·5 mg protein was loaded on each gel and the gels were stained by Coomassie brilliant blue.

Data analysis. The digitized gel images were imported into PDQuest (version 6.0; Bio-Rad) and were used for detection and quantification of the spot intensities, gel matching and statistical analysis. All spots were manually checked to exclude spots of low quality, e.g. spots in the periphery of the gel or in streaky areas. The analysis included spots that were either unique to one set of gels or changed in intensity by a factor of at least three. To correct for overall intensity differences between the gels, each gel was normalized to the standard according to the total quantity in valid spots.

Protein identification and MALDI-TOF. Proteins of interest were excised in 1 × 1 mm pieces that were destained in 0·2 M ammonium bicarbonate in 50 % (v/v) acetonitrile for 1 h at 37 °C, followed by a second wash in 0·05 M ammonium bicarbonate in 50 % (v/v) acetonitrile (1 h, 37 °C). The gel pieces were dried by Speedvac and reswelled in 5 µl buffer containing 0·2 M ammonium bicarbonate and 0·1 µg trypsin µl^{-1} (sequencing grade modified trypsin; Promega) for 10 min before incubation at 37 °C overnight covered by 20 µl 0·2 M ammonium bicarbonate. The cleavage was stopped by adding 0·5 µl triflouracetic acid (TFA) and peptides were extracted two times in a solution containing 60 % (v/v) acetonitrile and 0·1 % TFA followed by extraction two times in 40 % (v/v) acetonitrile and 0·1 % TFA. Peptides were concentrated by speedvac and dissolved in 13 µl 1 % acetic acid and purified using ZipTip$_{C18}$ (Millipore). Purified peptides were applied to an AnchorChip (Bruker) and analysed by MALDI-TOF (Bruker Daltronic Mass Analyser Reflex III) using α-4-hydroxycinnamic acid as matrix. External calibrations were made using a mix of peptides with known molecular masses and internally by using known tryptic fragments of trypsin. A search for protein candidates was made by using the apparent peptide masses, pI and molecular masses in the MS-Fit software at http://prospector.ucsf.edu/. A MOWSE score better than 1500 was judged to be significant and the whole procedure (from gel excision) was repeated to ensure that the correct protein was identified.

RESULTS AND DISCUSSION

Growth and ATP consumption during anaerobic conditions

M. tuberculosis cultures were grown to the transition to stationary phase, then a subset of culture flasks was shifted to anaerobic conditions and further incubated for 16 days

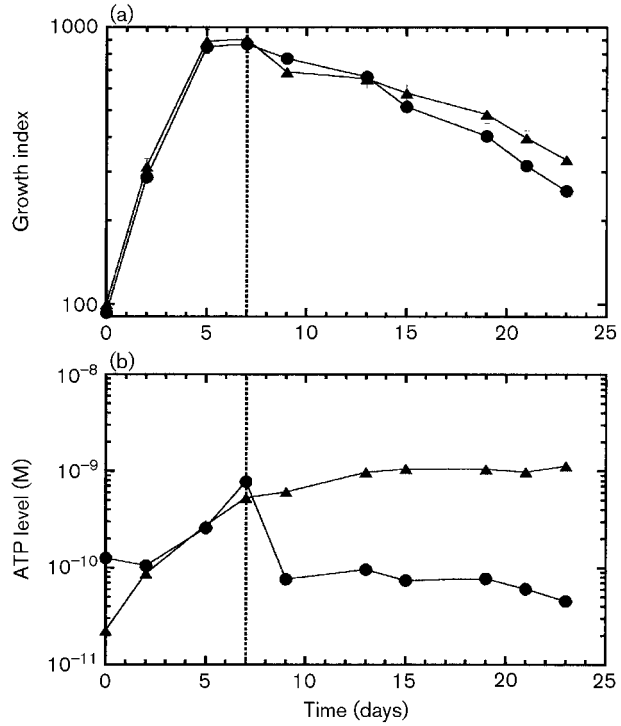

Fig. 1. Growth index (a) and ATP levels (b) of *M. tuberculosis* Harlingen cultured in Bactec vials. The vertical dashed line indicates the time point when half of the vials were shifted to anaerobic gas phase. Triangles, O_2; circles, N_2.

(Fig. 1a). The BACTEC readings were similar between shifted and unshifted cultures, although slightly lower values were obtained for the shifted cultures after a total of 23 days. However, BACTEC shows accumulated data and the amount of $^{14}CO_2$ at a certain time point depends on the history of the culture and does not provide a measurement of metabolism at the time of sampling. Also, a decrease in BACTEC readings over time may indicate that the substrate has become limiting and may thus not be an accurate measure of metabolic activity. To obtain a more accurate view of what impact the shift had on metabolic activity, the ATP content of the cells was determined using a bioluminescence assay (Nilsson *et al.*, 1988). Before the shift the ATP levels increased at the same rate in both unshifted and shifted cultures (Fig. 1b). ATP levels continued to rise at a slower rate in the unshifted cultures during stationary phase, while in the shifted cultures the ATP levels fell immediately after the shift, leading to final levels about 30-fold lower than for the control cultures (Fig. 1b).

2-D PAGE analysis

About 1500 protein spots (pI range 4–7) were detected in *M. tuberculosis* strain Harlingen grown under aerobic conditions (Fig. 2a). After a shift to anaerobic conditions most spots were unchanged (Fig. 2b). Analysis of three independent experiments using PDQuest 2-D analysis software

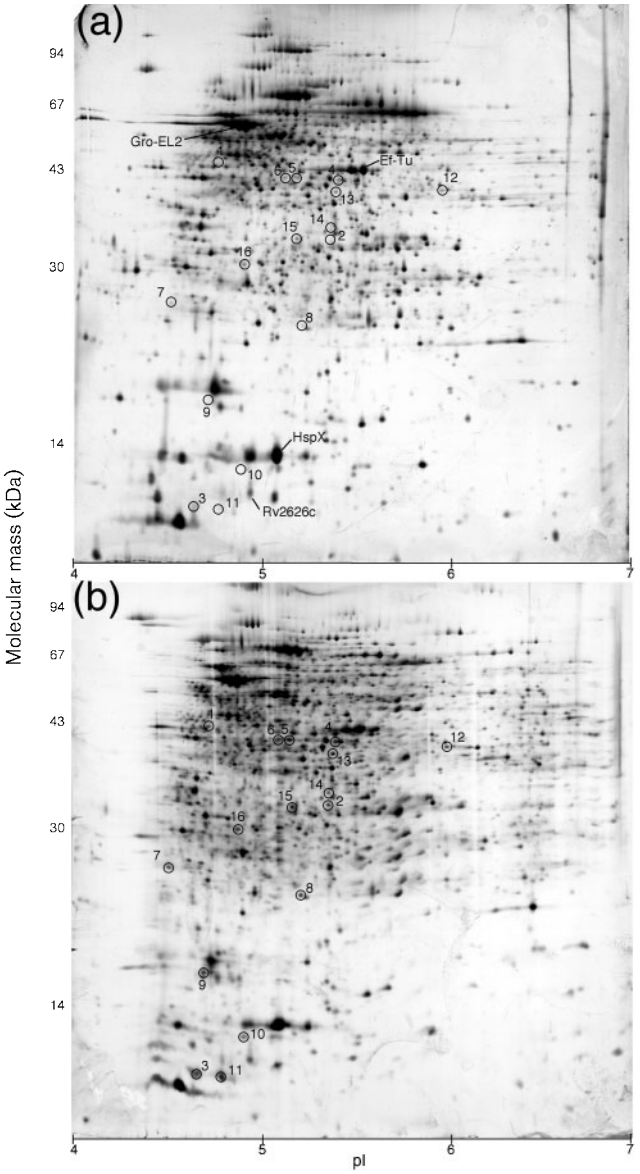

Fig. 2. 2-D polyacrylamide gels of *M. tuberculosis*. The panels show unshifted cultures (a) and those shifted to anaerobic gas phase (b). Circled spots indicate upregulated proteins (Table 1) and the proteins Gro-EL2, Ef-Tu, HspX, Rv2626c are indicated as reference spots.

revealed 13 unique spots and 37 spots that were greater than threefold more abundant during anaerobic conditions. Sixteen of these 50 spots were identified (Table 1). Either the remaining spots contained too small an amount of protein for analysis or they were in a position on the gel where they overlapped with other spots. One was identified as Rv2005c, a hypothetical protein with similarity to universal stress proteins (USPs). Two other proteins were identified as chaperones, Gro-EL2 and α-crystalline homologue HspX/14-kDa antigen (Table 1). According to the proteomic map of *M. tuberculosis*, Gro-EL2 is a major

Table 1. Identified proteins more abundant under anaerobic conditions

No.	Measured*		Calculated		Accession no. (identity)	Quantity† N_2 (CV‡)	Quantity O_2 (CV‡)	Upregulation (-fold)§
	Mass (kDa)	pI	Mass (kDa)	pI				
1	48	4·7	56·6	4·85	Rv0440 (GroEL2)	623 (35)	– (–)	Unique
2	33	5·2	30·9	5·53	Rv2005c (USP-like)	301 (37)	– (–)	Unique
3	11	4·7	16·1	5·00	Rv2031c (HspX)	1888 (36)	– (–)	Unique
4	45	5·2	44·3	5·27	Rv2246 (KasB)	1191 (39)	157 (5·3)	8
5	45	4·9	43·6	5·28	Rv0685 (Ef-Tu)	965 (23)	133 (27)	7
6	45	4·8	43·6	5·28	Rv0685 (Ef-Tu)	696 (23)	114 (7·1)	6
7	26	OR	25·9	4·68	Rv0560c (hypothetical)	748 (8·5)	135 (27)	6
8	24	5·0	22·9	5·13	Rv2503c (ScoB)	459 (38)	74 (0)	6
9	19	4·7	16·3	4·80	Rv2185c (hypothetical)	961 (18)	147 (76)	7
10	13	4·8	16·1	5·00	Rv2031c (HspX)	651 (20)	96 (17)	7
11	11	4·7	16·1	5·00	Rv2031c (HspX)	2047 (18)	213 (27)	10
12	44	OR	38·7	5·81	Rv2780 (Ald)	568 (23)	148 (48)	4
13	43	5·2	40·8	5·20	Rv2629 (hypothetical)	571 (24)	181 (25)	3
14	36	5·2	35·6	5·30	Rv3913 (TrxB2)	193 (33)	56 (15)	3
15	33	4·9	34·7	5·11	Rv0503c (CmaA2)	697 (29)	162 (5·1)	4
16	29	4·7	30·1	4·97	Rv3866 (hypothetical)	783 (6·3)	233 (12)	3

*OR, pI outside the calculation range on the gel.

†The mean normalized quantity is measured in OD units × area p.p.m.

‡CV, coefficient of variation, i.e. SD divided by the mean.

§Unique indicates a protein not detected in O_2 cultures.

immunodominant protein (cf. Fig. 2a with the web-based map at http://www.mpiib-berlin.mpg.de/2D-PAGE/EBP-PAGE/index.html) located at another spot position, showing that the Gro-EL2 spot identified here was a proteolytic degradation product. The α-crystalline homologue HspX/14-kDa antigen was also N-terminally sequenced (not shown) and identified as a proteolytic fragment of the native HspX/14-kDa protein (Fig. 2). The amounts of these proteolytic products were at least tenfold lower than the corresponding high-abundance full-length proteins. We furthermore identified elongation factor Tu, β-ketoacyl-ACP, succinyl-CoA : 3-oxoacid-CoA transferase, cyclopropane mycolic acid synthase 2, thioredoxin reductase, L-alanine dehydrogenase, Rv2629, Rv2185c, Rv0560c and Rv3866 (Table 1). Ef-Tu was identified at another spot position in the proteomic map with a higher molecular mass and significantly different pI, suggesting that the identified spot was a proteolytic degradation product or a result of other post-translational modifications (Fig. 2).

Analysing differential expression during dormancy and interpreting the result is not straightforward as dormancy and stationary phase are closely related. Dormancy can be defined as when the metabolic state of the cell is at its minimum, keeping only those functions that are necessary to persist and, upon environmental stimuli, activate growth. Stationary phase is a wider definition and refers normally to the cell-density-associated growth arrest in batch cultures caused by oxygen limitation, nutrient limitation, secondary metabolite production and pH changes, for example (cf. Wayne & Sohaskey, 2001). Many genes have been shown to respond similarly in various model systems for dormancy, e.g. entry into stationary phase, rapid shift to low O_2 concentrations and the introduction of low levels of NO (Table 2). In the experimental system used here both control and test cultures were harvested long after entry into stationary phase with the difference that the ATP level was 30-fold lower in the cultures purged with anaerobic gas. Our results indicate that there are few or no further changes in the expression of typical dormancy-related genes such as *hspX* in stationary phase when oxygen is absent (Table 2).

M. tuberculosis may lose viability if the shift to anaerobic or micro-aerophilic conditions is too abrupt (Wayne & Hayes, 1996). Sherman *et al.* (2001) performed experiments where BCG was shifted instantly to anaerobic/micro-aerophilic conditions during early exponential growth phase. Despite this apparently abrupt shift the cells were able to strongly induce the dormancy-associated *hspX* gene, suggesting that a majority of the cells were intact and viable. Also, these authors stated that viability of *M. tuberculosis* shifted to $<1\%$ O_2 remained high even after 3 weeks of hypoxia. In our experiments, shifts were made at the transition to stationary phase and although we cannot rule out a decreased viability, this shift more likely occurred when the cells were already exposed to a lowered oxygen pressure and thus more adapted than during early exponential phase. A rapid decrease of the intracellular ATP (or GTP) pool most probably leads to reduced RNA

Table 2. Comparison of dormancy-induced genes

Gene	Gene product/ putative function	Ambient air to 0·2 % O_2*† (-fold upregulation)	Ambient air to NO† (-fold upregulation)	Wayne dormancy model† (-fold upregulation)	This study
Rv0440	60 kDa chaperone 2				Unique‡
Rv0503c	Mycolic acid synthase				4
Rv0560c					6
Rv0571c		ND/2	4	3	
RV0572c		14/9	17	7	
Rv0573c		ND/1·3	2		
Rv0574c		4/3	5	5	
Rv0685	Elongation factor Tu				6‡
Rv1738		63/50	27	24	
Rv1812c		ND/2	2	8	
Rv1813c		15/13	18	22	
Rv2003c		11/12	14	6	
Rv2004c			2	8	
Rv2005c	USP-like	9/9	7	11	Unique
Rv2007c	Ferredoxin	22/24	16	18	
Rv2028c	USP-like	3/4	5	17	
Rv2029c	Phosphofructokinase II	12/12	16	23	
Rv2030c		19/11	19	48	
Rv2031c	α-Crystallin homologue	14/15	23	31	7–10/unique‡
Rv2032		44/45	31	24	
Rv2185c					7
Rv2246	β-Ketoacyl-ACP synthase				8
Rv2503c					6
Rv2623	USP-like	7/7	6	27	
Rv2624c	USP-like	44/20	17	5	
Rv2625c		6/7	6	5	
Rv2626c		37/41	15	57	No change§
Rv2627c		17/12	11	15	
Rv2628		5/5	8	23	
Rv2629		7/7	7	8	3
Rv2780	L-Alanine dehydrogenase				4
Rv3132c	Sensor histidine kinase	9/10	12	13	
Rv3133c	Two-component response regulator	14/12	14	12	
Rv3134c	USP-like	11/11	9	23	
Rv3866					3
Rv3913	Thioredoxin reductase				3

*Selected upregulated genes from Sherman *et al.* (2001). ND, Not detected.
†Selected upregulated genes from Voskuil *et al.* (2003).
‡Possibly proteolytic degradation products.
§Less than threefold change.

synthesis followed by rapid cessation of ribosomal and general protein synthesis, effectively reducing further growth. In our experiments the protein concentration of the *M. tuberculosis* cell pellets at harvest was threefold lower in the shifted cultures, which is in accord with the hypothesis that protein synthesis and growth rate is lowered in shifted cultures, while unshifted cultures most likely continued to grow for one to two cell generations. With respect to growth yield and ATP concentration, the specific ATP content per amount of protein would be tenfold less in the shifted cultures, still significantly lower than the unshifted ones.

One disadvantage with our protein approach may be that protein spots may represent proteolytic degradation products rather than upregulated proteins. First, it must be stressed that not all regulation occurs at the transcriptional level and proteolysis and other post-translational

modification may have a significant impact on cellular processes. Second, our estimation of general proteolytic activity was less than 10 %, but it is probably much lower. Thus, if the upregulated spots represented more stable proteins in the cell, there would not be more than a 10 % upregulation. Third, if the identified proteins were proteolytic degradation products, such as those found for HspX and GroEL2, these peptides would generally have significantly lower molecular masses. Twelve of our identified spots, excluding HspX and GroEL2, showed a mean apparent molecular mass difference of +3·9 % (range −4·9 to +16·5 %) of the predicted molecular mass, while the GroEL2 and HspX peptides showed 15 and 19/31 % reduction, respectively. The N-terminal sequences of the smallest HspX peptides identified were also determined and we recalculated the predicted molecular mass loss to 27 %, in accord with our estimated value of 31 %.

Stress proteins

HspX and USP Rv2623 have been found to be upregulated in stationary phase/hypoxic conditions in strain BCG and *M. tuberculosis* (Boon *et al.*, 2001; Cunningham & Spreadbury, 1998; Desjardin *et al.*, 2001; Florczyk *et al.*, 2001; Rosenkrands *et al.*, 2002; Sherman *et al.*, 2001; Tabira *et al.*, 1998; Voskuil *et al.*, 2003; Yuan *et al.*, 1996, 1998). The regulator *dosR* (Rv3133c) has been implicated in the control of 48 dormancy-associated genes, including *hspX*, Rv2623, Rv2626c, Rv2005c and Rv2629 (Boon & Dick, 2002; Park *et al.*, 2003). The expression of this 48-gene regulon was also induced by low concentrations of NO (Table 2). Eight USP homologues have been found in the genome of *M. tuberculosis* (Rv1636, Rv2028c, Rv2624c, Rv3134c, Rv1996, Rv2005c, Rv2026c and Rv2623) of which at least five have been found to increase during stationary phase/hypoxic conditions (O'Toole & Williams, 2003). The USP family is conserved in many organisms and studies in, for example, *Escherichia coli* have shown that *usp* mutants are less likely to survive growth arrest and DNA damage (Kvint *et al.*, 2003). Also, expression of *uspC*, *uspD* and *uspE* in *E. coli* was shown to be dependent on the stringent response as well as RecA, and mutants were sensitive to UV irradiation (Gustavsson *et al.*, 2000, 2002). Although the HspX, Rv2623 and Rv2626c products in *M. tuberculosis* are regarded as markers for dormancy and/or stationary phase, we did not detect any major difference in expression of HspX or Rv2626c, which appeared highly expressed under both conditions (Table 2 and Fig. 2). However, we found higher abundance of the dormancy-related proteins Rv2629 and Rv2005c (Tables 1 and 2). Thus, the expression of these commonly found dormancy markers suggests that the rapid introduction of anaerobic gas did not prevent the mounting of a dormancy programme by decreased viability, for example.

Thioredoxin reductase (Rv3913, TrxB2) has been found to be upregulated in *Mycobacterium smegmatis* during stationary phase (Murugasu-Oei *et al.*, 1999). Thioredoxin and thioredoxin reductase of *M. tuberculosis* have been shown to reduce peroxides and dinitrobenzenes with higher efficiency under anaerobic conditions (Zhang *et al.*, 1999). In *Streptomyces coelicolor* the *trxB* operon has been found to be controlled by SigR (Paget *et al.*, 1998) and its homologue in *M. tuberculosis* is designated SigH. Several studies have indicated that the sigma factors SigB, SigE, SigF and SigH in *M. tuberculosis* and *M. smegmatis* are involved in adaptation to several stresses, including heat shock and oxidative stress (Chen *et al.*, 2000; Fernandes *et al.*, 1999; Hu & Coates, 1999; Manganelli *et al.*, 1999). It has further been demonstrated that SigH, in response to these stresses, induces transcription of the genes encoding TrxB2, DnaK and ClpB (Raman *et al.*, 2001). As discussed by Zhang *et al.* (1999), it is possible that the Trx-TR system contributes to the antioxidant defence of *M. tuberculosis* during anaerobic or micro-aerophilic conditions.

Metabolic enzymes

The upregulated 40 kDa L-alanine dehydrogenase (Ald, Rv2780) was the first antigen reported to be produced by virulent *M. tuberculosis*, but not in the vaccine strain *M. bovis* BCG (Andersen *et al.*, 1992; Jungblut *et al.*, 1999). However, its gene is present both in virulent and avirulent strains. It is a functional L-alanine dehydrogenase and thus one of the few antigens with enzymic properties that makes it attractive for diagnostic and therapeutic interventions. Proteins in the culture filtrate of *M. tuberculosis* are the primary targets of the immune response (Harboe, 1992) and the 40 kDa protein is one of the earliest proteins detectable in the culture medium of *M. tuberculosis*, present as early as 4 days (Andersen *et al.*, 1992). The activity of the enzyme increases during dormancy development in *M. smegmatis*, in which the activity rapidly increases five-fold after depletion of oxygen. As oxygen becomes more limiting, the enzyme activity declines until it reaches a level about 3·5-fold above the baseline (Hutter & Dick, 1998). The *ald* transcript of *M. tuberculosis* H37Rv is overproduced under hypoxic conditions (Rosenkrands *et al.*, 2002; Sherman *et al.*, 2001) and under nutrient starvation conditions (Betts *et al.*, 2002). The suggested main function of this enzyme is the generation of alanine for protein and peptidoglycan synthesis. As alanine synthesis is coupled to NADH oxidation, it is also possible that the induction of alanine dehydrogenase activity might support the maintenance of the NAD pool when oxygen, as terminal electron acceptor, becomes limiting (Hutter & Dick, 1998).

Another metabolic enzyme that increased during anaerobisis was succinyl-CoA : 3-oxoacid-CoA transferase (ScoB, Rv2503c), which catalyses the reversible reaction where succinyl-CoA and a 3-oxoacid are converted to succinate and 3-oxoacyl-CoA, respectively.

Cell wall

Mycobacteria have an unusual cell wall in which mycolic acids play a critical role in structure and function. This structure confers to the bacteria resistance to chemical

injury, low permeability to antibiotics, resistance to dehydration and an ability to survive within the phagolysosomes of the macrophages (Barry *et al.*, 1998). During entry into dormancy, mycobacteria enlarge due to thickening of the cell wall (Cunningham & Spreadbury, 1998). There are at least three mycolic acid cyclopropane synthases (CmaA1, PcaA and CmaA2) that are responsible for the site-specific modifications of mycolic acids. The first one to be identified was CmaA1, based on its homology to the *E. coli* enzyme CFA synthase (Wang *et al.*, 1992). At least seven homologous genes have been identified in the genome sequence of H37Rv (Cole *et al.*, 1998). These are *cmaA2*, *mmaA1-4* and *umaA1-2*; they share 50–75 % identity with each other and are all thought to be SAM-utilizing methyl transferases. Another mycolic acid synthase, PcaA, was shown to be important for dormancy since *pcaA* knock-out strains lost their ability to persist, although not replicated in a mouse model (Glickman *et al.*, 2000). Studies in *M. tuberculosis* have shown that Rv0503c (CmaA2), found in this study to be fourfold upregulated during anaerobisis, is required for the synthesis of the *trans*-cyclopropane rings of keto- and methoxymycolates (Glickman *et al.*, 2001). Cyclopropanation of mycolic acids is a modification that is associated with pathogenic bacteria and is not common in the cell wall of saprophytic species such as *M. smegmatis* (Minnikin *et al.*, 1982). In wild-type *M. smegmatis*, less than 2 % of the mycolic acids are monocyclopropanated, whereas this increases to 25 % after induction of CmaA2 (George *et al.*, 1995). In addition, cyclopropane ring modifications have profound effects on the resistance of mycobacteria to oxidative stress (Yuan *et al.*, 1995) and the fluidity and permeability of the cell wall (George *et al.*, 1995). In *E. coli* and other species that cyclopropanate their plasma membrane, this modification occurs during the transition from active growth to stationary phase, and as a response to environmental conditions such as low pH, high incubation temperatures and low aeration rates (Grogan & Cronan, 1997; Wang & Cronan, 1994).

The upregulated protein β-ketoacyl-ACP synthase (KasB, Rv2246) is, together with KasA, involved in the synthesis of mycolic acids (Schaeffer *et al.*, 2001). KasA has been shown to specifically elongate palmitoyl-CoA to monounsaturated fatty acids averaging 40 carbons in length and overproduction of KasB in the presence of KasA leads to the production of even longer chains. The production of these chains is sensitive to isoniazid, thiolactomycin and triclosan *in vitro* (Slayden & Barry, 2002). Moreover, another study showed that KasAB is the target of thiolactomycin (Kremer *et al.*, 2000). However, since KasA is not the direct target of isoniazid, this effect may be indirect. Finally, *Mycobacterium marinum* KasB mutants (with transposon insertions in *kasB*) synthesized mycolic acids that were 2–4 carbons shorter than wild-type and grew poorly in macrophages (Gao *et al.*, 2003). Thus there appears to be an increase in full-length oxygenated mycolic acids during anaerobiosis and a requirement of long-chain mycolic acids during intracellular growth.

Protein Rv0560c shows similarity to SAM-utilizing methyl transferases and it is possible that Rv0560c is linked to synthesis of ketomycolates. Rv0560c has been shown to be induced by salicylate (Sun *et al.*, 2001), a compound that also increases oxygen consumption when added to *M. tuberculosis* cultures (Bernheim, 1940), and is clustered with other genes involved in ubiquinone biosynthesis (Sun *et al.*, 2001). The function of Rv3866 is not known, but it is located just upstream of the virulence-associated RD1 element on the *M. tuberculosis* chromosome (Sassetti & Rubin, 2003). Finally, PHI/PSI-BLAST searches revealed that Rv2185c shows weak similarity to polyketide cyclases.

In summary, we found about 50 proteins in a clinical isolate of *M. tuberculosis* that were unique or more abundant under anaerobic conditions and low ATP levels. The induction pattern was different from that during a shift from exponential growth to stationary phase and the induction ratios were moderate (3- to 10-fold induction). Newly found proteins were related to, for example, mycolic acid synthesis and oxidative stress. Their roles during the survival of dormant tubercle bacilli require further research.

ACKNOWLEDGEMENTS

We thank Marianne Ljungström for excellent technical assistance and Mats Andersson, MTC, Karolinska Institute for supervision and technical support of MALDI-TOF. This work was supported by the Swedish Heart Lung Foundation (D. I. A. and G. K).

REFERENCES

Andersen, A. B., Andersen, P. & Ljungqvist, L. (1992). Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis*. *Infect Immun* **56**, 1994–1998.

Barry, C. E., 3rd, Lee, R. E., Mdluli, K., Sampson, A. E., Schroeder, B. G., Slayden, R. A. & Yuan, Y. (1998). Mycolic acids: structure, biosynthesis and physiological functions. *Prog Lipid Res* **37**, 143–179.

Bernheim, F. (1940). The effect of salicylate on the oxygen uptake of the tubercle bacillus. *Science* **92**, 204.

Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A. & Duncan, K. (2002). Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol Microbiol* **43**, 717–731.

Boon, C. & Dick, T. (2002). *Mycobacterium bovis* BCG response regulator essential for hypoxic dormancy. *J Bacteriol* **184**, 6760–6767.

Boon, C., Li, R., Qi, R. & Dick, T. (2001). Proteins of *Mycobacterium bovis* BCG induced in the Wayne dormancy model. *J Bacteriol* **183**, 2672–2676.

Brosch, R., Gordon, S. V., Billault, A., Garnier, T., Eiglmeier, K., Soravito, C., Barrell, B. G. & Cole, S. T. (1998). Use of a *Mycobacterium tuberculosis* H37Rv bacterial artificial chromosome library for genome mapping, sequencing, and comparative genomics. *Infect Immun* **66**, 2221–2229.

Chen, P., Ruiz, R. E., Li, Q., Silver, R. F. & Bishai, W. R. (2000). Construction and characterization of a *Mycobacterium tuberculosis* mutant lacking the alternate sigma factor gene, *sigF*. *Infect Immun* **68**, 5575–5580.

Cole, S. T., Brosch, R., Parkhill, J. & 38 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.

Cunningham, A. F. & Spreadbury, C. L. (1998). Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton α-crystallin homologue. *J Bacteriol* **180**, 801–808.

Desjardin, L. E., Hayes, L. G., Sohaskey, C. D., Wayne, L. G. & Eisenach, K. D. (2001). Microaerophilic induction of the alpha-crystallin chaperone protein homologue (*hspX*) mRNA of *Mycobacterium tuberculosis*. *J Bacteriol* **183**, 5311–5316.

Dye, C., Scheele, S., Dolin, P., Pathania, V. & Raviglione, M. C. (1999). Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* **282**, 677–686.

Fernandes, N. D., Wu, Q. L., Kong, D., Puyang, X., Garg, S. & Husson, R. N. (1999). A mycobacterial extracytoplasmic sigma factor involved in survival following heat shock and oxidative stress. *J Bacteriol* **181**, 4266–4274.

Florczyk, M. A., McCue, L. A., Stack, R. F., Hauer, C. R. & McDonough, K. A. (2001). Identification and characterization of mycobacterial proteins differentially expressed under standing and shaking culture conditions, including Rv2623 from a novel class of putative ATP-binding proteins. *Infect Immun* **69**, 5777–5785.

Gao, L. Y., Laval, F., Lawson, E. H., Groger, R. K., Woodruff, A., Morisaki, J. H., Cox, J. S., Daffe, M. & Brown, E. J. (2003). Requirement for *kasB* in *Mycobacterium* mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. *Mol Microbiol* **49**, 1547–1563.

George, K. M., Yuan, Y., Sherman, D. R. & Barry, C. E., 3rd (1995). The biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. Identification and functional analysis of CMAS-2. *J Biol Chem* **270**, 27292–27298.

Glickman, M. S., Cox, J. S. & Jacobs, W. R., Jr (2000). A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. *Mol Cell* **5**, 717–727.

Glickman, M. S., Cahill, S. M. & Jacobs, W. R., Jr (2001). The *Mycobacterium tuberculosis cmaA2* gene encodes a mycolic acid trans-cyclopropane synthetase. *J Biol Chem* **276**, 2228–2233.

Grogan, D. W. & Cronan, J. E., Jr (1997). Cyclopropane ring formation in membrane lipids of bacteria. *Microbiol Mol Biol Rev* **61**, 429–441.

Gustavsson, N., Diez, A. & Nyström, T. (2000). The universal stress protein A of *Escherichia coli* is required for resistance to DNA damaging agents and is regulated by a RecA/FtsK-dependent regulatory pathway. *Mol Microbiol* **36**, 1494–1503.

Gustavsson, N., Diez, A. & Nyström, T. (2002). The universal stress protein paralogues of *Escherichia coli* are co-ordinately regulated and co-operate in the defence against DNA damage. *Mol Microbiol* **43**, 107–117.

Harboe, M. (1992). The significance of proteins actively secreted by *Mycobacterium tuberculosis* in relation to immunity and complications of mycobacterial diseases. *Int J Lepr Other Mycobact Dis* **60**, 470–476.

Hu, Y. & Coates, A. R. (1999). Transcription of two sigma 70 homologue genes, *sigA* and *sigB*, in stationary-phase *Mycobacterium tuberculosis*. *J Bacteriol* **181**, 469–476.

Hutter, B. & Dick, T. (1998). Increased alanine dehydrogenase activity during dormancy in *Mycobacterium smegmatis*. *FEMS Microbiol Lett* **167**, 7–11.

Jungblut, P. R., Schaible, U. E., Mollenkopf, H.-J. & 7 other authors (1999). Comparative proteome analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG strains: towards functional genomics of microbial pathogens. *Mol Microbiol* **33**, 1103–1117.

Kiers, A., Drost, A. P., van Soolingen, D. & Veen, J. (1997). Use of DNA fingerprinting in international source case finding during a large outbreak of tuberculosis in The Netherlands. *Int J Tuberc Lung Dis* **1**, 239–245.

Kremer, L., Douglas, J. D., Baulard, A. R. & 9 other authors (2000). Thiolactomycin and related analogues as novel anti-mycobacterial agents targeting KasA and KasB condensing enzymes in *Mycobacterium tuberculosis*. *J Biol Chem* **275**, 16857–16864.

Kvint, K., Nachin, L., Diez, A. & Nyström, T. (2003). The bacterial universal stress protein: function and regulation. *Curr Opin Microbiol* **6**, 140–145.

Mahairas, G. G., Sabo, P. J., Hickey, M. J., Singh, D. C. & Stover, C. K. (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J Bacteriol* **178**, 1274–1282.

Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F. R. & Smith, I. (1999). Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol Microbiol* **31**, 715–724.

Mattow, J., Jungblut, P. R., Muller, E. C. & Kaufmann, S. H. (2001). Identification of acidic, low molecular mass proteins of *Mycobacterium tuberculosis* strain H37Rv by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry. *Proteomics* **1**, 494–507.

Minnikin, D. E., Minnikin, S. M., Goodfellow, M. & Stanford, J. L. (1982). The mycolic acids of *Mycobacterium chelonei*. *J Gen Microbiol* **128**, 817–822.

Monahan, I. M., Betts, J., Banerjee, D. K. & Butcher, P. D. (2001). Differential expression of mycobacterial proteins following phagocytosis by macrophages. *Microbiology* **147**, 459–471.

Murugasu-Oei, B., Tay, A. & Dick, T. (1999). Upregulation of stress response genes and ABC transporters in anaerobic stationary-phase *Mycobacterium smegmatis*. *Mol Gen Genet* **262**, 677–682.

Nilsson, L. E., Hoffner, S. E. & Ansehn, S. (1988). Rapid susceptibility testing of *Mycobacterium tuberculosis* by bioluminescent assay of mycobacterial ATP. *Antimicrob Agents Chemother* **32**, 1208–1212.

O'Toole, R. & Williams, H. D. (2003). Universal stress proteins and *Mycobacterium tuberculosis*. *Res Microbiol* **154**, 387–392.

Paget, M. S., Kang, J. G., Roe, J. H. & Buttner, M. J. (1998). sigmaR, an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in *Streptomyces coelicolor* A3(2). *EMBO J* **17**, 5776–5782.

Park, H.-D., Guinn, K. M., Harrell, M. I., Liao, R., Voskuil, M. I., Tompa, M., Schoolnik, G. K. & Sherman, D. R. (2003). Rv3133c/*dosR* is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. *Mol Microbiol* **48**, 833–843.

Raman, S., Song, T., Puyang, X., Bardarov, S., Jacobs, W. R., Jr & Husson, R. N. (2001). The alternative sigma factor SigH regulates major components of oxidative and heat stress responses in *Mycobacterium tuberculosis*. *J Bacteriol* **183**, 6119–6125.

Rosenkrands, I., King, A., Weldingh, K., Moniatte, M., Moertz, E. & Andersen, P. (2000). Towards the proteome of *Mycobacterium tuberculosis*. *Electrophoresis* **21**, 3740–3756.

Rosenkrands, I., Slayden, R. A., Crawford, J., Aasgaard, C., Barry, C. E. & Andersen, P. (2002). Hypoxic response of *Mycobacterium tuberculosis* studied by metabolic labelling and proteome analysis of cellular and extracellular proteins. *J Bacteriol* **184**, 3485–3491.

Sassetti, C. M. & Rubin, E. J. (2003). Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* **100**, 12989–12994.

Schaeffer, M. L., Agnihotri, G., Volker, C., Kallender, H., Brennan, P. J. & Lonsdale, J. T. (2001). Purification and biochemical characterization of the *Mycobacterium tuberculosis* beta-ketoacyl-acyl carrier protein synthases KasA and KasB. *J Biol Chem* **276**, 47029–47037.

Sherman, D. R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M. I. & Schoolnik, G. K. (2001). Regulation of the *Mycobacterium tuberculosis* hypoxic response gene encoding α-crystallin. *Proc Natl Acad Sci U S A* **98**, 7534–7539.

Slayden, R. A. & Barry, C. E., 3rd (2002). The role of KasA and KasB in the biosynthesis of meromycolic acids and isoniazid resistance in *Mycobacterium tuberculosis*. *Tuberculosis* **82**, 149–160.

Sonnenberg, M. G. & Belisle, J. T. (1997). Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. *Infect Immun* **65**, 4515–4524.

Sun, Z., Cheng, S.-J., Zhang, H. & Zhang, Y. (2001). Salicylate uniquely induces a 27-kDa protein in tubercle bacillus. *FEMS Microbiol Lett* **203**, 211–216.

Tabira, Y., Ohara, N., Kitaura, H., Matsumoto, S., Naito, M. & Yamada, T. (1998). The 16-kDa α-crystallin-like protein of *Mycobacterium bovis* BCG is produced under conditions of oxygen deficiency and is associated with ribosomes. *Res Microbiol* **149**, 255–264.

Talbot, E. A., Perkins, M. D., Silva, S. F. & Frothingham, R. (1997). Disseminated bacille Calmette-Guerin disease after vaccination: case report and review. *Clin Infect Dis* **24**, 1139–1146.

Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R. & Schoolnik, G. K. (2003). Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med* **198**, 705–713.

Wang, A. Y. & Cronan, J. E., Jr (1994). The growth phase-dependent synthesis of cyclopropance fatty acids in *Escherichia coli* is the result of an RpoS(KatF)-dependent promoter plus enzyme instability. *Mol Microbiol* **11**, 1009–1017.

Wang, A. Y., Grogan, D. W. & Cronan, J. E., Jr (1992). Cyclopropane fatty acid synthase of *Escherichia coli*: deduced amino acid sequence, purification, and studies of the enzyme active site. *Biochemistry* **31**, 11020–11028.

Wayne, L. G. & Hayes, L. G. (1996). An *in vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun* **64**, 2062–2069.

Wayne, L. G. & Sohaskey, C. D. (2001). Nonreplicating persistence of *Mycobacterium tuberculosis*. *Annu Rev Microbiol* **55**, 139–163.

Wayne, L. G. & Sramek, H. A. (1994). Metronidazole is bactericidal to dormant cells of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **38**, 2054–2058.

Wong, D. K., Lee, B. Y., Horwitz, M. A. & Gibson, B. W. (1999). Identification of fur, aconitase, and other proteins expressed by *Mycobacterium tuberculosis* under conditions of low and high concentrations of iron by combined two-dimensional gel electrophoresis and mass spectrometry. *Infect Immun* **67**, 327–336.

Yuan, Y., Lee, R. E., Besra, G. S., Belisle, J. T. & Barry, C. E., 3rd (1995). Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **92**, 6630–6634.

Yuan, Y., Crane, D. D. & Barry, C. E., 3rd (1996). Stationary phase-associated protein expression in *Mycobacterium tuberculosis*: function of the mycobacterial alpha-crystallin homolog. *J Bacteriol* **178**, 4484–4492.

Yuan, Y., Crane, D. D., Simpson, R. M., Zhu, Y. Q., Hickey, M. J., Sherman, D. R. & Barry, C. E., 3rd (1998). The 16-kDa α-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc Natl Acad Sci U S A* **95**, 9578–9583.

Zhang, Z., Hillas, P. J. & Ortiz de Montellano, P. R. (1999). Reduction of peroxides and dinitrobenzenes by *Mycobacterium tuberculosis* thioredoxin and thioredoxin reductase. *Arch Biochem Biophys* **363**, 19–26.