Extracytoplasmic proteins of *Mycobacterium tuberculosis* – mature secreted proteins often start with aspartic acid and proline

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A surrogate expression system, based on fusions to the *phoA* bacterial reporter gene, was used to identify *Mycobacterium tuberculosis* genes that encode exported proteins and the promoter regions required for their expression in the heterologous host *Mycobacterium smegmatis*. To assess these results in the context of the complete *M. tuberculosis* genome sequence, the corresponding genes were identified and computational algorithms were employed to identify signal peptide (SP), transmembrane domain and membrane lipoprotein attachment motifs. This information was used to predict the subset of *M. tuberculosis* genes that encode exported proteins. Of the 34 genes identified by the *phoA* method, 22 were classified to encode potential soluble secreted proteins. Among these, 14 genes may encode novel secreted proteins. Six of the remaining 12 genes were predicted to encode membrane lipoproteins and an additional six to encode integral membrane proteins. Published observations of proteins proven to be secreted into *M. tuberculosis* culture filtrates were reviewed to further characterize the mycobacterial SP motif. It was concluded that mycobacterial SPs are comparable in size to Gram-positive SPs, but certain features are different. In particular, arginine was the predominant N-terminally positively charged amino acid in contrast to lysine in the Gram-positives. The hydrophobic transmembrane segment of the SP was dominated by alanine, in contrast to leucine. At the C-terminal end of the SPs, the (–3, –1) rule (AXA motif) holds, with alanine as the dominant amino acid in both positions, being most dominant in the (–1) position. A high proportion of mature sequences start with aspartic acid in the (+1) position and proline in the (+2) position – the DP motif. The authors propose that the DP sequence serves as a sorting signal, following translocation and cleavage by signal peptidase I. Alternatively, the DP motif may be part of the recognition site for the signal peptidase.

Keywords: *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, alkaline phosphatase A fusions, protein export, signal sequence

INTRODUCTION

A continuing challenge of the post-genomic era will be to integrate experimental data with the genome sequence. Most published annotation approaches for categorizing predicted ORFs of microbial genomes have been patterned after the functional categories defined for *Escherichia coli* by Riley (1993). Although this

approach has been exceptionally useful for initial gene groupings, its categorical approach limits each gene to a single assignment and fails to account for the complexities that are known to contribute to the biological properties of a gene. One proposal for improving this system is to add categorical depth to reflect each ORF's membership with regard to multiple properties, including subcellular localization, regulation, structural features, motifs and enzymic activities (Riley, 1998). The subcellular location has important biological significance and implications for vaccine and drug

Abbreviations: HMM, hidden Markov model; NN, neural network; PhoA, alkaline phosphatase A; SP, signal peptide; TM, transmembrane.

development strategies. In particular, proteins secreted across the cytoplasmic membrane are likely to be involved with cell wall biogenesis, mediate virulence or contain prominent antigenic determinants.

For *Mycobacterium tuberculosis*, the *phoA* reporter system has been established in a surrogate model and used to identify several exported proteins (Lim *et al.*, 1995). Fundamental to the successful identification of clones that confer positive PhoA activity are two features: (i) in-frame fusion with *phoA* identifies an ORF that is expressed by the cell; and (ii) enough of the N-terminal features of *M. tuberculosis* secreted proteins must be present to direct export of the chimeric protein across the cytoplasmic membrane.

This study was done to identify *M. tuberculosis* secreted proteins and to test the validity of computational model predictions. Proteins were evaluated for their potential to be located extracytoplasmically by one of three different criteria: (I) experimentally, by exhibiting PhoA positivity in *Mycobacterium smegmatis*; (II) computationally, by the presence of either a signal peptide (SP) or a transmembrane (TM) domain or a membrane lipoprotein attachment motif; and (III) by literature review to identify native *M. tuberculosis* proteins secreted into spent culture medium and which therefore are likely to possess authentic signal peptides.

METHODS

Strains and plasmid construction. *M. tuberculosis* strain 3609 is a clinical isolate that was provided by the San Francisco Department of Public Health. It was cultured in 7H10 medium (Difco) supplemented with bovine serum albumin and glucose (Jacobs *et al.*, 1991). *M. smegmatis*, strain mc²155, was a gift from William Jacobs (Albert Einstein College of Medicine). *E. coli* strain CC118 (*phoA*), was a gift from Colin Manoil (Genetics, University of Washington). Liquid cultures of mc²155 and CC118 were grown in Luria-Bertani (LB) broth at 37 °C with shaking; the medium for mc²155 was supplemented with 0.05% Tween 80 (Sigma). Toru Tobe (University of Tokyo, Japan) provided pBS.ATS, a derivative of the pBlue-script II KS cloning vector (Stratagene) (Sarmientos *et al.*, 1983). Douglas Young (Imperial College School of Medicine, London, UK) provided the shuttle vector pOLYG (Rauzier *et al.*, 1988).

A three-step cloning strategy was used to make the shuttle vector we used for creating the *phoA* fusions. The pJEM11 (Lim *et al.*, 1995) vector is similar to the vector described here. First, the 51 bp *Clal*–*XbaI* fragment of pOLYG was replaced with a 21 bp fragment formed by a pair of complementary oligonucleotides (5′-CTAGTACGTACGGTAACTT-3′ and 5′-CGAAGTTAACGCGTACGTA-3′) that contributed unique *SnaBI*, *MluI* and *HpaI* sites to the resulting plasmid, pAW99. The second step created pAW100 by inserting the *Bss*III cassette of pBS.ATS, which contains the Bluescript multicloning cassette flanked by transcriptional terminators, into the *MluI* site of pAW99. The third step, as described below, created pAW200 by inserting a modified *E. coli phoA* fragment into the *Bam*HI–*XbaI* sites of the pAW100 polylinker. We used PCR

amplification to modify the *E. coli phoA* gene in order to remove the promoter and the N-terminal portion of the coding region up to the eighth amino acid of the mature polypeptide. The oligonucleotide primers used for this purpose were 5′-GCCATTAAGTCTAGATGCTAACA-GCA-3′ and 5′-TGTTCTGGATCCATGGGCTGCTCAG-GGCGAT-3′. The resulting 1.4 kb fragment (designated '*phoA*') contains a *Bam*HI site near the N-terminus and an *XbaI* site 53 bp downstream from the '*phoA*' stop codon. Thus, the first three codons of the '*phoA*' gene were mutated from ENR (corresponding to codons 9–11 of the mature polypeptide) to DPW.

Creation of *phoA* fusions to *M. tuberculosis* DNA. *M. tuberculosis* genomic DNA was extracted as described by van Soolingen *et al.* (1991). To generate arbitrary small fragments for cloning, the DNA was partially digested by a mixture of pooled, frequent-cutting restriction enzymes (0.01 U µg⁻¹ of each enzyme): *AluI*, *DdeI*, *HaeIII*, *HincII*, *HinfI*, *MseI*, *MspI*, *RsaI*, *Sau3AI* and *Sau96I* (New England Biolabs) in a standard buffer (10 mM Tris/HCl, pH 7.9, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT and 100 µg ml⁻¹ BSA). A digestion of 10 min at 37 °C produced a distribution of fragments averaging about 500 bp. The DNA was extracted five times in phenol/chloroform (1:1, v/v), and the fragment ends were made blunt-ended by treatment with *Pfu* DNA polymerase (Stratagene). The DNA was extracted again in phenol/chloroform, precipitated in ethanol, washed in 70% ethanol, dried and dissolved in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) to 0.5 µg µl⁻¹.

Cloning adapters were made from a pair of complementary oligonucleotides (5′-pTCAGCTACTCAG-3′ and 5′-pATCC-TGAGTAGCTGA-3′). The double-stranded adapters were designed to form one blunt end (which ligates to *M. tuberculosis* fragments) and a 3-base (5′-ATC) protrusion on the opposite end which prevents self-ligation but promotes ligation into a *Bam*HI site that is partially G-filled. The adapter sequence, when translated in one orientation, adds five in-frame codons (Ser-Ala-Thr-Gln-Asp) when fused to the 3′ end of '*phoA*'. Translation of the adapter in the opposite orientation produces a stop codon in each of the three reading frames. Adapters were ligated onto the *M. tuberculosis* DNA fragments using T4 DNA ligase (8 U ml⁻¹, New England Biolabs) in a buffer designed for blunt-end ligations (50 mM Tris/HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 50 µM ATP and 25 µg ml⁻¹ BSA). After the ligation reaction, unincorporated adapters and small fragments (<200 bp) of *M. tuberculosis* DNA were separated from the larger fragments on a BioGel A-5m resin (Bio-Rad). The larger fragments were precipitated in ethanol and dissolved in 20 µl TE and ligated into the unique *Bam*HI site of pAW200. The resulting plasmid pool was electroporated into *E. coli* CC118, and the cells cultured on LB agar containing 200 µg hygromycin ml⁻¹. Approximately 80% of the transformed clones had inserts of *M. tuberculosis* DNA. The colonies of transformed *E. coli* were scraped from the plates and plasmids were isolated from the pool using a purification kit (Qiagen). The pooled plasmids (1 µg doses) were transformed into 50 µl of *M. smegmatis* mc²155, which had been prepared fresh for electroporation as described by Jacobs *et al.* (1991). Transformants were diluted and plated onto LB agar containing 50 µg hygromycin ml⁻¹ and 40 µg 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) ml⁻¹. The colonies were grown for 5 d on plates and kept at 37 °C in light-proof, humidified chambers. Colonies that developed a blue colour within the 5 d evaluation period

Table 1. Exported lipoproteins identified by PhoA fusions

Gene no.*	Total length†	Fusion length‡	SP length§	TM domains¶	Signal peptide	Alternative designations, comments
Rv0432	240	11, 39, 44, 107	32	1	MPKPADHRNH ⁺ AAVSTSVLSALFLGAGA ALLSA ⁺ CSSPQ	SodC, superoxide dismutase precursor (Cu-Zn)
Rv0934	374	40	23	1	MKIRLHT ⁺ LLAVLTAAPLLAAAG ⁺ C GSKP	PhoS precursor
Rv1009	362	108, 273	23	1	MLRL ⁺ VVGALLLVLAFAAG GYAVAA ⁺ CKTVT	Rpf homologue (Mukamolova <i>et al.</i> , 1998)
Rv1270c	244	80	24	1	MKHPPCSV ⁺ VAAATAILAVVLAIGG ⁺ C STEG	LprA, unknown function, homologues reported (Bigi <i>et al.</i> , 1997; Oftung <i>et al.</i> , 1997)
Rv1274	185	46	24	2	MRRKVR ⁺ RLTLAVSALVALF PAVAG ⁺ CSDSG	LprB, unknown function
Rv1368	261	50	38	1	MNGLISQACGSHRPPRSS ⁺ LGAVAILIAATLFATV VAG ⁺ CGKKP	LprF, unknown function

* The nomenclature from the *M. tuberculosis* H37Rv genome was used (Cole *et al.*, 1998).

† Length of the translated gene in amino acids.

‡ The fusion lengths of those proteins that were identified using PhoA fusion technology in this report are listed. The lengths given were counted from the predicted N-terminal start position of each protein.

§ Length of the predicted signal peptide counting from the predicted N-terminal start position of each protein.

¶ This column indicates how many TM domains were predicted within each protein including the signal peptide using the Transmembrane Prediction Server. Only TM segments with a score of >1000 were considered significant. Proteins with more than one TM domain also had TM domains within the mature polypeptide.

|| The N-terminal region of each protein is shown in single-letter code. The positively charged residues, K and R, are marked with a '+' sign above the sequence and the negatively charged residues, D and E, are marked with a '-' sign. Putative cleavage positions for the signal peptidase II are indicated by ↓. The two vertical bars (|) in each signal peptide mark the borders between the n-region and h-region, and the h-region and c-region, respectively.

were picked and restreaked to obtain single-colony isolates. The cycle of electroporating plasmids into *M. smegmatis* and screening for PhoA-positive colonies was repeated dozens of times until 300 separate blue colonies were collected.

We expected the screen to yield redundant representations of many different insert fragments because the cloned plasmids were propagated in *E. coli* before they were transferred to *M. smegmatis*. Redundant clones were identified by the restriction digestion patterns of the plasmids, using a panel of nine restriction enzymes (*Bam*HI, *Eco*RV, *Fsp*I, *Hind*III, *Hpa*I, *Mlu*I, *Pst*I, *Pvu*II and *Xba*I). We identified a putatively non-redundant set of 38 clones that formed the basis of all further analyses. In order to confirm that each of these non-redundant fusions expressed PhoA activity in *M. smegmatis*, plasmids from each clone were first electroporated back into mc²155, and single-colony isolates of each were harvested and propagated separately. PhoA activity was evaluated by comparing the colour to the reference strain, mc²155/pAW200, which was streaked onto a sector of every plate.

The nucleotide sequences of the *M. tuberculosis* DNA inserts were obtained by Southwest Scientific Resources, Inc. (Albuquerque) and Lofstrand Labs Unlimited (Bethesda), by dye terminator chemistry with fluorescent detection performed on an Applied Biosystems model 373A (PE Biosystems). Plasmids purified from *E. coli* were used as templates for the PCR reactions. A combination of four primers was used to do the sequencing in from the vector, one proximal and one distal on each side of each *M. tuberculosis* insert. The primer sequences were: 5'-TGCAGTAATATCGCCCTGA-3' (26 bp downstream from insert); 5'-CAGAGCGGCAG-TCTGATC-3' (72 bp downstream from insert); 5'-GATA-TCGAATTCCTGCAG-3' (23 bp upstream from insert); 5'-CTCGAGGTCGACGGTATC-3' (49 bp upstream from the insert).

Sequence data analysis. Sequences of *M. tuberculosis* proteins and ORFs were obtained from the Sanger Centre at <http://www.sanger.ac.uk/pub/tb/sequences>, from the EMBL 56 and GenBank 109 releases, and from SWISS-PROT 36, NBRF-PIR 57, GenBank translated release 109 and Prosite 15. Sequences were analysed using the DNASTar programs EditSeq, Protean, MegAlign and GeneMan (DNASTAR Inc.).

The TMPred program, which is available from <http://www.ch.embnet.org/software/TMPred/>, was used for prediction of TM helices and the orientation of TM segments in membrane proteins. The SignalP program V1.1 and the beta release of V2, which is available from <http://www.cbs.dtu.dk/services/SignalP/>, were used for SP motif and cleavage site predictions (Nielsen *et al.*, 1997). The neural network (NN) method is trained on datasets from SWISS-PROT release 29 (V1.1) or release 35 (V2). The dataset trained on the Gram-positive sample is most relevant for prediction of mycobacterial SPs. The second version of the SignalP program also includes a new method for identification of SPs, the hidden Markov model (HMM). In addition to predicting the cleavage site it also divides the SP into its three constituent parts: the n-region, which is the N-terminal part of the SP, generally characterized by having one or more positively charged amino acids, arginine (R) or lysine (K); the hydrophobic h-region which spans the cytoplasmic membrane; and the C-terminal part of the SP, the c-region, containing more polar amino acids and terminating with the conserved AXA motif which is the recognition site for signal peptidase I.

BLAST searches. To search for potential functions of the proteins, homologous proteins were found by the TBLASTN program in the databases 'All EMBL except EST' using PAM250 residue weight table (2.5 accepted mutations per residue). The computation was performed at the Swiss

Institute of Bioinformatics (SIB) using the BLAST network service.

RESULTS

Description of the *phoA* fusion experiments

In summary, a total of 38 independent fusions were obtained. Upon sequencing of these clones, 34 different genes were identified. Based on the features of the encoded gene products, as described below, we classified the proteins as soluble secreted proteins (22), membrane lipoproteins (6) or integral membrane proteins (6).

Six genes had significant TM segments in the N-terminal region and appropriately placed membrane lipoprotein attachment sites (ProSite motif PS00013) (Table 1). Rv0432 (*sodC*) was identified by four, and Rv1009 by two independent fusions (Table 1). This finding may indicate that these genes have promoters that give strong expression when cloned into *M. smegmatis*. One of the fusions with *sodC* was only 11 amino acids long, constituting the n-region of the SP. Leaderless PhoA does not contain a discrete domain that targets it for export; however it has been shown that the addition of positively charged amino acids N-terminally may be sufficient for its insertion into the translocation machinery (Prinz *et al.*, 1996). The short fusion with *sodC* supports the finding that the fusion of sequences with basic amino acids to leaderless PhoA may be sufficient for engaging it in the secretory pathway.

Six other genes encoded proteins with multiple predicted TM domains (>5); accordingly, these were classified as membrane proteins (Table 2). PhoA fusions can be used to assign the correct orientation of membrane protein TM helices (Manoil & Beckwith, 1986; Manoil *et al.*, 1988) because the phosphatase is enzymically active when fused to loops that project outside the cytoplasmic membrane. This information was used to predict the orientation of the six proteins described in Table 2. However, using *phoA* fusions to predict the proper folding of membrane proteins may have pitfalls. It has been reported that fusions near the N-terminal part of cytoplasmic loops may give false-positive signals, because they lack positively charged amino acids acting as topogenic signals (Prinz & Beckwith, 1994). Examination of our fusions showed that none was close to the N-terminus of the extramembranous loops. In addition, it is worth noting that the predicted orientations of the proteins described in Table 2 are based on only one PhoA fusion per molecule, and to assign the correct orientation and definition of all TM segments, several fusions at different points along the polypeptide chain are needed (Boyd *et al.*, 1993).

We identified 22 genes encoding proteins with strong TM domains N-terminally representing putative signal sequences (Table 3). Fourteen genes may encode novel secreted proteins. Seven coincided with previously described secreted proteins of the *M. tuberculosis* H37Rv genome (Rv0040c, Rv0129c, Rv1886c, Rv1980c, Rv2376c, Rv2878c and Rv3810). One gene coincided

with the unannotated O1mtb1 (Chubb *et al.*, 1998), which overlaps with Rv0192. In contrast to O1mtb1, Rv0192 does not have a putative SP sequence, and the codon usage of O1mtb1 is more optimal. To provide a more reliable characterization of the mycobacterial SP, we extended Table 3 to contain 15 additional proteins, which have been reported in the literature to encode exported proteins of the *M. tuberculosis* complex. These proteins were included in this dataset based on published data on N-terminal sequences or because they had been identified by PhoA or β -lactamase fusions by other authors. Table 3 thus contains a description of 37 probable secreted proteins. This dataset was used in the analysis that follows.

Determination of signal peptidase I cleavage sites

Possible cleavage sites for signal peptidase I were determined using the NN and the HMM methods. Comparison of the results showed that they predicted the same cleavage site in 21 of the 37 proteins in Table 3 whereas they diverged for the remaining 16. In all but one (Rv3810) of these examples, the HMM method predicted longer SPs than the NN method. These results were compared to the experimentally determined cleavage sites ($n = 16$). The NN and HMM methods identified the correct cleavage sites in 10 and 12 of the 16 proteins respectively. The two methods were in agreement in 9 of these examples. In 3 examples (Rv2376c, Rv2878c and Rv3036c) neither of the methods predicted the experimentally determined cleavage sites.

In some cases, the NN method predicts several alternative cleavage sites. The most notable example of this was Rv3803c (MPT51, fbpD). There are two very strong predictions of cleavage sites at position 27 and 34, of which the former yields a maximal C-score of 1.0 whereas the latter, which gave a C-score of 0.856, correctly predicted the experimentally determined cleavage site. In Rv3804c (Ag85A, FbpA), there are several possible AXA motifs and the NN method also predicts several possible cleavage sites with similar strengths, but the highest scoring one was not corroborated by the experimentally determined cleavage site. Because the new version of the SignalP program has been trained on a larger set of proteins, the old and new versions of SignalP predicted different optimal cleavage sites in Rv3804c. However, neither version identified the experimentally determined cleavage site for this protein.

Prediction of TM domains in the secreted protein dataset

Analysis of the predicted strength of the N-terminal TM domains in the proteins listed in Tables 1 and 3 showed peak values of 1994 ± 712 (2 SD) ($n = 43$) using the TM prediction program. Some of the secreted proteins also had significant TM domains within their mature parts. Each of the three antigen 85 complex proteins has a strongly hydrophobic region in the middle of the molecule, residues ~ 160 to ~ 180 . Nonetheless, all of these proteins in their native states are completely

Table 2. Putative membrane proteins identified by PhoA fusions

See also the footnotes to Table 1 for explanations of the columns.

Gene no.	Total length	Fusion length*	TM domains	Predicted orientation†	Alternative designations, comments
Rv1004c	419	60	6	N-in, C-in	Similarities with elastin
Rv1481	335	178	5	N-in, C-out	Unknown
Rv2196	549	146	9	N-out, C-in	Component of the ubiquinol-cytochrome <i>c</i> reductase complex
Rv2563	388	207	5	N-out, C-in	Unknown
Rv3157	553	71	12	N-out, C-out	'NuoM' subunit M of the NADH-CoQ reductase
Rv3252c	416	51	6	N-in, C-in	AlkB, non-haem iron monooxygenase

* The fusion lengths of those proteins that were identified using PhoA fusion technology in this report are listed in this column. The lengths given were counted from the predicted N-terminal start position of each protein.

† Indicates whether the N-terminal and C-terminal ends of the proteins were predicted to be on the inside or outside of the cytoplasmic membrane.

soluble, demonstrating that soluble proteins may have at least one strong TM domain prediction. The antigen 85 complex has been found to consist of mycolyl-transferases (Belisle *et al.*, 1997) and the hydrophobic part might be within an active site that binds and transfers mycolic acids. Rv1274 also contains a TM domain within the mature part of the protein (residues 76–99). Whether this domain adopts a TM helix conformation must be determined experimentally.

Characteristics of the mycobacterial SP

Altogether 28 secreted proteins were selected from Table 3 to determine general characteristics of the *M. tuberculosis* SP. Proteins with known primary N-terminal amino acid sequences and/or proteins where the NN and the HMM methods predicted the same cleavage site were selected. The HMM method was used to determine the lengths of the n-, h- and c-regions of the SPs. The median SP length was 32 residues (range 21–49), the median n-region was 9 amino acids long (range 4–24), the median h-region was 16 amino acids (range 13–17) and the median c-region was 7 amino acids long (range 4–26). These figures are comparable to the lengths of SPs from Gram-positive bacteria (von Heijne & Abrahmsen, 1989). The marked variability in the lengths of the c-regions was due to three proteins with unusually long sequences (Rv2301, Rv2376c, Rv3033).

In terms of overall amino acid composition, the n-region of *M. tuberculosis* SPs is characterized by a high arginine content (22%) and a relatively lower amount of lysine (6.0%). The arginine-rich n-regions are most likely explained by the high G + C content, which favours arginine because it is encoded by G + C-rich codons whereas lysine is encoded by G + C-poor codons. The overall amino acid composition of the h-region of the 28 selected signal sequences was 28% alanine, 18% leucine and 17% valine. In comparison *Bacillus* has only 10% alanine. Other bacterial SPs have generally more leucine (25–35%). The overall composition of the c-region of

the selected 28 signal sequences showed that the most abundant amino acids were: alanine, 33%; glycine, 14%; valine, 10%; and proline, 9.4%.

Analysis of the preferred amino acids in the positions around the cleavage site showed that the mycobacterial SPs generally fulfil the (–3, –1) rule, with alanine in 19 of 28 SPs in position –3 and alanine in 23 of 28 in the –1 position.

The DP motif

Aspartic acid (D) was the first (+1 position) amino acid of the mature sequence in 13 of 28 (46%) entries in the dataset and proline (P) was found in position +2 in 15 of 28 (54%) of these secreted proteins. In 12 of 28 (43%) of these, DP occurred simultaneously. The frequency of DP in any position of the 100 first proteins of *M. tuberculosis* was found to be less than 0.5%. DP as a motif in the two first positions of mature secreted proteins has not been described in other bacterial species.

Functional characterization of secreted proteins

We performed BLAST searches on our selected set of proteins in order to make predictions about possible functions. Additional information was obtained for some of the proteins that was not available when the *M. tuberculosis* genomic sequence was published.

A relatively large fraction (56%) of the lipoproteins in Table 1 and the secreted proteins listed in Table 3 are characterized as unknown. There were 19 previously characterized antigens, of which 10 have unknown function. Three proteins are potential virulence factors: Rv0590, Rv1566c and Rv2875. Rv1566 is a third homologue in *M. tuberculosis* to *Listeria monocytogenes* p60 invasion associated proteins (Kohler *et al.*, 1990; Kuhn & Goebel, 1989). Rv2875 (MPT70) has previously been reported to be homologous to osteoblast specific factor 2 (Ulstrup *et al.*, 1995) and may be

Table 3. Identification of signal peptides in previously and presently identified proteins

See also the footnotes to Table 1 for explanations of the columns.

Gene no.*	Total length	Fusion length†	SP length‡	Cleavage prediction§	TM domains	Signal peptide¶	Alternative designations, comments
Rv0012AS	247	87	32	HMM/NN	2	MIDRRRS ⁺ AWRF ⁺ [SVPLVCLLAGLLAA ⁺]THGVSG ⁺ GTEIR ⁺	Unknown function
Rv0040c	310	167	32	HMM/NN	1	MIQIARTWR ⁺ [VFAGGMATGFIGVVLVT ⁺]AGKASA ⁺ DPLLP ⁺	MTC28, unknown function
Rv0062	380	72	65/71	NN&HMM	1	...35 residues...LRHYFARV ⁺ SSAIGSVAR ⁺ WIVPLL ⁺ [GVAAVA ⁺]SIGVIA ⁺ DPVRV ⁺	CelA, possible cellulase
Rv0116c	251	178	28/47	NN&HMM	1	MRRVVR ⁺ [LSVVVAITLMLTA ⁺]ESVSIATA ⁺ [AVPPLQPIPGVASVSPANG ⁺]AVVGV ⁺	Unknown function
Rv0125	355	—	32	HMM/NN/NTS	1	MSNSRRRS ⁺ RLW ⁺ [SWLLSVLAAVGLGL ⁺]ATAPAAQA ⁺ APPAL ⁺	PepA, MTB32A, trypsin-like serine protease (Skeiky <i>et al.</i> , 1999)
Rv0129c	340	65	46	HMM/NN/NTS	2	MTFFEQ ⁺ VRRLRSAATTLP ⁺ RR ⁺ [LAIAAMGAVLVYGLVGT ⁺]FGGPATAGA ⁺ FSRPG ⁺	Ag 85C, FbpC, mycyloltransferase (Nagai <i>et al.</i> , 1991; Wiker <i>et al.</i> , 1990; Chubb <i>et al.</i> , 1998)
Rv0283AS	481	90	44/51	NN&HMM	1	MHDTFRLV ⁺ DP ⁺ L ⁺ TR ⁺ QSR ⁺ [AVLMGV LIVITGLIGS ⁺]FVFS ⁺ LRP ⁺ NGQA ⁺]GSNAVLA ⁺ DRSTA ⁺	Unknown function, ATP/GTP-binding site PS00017 motif A
Rv0431	164	75	34/57	NN&HMM	1	MNERVP ⁺ DS ⁺ SGLPL ⁺ R ⁺ [AMVMVLLFLGVVFLLLV ⁺]WQA ⁺]LGSSPNS ⁺ EDDSSAISTMTTTTAA ⁺	Tuberculin-related peptide
Rv0590	275	—	29	HMM/NN	1	MKTTGT ⁺ TIK ⁺ [LGIVLVLVSFTVMI ⁺]IVVFG ⁺ QVRFH ⁺	Mce2B (Chubb <i>et al.</i> , 1998)
Rv0982	504	82	49	HMM/NN	2	MWWFR ⁺ RRDRAP ⁺ LRATSS ⁺ LSL ⁺ R ⁺ W ⁺ [MLLAMSVMAMVVVLS ⁺]FAVYAVISA ⁺]ALYS ⁺ D	Putative two-component sensor
Rv1174c	110	—	28	HMM/NN/NTS	1	MRLSL ⁺ [TALSAGVGAVAMSLTV ⁺]GAGVASA ⁺ DPVDA ⁺	SA-5K, unknown function (Coler <i>et al.</i> , 1998; Freer <i>et al.</i> , 1998)
Rv1435c	202	—	33	HMM/NN	1	MTLMAIV ⁺ NRFN ⁺ K ⁺ [VIAGAGLFAAAIAL ⁺]SPDAAA ⁺ DP ⁺ LMT ⁺	Unknown function (Chubb <i>et al.</i> , 1998)
Rv1566c	230	72	30	HMM/NN	1	MKRS ⁺ MKS ⁺ [GSFAIGLAMMLAPMV ⁺]APGLAAA ⁺ DP ⁺ ATR ⁺	Homology with <i>M. avium</i> invasin 1 (inv1)
Rv1810	118	97	35	HMM/NN	1	MQLQRT ⁺ MG ⁺ QCRPM ⁺ R ⁺ [MLVALLLSAATMIGL ⁺]AAPGKA ⁺ DP ⁺ TGD ⁺	Unknown function
Rv1815	221	—	27	HMM/NN/NTS	2	MVRLV ⁺ PR ⁺ [AFAATVALLAAGF ⁺]SPATASA ⁺ DP ⁺ VLV ⁺	Protein F, unknown function (Sonnenberg & Belisle, 1997)
Rv1860	325	—	39/60	NN/NTS&HMM	1	MHQVD ⁺ PNL ⁺ TR ⁺ RK ⁺ GR ⁺ [LAALAIAMASASLVTV ⁺]AVPATANA ⁺ DEEPA ⁺ ...↓	ModD, MPT32, Apa precursor (Nagai <i>et al.</i> , 1991; Romain <i>et al.</i> , 1993)
Rv1886c	325	59	40	HMM/NN/NTS	2	MTDVS ⁺ RKIRAW ⁺ GRRL ⁺ [MIGTAAAVVLPGLVGL ⁺]AGGAATAGA ⁺ FSRPG ⁺	Ag 85B, FbpB, mycyloltransferase (Nagai <i>et al.</i> , 1991; Wiker <i>et al.</i> , 1986)
Rv1910c	197	54	22/33	NN&HMM	1	MAHAF ⁺ HRF ⁺ [ALAILGLALPVALV ⁺]AYGNGDS ⁺ RKA ⁺]APLAP ⁺	Unknown function
Rv1926c	159	—	29	HMM/NN/NTS	1	MKLTT ⁺ MIK ⁺ [TAVAVVMAAATATF ⁺]AAPVALA ⁺ AYPIT ⁺	MPT63, unknown function (Nagai <i>et al.</i> , 1991)
Rv1980c	228	108	23	HMM/NN/NTS	1	MRIK ⁺ [IFMLVTA ⁺ VLLCC ⁺]SGVATA ⁺ APKTY ⁺	MPT64, unknown function (Harboe <i>et al.</i> , 1986)
Rv1984c	217	—	30/32	NN&HMM/NTS	2	MTPRS ⁺ LV ⁺ R ⁺ [IVGVVATTALVSA ⁺]PAGGRAA ⁺ HA ⁺]DPCSD ⁺	CFP21, putative cutinase (Welding <i>et al.</i> , 1998)
Rv2253	167	99	27/28	NN&HMM	1	MSGHR ⁺ KK ⁺ [AMLALAAASLAATL ⁺]APNAVA ⁺]A ⁺ AEPSW ⁺	Unknown function
Rv2277c	301	39	22/37	NN&HMM	1	MPGRF ⁺ [TVALVIALGGTCGV ⁺]ADA ⁺]LPLGQTDDPMIVAH ⁺ R ⁺]AGTRD ⁺	Putative glycerolphosphodiesterase
Rv2301AS	230	—	32/43	NN&HMM/NTS	1	MNDLL ⁺ TR ⁺ R ⁺ [LLTMGAAAAMLA ⁺ AVLL ⁺]TPITVPA ⁺]GYPGAVAPATA ⁺ ACPDA ⁺	CFP25, putative cutinase (Welding <i>et al.</i> , 1998)
Rv2376c	168	49	21/48/53	NN&NTS &HMM	1	MK ⁺ MVKS ⁺ [IAAGLTAA ⁺ AAIGAAA ⁺]A ⁺]GVTSIMAGGPVVYQM ⁺ QPVVFGAPLPL ⁺]DPASA ⁺]PDVPT ⁺	MTB12, unknown function (Webb <i>et al.</i> , 1998)
Rv2875	193	—	30	HMM/NN/NTS	1	MKV ⁺ KNT ⁺ [IAATSFAAAGLAALAVA ⁺]VSP ⁺ PA ⁺ AA ⁺]GDLVG ⁺	MPT70, OSF-2 homologue (Harboe <i>et al.</i> , 1986)
Rv2878c	173	130	37/38	NN/HMM&NTS	1	MSL ⁺ RLVSP ⁺ IKAFAD ⁺]GIVAVAIAVVL ⁺ MFGLA ⁺]NT ⁺ PRAVA ⁺]A ⁺]DERLQ ⁺	MPT53, thioredoxin-like protein (Nagai <i>et al.</i> , 1991; Wiker <i>et al.</i> , 1999a)
Rv3033	182	83	40	NN/HMM	1	MAHSIV ⁺ RT ⁺ [LLASGAATALIAIPTAC ⁺]SFSIGTSHSHSVSKA ⁺]EVARQ ⁺	Unknown function
Rv3036c	227	—	22/23/36	NTS&NN&HMM	1	MRYL ⁺]IATAVLVAVVLVGVW ⁺]PAAG ⁺]AP ⁺]PSCAGLGGTVQA ⁺]GQICH ⁺	Protein B, MPT64 homologue (Sonnenberg & Belisle, 1997)
Rv3310	299	248	36/46	NN&HMM	1	MLRGIQALS ⁺ RPL ⁺ TRV ⁺ R ⁺]ALAVIGVLAASLLASWV ⁺]GA ⁺]VPQVGLAASA ⁺]LPTFA ⁺	Putative acid phosphatase
Rv3492c	160	46	21	NN/HMM	1	MRL ⁺]ISVAYALMVAT ⁺ IV ⁺]GLSA ⁺]AGGWF ⁺	Unknown function, PS00213 lipocalin signature
Rv3759c	315	—	30	NN/HMM	1	MRMLRR ⁺ LR ⁺ R ⁺]ATVAAAVWLATVCLV ⁺]ASCANA ⁺]DPLGS ⁺	ProX, probable osmoprotection protein (Chubb <i>et al.</i> , 1998)

Table 3 (cont.)

Gene no.*	Total length	Fusion length†	SP length‡	Cleavage prediction§	TM domains	Signal peptide¶	Alternative designations, comments
Rv3803c	299	–	26/33	NN&HMM/NTS	1	MKGRSALLRALW IAALSFLGLGGVAVA AEPTAKA APYEN	MPT51, FbpD (Nagai <i>et al.</i> , 1991)
Rv3804c	338	–	34/43	NN&HMM/NTS	2	MQLVDRVRGAVTGMSSRL VVGAVGAALVSLGVGA VVGGTATAGA FSRFG	Ag85A, FbpA, mycolyltransferase (De Bruyn <i>et al.</i> , 1987; Nagai <i>et al.</i> , 1991; Wiker <i>et al.</i> , 1990; Chubb <i>et al.</i> , 1998)
Rv3810	284	23	22/28	HMM&NN	1	MPNRRRRK LSTAMSAVAAL AVA SPCAYF LVYES	PIRG/Erp unknown function, putative cell surface precursor (Lim <i>et al.</i> , 1995)
Not assigned	100	36	35	NN/HMM	1	MSRWKQGWTRG SLFAALNIAAVVAVLML GAGVAVA DPDAA	O1mb1 (Chubb <i>et al.</i> , 1998), overlaps with Rv0192
Not assigned	–	–	32	NN/HMM	1	MNRIVA PAAASVVVGLLLGAAA IFGVTLMVQQ DKKPP	pExp59 (Lim <i>et al.</i> , 1995)

* AS, alternative start; indicates that a different start position than the annotated one was used.

† The fusion lengths of those proteins that were identified using PhoA fusion technology in this report are listed in this column. The lengths given were counted from the predicted N-terminal start position of each protein.

± Length of the predicted or experimentally verified signal peptide counting from the predicted N-terminal start position of each protein.

§Indicates how each of the cleavage positions for the signal peptidase I was determined: HMM, hidden Markov model; NN, neural network method; NTS, determined experimentally by N-terminal sequencing of the native purified protein. The most upstream prediction of the cleavage position is mentioned first. Different predictions are separated by '&'. When separated by slashes (/), the methods predict the same cleavage position.

¶ Experimentally verified cleavage positions as determined by N-terminal sequencing of the mature native protein are indicated by an asterisk (*).

|| The listed references contain information about N-terminal amino acid sequences of native proteins except for two references (Chubb *et al.*, 1998 and Lim *et al.*, 1995) which were included due to identification by PhoA or β -lactamase fusions respectively.

relevant for development of bone tuberculosis. Four proteins are thought to be involved in cell wall biogenesis (the antigen 85 complex and MPT51, FbpA–D), and nine proteins are probable enzymes (superoxide dismutase, a cellulase, a protease, two cutinases, glycerolphosphodiesterase, a thioredoxin-like protein, a protein possibly involved in hexuronate catabolism and a putative acid phosphatase). Proteins secreted by Gram-positive bacteria can serve as interbacterial signalling molecules, and one of the lipoprotein fusions found in this study (Rv1009) was recently identified as one of five genes in *M. tuberculosis* showing homology to the resuscitation promoting factor (Rpf) of *Micrococcus luteus* (Mukamolova *et al.*, 1998).

Among the membrane proteins, Rv2196 was reported to have low similarity to cytochrome *b* subunits. We also found that it shares strong homology to a component of the ubiquinol–cytochrome *c* reductase complex of *Streptomyces antibioticus*, and Rv3252c was highly similar to alkane-1 monooxygenase of *Pseudomonas aleovorans*.

DISCUSSION

Characterization of exported proteins in pathogenic mycobacteria is important because it will provide a better understanding of the signals that direct proteins to the cell membrane, to the cell wall or to the surrounding milieu as soluble secreted products. Many

of the exported proteins have key enzymic functions for building the mycobacterial cell wall, such as the antigen 85 complex, but the majority has not been functionally characterized yet. A complete list of exported proteins in mycobacteria is needed to obtain a more comprehensive picture of proteins involved in cell envelope biogenesis, immunogenicity and virulence. Potential candidates of the latter group are the proteins encoded in the four operons associated with mammalian cell entry (Arruda *et al.*, 1993; Cole *et al.*, 1998; Wiker *et al.*, 1999b).

The PhoA fusion data presented in this report provide experimentally derived information about the identification and characterization of *M. tuberculosis* secreted proteins. Comparison of this empirical dataset with informatics-based predictions highlights both the potential and current limitations of the latter. For example, recognition of an authentic SP may depend on correct identification of the initiation codon. In some cases the annotated start codon may be located downstream of the actual start codon, as for Rv2301, which was annotated without the n-region of the SP. In other instances the correct start codon may be found further downstream. A new start site was identified in Rv1270c that is 39 bp downstream of the previous one. Likewise, the *phoA* fusion data also suggest that the correct start codons and RBSs of Rv0012 and Rv0283 are located downstream of those specified by the genome database. Rv0062 is an exception as it has a long N-terminal positively charged piece, but there is no obvious

downstream RBS allowing an alternative interpretation. As a result, the putative SP would be unusually long.

The NN and HMM methods are not only designed to identify SPs, but also to predict the cleavage site for signal peptidase I. Our comparison with experimental data on known secreted mycobacterial proteins demonstrated good agreement with the general accuracy of prediction of cleavage sites by the NN and HMM methods (Nielsen & Krogh, 1998), which are reported to give about 70% correct determinations. Our results suggest however that the HMM method gives better predictions than the NN method when applied to mycobacterial signal sequences. Relatively few mycobacterial proteins were included for training the NN method. Inclusion of proteins from the dataset presented here may improve the predictive accuracy of the NN method when applied to mycobacterial sequences.

For identification of secreted proteins, genomic data may be screened using algorithms for SP predictions (Gomez *et al.*, 2000). However, due to inherent problems with the correct identification of initiation codons, the TM prediction server provides an attractive alternative for screening purposes to identify proteins with hydrophobic stretches in their N-terminal parts. Sequences that do not comply with the general characteristics of SPs may then be analysed more closely to reveal whether there are alternative start codons. Our results with the TM predictions in signal peptides show that they are strongly hydrophobic and give much higher scores (>1000) than the cut-off value (>500) suggested by the providers of the program. We find that TM predictions are sensitive and possibly ideal for screening purposes to identify novel secreted proteins in genomic datasets.

The *M. tuberculosis* genome is thought to contain a similar number of secreted proteins cleaved by signal peptidases I or II (~90 in each group) (Cole *et al.*, 1998). Assuming that this estimate is correct, Table 3 contains ~40% of the total number of soluble secreted proteins of *M. tuberculosis*, and our fusions would have picked out ~23% of the total number of secreted proteins processed by signal peptidase I. In comparison, ~7% of the exported lipoprotein genes were obtained. Our fusion experiments thus more readily identified genes encoding putative soluble secreted proteins than genes encoding lipoproteins or membrane proteins. This bias could indicate that PhoA activity is more readily detected in fusion proteins that are released from the bacterial cell. Alternatively, the number of proteins processed by signal peptidase I may have been underestimated and could be considerably more numerous (estimated ~200–300).

Our analysis has revealed a novel motif (found in 12 of the presently recognized soluble secreted proteins), consisting of aspartic acid and proline (DP) in the first and second positions, respectively. The role of the DP motif is not known. It is possible that these amino acids are part of the recognition site for the signal peptidase I. However, because a range of different amino acids occur

at these two positions in the other secreted proteins, considerable permissiveness must be allowed at the first and second positions of the mature sequence. Alternatively, the DP motif might function as a sorting signal that directs proteins to a particular compartment following translocation. A similar feature is well characterized in *E. coli* and *Klebsiella oxytoca*, where an aspartic acid in position +2 determines the inner membrane association of two lipoproteins (NlpA and pullulanase respectively) (Yamaguchi *et al.*, 1988). Further experimental work is needed to determine if DP at the mature N-terminus is a post-translocational sorting signal.

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