Identification of *Mycobacterium tuberculosis* signal sequences that direct the export of a leaderless β -lactamase gene product in *Escherichia coli*

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Proteins secreted by *Mycobacterium tuberculosis* may play a key role in virulence and may also constitute antigens that elicit the host immune response. However, the *M. tuberculosis* protein export machinery has not been characterized. A library of *M. tuberculosis* H37Rv genomic DNA fragments ligated into a signal sequence selection vector that contained a leaderless β -lactamase gene and an upstream Tac promoter was constructed. Transformation of *Escherichia coli* with the *M. tuberculosis* DNA library and selection on plates containing 50–100 μ g ampicillin ml⁻¹ resulted in the identification of 15 Amp^r clones out of a total of 14 000 transformants. Twelve of the β -lactamase gene fusions conferred high levels of Amp^r (up to 1 mg ampicillin ml⁻¹); insert sizes ranged from 350 to 3000 bp. Of ten inserts that were completely sequenced, two were identified as fragments of the genes for *M. tuberculosis* antigens 85A and 85C, which are the major secreted proteins of this pathogen. Seven of the remaining inserts were $\geq 97\%$ identical to hypothetical ORFs in the *M. tuberculosis* genome, one of which encoded a protein with 35% identity to a low-affinity penicillin-binding protein (PBP) from *Streptomyces clavuligerus*. Four of the seven hypothetical ORFs encoded putative exported proteins with one or more membrane interaction elements, including lipoprotein attachment sites and type I and II transmembrane (TM) segments. All of the inserts encoded typical signal sequences, with the exception of a possible type II membrane protein. It is concluded that expression of β -lactamase gene fusions in *E. coli* provides a useful system for the identification and analysis of *M. tuberculosis* signal-sequence-encoding genes.

Keywords: tuberculosis, *Mycobacterium*, signal sequences, β -lactamase, export

INTRODUCTION

Mycobacterium tuberculosis is an aggressive intracellular pathogen that preferentially invades mononuclear phagocytes and establishes persistent intracellular infec-

tions that are difficult to eradicate (Fenton & Vermeulen, 1996). There is evidence that these events are accompanied by the active secretion of proteins and other molecules by the pathogen. Comparative analyses by two-dimensional electrophoresis of proteins produced by intracellular versus broth-grown bacteria have revealed distinct differences, with intracellular bacteria producing proteins not evident during *in vitro* growth, some of these proteins being secreted (Lee & Horwitz, 1995). In addition to these specialized proteins, *M. tuberculosis* sheds a large array of proteins regardless of growth conditions, as revealed by extensive analyses of

Abbreviations: MCS, multiple cloning site; PBP, penicillin-binding protein; TM, transmembrane.

The GenBank accession numbers for the sequences reported in this paper are AF017098 (plasmid pGB14T-P), AF017099 (pGB14T-V), AF017100 (pGB14T-X), AF017101 (pGB14T-O2), AF017102 (pGB14T-B) and AF017103 (pGB14T-O1).

Fig. 1. Construction of plasmids pGB14T and pGB14TC. A PCR-amplified Tac promoter, using pGEX-2T as template, was ligated into the EcoRI site in the MCS of pGB14, producing pGB14T. A CfoI-digested fragment of pBR329, containing the Cm^r gene, was ligated into an HpaI site located in the Em^r gene of pGB14T to generate pGB14TC. In both pGB14T and pGB14TC, note the presence of a leaderless β -lactamase gene, an upstream Tac promoter and an interposed MCS. Asterisks indicate unique restriction sites.

culture filtrates (Andersen, 1994; Sonnenberg & Belisle, 1997). Many of these are true secreted proteins (Wiker *et al.*, 1991) and are antigenic *in vivo* (Orme *et al.*, 1993), and some may prove of value in the design of subunit vaccines (Andersen, 1994; Horwitz *et al.*, 1995). However, at least two issues regarding secreted *M. tuberculosis* proteins remain unresolved. First, what roles, if any, do secreted proteins play in pathogenesis, particularly as virulence factors in cell invasion and intracellular survival? Second, what is the nature of the protein export machinery responsible for the secretion of these proteins?

Three types of protein export pathway have been identified in eubacteria. The best studied is the classical, *sec*-dependent, general secretory pathway that exports proteins with characteristic N-terminal signal peptides and that is probably the major protein export pathway in both Gram-negative and Gram-positive bacteria (Pugsley, 1993; Salmond & Reeves, 1993). This pathway is also referred to as the type II secretion system. In addition, subsets of secreted proteins are exported by type I and type III secretion systems, which are *sec*- and signal-sequence-independent (Salmond & Reeves, 1993).

The type III system is of particular interest because of its involvement in the export of virulence-associated proteins in diverse bacterial pathogens (Salmond & Reeves, 1993; Dreyfus *et al.*, 1993; Silhavy, 1997). To date, none of these pathways has been characterized in mycobacteria.

The classical *sec*-dependent (type II) pathway is presumed to exist in mycobacteria because a number of secreted proteins have been found to be the products of genes that code for typical N-terminal signal sequences (Young *et al.*, 1992; Orme *et al.*, 1993). Moreover, recently a variety of signal-sequence-encoding genes have been identified by fusions with a leaderless alkaline phosphatase (*phoA*) gene and expression in a suitable mycobacterial host (Lim *et al.*, 1995). In this study, we have extended this approach by investigating the usefulness of gene fusions with a leaderless β -lactamase gene and expression in *Escherichia coli*. Selection for export-competent signals by both PhoA and β -lactamase fusions depends on the principle that the enzyme must be translocated across the cytoplasmic membrane into the periplasm; PhoA is inactive in the cytoplasm, and cytoplasmic β -lactamase cannot protect cells against

ampicillin-induced lysis (Broome-Smith *et al.*, 1990). However, β -lactamase gene fusions have been shown to provide an informative alternative to *phoA* fusions in analysing the membrane topology of secreted proteins in bacteria and in selecting for diverse signal-sequence-encoding genes (Broome-Smith & Spratt, 1986; Smith *et al.*, 1987; Broome-Smith *et al.*, 1990; Prinz & Beckwith, 1994; Reeves *et al.*, 1994).

We report that gene fusions with a leaderless β -lactamase are indeed useful in identifying signal-sequence-encoding *M. tuberculosis* genes and we demonstrate that *M. tuberculosis* signal sequences are able to direct the secretion of β -lactamase in a Gram-negative organism, *E. coli*.

METHODS

Bacteria, plasmids and culture conditions. All plasmid and library constructions and signal sequence selections were performed in *E. coli* XL-1 Blue (*supE44 hsdR17 recA2 gyrA46 thi relA1 lac F'*) (Sambrook *et al.*, 1989) as host strain. pGB14 was a gift from Jan M. van Dijk (Groningen Biomolecular Sciences and Biotechnology Institute, Haren, The Netherlands); pGB14T and pGB14TC were generated as described below. pGEX2T was from Pharmacia; pBluescript (pBS) and pBR329 were from Stratagene. *E. coli* transformed with pGB14T or pGB14TC was grown on LB-agar plates containing 200 μ g erythromycin ml⁻¹ or 30 μ g chloramphenicol ml⁻¹, respectively. Screening for *M. tuberculosis* DNA- β -lactamase gene fusions was performed on LB-agar plates containing 70 μ g IPTG ml⁻¹ and 50–1000 μ g ampicillin ml⁻¹.

Plasmid constructions. pGB14 is an export signal selection vector that is based on pUC13 and contains a 5'-truncated *E. coli* TEM β -lactamase gene (lacking the signal sequence, translational start site and promoter), an erythromycin resistance (Em^r) gene and a multiple cloning site (MCS) upstream of the β -lactamase gene (Smith *et al.*, 1987; Perez-Martinez *et al.*, 1992) (Fig. 1). pGB14T was constructed by cloning a Tac promoter into the unique *EcoRI* site in the MCS of pGB14. The Tac promoter with suitable *EcoRI* sites was generated by PCR amplification of a 114 bp fragment of the Tac promoter in pGEX2T, using the forward primer Tac1B (5'-AGATCTGAATTCGGTTCTGGC-3') and reverse primer Tac2 (5'-CAGCTGGAATTCGTGTGAAAT-3'). pGB14TC is a derivative of pGB14T in which the Em^r gene has been replaced with a chloramphenicol resistance (Cm^r) gene. pGB14T was digested with *HpaI* at a site located within the Em^r gene and ligated to a *CfoI*-digested, 1.2-kb Cm^r gene from pBR329 (Fig. 1).

Construction of *M. tuberculosis* genomic libraries. Genomic DNA from *M. tuberculosis* H37Rv (a gift from John T. Belisle, Colorado State University, Fort Collins, CO, USA) was digested with *Sau3A* and ligated into pGB14T or pGB14TC digested with *BamHI* and treated with calf intestinal phosphatase, at an insert to vector ratio of 2:1 (w/w); alternatively, the genomic DNA was digested with either *Hae3* or *RsaI* and ligated into *SmaI*-digested vectors. Ligation mixtures were transformed into *E. coli* XL-1 Blue rendered competent by the CaCl₂/MgCl₂ method, or by electroporation, and signal sequence- β -lactamase gene fusions were selected by direct plating of the transformed *E. coli* on 50–100 μ g ampicillin ml⁻¹.

DNA methods and sequence data analyses. DNA manipulations were carried out using standard techniques (Sambrook

Table 1. Internet servers used in the analysis of *M. tuberculosis* DNA- β -lactamase gene fusions

Server	Description	URL
Database searches		
NCBI BLAST	National Center for Biotechnology Information; gapped BLASTP/BLASTN/TBLASTN searches of all non-redundant peptide and nucleotide sequence databases	http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=0
TIGR TBLASTN	TBLASTN searches of TIGR contigs	http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-tigrbl
TIGR BLASTN	BLASTN searches of Sanger and TIGR contigs	http://ziggy.sanbi.ac.za/tb/tbsearch.htm
Sanger Centre <i>M.tb.</i> BLAST	BLAST searches of Sanger Centre TB database	http://www.sanger.ac.uk/Projects/M_tuberculosis/blast_server.shtml
MycDB BLAST	BLAST searches of Integrated <i>Mycobacterium</i> Database (Institut Pasteur)	http://kiev.physchem.kth.se/MycDB/seqsearch_form.html
Sequence analysis tools		
ExPASy	Expert Protein Analysis System; general protein analysis tools	http://expasy.hcuge.ch/www/tools.html
TMPred	TM domain predictions; under ExPASy tools	http://ulrec3.unil.ch/software/TMPRED_form.html
ExPASy compute MW	Molecular weight predictions; under ExPASy tools	http://expasy.hcuge.ch/ch2d/pi_tool.html
SignalP	Signal peptide motif and cleavage site prediction	http://www.cbs.dtu.dk/services/SignalP/
Genestream ALIGN	Sequence alignment tool	http://genome.eerie.fr/bin/align-guess.cgi

et al., 1989), or modified as described previously (Da Silva Tatley & Steyn, 1993). All DNA sequencing was performed on double-stranded plasmid DNA using the Sequenase protocol (version 2.0, USB), or by automated sequencing on an ALF Express Sequenator. *M. tuberculosis* DNA inserts were sequenced directly in plasmids pGB14T or pGB14TC using primers Tac1B (forward) and Bla (reverse, complementary to 5' end of TEM β -lactamase, 5'-CAGCATCTTTTACTTTC-3'), or after subcloning *EcoRI*/*XbaI*-digested inserts into pBS and using universal end-labelled primers. Insert DNA sequences were translated in three reading frames (forward direction with respect to the β -lactamase gene) and the ORF in-frame with the β -lactamase gene (Fig. 2) was identified. Insert ORFs were then searched against sequence databases and analysed for sequence motifs using the Internet servers listed in Table 1. The primary databases were the *M. tuberculosis* genome sequences released by The Institute for Genomic Research (TIGR) and the Sanger Centre.

Western blot analyses of fusion proteins. pGB14T(H37Rv)-transformed, ampicillin-resistant (Amp^r) *E. coli* were grown in LB broth containing 100 μ g ampicillin ml^{-1} to OD_{600} 0.5, induced with 0.3 mM IPTG for 2 h and centrifuged at 4000 r.p.m. The supernatants were concentrated 16-fold in a Centricon (30 kDa cut-off; Amicon) and saved as the culture filtrate fraction. The cells were divided into three fractions. (a) The pellet was resuspended in buffer A (20 mM Tris/HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA), frozen at $-20^\circ C$, thawed and sonicated (six 15 s pulses) and centrifuged at 8500 r.p.m., after which the supernatant was saved as the soluble cell fraction. (b) The pellet from (a) was resuspended in buffer A and saved as the insoluble cell fraction. (c) The cells were resuspended in 30 mM Tris/HCl, pH 8.0, containing 20% (w/v) sucrose, adjusted to 1 mM EDTA, incubated with shaking for 10 min at room temperature, centrifuged at 8500 r.p.m. for 10 min and the pellet resuspended in ice-cold 5 mM $MgSO_4$; after incubation on ice with shaking for 10 min, the suspension was centrifuged as before and the supernatant was saved as the periplasmic fraction.

The various cell fractions were mixed with SDS sample buffer and resolved by SDS-PAGE on a 10% (w/v) polyacrylamide gel. Electrophoresed proteins were transblotted onto Hybond-C nitrocellulose filters (Amersham) under standard conditions (see Hoppe *et al.*, 1997). The filters were incubated in blocking buffer (50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 5%, w/v, skimmed milk powder, 0.1% Tween 20) for 1.5 h at room temperature, and then incubated with rabbit anti-ampicillinase IgG (CP Laboratories; 1:1000 in blocking buffer) for 1 h at room temperature. The blots were washed in blocking buffer without skimmed milk powder (TBS/Tween), incubated with peroxidase-conjugated anti-rabbit IgG (ECL Western blotting system, Amersham; 1:1500 in TBS/Tween) for 1 h at room temperature, washed again in TBS/Tween, and then treated with ECL detection reagents and exposed to photographic film, as recommended by the manufacturer.

RESULTS

Construction of plasmids pGB14T and pGB14TC

pGB14 has proven useful in identifying signal-sequence-encoding genes from the Gram-positive bacteria *Bacillus subtilis* and *Lactococcus lactis* by selection in an *E. coli* background (Smith *et al.*, 1987; Perez-Martinez *et al.*, 1992). However, this vector may not function well with *M. tuberculosis* inserts because the leaderless β -lactamase gene in pGB14 also lacks a promoter and myco-

bacterial promoters are, in general, poorly recognized by the *E. coli* transcriptional apparatus (Das Gupta *et al.*, 1993). We therefore introduced the efficient, IPTG-inducible *E. coli* Tac promoter, to generate pGB14T. Selection for pGB14-transformed *E. coli* depends on erythromycin resistance, which is a poor marker in Gram-negative organisms; we found that even at 200 μ g erythromycin ml^{-1} , selection for true Em^r proved difficult due to a high rate of spontaneous low-level resistance. Hence we constructed pGB14TC as an alternative vector, in which the Em^r gene was interrupted by a Cm^r cassette. As expected, growth on chloramphenicol facilitated selection for *E. coli* (pGB14) transformants.

Construction in *E. coli* of a library of β -lactamase fusions with *M. tuberculosis* genomic DNA fragments

Genomic DNA from *M. tuberculosis* H37Rv digested with *Sau3A*, *Hae3* or *RsaI* was ligated into the MCS of pGB14T and pGB14TC between the Tac promoter and the leaderless β -lactamase gene. The highest transformation efficiency of *E. coli* with the ligation products was achieved with electroporation (10–100-fold greater than transformation of $CaCl_2$ -competent cells). However, even with electroporation, transformations were only in the order of 2×10^7 c.f.u. per μ g pGB14T(H37Rv) or pGB14TC(H37Rv) DNA. Transformation with the pGB14T or pGB14TC vectors alone gave similar results, compared to approximately 10^9 c.f.u. per μ g for pBS, indicating that pGB14-based vectors are inefficient transformation substrates.

Generally, *E. coli* transformed with pGB14T(H37Rv) or pGB14TC(H37Rv) were selected directly on plates containing 50–100 μ g ampicillin ml^{-1} , and an aliquot of each batch of transformants was grown on plates containing 200 μ g erythromycin ml^{-1} or 30 μ g chloramphenicol ml^{-1} , respectively, to assess transformation efficiencies. All Amp^r clones were tested on erythromycin or chloramphenicol plates, as appropriate, to select for true transformants. From a total of 6000 Em^r clones and 8000 Cm^r clones, 10 Amp^r/Em^r and 5 Amp^r/Cm^r transformants, respectively, were isolated.

Analysis of *M. tuberculosis* DNA- β -lactamase fusions conferring Amp^r in *E. coli*

Twelve of the Amp^r clones were tested for their level of Amp^r and all were resistant to up to 1 mg ampicillin ml^{-1} on LB plates. Insert sizes ranged from 350 to 3000 bp. Ten inserts from Amp^r/Em^r and Amp^r/Cm^r clones were sequenced and the results are summarized in Table 2. To facilitate analysis, insert DNA sequences were translated and the ORF in-frame with the β -lactamase gene was identified. Comparison of the deduced amino acid sequences with known sequences in databases enabled complete identification in all cases (Fig. 2). pGB14T-L4 and pGB14TC-W were found to be identical to the *M. tuberculosis* antigen 85A and 85C genes, respectively (Fig. 2a, b). pGB14T-L4 showed 100% identity over 180 amino acid residues with

Table 2. Characteristics of *M. tuberculosis* DNA- β -lactamase gene fusions that confer Amp^r in *E. coli*

Plasmids containing β -lactamase gene fusions that confer Amp^r in *E. coli* were purified, analysed by restriction mapping and sequenced as indicated. Sequence data were searched for homologies to sequences in the *M. tuberculosis* genome (see Table 1). All ten transformants were able to grow on agar plates containing 1000 μ g ampicillin ml⁻¹.

Recombinant plasmid	Insert size (bp)	G + C content (mol %)	Identity*	Size of mature protein (kDa)†
pGB14T-L4	921	67.2	Ag85A	32
pGB14TC-W	350	61.4	Ag85C	32
pGB14T-P	926	69.7	Hypothetical ORF, probable PBP (Pmtb1)	52
pGB14T-B	890	56.9	Hypothetical ORF (Bmtb1)	26
pGB14TC-X	928	62.4	Hypothetical ORF (Xmtb1)	13
pGB14TC-V	604	61.5	Hypothetical ORF (Vmtb1)	15
pGB14T-O2	914	62.2	Hypothetical ORF (O2mtb1)	30
pGB14T-O1	500	57.7	Hypothetical ORF (O1mtb1)	7.8
pGB14T-B3	500	57.7	Identical to O1mtb1	7.8
pGB14T-Q5	~ 3000	54.5	<i>E. coli</i> cyt. C biogen. protein	33

* Identities ($\geq 97\%$) to *M. tuberculosis* genes. In parentheses are our designations (see Fig. 2 for further information).

† Predicted molecular masses of putative proteins after cleavage of potential signal peptides.

Ag85A; pGB14TC-W showed 100% identity over 40 residues with Ag85C. Both inserts included the entire signal sequence, translational start site and upstream ribosome-binding site.

A group of four inserts was found to be homologous to mycobacterial genes that comprise hypothetical ORFs encoding one or more potential membrane-interaction domains (in addition to the signal peptides) (Fig. 3).

The first insert, pGB14T-P, was 98% identical (176 out of 180 amino acid residues) to the product of an unannotated, hypothetical ORF at positions 4244–3573 on contig gmt18 of the TIGR *M. tuberculosis* genome, which we have designated Pmtb1 for ease of reference (Fig. 2c). Pmtb1, in turn, was 75% identical (168 out of 225 amino acid residues) to the product of an unannotated, hypothetical ORF on cosmid B1529 (positions 19336–20019) of the *Mycobacterium leprae* genome (GenBank accession number L78824) (Pmlep1 in Fig. 2c). Using the recently released gapped BLAST protein database search program (BLASTP 2.0.2, Table 1), Pmtb1 was found to be 35% identical to PcbR, a low-affinity penicillin-binding protein (PBP) from *Streptomyces clavuligerus* (Paradkar *et al.*, 1996; GenBank accession number U56256). A lower degree of identity (31%) was found for two *M. tuberculosis* ORFs also annotated as low-affinity PBPs (MTCY10H4.16c and MTCY270.05; EMBL accession numbers Z80233 and Z95388). Both Pmtb1 and Pmlep1 contained potential membrane lipoprotein lipid attachment sites (as predicted by GCG motifs; see also Pugsley, 1993); Pmtb1 contained a potential TM domain near its C terminus (Fig. 3a).

The second insert, pGB14TC-V, was 98% identical (83 out of 84 residues) to the protein encoded by an annotated, hypothetical ORF (MTCY493.19) on *M. tuberculosis* cosmid Y493 (positions 26656–27264, EMBL accession number Z95844), which we have designated Vmtb1 (Fig. 2d). Vmtb1 was a 202 amino acid preprotein that contained an extended apolar, glycine- and proline-rich central domain (residues 60–156) that was not typical of a TM domain but may participate in hydrophobic interactions (Fig. 3b).

The third insert, pGB14TC-X, was a chimera consisting of two unrelated DNA fragments ligated at a *Hae*III site. The 5' fragment encoded a signal peptide that was 100% identical (23 out of 23 residues) to the product of an unannotated, hypothetical ORF in contig gmt234 (positions 5559–5627) of the TIGR *M. tuberculosis* genome, referred to here as Xmtb1 (Fig. 2e). Xmtb1 was, in turn, 75% identical (104 out of 137 residues) to a hypothetical, 16.9 kDa *M. leprae* protein (SWISS-PROT accession number P54880) (Xmlep1 in Fig. 2e). Further, Xmlep1 was 44% identical (65 out of 147 residues) to a hypothetical, 15.9 kDa *M. tuberculosis* protein (SWISS-PROT accession number Q11170) (Xmtb2 in Fig. 2e), which, lastly, was 42% identical (59 out of 142 residues) to a third hypothetical *M. tuberculosis* protein (MTCY04D9.16c on cosmid SCY04D9, EMBL accession number Z84725) (Xmtb3 in Fig. 2e). Two of the homologues, Xmlep1 and Xmtb3, contained putative TM domains near their C termini (Fig. 3c).

The fourth insert in this group, pGB14T-O2, was 100% identical to the product of an unannotated, hypothetical ORF in *M. tuberculosis* contig gmt551 (positions

Fig. 2. Sequence alignments of predicted proteins encoded by export-competent *M. tuberculosis* gene fusions. In each case (a-h), *M. tuberculosis* insert sequences identified with the pGB14T and pGB14TC selection vectors are shown above their respective homologues retrieved from mycobacterial databases (Table 1). Sequences are in the single-letter amino acid code. Only one line of sequence per insert is shown, truncated as necessary (by the number of residues indicated), ending in the underlined sequence (G)DPLESTAQAQ (spacer sequence at the fusion junction) followed by *ppetl*... (start of mature β -lactamase, in lower-case italics). Hydrophobic sequences that represent potential signal peptides or TM domains are in bold; arrows indicate probable signal peptide cleavage sites in the native proteins, as predicted by SignalP (Table 1). Sequence nomenclature is our own. Sources are as follows (in parentheses are accession numbers; gb, GenBank; emb, EMBL; sp, SWISS-PROT): (a) Ag85A (gb M27016); (b) Ag85C (sp P31953); (c) Pmtb1, unannotated ORF, contig gmt18, 4244-3573 (TIGR); Pmlep1, unannotated ORF, *M. leprae* cosmid B1529, 19336-20019 (gb L78824); (d) Vmtb1, ORF labelled MTCY493.19 on cosmid Y493, 26656-27264 (emb Z95844); (e) (note that pGB14T-X was a chimera, the 5' sequence encoding a signal peptide that was identical to that of Xmtb1; the 3' sequence, in italics, was not analysed further) Xmtb1, unannotated ORF, contig gmt234, 5559-5627 (TIGR); Xmllep1, ORF labelled U1740W on *M. leprae* cosmid B174, complement 24139-24603 (gb U15183); Xmtb2, ORF labelled CY20G9.33, on cosmid SCY20G9, 31159-31602 (emb Z77162); Xmtb3, ORF labelled MTCY04D9.16c, on cosmid SCY04D9, 14771-15199 (emb Z84725); (f) O2mtb1, unannotated ORF, contig gmt551, 5197-4167 (TIGR); (g) (pGB14T-B was also chimeraic; 3' sequence, in italics, was not analysed) Bmtb1, ORF labelled MTCY19H5.32c on cosmid SCY19H5, 24949-25776 (emb Z97182); Bmtb2, ORF labelled MTCI28.10 on cosmid I28, 8981-10021 (emb Z97050); Bmlep1, unannotated ORF on *M. leprae* cosmid B983, 9760-10797 (gb L78828); (h) O1mtb1, unannotated ORF on contig gmt374, 1342-1040 (TIGR).

Fig. 3. Hydropathy plots of putative *M. tuberculosis* exported proteins with possible membrane-interaction domains. Identities of the sequences are described in Fig. 2. Plots were drawn with the program TMPred (Table 1). Combined weight matrix scores of >500 indicate possible TM regions. (a) Pmtb1, (b) Vmtb1, (c) Xmtb3, (d) O2mtb1.

5197–4167) (O2mtb1 in Fig. 2f). O2mtb1 did not contain a typical cleavable signal sequence. A stretch of 21 hydrophobic and apolar residues that could serve as an export signal was preceded by at least 37 N-terminal residues, including three negatively charged residues and a cluster of five arginines (Fig. 2f), which is very uncharacteristic of cleavable signal sequences (Pugsley, 1993). O2mtb1 may be a type II TM protein with an uncleaved signal peptide acting as a true TM segment (Fig. 3d). Apart from this exception, all Amp^r-conferring β -lactamase gene fusions that were sequenced coded for readily identifiable signal sequences, defined as N-terminal extension sequences that direct export across the cytoplasmic membrane and characterized by shared physico-chemical features (Pugsley, 1993; Izard & Kendall, 1994) (Fig. 2). Thus, Pmtb1, Vmtb1, Xmtb1, O2mtb1 and homologues represent a group of hypothetical exported mycobacterial proteins that contain putative TM, hydrophobic, or membrane-interaction domains.

As was found for pGB14T-P and pGB14TC-X, pGB14T-B was homologous to several mycobacterial genes. pGB14T-B was 100% identical (50 out of 50 residues) to a hypothetical *M. tuberculosis* protein (MTCY19H5.32c on cosmid SCY19H5, EMBL accession number Z97182) (Bmtb1 in Fig. 2g), which was 65% identical (168 out of 257 residues) to a second hypothetical *M. tuberculosis* protein (MTCI28.10 on cosmid I28, EMBL accession number Z97050) (Bmtb2 in Fig. 2g). Bmtb2, in turn, was 75% identical (260 out of 346 residues) to the product of

an unannotated, hypothetical ORF in the *M. leprae* genome (cosmid B983, positions 9760–10797, GenBank accession number L78828; Bmlep1 in Fig. 2g). The functions of each of these putative exported proteins is unknown. Of note is that Bmtb2 contained four potential N-linked glycosylation sites (NXS/T) typical of eukaryotic exported proteins.

The insert in pGB14T-O1 was cloned independently on two occasions, as pGB14T-O1 and -B3, perhaps because it encoded an extremely favourable signal peptide (Fig. 2h), or because multiple copies of the gene were present in the genome. pGB14T-O1 was 100% identical (44 out of 44 residues) to the product of an unannotated, hypothetical ORF in *M. tuberculosis* contig gmt374 (positions 1324–1040) in the TIGR genome (O1mtb1 in Fig. 2h). Lastly, the insert in pGB14T-Q5 (at 3000 bp the largest) was found to be 100% identical (193 out of 193 residues) to the *E. coli* cytochrome C-type biogenesis protein CCMH precursor, which has an excellent N-terminal signal peptide (not shown). This is presumably an *E. coli* chromosomal DNA contaminant that was copurified and codigested with the pGB14T plasmid preparation prior to *M. tuberculosis* DNA insert ligation.

Immunodetection of β -lactamase fusions

Cell fractions of Amp^r *E. coli* transformed with the clones described in Fig. 2 were probed by Western blotting using an anti-TEM β -lactamase antibody.

Fig. 4. Western blots of Amp^r, pGB14T(H37Rv)-transformed *E. coli* fractions probed with an anti- β -lactamase antibody. *E. coli* cell fractions were prepared as described in Methods. (a) Periplasmic (peri) and culture filtrate fractions, respectively, of *E. coli* transformed with pGB14T-P (P peri, P filtrate), -O2 (O2 peri, O2 filtrate), -O1 (O1 peri, O1 filtrate) and pBS (pBS peri, pBS filtrate). (b) Insoluble fractions of *E. coli* transformed with the indicated vectors; no band was seen in the pGB14T-O1 lane on this blot. The arrow on the right indicates the position of an immunoreactive band in the pGB14T-B lane that was seen clearly on the original blot. Molecular mass marker positions (in kDa) are indicated.

Analysis of the periplasmic and culture filtrate fractions revealed fusion proteins with relative mobilities that in most cases were in good agreement with predicted molecular masses for the mature proteins after signal peptide cleavage, as follows (predicted masses in parentheses): pGB14T-W, 32 kDa (31); -P, 45 kDa (49); -V, 35 kDa (35); -X, 34 kDa (34); -O2, 31 kDa (30); -O1, 32 kDa (30.5) (Fig. 4a; pGB14T-W, -V and -X not shown). No immunoreactive band was detected in extracts of *E. coli* transformed with vector (pGB14T) alone; as positive control, pBS-transformed *E. coli* extracts revealed a protein of the expected molecular mass (30 kDa; predicted, 29 kDa) (Fig. 4a). Two of the transformants gave fusion proteins with smaller than expected masses: pGB14T-L4 revealed a protein of 38 kDa (predicted, 46.3) and pGB14T-B gave 35 kDa (39.5) (Fig. 4b). This discrepancy may have resulted from signal peptide cleavage at an alternative site or from secondary proteolytic processing of the mature fusion protein. Anomalous low fusion protein masses have been observed previously with α -amylase and PhoA fusions (Smith *et al.*, 1987; Lim *et al.*, 1995). Western blot analysis of the insoluble (Fig. 4b) and soluble (not shown) cell fractions revealed fusion proteins identical

in size to those seen in the periplasmic and culture filtrate fractions. Hence we were unable to detect the unprocessed pre-forms, except as occasional faint traces, including a 32 kDa immunoreactive band in the pBS fraction that presumably corresponded to the unprocessed form (faintly visible in Fig. 4b). We conclude that under these experimental conditions, signal peptide cleavage was so efficient that the vast majority of detectable fusion proteins was processed.

DISCUSSION

The mycobacterial protein export machinery has not been characterized. Although they are formally considered as members of the Gram-positive family, mycobacteria have unusual cell walls that contain unique features. Most notable is the presence of an arabinogalactan-mycolic acid superpolymer that is covalently linked, and external, to the peptidoglycan. The mycolic acids of this superpolymer have been proposed to constitute the inner leaflet of a pseudo outer membrane; the outer leaflet is provided by various non-covalently associated phospholipids and glycolipids (Brennan & Draper, 1994). Thus, the mycobacterial cell wall bears a superficial resemblance to the lipid-rich Gram-negative envelope, although the mycobacterial envelope is markedly waxy and much less fluid (Liu *et al.*, 1996). It can be anticipated, therefore, that some of the special problems of protein export in Gram-negative organisms are also encountered in mycobacteria, particularly with regard to targeting to various cell wall compartments, including complete secretion into the extracellular milieu (Pugsley, 1993). These considerations notwithstanding, it seems likely that the first step in protein translocation, export across the cytoplasmic membrane, which is common to the general secretory pathways of both Gram-positive and Gram-negative organisms, is also shared by mycobacteria. Indeed, many of the genes encoding secreted mycobacterial proteins encode typical N-terminal signal sequences (Young *et al.*, 1992), indicating that mycobacteria probably possess a standard Sec-dependent export pathway. A search of annotated genes in the *M. tuberculosis* genome (Sanger Centre) revealed homologues of *secA*, *secB*, *secD*, *secE* and *secF* [EMBL accession numbers and loci Z95121 (MTCY20B11.15c), Z79701 (MTCY277.40), Z77724 (MTCY227.14), Z92772 (MTCY20H10.19) and Z77724 (MTCY227.15), respectively]. The *Mycobacterium bovis* BCG *secY* has been reported (GenBank accession number U77912) and we found the *M. tuberculosis* homologue on TIGR contig gmt7540 (positions 12060–13382).

In efforts to gain further insights into protein secretion in *M. tuberculosis* we have explored the usefulness of a signal sequence selection vector for the identification and characterization of mycobacterial signal-sequence-encoding genes in an *E. coli* host. We have exploited a vector relying on β -lactamase gene fusions, which has proved useful in identifying *B. subtilis* and *L. lactis* signal sequence genes in transformed *E. coli* (Smith *et al.*, 1987; Perez-Martinez *et al.*, 1992). After modifying the vector by inserting a Tac promoter upstream of the

leaderless β -lactamase gene, we were able to isolate a variety of signal sequence genes from an *M. tuberculosis* genomic DNA library. The ability of the selection vector to successfully identify *bona fide* secreted proteins was evident from clones pGB14T-L4 and -W that contained fragments of the Ag85A and Ag85C genes, respectively, which together with Ag85B constitute the major secreted proteins of the *M. tuberculosis* complex (Content *et al.*, 1991). Ag85 proteins account for more than 30% (by weight) of the total extracellular proteins in *M. tuberculosis* culture filtrates (Horwitz *et al.*, 1995).

All but one of the Amp^r β -lactamase gene fusions that were sequenced were found to encode clearly recognizable signal sequences; the exception (pGB14T-O2) appeared to encode a sequence more typical of type II TM proteins with uncleaved signal peptides. All of the gene fusions tested conferred wild-type levels of Amp^r (up to 1 mg ampicillin ml⁻¹ on agar plates), indicating that the *M. tuberculosis* genome contains a number of genes encoding signal sequences that satisfy all the requirements of the *E. coli* Sec-dependent export machinery. This conclusion is supported by previous data demonstrating that the secreted *M. bovis* protein MPB70 was efficiently secreted into the periplasm of *E. coli* transformed with the entire MPB70 gene, including the mycobacterial ribosome-binding site, start codon and signal peptide (Hewinson & Russell, 1993). The MPB70 signal peptide also directed the export of the 19 kDa *M. bovis* antigen in *E. coli* (Hewinson *et al.*, 1996a). The nature of the selection system used in our experiments will be biased towards the selection of *E. coli*-compatible signal sequences and provides no information on the presence of atypical signal sequences and/or export pathways in *M. tuberculosis*. Nevertheless, these results argue strongly that a subset of proteins secreted by *M. tuberculosis* is exported by a Sec-dependent pathway which has characteristics similar to the *E. coli* machinery. This is consistent with the notion that despite differences in detail, the sec-dependent pathways of Gram-positive and Gram-negative bacteria are broadly similar (Pugsley, 1993; Salmond & Reeves, 1993; Bolhuis *et al.*, 1996).

All of the signal-peptide-encoding DNA fragments sequenced could be matched with database sequences. Nine of ten gave perfect matches with sequences in the *M. tuberculosis* genome (TIGR and Sanger Center databases), whereas one (pGB14T-Q5) contained an *E. coli* chromosomal DNA contaminant. Strikingly, apart from the Ag85 proteins that were recently shown to be mycolyltransferases (Belisle *et al.*, 1997), only one of the other putative exported proteins shown in Fig. 2 could be assigned a function, despite extensive BLAST searches against all of the major databases. Pmtb1 was an unannotated hypothetical ORF that after a gapped BLAST search was found to be 35% identical to a low-affinity PBP (PcbR) from *S. clavuligerus*, a protein that belongs to the high-molecular-mass group B PBPs (Paradkar *et al.*, 1996). Low-affinity PBPs of this type may play an important role in β -lactamase resistance in actinomycetes (Paradkar *et al.*, 1996). Further analysis

revealed that Pmtb1 was 31% identical to two annotated *M. tuberculosis* low-affinity PBPs, MTCY10H4.16c and MTCY270.05, which were 23% identical to each other. In contrast to the latter two, Pmtb1, and its *M. leprae* homologue Pmlep1, contained a signal peptide with features typical of a lipoprotein lipid attachment site (Fig. 2; see Pugsley, 1993). High-molecular-mass PBPs typically contain N-terminal hydrophobic domains that probably function as uncleaved signal peptides and membrane anchors, as has been inferred for PcbR (Paradkar *et al.*, 1996); similar sequences were present in the putative *M. tuberculosis* PBPs MTCY10H4.16c and MTCY270.05. Interestingly, Pmtb1 was only 6 kb removed from the gene encoding MPT83 (on contig gmt18 in the TIGR *M. tuberculosis* genome), which is a known lipoprotein that is expressed by members of the *M. tuberculosis* complex and which is homologous to, and in turn only 2.4 kb upstream of, the gene for the secreted *M. tuberculosis* protein MPT70 (Hewinson *et al.*, 1996b; Vosloo *et al.*, 1997). The functions of MPT83 and MPT70 are unknown, but expression of MPT83 may be elevated during an *in vivo* *M. tuberculosis* infection (Hewinson *et al.*, 1996b), and MPB70 is an immunodominant *M. bovis* antigen (Hewinson & Russell, 1993).

Further interesting observations that emerged from analyses of putative proteins encoded by the ORFs identified in our screen (Fig. 2) include the following. Of a set of four homologues, Xmlep1 and Xmtb3 contained a possible TM domain near their C termini, making them potential type I TM proteins. Together with O2mtb1, a potential type II TM protein, and Vmtb1, a protein containing an extended glycine- and proline-rich apolar domain, Pmtb1, Pmlep1, Xmlep1 and Xmtb3 constitute a group of exported proteins that are potentially associated with the cell membrane or other compartments of the lipid-rich mycobacterial cell wall (Fig. 3). Finally, also of interest was the presence of four potential N-linked glycosylation sites in Bmtb2. The existence of O-linked glycoproteins in mycobacteria is now well-established (Dobos *et al.*, 1996; Herrmann *et al.*, 1996) and there is evidence for N-linked carbohydrates in the secreted 19 kDa antigen of *M. tuberculosis* (M. Ward, W. Blackstock, M.-P. Gares, D. B. Young & C. Abou-Zeid, personal communication). The significance of this form of glycosylation in mycobacteria remains to be determined. It is noteworthy that the major outer-membrane protein of *Chlamydia trachomatis* contains N-linked high-mannose type oligosaccharides that are required for binding and infection of HeLa cells *in vitro* (Kuo *et al.*, 1996). We have shown recently that surface-exposed glycoconjugates mediate direct and opsonin-enhanced binding of *M. tuberculosis* to various mammalian cells *in vitro* (Hoppe *et al.*, 1997; Cywes *et al.*, 1997). Thus the possible involvement of mycobacterial glycoproteins in such processes warrants further investigation.

It has been shown that alkaline phosphatase (*phoA*) gene fusions are useful in studying protein export in mycobacteria (Timm *et al.*, 1994) and that this system

allows identification of *M. tuberculosis* genes encoding secreted proteins in an *M. smegmatis* host (Lim *et al.*, 1995; Jackson *et al.*, 1997). *PhoA* reporter constructs gave a yield of 0.09% Amp^r transformants from an *M. tuberculosis* genomic DNA library (Lim *et al.*, 1995), compared to our rate of 0.11%. Enhanced vector design enabling inserts to be cloned in all three reading frames with respect to *phoA* and optimization of mycobacterial genomic DNA digestions reportedly improved the yield of export-competent inserts to 1.5% of transformants; a complete analysis of these inserts has still to be presented (Mdluli *et al.*, 1995).

Compared to *phoA* gene fusions, the spectrum of signal sequence genes identified with β -lactamase fusions may be different because selection is based on antibiotic resistance with the latter versus a colour reaction with the former (Smith *et al.*, 1987). The advantages of β -lactamase fusions in contrast to *PhoA* fusions as membrane protein topology probes have been reviewed (Broome-Smith *et al.*, 1990). Particularly notable for the purposes of selection for export-competent sequences is the fact that β -lactamase fusions allow direct selection whereas *PhoA* fusions require screening of all colonies. *PhoA* fusions also have the disadvantage that some cytoplasmic fusions display weak enzymic activity, essentially giving false positives (Broome-Smith *et al.*, 1990). In our protocol we potentially selected against fusions that were poorly translocated by direct selection of all transformants at high ampicillin levels. Since precursor forms of β -lactamase have reduced activity (Roggenkamp *et al.*, 1985; Perez-Martinez *et al.*, 1992), we may have selected for fusions that were processed efficiently by the *E. coli* signal peptidase to generate mature, soluble proteins (Fig. 4). Moreover, by using conditions for inducing the strong *Tac* promoter, we probably selected against the recovery of β -lactamase fusions with multiple membrane-spanning, polytopic membrane proteins, which are toxic when over-expressed in *E. coli*. These considerations probably account for the exclusive identification of *M. tuberculosis* gene fragments encoding typical N-terminal signal sequences in our screen. Interestingly, none of the reported *M. tuberculosis* signal-sequence-encoding genes isolated by *phoA* gene fusions (Lim *et al.*, 1995; Mdluli *et al.*, 1995; Jackson *et al.*, 1997) appears to match those identified by β -lactamase gene fusions described here.

With the total sequencing of the *M. tuberculosis* genome virtually complete (Williams, 1998), the utility of both β -lactamase and *phoA* gene fusions lies not so much in identifying mycobacterial signal-sequence-encoding genes *per se*, but rather in testing the export competence of putative signal sequences in various bacterial hosts to probe for similarities between mycobacterial, Gram-negative and Gram-positive export pathways. The preliminary characterizations of export-competent β -lactamase and *phoA* gene fusions reported here and elsewhere (Lim *et al.*, 1995; Mdluli *et al.*, 1995; Jackson *et al.*, 1997) are first attempts at throwing some light on this important question. In view of the strong likelihood

that exported proteins play a key role in mycobacterial virulence and in potentially eliciting a protective host immune response (Lee & Horwitz, 1995; Orme *et al.*, 1993; Andersen, 1994; Horwitz *et al.*, 1995), an understanding of the structure, function and regulation of the *M. tuberculosis* protein export machinery may prove decisive in the design of novel preventive and therapeutic strategies for tuberculosis.

ACKNOWLEDGEMENTS

This work was supported by funds provided under the Glaxo Wellcome Action TB initiative and by the University of Cape Town. We thank Jan Maarten van Dijk for the vector pGB14 and John T. Belisle for the *M. tuberculosis* H37Rv genomic DNA. We are indebted to Di James for the automated DNA sequencing.

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Received 12 August 1997; revised 2 February 1998; accepted 17 February 1998.