Comparison of the proteome of *Mycobacterium tuberculosis* strain H37Rv with clinical isolate CDC 1551

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The genome sequences of two virulent strains of *Mycobacterium tuberculosis* (H37Rv and CDC 1551) are now available. CDC 1551 is a recent clinical isolate and H37Rv is a commonly used lab strain which has been subject to *in vitro* passage. The two strains have been shown to display differing phenotypes both *in vivo* and *in vitro*. The proteome of the two strains grown in liquid culture were examined over time to determine whether there are any major differences between them at the protein level and the differences were compared to the genome data. Total cell lysates of the two strains were analysed by two-dimensional electrophoresis. Approximately 1750 protein spots were visualized by silver staining and the protein profiles of the two strains were found to be highly similar. Out of a total of 17 protein spot differences, seven were unique to CDC 1551 and three to H37Rv. Two further spots showed increased intensity in H37Rv, one spot showed differing vertical mobility between the strains and four showed differing spot intensities with time. Twelve of the spot differences were identified using mass spectrometry; however, no obvious association with phenotype could be deduced. When genome differences were analysed and related to the proteome differences, a mobility shift identified in the MoxR protein could be explained by a point mutation at the gene level. This proteome analysis reveals that, despite having been maintained under vastly different conditions, namely *in vitro* passage and *in vivo* transmission, these two strains have remained highly similar.

Keywords: mycobacteria, tuberculosis, proteome, two-dimensional gel electrophoresis, genome

INTRODUCTION

The complete genome sequence of *Mycobacterium tuberculosis* H37Rv has recently been published (Cole *et al.*, 1998). Although not yet totally complete, the sequence of a second strain, CDC 1551, a recent clinical

isolate (Valway *et al.*, 1998), has also been made publicly available via the internet (<http://www.tigr.org/tdb/CMR/gmt/htmls/SplashPage.html>). The two genomes show > 99% identity at the nucleotide level (Delcher *et al.*, 1999) but the strains display phenotypic differences which might be explained by changes at the protein-expression level.

M. tuberculosis H37Rv is the most commonly studied laboratory strain and was first isolated in 1905. Since this time it has been continually passaged and, as its virulence in humans is unknown, there have been concerns as to whether this may have led to attenuation of virulence (Jacobs *et al.*, 1996). However, it has maintained virulence in animal models. In contrast, CDC 1551 is a recent clinical isolate which was responsible for an outbreak of TB in a rural area of the

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Abbreviations: 2D, two-dimensional; BCG, bacille Calmette–Guérin; IPG, immobilized pH gradient; MALDI, matrix-assisted laser desorption-ionization; PGRS, polymorphic G + C rich sequence; PE, Pro-Glu; PPE, Pro-Pro-Glu; TOF, time-of-flight.

USA in 1995 (Valway *et al.*, 1998). Due to the high rate of skin-test conversion that occurred and the large skin-test response to purified protein derivative of tuberculin (PPD) of those infected, CDC 1551 was considered to be unusually infectious in man. In addition, evaluation of the growth of CDC 1551 in lungs of mice 20 d after aerosol infection gave 100-fold higher numbers of bacilli compared to the numbers of bacilli isolated from the lungs of mice infected with the *M. tuberculosis* laboratory strain Erdman (Valway *et al.*, 1998). As a result of these findings, CDC 1551 was assumed to be highly virulent and was selected for sequencing by the National Institutes of Health (Jacobs *et al.*, 1996).

More recently, however, the growth of CDC 1551 has been compared to both H37Rv, the Erdman strain and two clinical isolates in a mouse aerosol infection model (Manca *et al.*, 1999). These studies confirmed CDC 1551 replicates to higher number in mice than Erdman but showed it to have a comparable growth rate to H37Rv and the two additional recent clinical isolates. This study also found CDC 1551 induces granulomatous differentiation and high cytokine levels in the lungs of infected mice at an earlier time point than any of the other strains studied. When human monocytes were infected with CDC 1551, larger amounts of cytokines were produced than in monocytes infected with H37Rv, although there were similar numbers of intracellular organisms (Manca *et al.*, 1999). It was therefore suggested that CDC 1551 is not more virulent but induces a more rapid and vigorous immune response (Manca *et al.*, 1999). The two strains have also been compared in a rabbit inhalation model (Bishai *et al.*, 1999) where it was shown that CDC 1551 has no greater ability either to induce visible pulmonary tubercles or to multiply within these tubercles.

Comparison of the proteome of strains may therefore explain some of these phenotypic differences and may shed light on the mechanism of survival of *M. tuberculosis* within the host. This information is crucial for the design of new anti-tubercular vaccines and drugs. Since the virulence of *M. tuberculosis* of H37Rv in man is unknown, comparison of its proteome with that of a recent clinical isolate will also test its relevance for use in studies aimed at the combat of human disease. Several recent studies of the proteome of *M. tuberculosis* have been reported including the analysis of cell lysates, culture-filtrate proteins and the response of *M. tuberculosis* to different environmental conditions (Lee & Horwitz, 1995; Garbe *et al.*, 1996, 1999; Sonnenberg & Belisle, 1997; Urquhart *et al.*, 1997, 1998; Weldingh *et al.*, 1998; Wong *et al.*, 1999). The most comprehensive study to date, in terms of protein spot identification, compared two strains of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) and the *M. tuberculosis* strains H37Rv and Erdman (Jungblut *et al.*, 1999). These data have been deposited in a database accessible via the internet (Mollenkopf *et al.*, 1999; <http://www.mpiib-berlin.mpg.de/2D-PAGE/>). Most recently, subcellular fractions of *M. tuberculosis* H37Rv have been analysed by two-dimensional (2D) PAGE and several proteins

identified (Rosenkrands *et al.*, 2000). These data are also available via the internet (<http://www.ssi.dk/en/forskning/tbimmun/tbhjemme.htm>).

In this study we have compared the protein profiles of cell lysates of *M. tuberculosis* H37Rv and CDC 1551, utilizing commercially available immobilized pH gradients (IPGs) of pH 4–7 and pH 3–10 over a three point time course. We have been able to resolve approximately 1750 distinct protein spots and show that the protein profiles of these two strains remain remarkably similar. Proteins which were identified as being different between the strains or differed with time have been characterized by MS (reviewed by Humphery-Smith *et al.*, 1997). In addition, we have compared differences seen experimentally at the protein level to those predicted from comparison of the two genome sequences.

METHODS

Bacterial culture. *M. tuberculosis* H37Rv (NCTC 7416) and CDC 1551 (I. Orme, Colorado State University, USA) were grown without shaking in 10 ml Middlebrook 7H9 media supplemented with 0.2% (v/v) glycerol, 10% albumin-dextrose-catalase (ADC) and 0.025% Tween 80 at 37 °C. Optical densities at 550 nm were measured on a plate reader (Victor) using 200 µl culture and adjusted for 1 cm path length.

Sample preparation for two-dimensional (2D) electrophoresis. Whole-cell extracts were prepared from *M. tuberculosis* by harvesting 2 × 10 ml culture for each strain after 5, 8 and 12 d growth. Cultures were pooled and pelleted by centrifugation (1900 g, 15 min). Cell pellets were washed twice in PBS/1% (v/v) Tween 80. Mycobacteria were resuspended in lysis buffer containing 0.3% (w/v) SDS, 200 mM DTT, 28 mM Tris/HCl, 22 mM Tris base, 1 mM PMSF and Complete protease inhibitor cocktail (Roche Molecular Biochemicals), and heat-killed at 80 °C for 20 min prior to bead disruption in a ribolyser (Hybaid). Lysates were then clarified by centrifugation and the supernatant removed. The protein concentration of each sample was estimated using the Bradford assay (Bradford, 1976).

2D electrophoresis. Isoelectric focusing sample loading buffer (8 M urea, 4%, w/v, CHAPS, 40 mM Tris base, 65 mM DTT, trace amount of bromophenol blue) was added to the samples and each separated using pH 4–7 and pH 3–10 (non-linear) 18 cm IPG strips (Amersham Pharmacia Biotech) in the first dimension. Total protein (50 µg) was loaded at the cathode and proteins focused as follows using a Multiphor II (Amersham Pharmacia Biotech): 300 V for 2 h, 1000 V for 1 h and 3500 V for 22 h. Second-dimension electrophoresis was carried out essentially as described by O'Farrell (1975), using large format (20 × 20 cm), 1.5 mm thick, 12% polyacrylamide gels. IPG strips were equilibrated prior to running on the second dimension as described (Bjellqvist *et al.*, 1993; <http://www.expasy.ch/ch2d/protocols/>) but using 0.375 M Tris/HCl, pH 8.8, and 15 min incubation times. Strips were then overlaid onto the second-dimension gels and sealed with 0.5% (w/v) agarose in cathode buffer, containing a trace amount of bromophenol blue. Anode and cathode buffers as described by Herbert *et al.* (1998) were used. For protein spot comparisons, gels were silver stained using an ammoniacal stain as described (Bjellqvist *et al.*, 1993; <http://www.expasy.ch/ch2d/protocols/>). For subsequent MS, gels were rerun and silver stained as described by Shevchenko *et al.* (1996a) with some

modifications (Betts & Smith, 2000). Gel images were digitized by scanning on a flat-bed scanner (Epson GT9000).

Sample preparation for mass spectrometry. Protein spots of interest were excised from the gel, reduced, carboxyamidated and digested *in situ* with trypsin as described by Jensen *et al.* (1999). After digestion overnight at 37 °C, samples were centrifuged and an aliquot of the supernatant taken for analysis by MALDI (matrix-assisted laser desorption-ionization) MS. Prior to nanoelectrospray analysis, peptides were extracted from the gel pieces as described by Jensen *et al.* (1999).

MALDI-MS. This was performed on a VG ToFSpec SE time-of-flight (TOF) mass spectrometer equipped with a delayed extraction ion source (Micromass). Samples were prepared largely as described by Jensen *et al.* (1996). A saturated solution of α -cyano-4-hydroxycinnamic acid in acetone was mixed in a 4:1 ratio (v/v) with a 10 g l⁻¹ solution of nitrocellulose (Trans-Blot transfer medium, 0.45 µm; Bio-Rad) in acetone/2-propanol (1:1, v/v). This was deposited on the stainless steel target in 0.6 µl aliquots, leaving a matrix/nitrocellulose surface by fast evaporation of the solvent. Aliquots (0.5 µl) were taken from the tryptic digest mixture and loaded into a 0.5 µl droplet of 5% (v/v) formic acid previously applied to the matrix. Samples were air dried at room temperature and washed with 1 µl 5% (v/v) formic acid prior to insertion into the instrument. Spectra were internally calibrated using the matrix ion at m/z 1060.10 and trypsin autolysis peaks at m/z 2163.06 and m/z 2289.15. Mono-isotopic masses were assigned and proteins identified by peptide mass fingerprinting using PepSea software (Protana) and a mass accuracy of 0.1 Da.

Nanoelectrospray MS. Dried digest mixtures were desalted prior to nanoelectrospray analysis. Pulled glass capillaries were packed with approximately 5 µl POROS R2 sorbent (PerSeptive Biosystems). Peptides were dissolved in 0.5% (v/v) formic acid, loaded on to the sorbent and washed with 5 µl 0.5% (v/v) formic acid. Samples were eluted with approximately 2 µl 1% (v/v) formic acid, 50% (v/v) methanol and 1 µl inserted into the spraying needle. Needles for electrospraying were made with a micropipette puller (Sutter Instrument) from borosilicate glass capillaries (Clark Electromedical Instruments) as described by Wilm & Mann (1994, 1996) and were gold coated in a vapour desorption instrument. Electrospray mass spectra were acquired on an API III triple quadrupole machine (Perkin-Elmer Sciex) equipped with a nanoelectrospray ion source (Wilm & Mann, 1994, 1996). Proteins were identified by the sequence tag approach using PepSea software (Protana).

Genome comparison. The MUMmer program (Delcher *et al.*, 1999) was used to align and compare the genome sequences of *M. tuberculosis* H37Rv and CDC 1551 using a MUMmer cut off of 50 bp. The genome sequences of H37Rv and CDC 1551 were downloaded from the Sanger Center, Hinxton, Cambridge, release date 17 June 1998 (http://www.sanger.ac.uk/Projects/M_tuberculosis/) and The Institute for Genome Research, release date 4 June 1999 (<http://www.tigr.org/tdb/CMR/gmt/htmls/SplashPage.html>), respectively.

RESULTS

Comparison of 2D protein patterns of *Mycobacterium tuberculosis* strains H37Rv and CDC 1551

Comparison of the protein patterns of H37Rv and CDC 1551 were made at selected time points. Cultures of each

Fig. 1. Growth curves for *M. tuberculosis* H37Rv (▲) and CDC 1551 (■) over the time course studied.

strain were grown in duplicate and pooled for 2D electrophoretic analysis. Duplicate gels were run for each sample and silver stained. Care was taken to ensure that all gels were stained to the same extent; however, variability was seen amongst very faint spots just at the silver-stain detection limit. These spots were therefore excluded from our comparisons. For subsequent identification of protein spots, gels were rerun, again in duplicate, and stained with a silver stain compatible with mass spectrometry though less sensitive than the initial ammoniacal stain used. This also served to confirm the reproducibility of spot differences observed in the initial analysis. The differences detailed here were found to be consistent with both silver-staining protocols used. Fig. 1 shows that the growth of the two strains was similar up until day 8. By day 12, however, CDC 1551 seemed to be entering stationary phase whilst H37Rv was still in late exponential phase. Despite this, the resulting protein spot patterns were found to be both highly reproducible and highly comparable between the two strains over all time points. The 2D protein patterns also varied little across the time course studied. Approximately 1500 protein spots were visualized on the pH 4–7 gels and 1100 spots on the pH 3–10 gels, with approximately 250 of these being unique to the pH 3–10 range and hence giving a combined total of 1750. Since the majority of proteins seemed to be in the acidic range, increased resolution of this area on the pH 4–7 gels significantly aided individual spot resolution and comparison of protein species within this region. One pH 4–7 gel representative of each time point for each strain is shown in Fig. 2.

Table 1 summarizes the protein spot differences and proteins identified. Only consistent differences obvious by visual examination have been included. A total of 17 consistent protein spot differences were seen, comprising both differences between the two strains and common differences between the three time points. Gel regions displaying spot differences between the strains are surrounded by boxes on Fig. 2 and shown in more detail in Fig. 3. Protein spots differing with time of culture are numbered on Fig. 2. Seven protein spots (spots 1–7) seemed to be present only in CDC 1551. Three spots (spots 8–10) seemed to be exclusive to H37Rv and a

Fig. 2. 2D gels of *M. tuberculosis* over the three-point time course. (a) H37Rv, day 5; (b) H37Rv, day 8; (c) H37Rv, day 12; (d) CDC 1551, day 5; (e) CDC 1551, day 8; (f) CDC 1551, day 12. Protein spots changing over the time course are ringed and their identities listed in Table 1. Regions where protein spots differ between strains are surrounded by boxes (labelled A–F) and shown in detail in Fig. 3. Key landmark proteins previously identified by MS are marked on (a).

Table 1. Protein spot differences observed for the time-point proteome analysis of *M. tuberculosis* H37Rv and CDC 1551

Spots not detected represented by –. Spots present represented by +. Increased spot intensity compared to other strain/time point represented by ++. Vertical mobility shift represented by M.

Spot no.	Protein	Short name	Rv no.	MT no.	MALDI mass mapping		H37Rv day 5	H37Rv day 8	H37Rv day 12	CDC 1551 day 5	CDC 1551 day 8	CDC 1551 day 12
					No. matched peptides	Sequence coverage (%)						
1	Probable alcohol dehydrogenase	–	Rv0927c	MT0954	4	16	–	–	–	+	+	+
2	Transcriptional regulator, MoxR homologue	MoxR	Rv1479	MT1526	15	42	–	–	–	+	+	+
3	Transcriptional regulator, MoxR homologue	MoxR	Rv1479	MT1526	20	63	–	–	–	+	+	+
4	Not identified						–	–	–	+	+	+
5	Not identified						–	–	–	+	+	+
6	Not identified						–	–	–	+	+	+
7	Not identified						–	–	–	+	+	++
8	Transcriptional regulator, MoxR homologue	MoxR	Rv1479	MT1526	4	12	+	+	+	–	–	–
9	Transcriptional regulator, MoxR homologue	MoxR	Rv1479	MT1526	13	45	+	+	+	–	–	–
10	Phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide isomerase	HisA	Rv1603	MT1639	3	22	+	+	+	–	–	–
11	Alkyl hydroperoxide reductase chain C	AhpC	Rv2428	MT2503	12	79	++	++	++	+	+	+
12	Alkyl hydroperoxide reductase chain C	AhpC	Rv2428	MT2503	4	31	++	++	++	+	+	+
13	Ribosome recycling factor	Rrf	Rv2882c	MT2949	7	51	M	M	M	M	M	M
14	Putative Hsp20	–	Rv0251c	MT0265	5	47	+	++	++	+	++	++
15	Not identified						+	++	++	+	++	++
16	16 kDa antigen, α -crystallin homologue	HspX	Rv2031c	MT2090	7	57	+	+	++	+	+	++
17	16 kDa antigen, α -crystallin homologue	HspX	Rv2031c	MT2090	6	37	+	+	++	+	+	++

further two (spots 11 and 12) were of increased intensity in H37Rv relative to CDC 1551. One spot (spot 13) showed differing vertical mobility between the two strains. Proteins were identified using a hierarchical approach (Shevchenko *et al.*, 1996b), combining peptide mass fingerprinting (Henzel *et al.*, 1993; James *et al.*, 1993; Mann *et al.*, 1993; Pappin *et al.*, 1993; Yates *et al.*, 1993) by MALDI-TOF MS as a rapid first-pass technique with tandem mass spectrometric sequencing by nano-electrospray (Shevchenko *et al.*, 1996a; Wilm *et al.*, 1996) used for any protein spots remaining unidentified. Proteins identified were assigned the appropriate Rv numbers, relating to the H37Rv genome (Cole *et al.*, 1998), and corresponding MT numbers, relating to the CDC 1551 genome (<http://www.tigr.org/tdb/CMR/gmt/htmls/SplashPage.html>).

Of the 17 spots analysed, 12 were identified unambiguously by MALDI-MS alone. Details of the number of matched peptides and the percentage sequence coverage obtained for each protein spot are given in Table 1. Criteria used to determine a positive identification were at least three matching peptides with 0.1 Da mass accuracy. The pI and M_r of the identified protein were also checked against the spot position on the gel. Further analysis of the remaining spots by nanoelectrospray failed to give an identification. Mass spectrometry analysis of these differences revealed that four spots (2, 3, 8 and 9) (Fig. 3a) were mobility variants of the transcriptional regulator MoxR (Rv1479/MT1526). These varied in their comparative presence at each position between the strains, with H37Rv displaying two forms and CDC 1551 a different two, and

may be explained by either amino acid changes or post-translational modifications. The most intense form present in H37Rv was observed in a more basic position on the gel relative to the most intense form present in CDC 1551. The other H37Rv spot corresponding to this protein was also in a more basic position than the second CDC 1551 spot. Mobility variants of this protein have also been observed between the strains H37Rv and Erdman (Jungblut *et al.*, 1999), with the protein being shifted to a more acidic position in the Erdman strain. One spot, identified as ribosome recycling factor (Rv2882c/MT2949), showed a marked vertical mobility shift between the two strains, being consistently in a higher M_r position on the H37Rv gels (spot 13) (Fig. 3b). Spot 1 (Fig. 3b), corresponding to Rv0927c, a probable alcohol dehydrogenase, was absent from the H37Rv proteome, whilst spot 10 (Fig. 3d), corresponding to phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide isomerase (HisA; Rv1603/MT1639), was absent from the CDC 1551 proteome. Two spots corresponding to alkyl hydroperoxide reductase chain C (AhpC; Rv2428/MT2503) were identified and both were decreased in intensity in CDC 1551 (spots 11 and 12) (Fig. 3d). This protein has also been found to be decreased in intensity in H37Rv relative to *M. bovis* BCG Chicago (Jungblut *et al.*, 1999).

Four proteins (spots 14–17) showed increased intensity over time in both strains (Fig. 2). Spot 14 was identified as the heat-shock protein, Hsp20 (Rv0251c/MT0265) and showed increased spot intensity on days 8 and 12 compared to day 5. Spots 16 and 17 were identified as the 16 kDa antigen (alpha-crystallin) (Rv2031c/MT2031c)

Fig. 3. Gel sections showing differences in protein spots detected between *M. tuberculosis* H37Rv and CDC 1551. Boxed regions (A–F) indicated on Fig. 2 are displayed in panels (a)–(f) respectively. Spot identities are listed in Table 1.

and showed increased intensity on day 12 compared to days 5 and 8.

Comparison of proteome data with differences observed in the genomes of *M. tuberculosis* H37Rv and CDC 1551

Comparison of the genome sequences of the two strains was carried out using the MUMmer program (Delcher

et al., 1999). This enabled the mapping of single nucleotide changes and insertions and deletions present in each genome relative to the other. The majority of differences were found to be single nucleotide changes. Approximately 1000 of these were identified. One of the observed proteome differences correlates with a single nucleotide change. This occurs in the *moxR* gene (Rv1479/MT1526), where there is a base change from C in H37Rv to A in CDC 1551, resulting in a predicted

Table 2. Predicted protein differences resulting from genomic sequence present in H37Rv but not CDC 1551

Genome position*		Size (bp)	Rv no.	M_r †	pI†	MT no.	M_r †	pI†	Protein‡
Start	End								
32351	32388	37	Rv0029	39559	7.88	MT0033	17082	9.02	Conserved hypothetical
32720	32725	4							
183857	183861	3	Rv0155	37693	4.90	—	—	—	Transhydrogenase alpha subunit
333547	339002	5452	Rv0278c	81904	11.16	—	—	—	PE-PGRS
			Rv0279c	68986	4.06	—	—	—	PE-PGRS
427358	427373	15	Rv0355c	327030	4.11	MT0370	324564	4.11	PPE
836082	840993	4910	Rv0745	18648	11.53	MT0772	28088	11.53	Conserved hypothetical
			Rv0746	64158	3.97	—	—	—	PE-PGRS
			Rv0747	65407	4.43	—	—	—	PE-PGRS
			Rv0748	9114	9.92	MT0772	28088	11.53	Conserved hypothetical
886543	887417	874	Rv0792c	28959	8.54	MT0817	67988	6.38	GntR transcriptional regulator
			Rv0793	11192	5.61	—	—	—	Conserved hypothetical
			Rv0794c	52476	5.61	MT0817	67988	6.38	Dihydrolipoamide dehydrogenase
1212121	1212199	78	Rv1087	62469	4.18	—	—	—	PE-PGRS
1217504	1218158	58	Rv1091	68317	4.19	MT1123	51726	4.02	PE-PGRS
2163788	2163926	138	Rv1917c	140612	3.75	MT1968	146131	3.84	PPE
2165426	2165502	76							
2180804	2180826	22	Rv1928c	27031	5.46	—	—	—	Short-chain alcohol dehydrogenase
2357283	2357286	3	Rv2098c	36529	4.34	MT2159	41978	4.33	PE-PGRS
			Rv2099c	5510	4.32				
2372491	2372548	57	Rv2112c	60129	5.70	MT2172	58256	5.47	Conserved hypothetical
2714308	2714808	500	Rv2416c	44418	5.97	MT2489	51387	6.37	Conserved hypothetical
3054718	3054931	213	Rv2741	44210	4.40	MT2812	43562	5.56	PE-PGRS
3171570	3171624	54	Rv2859c	32410	5.39	MT2927	32684	5.86	Conserved hypothetical
3663929	3663992	63	Rv3281	19014	4.48	MT3380	16887	4.54	Conserved hypothetical
3730578	3735861	5283	Rv3343c	250346	4.09	MT3446	34947	5.94	PPE
						MT3447	56229	6.89	
3739705	3739894	189	Rv3345c	129385	9.58	MT3449	126768	9.52	PE-PGRS
3933523	3936659	3136	Rv3508	147625	3.70	MT6312	194124	10.08	PE-PGRS
3955465	3956104	639	Rv3518c	44399	4.70	MT3619	41439	4.72	Cytochrome P450
			Rv3519	25960	9.66	MT3620	7827	10.81	Unknown
4359144	4359163	19	Rv3879c	74492	5.33	MT3993	73908	5.33	Unknown

* Genome position of genomic sequence present in H37Rv but not CDC 1551.

† Predicted M_r and pI values calculated from the ExPASy compute pI/Mw tool web site (http://www.expasy.ch/tools/pi_tool.html).

‡ Annotations taken from the TubercuList web site, Institute Pasteur (<http://genolist.pasteur.fr/TubercuList/>).

amino acid change from histidine-26 in H37Rv to asparagine-26 in CDC 1551 and consequently differences in the predicted pI and M_r values of the two proteins. This point mutation is contained within the N-terminal tryptic peptide of the protein. However, this peptide was not mapped during the MALDI analysis to confirm the amino acid change. This can be explained by the fact that the peptide, minus the first methionine residue, has a calculated M_r of 4123.94 Da or 4101.91 Da for H37Rv or CDC 1551 respectively, meaning that it falls outside the range scanned during our MALDI analysis, as we routinely only cover a range up to m/z 4000. The predicted pI value (http://www.expasy.ch/tools/pi_tool.html) for the H37Rv protein is 5.96 and that for the CDC 1551 protein is 5.89. This is reflected in the 2D gel spot pattern, where the major MoxR spot in H37Rv (spot 9) was shifted to a more basic position relative to the major CDC 1551 MoxR spot (spot 3) (Fig. 3a). This doesn't however explain the presence of a second form of MoxR in each strain, which could be caused by post-translational modification. The predicted M_r values for the two proteins are 40762 Da and 40739 Da for H37Rv and CDC 1551 respectively. The resolution of the gel in the M_r range is not sufficient for this difference to be observed. It is also interesting to note that, although not

containing a single nucleotide change itself, the *hisA* gene (Rv1603/MT1639) lies directly downstream of *hisH* (Rv1602/MT1638), which contains a single nucleotide change resulting in an amino acid difference at the C terminus of the protein. We have shown a spot corresponding to HisA to be absent from the CDC 1551 proteome but present in the H37Rv proteome.

Insertions or deletions in the genome which result in predicted pI or M_r differences at the protein level between the two strains are summarized in Tables 2 and 3. We have limited our comparison to regions which appeared to be unique to each genome and were at least 3 bp in size. Insertion elements differing between the two strains have not been included in Tables 2 and 3. The majority of unique coding material found only in the H37Rv genome, with respect to CDC 1551, affected insertion elements or the acidic, glycine-rich proteins of the PE (Pro-Glu) and PPE (Pro-Pro-Glu) protein families which often contain multiple copies of the polymorphic G + C rich sequences (PGRSs) or major polymorphic tandem repeats, respectively (Cole *et al.*, 1998). Our analysis found eight ORFs completely unique to H37Rv, five of these belonging to the PE-PGRS family (Rv0278c, Rv0279c, Rv0746, Rv0747 and Rv1087) and one being a

Table 3. Predicted protein differences resulting from genomic sequence present in CDC 1551 but not H37Rv

Genome position*		Size (bp)	Mt no.	M_r †	pI†	Rv no.	M_r †	pI†	Protein‡
Start	End								
150883	151063	180	MT0132	46525	5.04	Rv0124	40891	4.90	PE-PGRS
477981	479339	1358	MT0415	16135	6.27	Rv0403c	15261	6.64	Small membrane protein
619256	619340	84	MT0556	49170	4.17	Rv0532	50835	4.39	PE-PGRS
1111473	1111488	15	MT1033	40686	6.07	Rv1004c	38754	4.78	Possible exported protein
1432687	1432743	56	MT1328	54536	9.13	Rv1290c	55996	9.42	Unknown
1470228	1471902	1674	MT1360	57889	9.59	Rv1319c	58098	9.79	Possible adenylate cyclase
			MT1361	60720	9.76				
1601854	1601860	6	MT1478	4820	4.79	Rv1434	4606	5.09	Unknown
1602221	1602242	21	MT1479	20494	5.02	Rv1435c	18488	4.10	Hypothetical conserved
1874909	1874919	10	MT1707	65021	6.21	Rv1668c	40339	9.57	Probable ABC transporter
1963756	1963916	160	MT1796	109674	4.16	Rv1753c	104650	4.16	PPE
1968420	1975229	6809	MT1799	57066	6.35	Rv1755c	29970	7.13	Phospholipase C
			MT1800	41288	10.04	—	—	—	Glycosyl transferase
			MT1801	40756	4.90	—	—	—	Oxidoreductase
			MT1802	99509	6.62	—	—	—	Membrane protein
2120399	2120414	15	MT1931	48900	8.10	Rv1882c	30010	9.61	Probable dehydrogenase
						Rv1883c	17281	8.71	Conserved hypothetical
2150367	2150634	276	MT1968	146131	3.84	Rv1917c	140612	3.75	PPE
2255757	2260757	5000	MT2080	26192	8.89	—	—	—	Conserved hypothetical
			MT2081	34444	8.18	—	—	—	Conserved hypothetical
			MT2082	177866	5.48	Rv2024c	57274	5.65	Conserved hypothetical
2619675	2621718	2043	MT2420	10000	4.97	—	—	—	Conserved hypothetical
			MT2421	11778	4.98	—	—	—	Conserved hypothetical
			MT2422	40092	4.50	—	—	—	PPE
			MT2423	59491	4.05	Rv2353c	34069	4.22	PPE
2852392	2853048	656	MT2619	27086	6.19	—	—	—	Putative lipoprotein
			MT2620	27112	5.96	Rv2544	24175	6.19	Putative lipoprotein
2980704	2980806	102	MT2754	31680	4.47	Rv2680	22555	4.51	Conserved hypothetical
3361395	3362754	1359	MT3098	12334	4.24	Rv3018c	42997	4.08	PPE
			MT3101	31983	4.17				
3514231	3516381	2150	MT3247	57661	4.19	Rv3159c	56586	4.22	PPE
			MT3248	66937	4.51				
4022344	4022455	111	MT3715	29829	12.00	Rv3611	23815	11.93	Unknown

* Genome position of genomic sequence present in CDC 1551 but not H37Rv.

† Predicted M_r and pI values calculated from the ExPASy compute pI/Mw tool web site (http://www.expasy.ch/tools/pi_tool.html).

‡ Annotations taken from the TubercuList web site, Institute Pasteur (<http://genolist.pasteur.fr/TubercuList/>) or from the TIGR *M. tuberculosis* genome database (<http://www.tigr.org/tdb/CMR/gmt/htmls/SplashPage.html>) for ORFs unique to CDC 1551.

hypothetical protein (Rv0793). Rv0278c and Rv0279c are contained within a 5452 bp region which is missing from CDC 1551. Similarly, Rv0746 and Rv0747 are contained within a 4910 bp region which is deleted in CDC 1551 relative to H37Rv. Part of Rv0748 is also contained within this deleted region. As a result of this large deletion the ORF predictions in this region differ between the two strains. Rv0745 is present at the DNA level in CDC 1551; however, the corresponding ORF in CDC 1551 (MT0772) has a different predicted start site and is frame-shifted. MT0772 is predicted to span the deleted region and its C terminus corresponds to that of Rv0748. Deletions in CDC 1551 in the region of the remaining two ORFs (Rv0155 and Rv1928c) seem to result in frameshifts, producing numerous stop codons, which are likely to mean that these regions are non-coding in CDC 1551.

The CDC 1551 genome seems to contain nine unique ORFs. Eight of these are contained within three separate large deletions from H37Rv. MT1800–MT1802 lie within a 6809 bp stretch of DNA which is absent from H37Rv. This region corresponds to RvD2 which has been found to be deleted in H37Rv with respect to *M.*

bovis and *M. bovis* BCG (Gordon *et al.*, 1999), and also the attenuated *M. tuberculosis* strain H37Ra (Brosch *et al.*, 1999). MT1800 is annotated as a glycosyl transferase and a BLASTP search of a non-redundant protein database showed it to have significant similarity to sugar transferases and proteins involved in lipopolysaccharide biosynthesis. MT1801 is annotated as an oxidoreductase and shows considerable homology to eukaryotic sulphite oxidase genes. MT1802 is annotated as a membrane protein belonging to the MmpL family. This region also contains MT1799, which is a full-length version of phospholipase C (*plcD* gene) that is interrupted by an IS6110 insertion in H37Rv (Rv1755c). MT2080 and MT2081 are both annotated as hypothetical and lie within a 5000 bp region deleted in H37Rv relative to CDC 1551. This region is also present in *M. bovis* and *M. bovis* BCG (Gordon *et al.*, 1999). MT2082, also in this region, is an extended version of the H37Rv gene Rv2024c which bears homology to a hypothetical protein from *Helicobacter pylori* (TrEMBL accession no. O25380). MT2420–MT2422 are contained within a 2043 bp region deleted from H37Rv. Eight further genes in this region have been found to be deleted in *M. bovis* and *M. bovis* BCG (Behr *et al.*, 1999; Gordon *et al.*,

1999). All three ORFs are annotated as hypothetical; however, MT2420 shows homology to proteins of the ESAT-6 family (Berthet *et al.*, 1998; Tekaia *et al.*, 1999). Immediately upstream is MT2421 which shows 95 % identity with the QILSS family of proteins, so called because of a distinctive QILSS motif at the C terminus. In the *M. tuberculosis* H37Rv genome this family consists of six members all of which occur upstream of ESAT-6 ORFs (Tekaia *et al.*, 1999). MT2422 shows a high level of homology to MT2419/Rv2352c, which encodes a PPE protein. Upstream of this region in the H37Rv sequence are two ORFs (Rv2354 and Rv2355) not present in CDC 1551, which code for a transposase of an IS6110 insertion element. This seems to have disrupted the gene immediately upstream (Rv2353c) which corresponds to 5' truncation of MT2423.

The remaining predicted protein differences between the two strains are the result of small insertions or deletions at the DNA level, giving significant changes in predicted M_r and pI at the protein level. None of the protein spot differences observed in this study correspond to predicted changes resulting from these insertions or deletions. However, it is possible that the four remaining unidentified proteins correspond to some of these predicted protein differences.

DISCUSSION

The availability of genome sequence data for two virulent strains of *M. tuberculosis* (Cole *et al.*, 1998; <http://www.tigr.org/tdb/CMR/gmt/htmls/SplashPage.html>) has facilitated the study of the proteome of this organism. Here we describe the comparison of the proteome of these two strains, a lab strain (H37Rv) and a recent clinical isolate (CDC 1551). After its outbreak in 1995, initial reports suggested that CDC 1551 was a highly virulent strain of *M. tuberculosis* based on the high rate of skin-test conversion in contacts to the index case, the unusually large PPD (purified protein derivative of tuberculin) reactions observed and its increased growth in mice compared to *M. tuberculosis* Erdman (Valway *et al.*, 1998). Due to serial passage and, more importantly, its unknown virulence in humans, concerns have been raised as to the relevance of *M. tuberculosis* H37Rv as a model for the study of human disease (Jacobs *et al.*, 1996). More recently both *in vivo* and *in vitro* models have been used to compare the growth of, and host response to, *M. tuberculosis* CDC 1551, H37Rv, Erdman and two other clinical isolates (Manca *et al.*, 1999). This study led to the conclusion that CDC 1551 is not more virulent than other strains of *M. tuberculosis* but induces a more vigorous host response.

In our proteome comparison of these strains, it was hoped that any protein differences seen may give some insight into the reasons for the differing phenotypes observed, aiding the design of new drugs and vaccines against tuberculosis. Additionally, these studies provide a useful background reference for future proteome

experiments and, in comparing its protein profile to that of a recent clinical isolate, test the relevance of H37Rv as a model for human infection.

Strains were compared over a three-point time course. This is an important precursor to further proteome analyses. The protein patterns of the two strains were stable and highly similar over the time course studied. Only 13 protein spots were seen to differ between the two strains. This is consistent with the comparison of *M. tuberculosis* H37Rv and Erdman, which revealed 18 variant proteins (Jungblut *et al.*, 1999). Four of the variants described here correspond to different mobility variants of the MoxR protein, which is a probable regulatory protein involved in the formation of active methanol dehydrogenase. Mobility variants of MoxR were also observed in the comparison of H37Rv with Erdman (Jungblut *et al.*, 1999). Genome sequence comparison of the *moxR* gene in H37Rv and CDC 1551 revealed a single nucleotide change, resulting in a predicted amino acid difference and consequent pI change, explaining the presence of mobility variants of this protein. The *M. tuberculosis* Erdman genome has not been sequenced. However, in the light of our findings, it is likely that the mobility variants of MoxR, described by Jungblut *et al.* (1999), are also a result of a single nucleotide change, and hence amino acid difference, in this protein between H37Rv and Erdman. A spot corresponding to Rv0927c/MT0954, a probable short-chain alcohol dehydrogenase, was found to be present on gels of CDC 1551 but not H37Rv. The gene encoding this protein is, however, present in the H37Rv genome. Similarly, a spot corresponding to HisA, an isomerase which catalyses the fourth step in the histidine biosynthetic pathway, was present on gels of H37Rv but not CDC 1551, although the corresponding gene is present in the CDC 1551 genome. The *hisA* gene forms part of an operon of histidine biosynthetic genes. The levels of the other proteins encoded within this operon, however, were not detected as differences in this study. These differences are most likely to be explained either by subtle expression-level differences between the two strains at the point in time studied, meaning that the absent protein spots are present but below the silver-stain detection limit, by post-translational modification or by degradation during sample preparation. Interestingly, AhpC was found to be increased in intensity in H37Rv relative to CDC 1551. This protein has also been found to be increased in intensity in *M. bovis* BCG compared to *M. tuberculosis* H37Rv (Jungblut *et al.*, 1999).

The protein profile of each strain also seemed similar across the three time points studied, with only a small number of protein spots changing in intensity with time. Two of these were identified as the 16 kDa antigen (α -crystallin), which has been shown to be upregulated as the bacterium enters stationary phase (Yuan *et al.*, 1996). However, this protein is also found as an intense spot higher up the gel (Fig. 2a) so it is likely that these spots are due to degradation or post-translational processing. This observation has been made previously

(Sonnenberg & Belisle, 1997), where lower molecular mass spots were found to correspond to N-terminal truncation or degradation by Edman sequencing. The intensity of the spot representing the main form of this protein does however seem to be increased somewhat on day 12 relative to days 5 and 8. Another heat-shock protein, Hsp20, also shows increased intensity on days 8 and 12 in both strains.

Of the differences that we have identified in this proteome comparison, only those in the MoxR protein relate to those predicted from the genome comparison. There are several explanations for this discrepancy. Firstly, some of the proteins predicted to differ between strains lie outside the region resolved on our 2D gels; that is a M_r above 100 kDa or below 10 kDa, or a pI above 10.0. Secondly, we have resolved approximately 1750 protein spots. At best this accounts for 44% of the 3924 predicted genes in *M. tuberculosis* H37Rv. However, some spots visualized here appear to be spot series of the same protein, perhaps caused by amino acid modifications during sample preparation. Whilst it is extremely unlikely that all predicted proteins are expressed at the same point in time, it is likely that certain low-abundance proteins are masked by the more abundant species on our gels, particularly in the acidic region where the majority of mycobacterial proteins seem to lie, or are below the detection limit of silver staining. It has been suggested that proteins present at less than 1000 copies per cell cannot currently be detected by 2D electrophoresis (Wilkins *et al.*, 1998). The limitations of silver staining must also be considered. Although the ammoniacal method utilized for comparison purposes here is extremely sensitive and care was taken to ensure equal intensity of gel staining, variation between protein spots at the silver-stain detection limit was observed and these low-level differences were not included in our comparison. The dynamic range of silver staining is also limited and certain proteins have been shown to stain poorly or not at all. Fluorescent stains, compatible with MS, have recently been developed and now approach the sensitivity of silver staining whilst overcoming the issues of dynamic range and reproducibility.

Some of the proteins predicted to differ are membrane proteins and it is very likely that these hydrophobic proteins are underrepresented on our gels as standard 2D techniques, such as those used in our study, often result in substantial losses of hydrophobic proteins due to solubility problems during sample preparation, precipitation in the first-dimension IPG or inefficient transfer to the second-dimension gel (Adessi *et al.*, 1997; Wilkins *et al.*, 1998). There have recently been a number of methods reported which improve protein solubilization in 2D electrophoresis (Rabilloud *et al.*, 1997; Herbert *et al.*, 1998; Molloy *et al.*, 1998; Santoni *et al.*, 1999). Such methods are likely to be required in order to visualize more of the mycobacterial proteome. To this end, it may also be necessary to study subcellular fractions of *M. tuberculosis* as described recently by Rosenkrands *et al.* (2000). Another approach may be to utilize several overlapping narrow-pH-range IPGs to

build up a composite proteome map and increase spot resolution (Cordwell *et al.*, 2000).

Lastly, many of the predicted differences are within PE or PPE genes, for which there is little functional information available and it is still unclear as to whether they are all expressed and at what level. The recently published mycobacterial 2D PAGE internet database, which contains information on approximately 260 proteins identified from 2D gels of *M. tuberculosis* and *M. bovis* BCG by MS, does not include any PE or PPE proteins (Mollenkopf *et al.*, 1999; <http://www.mpiib-berlin.mpg.de/2D-PAGE/>) However, inspection of the amino acid sequence of many of these proteins reveals them to have few trypsin cleavage sites. This would make identification by MS following tryptic digestion, as routinely used, almost impossible. Since the PE and PPE families of proteins have been implicated in antigenic variation in *M. tuberculosis* (Cole *et al.*, 1998), it is interesting to speculate that the variation in these proteins between the two strains may be significant with regard to their differing immunogenic properties (Manca *et al.*, 1999).

The genome comparison also reveals that CDC 1551 contains an extra gene (MT2420) that bears homology to the ESAT-6 family. The ESAT-6 protein is a major T-cell antigen which has been purified from *M. tuberculosis* culture filtrates (Sorensen *et al.*, 1995) and is recognized strongly by a high percentage of tuberculosis patients (Mustafa *et al.*, 1998; Ulrichs *et al.*, 1998; Ravn *et al.*, 1999). More recently, two further members of this family have been identified within culture filtrates and shown to induce interferon gamma release from lymphocytes isolated from tuberculosis patients (Skjot *et al.*, 2000). There are 14 members of the ESAT-6 family within H37Rv and all are situated in genomic loci showing strongly conserved structure and organization (Tekaiia *et al.*, 1999). Each of these genes are conserved in CDC 1551. It is possible that the extra copy of this gene in CDC 1551 may contribute to the increased host immune response to this strain (Manca *et al.*, 1999). Our study was focused on the analysis of cellular proteins. It is likely that an analysis of the culture filtrate would be required to reveal differences in the secreted antigens such as the ESAT-6 family of proteins. Our current studies are now directed toward the analysis of membrane and secreted proteins from *M. tuberculosis*.

Of the 17 silver-stained spots analysed here, 12 were identified by MALDI-MS alone giving a success rate of about 70%. The availability of the complete genome sequence of *M. tuberculosis* means that, in theory, it should be possible to identify the majority of proteins by peptide mass fingerprinting using the MALDI data alone. We have used a protein loading of approximately 50 µg for our analyses and pooled spots from duplicate gels. The protein spots which remain unidentified are low in abundance as judged by the silver-stain intensity and it is therefore likely to be advantageous to increase protein loading on gels for subsequent mass spectrometry analysis. Another alternative is to use HPLC

combined with tandem mass spectrometry (LC/MS/MS) for the analysis of these protein spots. We have recently found this method to have increased sensitivity over the nanoelectrospray technique employed in this study (unpublished results).

In conclusion, we have performed a rigorous proteome comparison of two virulent strains of *M. tuberculosis* for which there is genome sequence available. The proteome patterns of the two strains grown in broth are highly similar which is in accordance with their high sequence similarity. This suggests that *M. tuberculosis* H37Rv is indeed a valid laboratory model for the study of the mechanisms of human disease. To gain more insight into the differing phenotypes displayed by these two strains it may be necessary to perform proteome analysis on mycobacteria isolated from conditions where these phenotypes are observed; for example macrophages or animal models of infection. In contrast to the genome level, differences seen at the proteome level are likely to reflect variability in translational control and post-translational modification between the strains. Despite the limitations discussed here, 2D gel-based proteomics remains a powerful approach toward the functional understanding of the proteins encoded within the mycobacterial genome. Technologies are constantly being improved and the problems associated with 2D gels are being eliminated. This study provides a useful and necessary background reference for future studies where the genes responsible for virulence, intracellular survival or drug resistance may be identified through the analysis of mutant strains or mycobacteria subjected to differing environmental conditions. In turn, this may provide us with targets for the development of novel therapies against tuberculosis.

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