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Immunological diversity within a family of cutinase-like proteins of *Mycobacterium tuberculosis*

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Abstract

Secreted proteins of *Mycobacterium tuberculosis* play key roles in the assembly of the mycobacterial cell wall, with many being major targets of the host immune response. To date, meaningful characterization of a significant proportion of this important group of proteins is lacking. Among the group of putatively secreted proteins of *M. tuberculosis* are 7 cutinase-like proteins (CLP), not previously characterized in terms of their immunogenicity or vaccine protective efficacy. Although the CLP vary in the degree of homology with one another, they all share a similar active catalytic triad, closely homologous to that of the cutinase of *Fusarium solani*. By construction of DNA vaccines of all 7 CLP, and expression and purification of soluble, recombinant CLP, this study addresses the immunological responses to these proteins. Clp1, 2, 3 and 6 were found to elicit significant IFN- γ secretion in DNA immunized mice, with the antigens also demonstrating specificity in terms of CLP-generated T cell IFN- γ release, with minimal cross reactivity of humoral responses. Finally, following delivery of DNA vaccines, Clp1, 2 and 6, conferred a moderate yet reproducible and significant level of protection in a murine aerosol model of *M. tuberculosis* infection.

Keywords

Tuberculosis; Cutinase; Vaccine; Immunology

1. Introduction

Mycobacterium tuberculosis continues to be one of the leading causes of death owing to an infectious disease. Approximately 2 billion people are infected with the bacteria with 8 million developing active disease per year [1,2]. In order to understand the process of *M. tuberculosis* pathogenesis and host immune activation, a great deal of attention has been given to those proteins destined for secretion. This has arisen from the fact that a large number of proteins are secreted from *M. tuberculosis* at key stages of host infection, such as within the macrophage phagosome, as well as during *in vitro* culture [3]. An array of key virulence determinants and immunogens are secreted by many pathogenic bacterial species. Examples of these include the well described and intricate systems of Gram-negative bacteria but also

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other intracellular, Gram-positive bacteria. *M. tuberculosis* possesses multiple systems dedicated to the export of proteins, secreting at least 250 [4,5]. In addition to the classical SecA dependant system, by which the well described, immunogenic antigen 85 complex is exported [6], *M. tuberculosis* also contains a second SecA protein, namely SecA2, which is necessary for full virulence through the translocation of SodA in response to oxidative stress [7]. Additionally, the SecA2 pathway is necessary for replication of the bacterium in murine macrophages *ex vivo* [8]. Furthermore, *M. tuberculosis* possesses an additional novel secretion mechanism, encoded by the ESX-1 locus, by which other important virulence determinants and immunologically dominant antigens are secreted by *M. tuberculosis*, i.e. early secreted antigen T cell 6 (ESAT-6) and culture filtrate protein 10 (CFP10) [9]. Although advances have been made in appreciating the repertoire of secreted proteins by analysis of the proteome [10,11], these methods are limited to the identification of proteins expressed under particular experimental *in vitro* conditions, which may not fully reflect the extent of secretion possibilities that may occur during *in vivo* survival. To utilize the abundant information offered by the annotated genome sequence [12] we have undertaken a bioinformatic approach to identify proteins with the potential for secretion in any condition. Our analysis identified a family of 7 cutinase-like proteins (CLP), 6 with putative secretion signals and sharing 17–63% homology with one another. All CLP have a similar predicted functional site as that of the well characterised cutinase from *Fusarium solani* [13], a feature which also had them grouped in a recent separate study [14]. Cutinases are serine hydrolases, which cleave complex glycolipid polymers, and are important secreted virulence factors in fungal pathogens of plants [15]. The role of bacterial cutinases, however, is not well described, with those identified to date being largely confined to saprophytic species and those pathogenic to plants, such as the pseudomonads [16,17]. We have prepared DNA vaccines expressing all 7 family member proteins and tested their ability to confer protection in a murine aerosol TB challenge model. Four of the *M. tuberculosis* CLP were selected and purified following expression in *Escherichia coli* and utilized to examine immunogenic potential. These studies reveal immunological diversity between the members of this family.

2. Materials and methods

2.1. Bacterial growth conditions

M. tuberculosis H37Rv (ATCC 27294) was grown in Proskauer and Beck liquid medium for 14 days and *M. bovis* was grown in Middlebrook 7H9 broth supplemented with ADC (Difco Laboratories, Detroit, MI) for 14 days at 37 °C. The bacteria were enumerated on OADC enriched Middlebrook 7H11 agar and stored in 30% glycerol/Phosphate buffered saline (PBS) at –70 °C. For plasmid preparations, *Escherichia coli* was grown in Luria-Bertani (LB) broth or on LB agar. Ampicillin (100 µg/ml) was supplemented as necessary.

2.2. Bioinformatic procedures

The proteome of *M. tuberculosis* H37Rv was downloaded from the Sanger site at <http://www.sanger.ac.uk/Projects/Mtuberculosis/> and batch submitted to the SignalP server [18] (<http://www.cbs.dtu.dk/services/SignalP/>) to identify putative signal peptides indicative of secretion using both the neural network and hidden Markov models of prediction applied to Gram-positive bacteria. The proteome was compared to the COG and CDD databases [19, 20] using the program rpsblast [21] and to the non-redundant protein database of the National Centre for Biotechnology Information using the program blastp with the low complexity filter off. The output of all programs above was piped into a hyperlinked Excel spreadsheet by tailored programs written in Visual Basic 6.0 (Microsoft Corp., Redmond, CA). The program clustalW [22] was used to align the protein sequences and to produce the phylogenetic tree.

2.3. DNA vaccines

The individual *clp* genes were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR with primers listed in Table 1. Amplified products were cloned directly into VR1002-TOPO [23], a mammalian expression vector modified from VR1020 [24], which utilizes the tissue plasminogen activator signal peptide and the human cytomegalovirus promoter for expression. Clones were sequenced prior to use to confirm integrity and orientation.

2.4. Protein expression and purification of inclusion bodies

CLP were cloned without putative signal sequences, when present, into an *E. coli* expression vector (pET19b, Novagen) following amplification of each from H37Rv genomic DNA using the primers listed in Table 1. Protein expression was driven by the T7 promoter, induced with 0.5 mM IPTG and incubated at 37 °C for 4–16 h depending on protein. This resulted in over-expression of N-terminally His-tagged cytoplasmic proteins, which aggregated as insoluble inclusion bodies. Inclusion bodies containing the recombinant proteins were purified as follows. The cell pellet recovered from an overnight, *E. coli* BL21 (DE3) expression culture was resuspended in 20 ml buffer (50 mM HEPES pH 7.5, 0.5 M NaCl, 10 mM EDTA, 5 mM DTT, 0.35 mg/ml lysozyme, 1 mM PMSF) per litre of culture and slowly agitated at RT for 30 min. Triton-X-100 was added (1%, v/v) and cells ruptured with 3 freeze/thaw cycles in liquid nitrogen. Chromosomal DNA was sheared by sonication and extract treated with DNase I (0.03 mg/ml) for 2 h at 37 °C. Inclusion bodies were pelleted following centrifugation at 30,000 × *g* for 30 min and washed 2–3 times (PBS with 1% Triton-X-100 and 1 mM EDTA), spinning as above between each wash. Following the final spin, the pelleted inclusion bodies were solubilised in 2 ml of buffer (50 mM HEPES pH 7.5, 6 M guanidine HCl, 25 mM DTT) for a minimum of 2 h. In order to remove remaining aggregates, a final high speed spin (100,000 × *g*) was necessary. Solubilised inclusion bodies were purified by Talon (Clontech), Co²⁺ charged immobilized metal affinity chromatography (IMAC) according to manufactures instructions. Briefly, a batch/gravity format was conducted where His-tagged proteins are bound and washed to the resin in ‘batch’ with gentle agitation. Following this the resin was transferred to a 20 ml disposable column for one last wash and elution by ‘gravity’. The His-tagged proteins were eluted by the addition of 150 mM imidazole containing buffer.

2.5. Protein refolding

Proteins were refolded by a rapid dilution (20×), at 4 °C, into a set of 15 buffers prepared as described in the Quick Fold Protein Refolding kit manual (AthenaES, Baltimore). Proteins were then analyzed for solubility to identify the protein specific, optimal refolding conditions. A buffer composed of 50 mM Tris (pH 8.5), 9.6 mM NaCl, 0.4 mM KCl, 0.4 M sucrose, 0.05% PEG 3550, 0.5% Triton-X-100, 2 mM MgCl₂, 2 mM CaCl₂, with 1 mM GSH and 0.1 mM GSSH was selected to fold all proteins. Proteins were allowed to fold overnight with gentle stirring.

2.6. PAGE and Western blot

Protein samples were separated on a 12% SDS-polyacrylamide resolving gel and stained with coomassie blue. For Western blot, equivalent amounts of the protein samples were loaded along with prestained molecular weight standards (BioRad) and transferred to PVDF membrane. Blots were probed with anti-His (Qiagen) or anti-CLP (1:100) antibodies as required. HRP-conjugated secondary antibodies were used and blot developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and visualized on a Kodak 4000 mm Image Station (Eastman Kodak, Rochester, NY).

2.7. Immunization

Six to eight weeks old C57BL/6 female mice were obtained from Animal Resources Centre (Perth, Australia) and maintained in specific pathogen free conditions. Mice were immunized with 100 µg DNA vaccine encoding each CLP (1, 2, 3, 4, 5, 6, 7) or control vector, pcDNA3 (Invitrogen), by intramuscular (im) injection of 50 µl into each tibialis anterior muscle. Control mice were immunized subcutaneously with *Mycobacterium bovis* (bacille Calmette Guerin, BCG) at least 100 days prior to challenge at a dose of 5×10^5 CFU.

2.8. *M. tuberculosis* challenge

Six weeks following final DNA immunization, mice were challenged with aerosol *M. tuberculosis* H37Rv with an infective dose of approximately 100 CFU per lung using a Middlebrook airborne infection apparatus (Glas-Col, Terre Haute, Ind.). At four weeks post challenge, selected organs were homogenized, and plated as 10-fold dilutions on supplemented Middlebrook 7H11 Bacto agar.

2.9. Antibody measurement

Serum CLP-specific antibody levels were detected by ELISA as previously described [25]. Briefly, plates were coated with purified recombinant Clp1, 2, 3 or 6 (2 µg/ml) and sera of immunized and control mice was titrated five-fold to completion. Levels of anti-CLP Ab were detected with alkaline phosphate conjugated IgG, and subsequent development with *n*-nitrophenyl phosphate (1 mg/ml) (Sigma). The mean absorbance plus three standard deviations of normal mouse sera, diluted at 1:100, was adopted as the cut-off absorbance for determining antibody titers.

2.10. ELIspot for IFN-γ-producing cells

Antigen-specific IFN-γ secreting cells were measure by ELIspot. The anti-IFN-γ antibody, AN18 (Endogen, Woburn, MA) was coated onto 96 well, Immobilon-P plates (Millipore, Bedford, MA). Splenocyte samples were seeded at 2×10^5 cells per well and incubated with purified Clp1, 2, 3 or 6 (10 µg/ml) or media alone. Following 16 h of incubation at 37 °C, the plates were washed and incubated for 1 h with biotinylated anti-IFN-γ antibody, XMG 1.2 (Endogen) followed by streptavidin–alkaline phosphate conjugate. The number of spot forming cells were enumerated with an automated ELIspot reader system (AID, Strasburg, Germany)

2.11. Statistical analysis

Statistical analysis of the results from log₁₀ transformed data was carried out using analysis of variance (ANOVA). Fisher's Protected Least Significant Difference ANOVA post hoc test was used for pair-wise comparison of multi-grouped data sets. Differences with $p < 0.05$ were considered to be significant.

3. Results

3.1. Cutinase identification and classification

The signalP server [18] was queried to identify the presence of putative secretion signals in the *M. tuberculosis* proteome. Those proteins determined to have secretion potential were grouped by possible functional similarities. Seven proteins clustered with homologies ranging from 17 to 63% at an amino acid level (Table 2). Two of these proteins have been previously identified in filtrates of *M. tuberculosis* cultures, i.e. Cfp21 (Rv1984c) and Cfp25 (Rv2301), which are reported here as Clp1 and Clp2 respectively [26]. These proteins, as other members of the family, share homology to a fungal cutinase and have therefore been classed as a family of cutinase-like proteins.

3.2. Expression and purification

Four CLP were cloned into an *E. coli* expression vector that resulted in an N-terminally His tagged protein. Following over-expression, purification of chemically solubilized CLP was achieved using Co²⁺ charged immobilized metal affinity chromatography (IMAC). The CLP were then refolded in a buffer series, which varied in their components, so as to address critical and unique physiochemical refolding conditions for the proteins, including pH, oxidation state, ionic strength, as well as the presence of chaotropic and polar agents. This resulted in refolded, soluble and pure recombinant proteins (Fig. 1), allowing further characterization of the individual proteins immunologically.

3.3. Immunogenicity

The humoral response to immunization with DNA vaccines expressing CLP was analyzed by Western blot and an anti-Clp IgG antibody ELISA. Western blot analysis of the four purified CLP demonstrated specificity of the anti-CLP antisera. When blots of purified Clp1, Clp2, Clp3 and Clp6 were probed with antiserum raised against the product of each CLP DNA vaccine, only the protein specific for its own immune serum was detected indicating a lack of cross reactivity between the antiserum, at least at the limits of detection by Western blot (Fig. 2). A more accurate evaluation of the specificity of serological responses was determined by IgG antibody ELISA and were also shown to be distinct and specific for each antigen (Table 3). High titers were observed for all four CLP with Clp1 and Clp3 being the highest with titers greater than 10,000. Sera from *clp1*, *clp2*, and *clp3* immunized mice demonstrated IgG titers at least 20 fold higher for their respective antigens compared to the next highest cross reacting antigen, indicating a surprising degree of specificity for proteins of the same enzymatic class that share a significant level of homology (39–49%) between them.

For cell-mediated immunological responses spleens were harvested from DNA vaccinated mice and antigen specific T cells determined by an *ex vivo* ELISpot assay. DNA immunization stimulated a strong T cell response, with the most vigorous IFN- γ -secreting T cell responses being elicited predominately for their specific recall antigen (Fig. 3). In particular, *clp1* and *clp2* immunized mice both responded with approximately 1000 IFN- γ cells per 10⁶ splenocytes when recalled to Clp1 and Clp2 respectively (Fig. 3A and B), indicating that 0.1% of splenocytes were antigen specific T cells. Immunization with *clp3* and *clp6* also generated specific IFN- γ -secreting T cells, albeit to a lesser degree than those with *clp1* and *clp2*. The T cells of *clp3*-immunized mice were stimulated by Clp3 and Clp6 with the reciprocal relationship existing for *clp6* immunized mice also, in that splenocytes respond strongly to Clp3 and Clp6. This suggests that a dominant T cell epitope in C57BL/6 mice is shared between Clp3 and Clp6.

3.4. CLP as protective antigens

Many of the secreted proteins of *M. tuberculosis* tested as possible vaccine antigens have proven to be protective, of which the best-known examples are members of the antigen 85 complex and ESAT-6. To determine if the individual CLP could confer protective immunity, C57BL/6 mice were immunized with DNA vaccines encoding each of the 7 genes. Plasmid *clp1*, 2, and 6 immunized mice repeatedly demonstrated a significant level of protection as evidenced by a reduced bacterial load in the lungs, up to a 0.5 log₁₀ reduction, compared to mice immunized with control plasmid ($p < 0.001$) (Fig. 4). Immunization with plasmid *clp3*, *clp4*, *clp5* and *clp7* did not induce a consistently significant level of protection in biological repeats of the experiment (data not shown). Furthermore, immunization with plasmids encoding CLP genes did not prevent dissemination of *M. tuberculosis* into the spleens of infected mice.

4. Discussion

In the present study we have initiated the immunological characterization of the putatively secreted family of cutinase-like proteins of *M. tuberculosis* and revealed immunological diversity between its members. Some CLP elicit distinct immune responses in both the cell mediated and humoral compartments and some members are shown to protect mice partially from challenge with virulent *M. tuberculosis*.

The importance of various secreted gene products of *M. tuberculosis*, in terms of immunogenic potential is established, with some being frequently included in multi-component vaccines, such as Ag85, ESAT-6 and CFP10 [27–29]. The success of these secreted proteins in vaccines drives the search for further immunogenic and protective antigens. By utilizing an efficient set of bioinformatic algorithms we highlighted the family of cutinase-like proteins from *M. tuberculosis*, and produced soluble, refolded recombinant CLP allowing the characterization reported here.

The first description of cutinase-like proteins in *M. tuberculosis* were as short term (7 day), culture filtrate proteins (CFP), Cfp21 and Cfp25, here termed Clp1 and Clp2 respectively [26]. These antigens were potent inducers of IFN- γ from memory T lymphocytes from *M. tuberculosis*-infected mice and elicited strong delayed type hypersensitivity response in *M. tuberculosis*-sensitized guinea pigs [26]. Subsequent studies revealed that Clp1 vaccination resulted in cytotoxic T lymphocyte activity in mice [30] and further, one or both of these antigens were also recognized by tuberculosis patients [31,32]. The current study demonstrates that Clp1, Clp2, Clp3 and Clp6, when delivered as DNA vaccines, induced potent IFN- γ secreting T lymphocyte and strong specific IgG responses in immunized mice. Overall, the T cell response was specific to the individual CLP, with limited cross reactivity. For example, splenocytes from mice immunized with Clp1 did not respond to reactivation with the other CLP. Clp3 and Clp6, however, did appear to share a T cell epitope in H-2^b mice, even though these two proteins have the least aa homology within the group (Table 1). Regions of high homology do exist between the proteins, and further experimental work is required to determine if one or more of these regions may contain this epitope.

Direct evidence for secretion of the CLP family members is lacking. Secretion of Clp1 and Clp2 into the culture supernatant has been demonstrated previously [26], and indeed this observation has been confirmed by us by immunoblotting with anti-CLP antiserum (results not shown). However we were not able to detect other CLP in *M. tuberculosis* CFP. Clp4 has recently been reported to be secreted into culture supernatant by *Mycobacterium smegmatis* yet reside only within the cell wall of *M. tuberculosis* [14]. One member of the family, Clp5, lacks the classical secretion signal and would appear to be restricted to the cytoplasm unless secreted by another mechanism. Secretion of several CLP may be occurring at a very low concentration, below the detection possible offered by the anti-CLP antibodies generated by DNA vaccination. Further work is needed with improved reagents and more sensitive assays to clarify the actual secretion potential of these proteins and if so, by which mechanism.

DNA vaccination has been demonstrated to result in legitimate protein folding within the cells that take up and express the vaccine vector, particularly for viral antigens [33,34]. Although conformational epitopes might exist, the IgG epitopes of the CLP would be polyclonal and predominantly linear. This assumption is made based on the Western blot containing reduced proteins, thereby exposing linear epitopes only. Conformational epitopes may represent some of the labeling seen in the ELISA analysis as these were performed with *in vitro* refolded proteins as target.

The positive immunogenicity seen translated into protection when DNA immunized mice were challenged with virulent *M. tuberculosis*. DNA immunization with plasmids expressing Clp1,

Clp2 and Clp6 consistently stimulated a significant, albeit a moderate, level of protection against aerosol *M. tuberculosis* challenge similar to that observed with DNA vaccines expressing other *M. tuberculosis* proteins [35]. Further this level correlates with protection reported when Clp1 was administered as protein, adjuvanted with DDA [30]. CLP have also been included in multivalent vaccines. Immunization with vaccines containing Clp1 as a component, either as DNA [28] or protein delivered with monophosphoryl lipid (MPL) and/or dimethyl-dioctadecylammonium (DDA) [29], stimulated protective immunity against *M. tuberculosis*. A multivalent protein vaccine containing Clp2 also stimulated a similar degree of protection [31]. It is however difficult to ascertain the contribution of the CLP in these studies as they were partnered by well defined, immuno-dominant mycobacterial antigens. We believe this study demonstrates that the CLP may have played a role in conferring protection in these previous studies and taken together, indicate the potential of CLP as future vaccine components. Further work is underway to establish whether the CLP members can demonstrate superior protective effects when administered as either single or multiple proteins with the appropriate adjuvants.

The real value of subunit vaccines, such as DNA encoded vaccines, may be in their use as boosting vaccines following BCG immunizations [36]. It is interesting to note that not all CLP are present in all strains of BCG. Clp1 is encoded within RD2, a 10.7 kb chromosomal region deleted from the original BCG Pasteur strain sometime after its derivation [37]. With the exception of Clp1, BCG Pasteur (ATCC 35734) encodes all CLP, so their use as homologous boosting antigens may reactivate effective memory immune responses. Including Clp1 in a subunit vaccine would potentially have a similar effect in those vaccinated with RD2 containing BCG strains but further, may act as a priming antigen when used as a heterologous boost in BCG Δ RD2 vaccinated individuals. Further work is required to define the potential use of CLP as BCG boosting vaccines.

Unlike the Antigen 85 complex, which are known to associate with the mycobacterial cell wall where they are required for its assembly [38], the functions of most CLP are not yet defined. Homology searches identify the CLP as potential serine esterases belonging to the α/β -hydrolase superfamily [39] and as such may possess lipolytic and/or esterase activities. Phospholipase A activity has recently been attributed to Clp4 [14], indicating yet a further potential substrate range for the CLP family. Due to their secretion potential it could be hypothesized that one or more CLP are involved in synthesis or maintenance of the cell wall and/or nutrient scavenging, thereby having a direct role in the virulence of *M. tuberculosis*. The number of CLP in the *M. tuberculosis* genome and the conservation of this enzymatic class in other Mycobacteria may indicate an important role for these proteins and might therefore highlight their potential as vaccine constituents or drug targets and is the subject of ongoing work.

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Fig. 1. Purified, refolded recombinant CLP. Coomassie stained CLP shown following *E. coli* expression and HIS-tag purification. Lane 1: Clp1; lane 2: Clp2; lane 3: Clp3; lane 4: Clp6.

Fig. 2.

Antigen specificity determined by Western blot. (a) Western blot of Clp1, 2, 3 and 6 loaded in lanes 1–4 respectively and probed with anti-Clp2. (b) Composite figure of the positive detection of Clp1, 3 and 6 resulting from their relative immune sera. As in (a), no detection of other CLP family members was observed with antisera from another immunizing antigen (lanes not shown). Molecular weight standards (M) with sizes (kDa) are shown to the left.

Fig. 3.

Antigen-specific T cell responses from CLP immunized mice. IFN- γ secreting cells were enumerated by ELISpot following recall to antigens at 10 μ g/ml, for mice immunized with DNA vaccines expressing: (A) Clp1; (B) Clp2; (C) Clp3; (D) Clp6. The IFN- γ T cell responses in splenocytes from control vector immunized mice, stimulated with each of the CLP antigens were subtracted from the corresponding samples of DNA vaccine immunized mice. These values were: Clp1 protein, 190; Clp2 protein, 42; Clp3 protein, 194; Clp6 protein, 254. Data are the means and standard errors for five mice and are representative of one of two experiments. Statistical significance was determined by ANOVA; ** $p < 0.001$, *** $p < 0.0001$, NS; not significant, ND; not detected

Fig. 4.

CLP DNA vaccination protects mice from TB challenge. Immunization with DNA vaccines expressing the Clp1, 2 and 6 confers partial protection against *M. tuberculosis* H37Rv infection following aerosol challenge. Parental vector (Cont) and BCG were used as negative and positive controls respectively. Data are the means $\pm$ S.E.M. for five mice and are representative of duplicate experiments. Statistical significance was determined by ANOVA; * $p < 0.001$, ** $p < 0.0001$.

Table 1

Primers used in DNA vaccine construction and recombinant protein expression

Primer	Sequence 5'–3'	1st CLP aa
C1dnaFOR	GAT CCGTGTTCTGGACATCGCG	D33
C1dnaREV	ACAACAGTCTTTGATCATCCGGCG	
C2dnaFOR	GCCT GCCCCGACGCCG	A33
C2dnaREV	GAAAGCGTGAAGCAAAGACTCGC	
C3dnaFOR	GAT GGATGCCCCGGACGCCAAG	D27
C3dnaREV	TCACCCCGATGAACCTCATGACGC	
C4dnaFOR	CCAC CGGCATCGGGGGGTGCC	P41
C4dnaREV	GGCGGCGCAGCCGTTGCTTAGCC	
C5dnaFOR	ATG CCGGGGCGGTTTCAGAG	M1
C5dnaREV	TCACGACGTCCGATCGGTG	
C6dnaFOR	CTG GTTCATCGTGGCCGTGGTG	L27
C6dnaREV	CGCCGCCTAGCGTCCATCG	
C7dnaFOR	GCG TCGGAGGACTGTTTCGTC	A11
C7dnaREV	TGGGGTGGCAGCGCAGTTC	
C1protFOR	TGCGG ATCCGTGTTTCGGAC	A32
C1protREV	ATT GGATCC AGCGGTATAGGGACAACAGTC	
C2protFOR	ATT GGATCC CA CTG CAGCCTGCCCCG	T31
C2protREV	ATT GGATCC TATTGCAGCTTTCCGGC	
C3protFOR	ATT CTCGAGGTT GCCTTCGCCGATGG	V23
C3protREV	ATT CTCGAGT CACCCCGATGAACCTCATGAC	
C6protFOR	AT GGATCC GGCCGTGGTGATCATGCTG	A31
C6protREV	AT GGATCC TAGCGTCCATCGTC	

Restriction sites used for cloning are underlined and the first CLP specific codon indicated in bold with the resultant amino acid and its position in the unprocessed protein also shown.

Table 2
Percentage homology of mature cutinase-like proteins (CLP) of *M. tuberculosis* as determined by clustalW

	Clp1	Clp2	Clp3	Clp4	Clp5	Clp6	Clp7
Rv1984c	Clp1	100					
Rv2301	Clp2	44	100				
Rv3451	Clp3	49	39	100			
Rv3452	Clp4	53	54	63	100		
Rv1758	Clp5	49	39	45	100		
Rv3802c	Clp6	20	22	21	18	100	
Rv3724	Clp7	49	38	46	56	27	100

Table 3

Serological responses of mice immunized with DNA vaccines expressing CLP

Mouse Serum	Protein antigens			
	Clp1	Clp2	Clp3	Clp6
Anti-Clp1	>10000	ND	3000	150
Anti-Clp2	250	1800	300	150
Anti-Clp3	500	ND	10000	ND
Anti-Clp6	ND	ND	2200	6500

Data represent the mean IgG titers for five mice, at a cut-off three times the level for non-immunized sera and are representative of two experiments.