

## Cutinase-like proteins of *Mycobacterium tuberculosis*: characterization of their variable enzymatic functions and active site identification

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**ABSTRACT** Discovery and characterization of novel secreted enzymes of *Mycobacterium tuberculosis* are important for understanding the pathogenesis of one of the most important human bacterial pathogens. The proteome of *M. tuberculosis* contains over 400 potentially secreted proteins, the majority of which are uncharacterized. A family of seven cutinase-like proteins (CULPs) was identified by bioinformatic analysis, expressed and purified from *Escherichia coli*, and characterized in terms of their enzymatic activities. These studies revealed a functional diversity of enzyme classes based on differential preferences for substrate chain length. One member, Culp1, exhibited strong esterase activity, 40-fold higher than that of Culp6, which had strong activity as a lipase. Another, Culp4, performed moderately as an esterase and weakly as a lipase. Culp6 lipase activity was optimal above pH 7.0, and fully maintained to pH 8.5. None of the CULP members exhibited cutinase activity. Site-directed mutagenesis of each residue of the putative catalytic triad in Culp6 confirmed that each was essential for activity toward all fatty acid chain lengths of nitrophenyl esters and lipolytic function. Culp1 and Culp2 were present only in culture supernatants of *M. tuberculosis*, while Culp6, which is putatively essential for mycobacterial growth, was retained in the cell wall, suggesting the proteins play distinct roles in mycobacterial biology.—West, N. P., Chow, F. M. E., Randall, E. J., Wu, J., Chen, J., Ribeiro, J. M. C., Britton, W. J. Cutinase-like proteins of *Mycobacterium tuberculosis*: characterization of their variable enzymatic functions and active site identification. *FASEB J.* 23, 1694–1704 (2009)

*Key Words:* secreted enzymes • activity

*MYCOBACTERIUM TUBERCULOSIS* CONTINUES to be one of the leading causes of death from an infectious disease, claiming 5500 lives each day (1). One-third of the world's population is estimated to be infected with the

pathogen, and resistance to multiple front-line drugs has emerged. The current vaccine against tuberculosis (TB), *Mycobacterium bovis* bacille Calmette Guerin (BCG), is failing to stop its spread, and new, more effective treatments are urgently required. As a result, TB has been declared a public health emergency. Secreted proteins of *M. tuberculosis* have attracted much attention in attempts to understand the molecular mechanisms of pathogenesis. Reasons for this include the fact that a large number of proteins are secreted from *M. tuberculosis* during *in vitro* culture and that many of these proteins are important for virulence, as they are secreted at key stages of infection, such as the macrophage phagosome (2), where live bacilli effectively arrest maturation and phagolysosomal fusion (3, 4). Several of these proteins perform important enzymatic functions, as membrane-bound or secreted proteins, and they can enhance mycobacterial survival and virulence. Among the most well known groups of secreted proteins to perform a vital role in the cell wall are the members of the Antigen 85 complex, which are essential mycolyl transferases required for the terminal transfer of mycolic acid during cell wall biosynthesis (5). Other secreted enzymes such as SapM and PtpA inhibit phagolysosomal fusion through their action on host molecules required for this process (6, 7). A further group of secreted enzymes known to perform important functions are the serine hydrolase/lipases, which may act as cell wall-associated virulence factors (8) or in triacylglycerol utilization under nutrient-limiting conditions (9), inferring a role that may occur during latent infection. Mining of the *M. tuberculosis* genome sequence (10) has highlighted the importance of lipid metabolism for mycobacteria with lipases being over-

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represented compared to other bacteria. For example, *Escherichia coli* possesses 50 enzymes involved in lipid metabolism, while the *M. tuberculosis* genome contains 5 times this number (11).

Seven cutinase-like proteins (CULPs), 6 of which have putative secretion signals, were selected for further functional studies, based on the outcome of a bioinformatic analysis. This family of putative enzymes are so-named because they share a similar predicted functional site as the well-characterized cutinase from *Fusarium solani*, a fungi pathogenic to plants (12), facilitating fungal invasion as a secreted virulence factor (13). Cutinases are hydrolytic enzymes with specificity for cutin, a complex glycolipid polymer consisting of hydroxy and epoxy fatty acids (14). Few examples of bacterial cutinases exist in the literature, with those identified to date being largely confined to saprophytic species and those pathogenic to plants, such as the pseudomonads (15, 16). Recently we have demonstrated different immune responses to CULP members, with three conferring protection as DNA vaccines against *M. tuberculosis* challenge in mice (17). In this study, the seven *M. tuberculosis* CULPs were expressed as recombinant *E. coli* proteins and purified for enzymatic characterization. There was functional diversity and variation in location within the CULP family, with none of the member proteins able to hydrolyze cutin, the natural substrate of a cutinase. The active site of Culp6, which is a putatively essential enzyme (18), was identified and the subcellular location of Culp6 was established for the first time. This novel secreted enzyme may represent a future drug target to control mycobacterial infections.

## MATERIALS AND METHODS

### Reagents

*E. coli* DH5 $\alpha$  was used for plasmid preparations during cloning experiments and grown in Luria-Bertani (LB) broth or on LB agar. Ampicillin (100  $\mu$ g/ml) or kanamycin (50  $\mu$ g/ml) was supplemented as necessary. H37Rv genomic DNA and *M. tuberculosis* cellular fractions were obtained from Colorado State University (Fort Collins, CO, USA).

### Bioinformatic procedures

Bioinformatic analyses was performed as described previously (17). Briefly, the proteome of *M. tuberculosis* H37Rv was batch submitted to the SignalP server (19) (<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 further compared to the COG and CDD databases (20, 21) using the program rpsblast (22) and also to the nonredundant protein database of the National Center for Biotechnology Information (NCBI), where blastp was utilized. Initial sequence alignment was performed with ClustalW (23), whereas final alignment of catalytic residues was done manually. Mega3 was used to obtain the phylogram using the neighbor-joining algorithm, bootstrapped 10,000 times (24).

### Protein expression and purification of inclusion bodies

CULPs were cloned without signal sequences into an *E. coli* expression vector (pET19b; Merck, San Diego, CA, USA) following amplification of each from H37Rv genomic DNA. This resulted in overexpression of N-terminally His-tagged cytoplasmic proteins. Cytoplasmic inclusion bodies consisting of the recombinant proteins accumulated following IPTG (0.5 mM) induction and were purified as described previously (17).

### Protein refolding

Refolding potential of each protein was screened in a set of 15 buffers, prepared as described in the Quick Fold Protein Refolding kit manual (AthenaES, Baltimore, MD, USA). A buffer representing optimal refolding conditions 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 MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, with 1 mM GSH and 0.1 mM GSSH was selected to fold all proteins. Proteins were rapidly diluted (20 $\times$ ) into refolding buffer and were allowed to fold overnight at 4°C with gentle agitation. Correct protein folding was analyzed by FPLC on a Sephadex-200 gel filtration column (GE Healthcare, Piscataway, NJ, USA). Refolded protein (100  $\mu$ l) was loaded to the column, and chromatography was performed at a flow rate of 0.5 ml/min. Eluted protein peaks were detected by UV absorption, and peak retention times were compared to a standard curve giving approximate molecular masses.

### Enzyme assays

The esterase and lipase potential of the CULPs were determined as described by Winkler and Stuckmann (25) with minor adaptations. The stock solutions of the substrates, *p*-nitrophenyl butyrate (pNPB), *p*-nitrophenyl laurate (pNPL), *p*-nitrophenyl myristate (pNPM), *p*-nitrophenyl palmitate (pNPP), and *p*-nitrophenyl stearate (pNPS) were obtained from Sigma (St. Louis, MO, USA) or MP Biomedicals (Irvine, CA, USA) and prepared in isopropanol to 10 mM. These substrate solutions were mixed 1:9 with a solution containing 50 mM sodium phosphate (pH 8.0), 2.3 mg/ml sodium deoxycholate, and 1 mg/ml gum arabic. To 10  $\mu$ l of enzyme solution (100  $\mu$ g/ml), 240  $\mu$ l of the above reaction mixture was added in a 96-well microwell plate, mixed, and incubated at 37°C for 30 min. The accumulation of *p*-nitrophenol was measured spectrophotometrically at 405 nm, and concentrations were calculated by comparisons to *p*-nitrophenol standards. Lipolytic activity as determined by Tween cleavage was performed as previously reported (26). Briefly, a reaction mix was prepared with 33 mM CaCl<sub>2</sub> and 0.33% Tween 20 in buffer (50 mM Tris for pH 7.0 and pH 8.0; 50 mM MES for pH 6.0). Fifty microliters of enzyme was added prior to 1 h incubation at 37°C. Two hundred fifty microliters was transferred to a 96-well microwell plate, and turbidity was assessed at 405 nm. Activity in this assay is reported as U/ $\mu$ g of protein, with 1 U defined as the amount of enzyme required to effect a change in OD (405 nm) of 0.01 under assay conditions.

Cutinase activity was determined as previously reported (27) with the following modification. Apple cutin, prepared from mature apples (28), served as the substrate. For a typical assay, 1 mg of enzyme and 100 mg of apple cutin were added into buffer (50 mM Tris-HCl, pH 7.5) in a final volume of 10 ml. The tube was shaken (125 rpm) for 18 h at 37°C for Clp1-7 and 60°C for *Thermobifida fusca* cutinase. At the end of reaction, the remaining cutin was removed by centrifugation. The resulting supernatant was acidified with acetic acid, and the released cutin monomers were extracted with chloroform. The organic soluble material was collected and dried

under a stream of nitrogen. The dried cutin monomers were converted to their corresponding methyl esters and then silylated with bis-(trimethylsilyl) trifluoroacetamide (BSTFA). The silylated methyl esters were dissolved in hexane and analyzed by Finnigan GC/MS 4610B on a 122-7032 DB-WAX 30 M capillary column (Thermo Finnegan, San Jose, CA, USA) with temperature programmed as following: 125°C for 5 min, 4°C/min to 250°C, 250°C for 15 min.

### Enzyme inhibition assays

Diethyl 4-nitrophenyl phosphate (paraoxon; Sigma) was solubilized in acetonitrile to 100 mg/ml. A working stock was prepared by dilution into 100% methanol to 10 mg/ml. Assay concentrations were then prepared by aqueous dilution. Equivalent methanol concentrations were tested for inhibition of enzyme activity and were found to have no effect (data not shown). Inhibitor was mixed 1:1 with 100 µg/ml enzyme solution and incubated at RT for 30 min. *p*-Nitrophenyl esterase assays were then performed as described previously: pNPB was used as the substrate for Culp1 and 4, while pNPL was used as the substrate for Culp6. Assays were carried out for 60 min at 37°C.

### Site-directed mutagenesis

Site-directed mutagenesis of Ser-175, Asp268 and His299 of Culp6 was carried out using the Stratagene QuikChange II kit (Stratagene, La Jolla, CA, USA) according to manufacturer's instructions. Briefly, template plasmid from Dam<sup>+</sup> *E. coli* DH5α was amplified using a pair of complementary primers containing the desired mutations by a non-strand-displacing, high-fidelity polymerase in a linear amplification. A methylation-sensitive restriction endonuclease, *DpnI*, was then added, degrading the methylated template but leaving the nicked, unmethylated copies containing the mutation. The non-linearized plasmids were then transformed into supercompetent *E. coli* XL1-Blue and screened by sequencing for the presence of the desired mutation.

### PAGE, antibody production, and Western blot analysis

Protein samples were separated by two-phase 4%/12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Mouse polyclonal anti-Culp1, 2 and 6 antibodies were raised for subcellular location experiments. Mice were immunized 3×, at 2-wk intervals, with 10 µg of antigen injected subcutaneously on the back. Antigen was delivered with 250 µg of dimethyl-dioctadecylammonium (DDA) and 25 µg monophosphoryl lipid (MPL) as adjuvant (29). Blood was collected 4 wk after final immunization and allowed to clot before serum was clarified by centrifugation. For Western blot, equivalent amounts of the protein samples visualized by

Coomassie staining were loaded along with prestained molecular mass standards (Bio-Rad, Richmond, CA, USA) and transferred to PVDF membrane. Blots were probed for polyhistidine tag with HisDetector Nickel-HRP (KPL, Gaithersburg, MD, USA) or anti-CULP (1:250) antibodies as required. Anti-mouse HRP-conjugated secondary antibodies were used; blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and visualized on a Kodak 4000MM Image Station (Eastman Kodak, Rochester, NY, USA).

## RESULTS

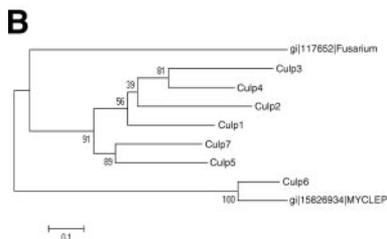
### CULP identification

The signalP server (19) was queried to identify the presence of putative secretion signals in the *M. tuberculosis* proteome. Proteins identified in initial analysis with possible functional similarities were grouped, revealing six proteins (Table 1) that clustered with homologies ranging from 17 to 63% at an amino acid level. Another protein, Culp5 (Rv1758), was added to the group based on sequence homologies, although the lack of a classic signal sequence indicates that it may be cytoplasmic in its subcellular location. A previous study (30) identified two of these proteins on silver-stained 2-dimensional electrophoretic gels of *M. tuberculosis* culture filtrates, named Cfp21 (Rv1984c) and Cfp25 (Rv2301), which are reported here as Culp1 and Culp2, respectively. These proteins, like other members of the family, share homology to the fungal cutinase, but until now, cutinase activity had not been empirically determined. Therefore, we have referred to these and the other members of the family as CULPs. The full-length CULPs of *M. tuberculosis* align with varying amino acid homology with the well-characterized cutinase of the fungi, *Fusarium solani*, and the sole *Mycobacterium leprae* homologue, ML0099, referred to here as CulpL. Of note is the conservation of the active catalytic triad between species. Each CULP contains the serine, aspartic acid, and histidine residues that comprise the active site, critical for functional *F. solani* cutinase (Fig. 1A). A comprehensive analysis of the protein sequences reveals significant homology about the catalytic triad of each. CULPs are α/β hydrolases (31), possessing the conventional catalytic triad comprised of Ser, Asp, and His, with the serine residue located within the con-

TABLE 1. Proteomic characteristics of the family of CULPs of *M. tuberculosis*

CULP	Rv number	Signal peptide	Cleavage	Length (aa)	M (kDa)	Mat M (kDa)	pI
Culp1	Rv1984c	+	32–33	217	21.8	18.6	5.52
Culp2	Rv2301	+	32–33	219	22.6	19.6	4.96
Culp3	Rv3451	+	26–27	247	24.9	22.3	5.79
Culp4	Rv3452	+	45–46	226	23.7	18.9	9.61
Culp5	Rv1758	–	–	174	17.9	17.9	4.7
Culp6	Rv3802c	+	26–27	336	35.5	32.5	6.5
Culp7	Rv3724	+	30–31	187	21.2	17.8	6.93

M, molecular mass; Mat M, mature molecular mass; pI, isoelectric point.



acid sequence divergence. Symbol gi| indicates the NCBI accession number for the *F. solani* and *M. leprae* (MYCLEP) proteins.

served pentapeptide, G-X-S-X-G (Fig. 1A, bar 1). Within all of the sequences shown in Fig. 1, the pentapeptide motif describing all of the sequences is G-[YF]-S-[QL]-G. The Tyr residue is the most common in position 2, phenylalanine being observed in Culp5, Culp6, and CulpL of *M. leprae*, while only Culp2 contains the leucine residue in position 4, all of the others containing asparagine. Uniquely, Culp5 and Culp6 have the pentapeptide sequence of a family VI-type lipase, *i.e.*, G-F-S-Q-G (32), as does the CulpL ortholog. The spatial organization of the active site region is also conserved, except that Culp6 and CulpL of *M. leprae* possess two extra amino acid blocks of ~15 aa each, one after the catalytic Ser and the other between the key Asp and His residues (Fig. 1A, bars 2 and 3). The first extra block is flanked by Gly, while the second is flanked by Pro, suggesting they may form extra loops in relation to the general enzyme framework. This may lead to an alternative conformation around the active site of Culp6, which further suggests that this enzyme has an alternative substrate to the other CULPs. The phylogram shows strong bootstrap support for orthology between Culp6 and the *M. leprae* CulpL, and suggests that the remaining *M. tuberculosis* are products of gene duplication, in particular the pairs Culp3/Culp4 and Culp7/Culp5 (Fig. 1B).

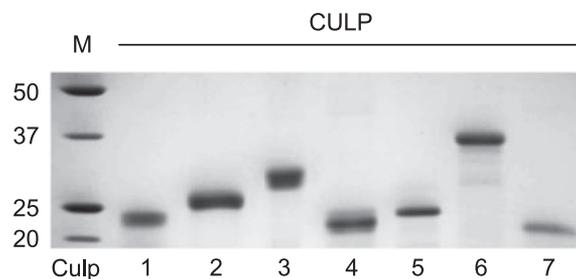
### Functional analysis

All seven CULPs were cloned and expressed as N-terminally His tagged recombinant *E. coli* proteins. Following overexpression, Co<sup>2+</sup>-charged immobilized metal affinity chromatography (IMAC) was utilized to purify the chemically solubilized CULPs. The CULPs were then refolded in a buffer series, which varied in their components in order to address critical and often unique physiochemical refolding conditions for the proteins. These included pH, ionic strength, oxidation state, and the presence of chaotropic and polar agents. The resultant proteins were pure, refolded, and soluble proteins by SDS-PAGE (Fig. 2), allowing further functional characterization of the individual proteins.

**Figure 1.** Alignment and phylogram of the CULPs of *M. tuberculosis*, *M. leprae*, and *F. solani*. A) Alignment of residues of the catalytic triad (indicated by arrowheads) and surrounding regions. Residues conserved in all proteins are indicated with black background; those shaded gray are identical in  $\geq 50\%$  of the proteins. Conserved cysteines are boldface in black background. Bar 1 indicates the conserved pentapeptide motif G-X-S-X-G containing the catalytic serine. Bars 2 and 3 indicate insertion of amino acids found in Culp6 and in the *M. leprae* ortholog, respectively. B) Bootstrapped phylogram from alignments of whole sequences. Numbers represent percentage bootstrap support. Scale bar indicates 10% amino acid sequence divergence. Symbol gi| indicates the NCBI accession number for the *F. solani* and *M. leprae* (MYCLEP) proteins.

Paradoxically, Culp5 migrates slower than Culp4, even though the predicted molecular masses are 17.9 and 18.9 kDa, respectively. This pattern of migration was consistently observed and may be attributed to the two proteins possibly binding different molar amounts of SDS.

To establish whether the *M. tuberculosis* CULPs function as enzymes, and if so to classify them to a specific enzymatic class, a series of functional assays were performed. Functional and structural properties of *F. solani* cutinase have been comprehensively examined, and the enzyme is well established as a serine hydrolase (12). Comparison of the protein sequences of the two species suggested that the enzymes might share functional characteristics. Therefore, the CULPs of *M. tuberculosis* were assessed for their esterase, lipase, and cutinase activities. To delineate the activities of the CULP specificity for substrate fatty acid chain length, and thereby allow a more definitive description in terms of enzymatic class, the enzymes were assessed for their capacity to cleave a range of fatty acid substrates. The substrates used were esters of *p*-nitrophenyl and butyric (C4), lauric (C12), myristic (C14), palmitic (C16), and stearic (C18) acids. Cleavage of these esters liberates *p*-nitrophenol, which can be measured spectrophotometrically (25, 33, 34). The CULPs demonstrated a divergence in optimal fatty acid chain length specificity, with Culp1 proving an effective esterase



**Figure 2.** Purified, refolded, recombinant CULPs. Coomassie blue-stained CULPs shown following *E. coli* expression and HIS-tag IMAC purification and separation by SDS-PAGE. Molecular mass standards (M) with sizes (kDa) are at left.

owing to its ability to cleave pNPB efficiently (Fig. 3). In fact, Culp1 had an average specific activity ( $1688 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ )  $\sim 40$ -fold that of Culp6 for the same substrate. Further, the activity of Culp1 decreased sharply as substrate chain length increased, with its specific activity reduced 20-fold for the substrate with which it displayed the next highest activity, *i.e.*, pNPL. The activity of Culp1 continued to decrease as fatty acid chain length increased. In comparison, although the overall activity of Culp6 declined after a peak for medium-length fatty acids (lauric acid), its activity was greater than Culp1 when the chain length was C16 or more. These results infer that Culp6 possesses lipolytic activity in addition to esterase activity. Culp4, whose homologue in *Mycobacterium smegmatis* had activity as a phospholipase A in a recent study (35), was able to effectively cleave butyric acid from the nitrophenyl ester with a mean specific activity of  $182 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ , ranking it only behind Culp1 as the most active esterase of the family. Cleavage of pNPP and pNPS was most efficiently achieved by Culp6, again indicating lipolytic activity, while Culp1 and Culp4 were also able to cleave the longer-chain length fatty acids, albeit to a lesser degree than Culp6. Culp2 and Culp3 performed weakly as esterases for pNPB (Fig. 3), while Culp5 and Culp7 demonstrated activities at or near background levels for all substrates tested, at pH ranging from 5.0–8.0 (results not shown). Also, performing these assays in reducing or oxidizing buffers did not appreciably alter their activities (results not shown).

To define further the lipase activities of Culp1, Culp4, and Culp6, the cleavage of polyoxyethylene sorbitans (Tweens) (26, 36) was measured. This assay is reported to be up to 36-fold more sensitive than the titrimetric assay and at least 4-fold more sensitive than the colorimetric assay of pNPP (37). We also extended this assay by varying the buffer system in order to examine enzyme activity in either basic or acidic conditions. The activities for these three CULPs were,

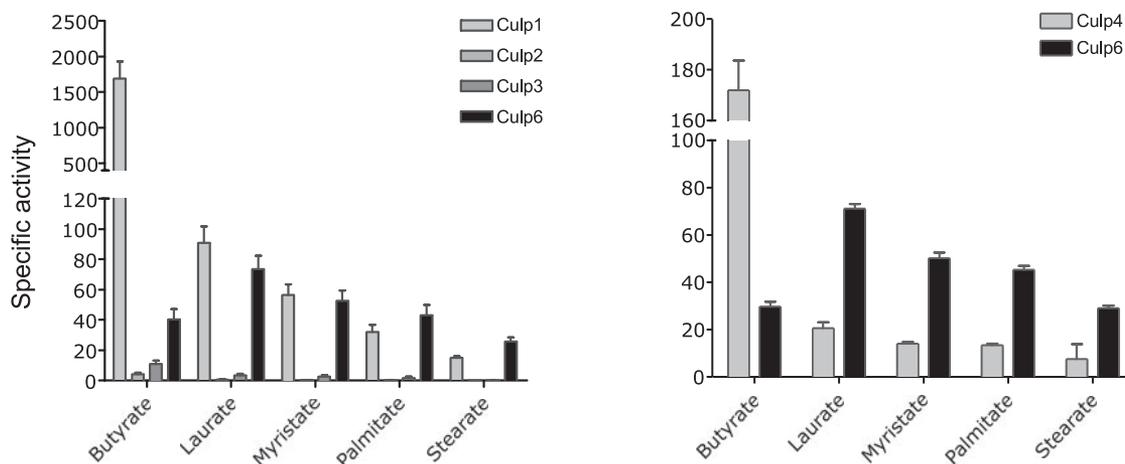
therefore, determined at pH 6, 7, and 8 (Fig. 4). When the CULPs were tested for the cleavage of polyoxyethylene sorbitan monolaurate (Tween 20), Culp6 demonstrated lipolytic activity up to 10-fold higher than either Culp1 or Culp4, and this activity was optimum at pH 8.0 (Fig. 4A). Culp1 was able to hydrolyze substrate to a maximum of  $1 \text{ U}/\mu\text{g}$  at a pH of 7.0 and 8.0, while Culp4 demonstrated a similarly low level of activity, but only at pH 7.0. No other CULP demonstrated lipolytic activity as measured by this assay at any pH, including Culp5, despite sharing a family VI-type lipase serine pentapeptide with that of Culp6 (results not shown).

To establish a pH profile of Culp6 activity, the cleavage of Tween 20 was examined over a broad pH range. Activity was highest at neutral to alkaline conditions, with performance dropping markedly below pH 6 (Fig. 4B). Interestingly, Culp6 lipase activity was maintained above pH 7.0, whereas a genuine lipase of *Candida rugosa*, in addition to having  $\sim 10$ -fold lower activity, was far less active above pH 7.0 (Fig. 4C).

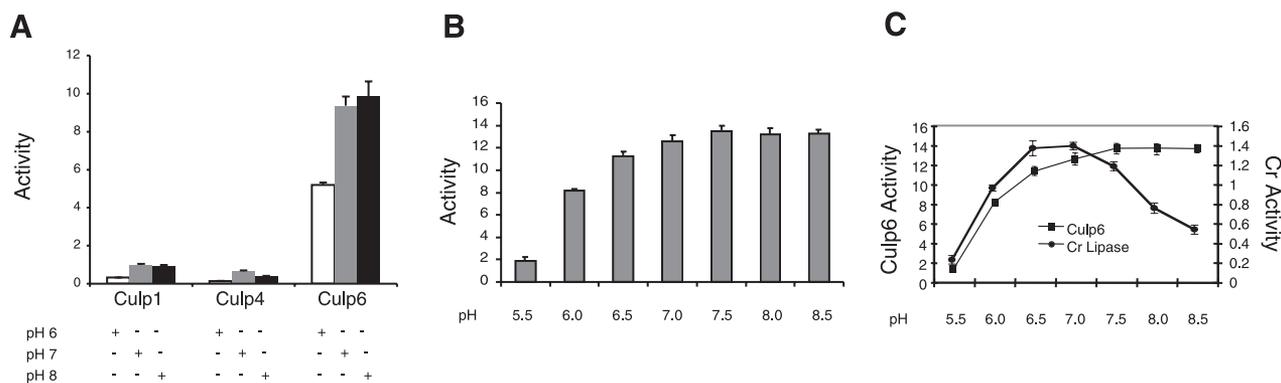
To establish whether the proteins were folded correctly or merely soluble aggregates, gel filtration was conducted by FPLC. Analysis of the elution chromatograms indicated that all of the proteins were soluble and eluted at expected molecular masses consistent with them being correctly folded, with the exception of Culp2, which demonstrated a majority of incorrectly folded protein (results not shown). It is possible that Culp2 aggregated under the conditions of gel filtration, yet we cannot rule out similar aggregation during enzyme analysis, resulting in apparent inactivity.

### Cutinase activity

Because of the presence of a cutinase motif comprising the putative active site of these proteins, a number of them have been named as cutinases in the annotated *M. tuberculosis* genome. As an absence of experimental



**Figure 3.** CULPs of *M. tuberculosis* possess divergent enzymatic function. Specific activity of CULPs demonstrates substrate specificity with fatty acid substrates of different lengths. Specific activity represents  $\text{pmol } p\text{-nitrophenol liberated} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  enzyme. Results are the means  $\pm$  SE of triplicate experiments performed at pH 8.0. Culp5 and Culp7 showed no activity in assays tested (not shown).



**Figure 4.** Lipolytic activity demonstrated by cleavage of polyoxyethylene sorbitan monolaurate (Tween 20). *A*) CULPs with activity for pNPP were tested at varying pH. *B*) Culp6 activity for Tween 20 performed from pH 5.5–8.5. Bars represent means  $\pm$  SE of triplicate experiments. *C*) Comparison of Culp6 activity (U/ $\mu$ g protein) to that of a purified lipase of *Candida rugosa* (Cr). Data points are means  $\pm$  SE of triplicate experiments.

evidence for this activity exists, we have defined them as CULPs. We sought to establish whether they do indeed possess cutinase activity, and thus we performed assays of cutin hydrolysis (Fig. 5). None of the 7 CULPs exhibited cutinase activity, as  $C_{16}$  and  $C_{18}$  hydroxyl fatty acids, the characteristic cutin components, were absent in the enzymatic reaction products (Table 2). These products were clearly present following hydrolysis of cutin by *T. fusca* cutinase, which served as the positive control (Fig. 5). The present nomenclature is, therefore, more appropriate.

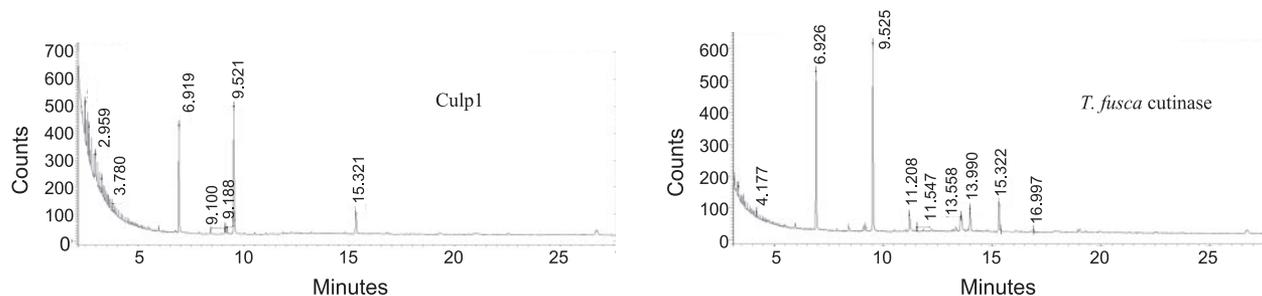
### Inhibition of enzyme activity

Esterases can be classified by the interaction with organophosphates, such as paraoxon. In this context, if an esterase can hydrolyze organophosphate esters, it is referred to as class A. If it is inhibited by organophosphate esters, it falls into class B and is classified as a carboxylesterase (EC 3.1.1). Paraoxon acts as an inhibitor by phosphorylating the hydroxyl group of the catalytic serine residue (38). *F. solani* cutinase is rapidly inhibited by low concentrations of paraoxon (39, 40), as are other esterases, such as porcine liver esterase (41). Lipases, such as that of *C. rugosa* used as a control in this study, are also inhibited by paraoxon, but this inhibition is reduced because of the protection of the catalytic serine by the lid domain of the enzyme (42).

We therefore tested the sensitivity of the *M. tuberculosis* CULPs to inhibition by paraoxon in order to establish their classification as A or B esterases and the level of exposure of their catalytic serine to the solvent. Inhibition of CULP activity by paraoxon was varied. Culp1 was almost completely inhibited (>99%) in its ability to cleave pNPB at high inhibitor concentrations, with an  $IC_{50} \sim 1.9 \mu\text{g/ml}$  (Fig. 6A). Culp4 was less susceptible to inhibition, with  $\sim 65$  times the inhibitor concentration needed to quench activity to the same degree ( $IC_{50} \sim 125 \mu\text{g/ml}$ ); a similar pattern of resistance to inhibition was seen for Culp6, where an  $IC_{50} \sim 65 \mu\text{g/ml}$  was observed for activity to pNPL (Fig. 6B). The rapid and complete inhibition of Culp1 by paraoxon firmly classifies this enzyme as a carboxylesterase with an active site readily accessible to the inhibitor. Culp4 and Culp6 can also be classified as carboxylesterases; however, the high concentrations of inhibitor required may indicate a shielding of the enzyme active site, as seen with the *C. rugosa* lipase.

### Active site identification of Culp6

The active site of Culp6 was defined by site-directed mutagenesis. Three mutant proteins were created, each with a single residue of the putative catalytic triad (*i.e.*, Ser-175, Asp268, and His299) being replaced with alanine. For each of the altered proteins, all activity was



**Figure 5.** Enzymatic reaction products from cutinase activity analysis. Example mass chromatograms for Culp1 and positive control, *T. fusca* cutinase, illustrating peak retention times (min).

TABLE 2. Enzymatic reaction products detected in assays for cutinase activity of *M. tuberculosis* CULPs

Cutin hydrolysis product	Reaction time (min)	<i>T. fusca</i> cutinase	Hydrolysis area (%)						
			Culp1	Culp2	Culp3	Culp4	Culp5	Culp6	Culp7
Hexadecanoic acid	6.926	30.94	24.25	37.92	25.11	40.26	32.44	27.09	23.18
9-Octadecenoic acid	9.1	0.912	1.55	5.1	0.512	0.571	1.757	0.349	0.492
9,12-Octadecadienoic acid	9.188	1.696	1.825	0.345	0.316	2.166	2.143	0.913	0.554
Octadecanoic acid	9.525	36	32.16	39.06	31.08	44.36	41.73	32.59	27.68
16-Hydroxyhexadecanoic acid	11.547	0.843	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18-Hydroxyoctadeca-9-enoic acid	13.017	1.97	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18-Hydroxyoctadeca-9,12-dienoic acid	13.558	7.18	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10,16-Dihydroxyhexadecanoic acid	13.99	5.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D., not determined.

lost for both short- and long-chain substrates (Fig. 7A). Further, lipase activity was eliminated, as measured by the cleavage of Tween 20 (Fig. 7B).

### Subcellular location of CULPs

Subcellular location and secretion potential are known for few CULPs. Culp1 and Culp2 have been identified

in *M. tuberculosis* culture filtrates (30), and Culp4 has been reported to be located in the cell wall of *M. tuberculosis* but not to be secreted into the culture supernatant (35). To establish the secretion status of Culp6 and to further characterize that for Culp1 and Culp2, high-titer antibodies were raised against the respective recombinant proteins and used to probe subcellular fractions of *M. tuberculosis* via immunoblotting. We confirmed the presence of Culp1 and Culp2 in culture filtrate and failed to detect appreciable levels either in the cell wall (Fig. 8) or the cytoplasm (not shown), illustrating that the majority of protein is destined for immediate secretion. By contrast Culp6 was present in the cell wall fraction alone (Fig. 8), with no detectable levels in the culture filtrate protein, despite possessing a typical signal sequence, as predicted by both the neural network and hidden Markov models. This finding strongly suggests a role for this enzyme in cell wall biosynthesis and/or maintenance.

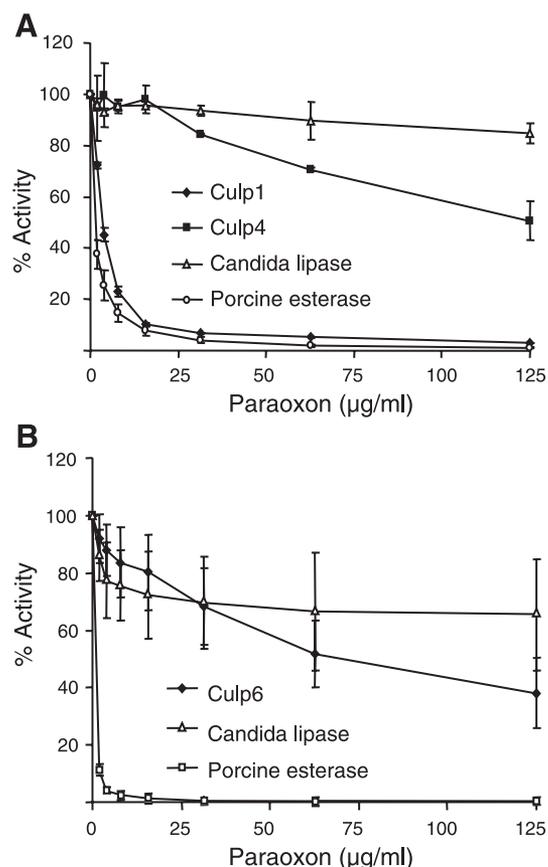
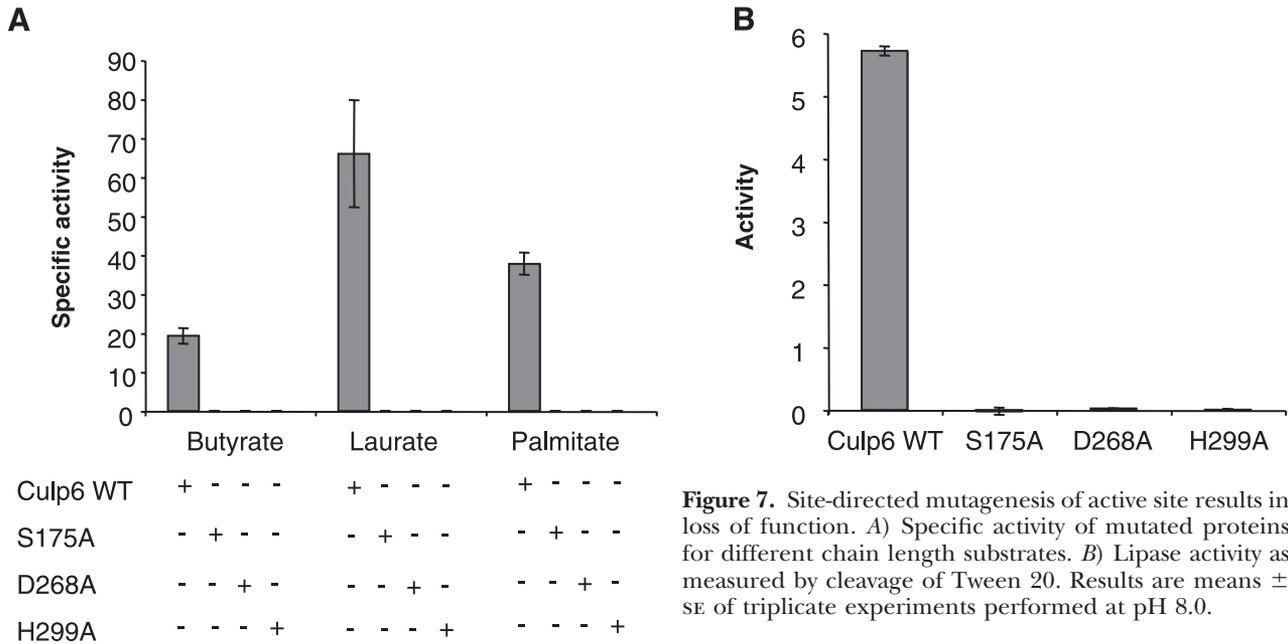


Figure 6. Dose-related paraoxon inhibition of CULPs. Graphs demonstrate dose-dependent inhibition of Culp1 and Culp4 (A) and Culp6 (B) to hydrolyze pNPB and pNPL, respectively. *Candida* lipase and porcine esterase are included as inhibition-resistant and inhibition-sensitive controls, respectively.

### DISCUSSION

This study aimed to characterize the CULPs of *M. tuberculosis*, a little-known family of putatively secreted proteins, some of which are emerging to perform important functions. Three members have strong enzyme activity as either esterases or lipases and are all carboxylesterases, as defined by enzyme inhibition. The study defined the active site of the enzyme family by site-directed mutagenesis of Culp6, which has been reported as essential for replication of *M. tuberculosis* (18), as well as its operational pH optimum and cellular location. This work also demonstrates that these proteins do not possess cutinase activity, as would be inferred by the annotation as “cutinases” in the genome.

Secreted gene products of *M. tuberculosis* are important for virulence and survival of the bacillus and pathogenesis of tuberculosis. For example, the mycolyl transferases of the Antigen 85 complex are critical for cell wall development (5) and are major targets of the immune responses in animal and human mycobacterial



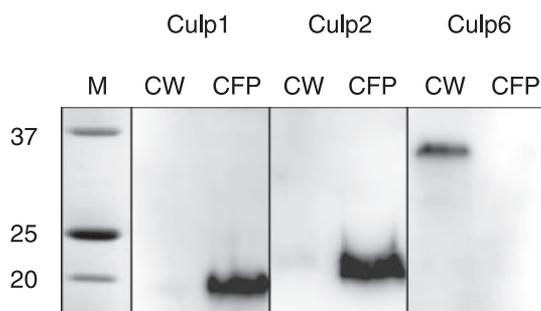
**Figure 7.** Site-directed mutagenesis of active site results in loss of function. *A*) Specific activity of mutated proteins for different chain length substrates. *B*) Lipase activity as measured by cleavage of Tween 20. Results are means  $\pm$  SE of triplicate experiments performed at pH 8.0.

infections (43, 44). The first secreted CULPs of *M. tuberculosis* to be identified were Culp1 and Culp2, which were present in short-term culture filtrate (30). These proteins were found to be potent inducers of IFN- $\gamma$  from memory T lymphocytes from *M. tuberculosis*-infected mice, and they elicited strong delayed type hypersensitivity responses in *M. tuberculosis*-sensitized guinea pigs. Subsequent studies revealed that one or both of these antigens were also recognized by T cells from tuberculosis patients (45, 46). Recently, we have demonstrated that Culp1 and Culp2 as individual antigens can confer protective immunity in mice against *M. tuberculosis* challenge (17). In addition to the potential use of Culp1 as a protective vaccine antigen, this study defines it as an esterase with high specific activity, rapidly hydrolyzing pNPB. As an enzyme secreted into the extracellular milieu and largely absent from the bacterial cytoplasm or cell wall, we suggest that Culp1 may interact with host molecules and contribute to the virulence and intracellular survival of *M. tubercu-*

*losis*, as demonstrated for other secreted enzymes. *Culp1* is unlikely to be essential for the viability of *M. tuberculosis*, as it is absent from the majority of *M. bovis* (BCG) vaccine strains, which lack the region of deletion 2 (RD2) containing the *culp1* homologue of *M. bovis* (47).

The secreted phosphatases, SapM and PtpA, and the serine/threonine protein kinase G, PknG (48), influence the survival of *M. tuberculosis* within macrophages by inhibiting phagosome maturation and acidification and host vacuolar protein sorting. This inhibition of the normal macrophage responses to phagocytosed bacteria has important consequences to the outcome of infection. Interestingly, although Culp2 displayed minimal enzyme activity in the assays performed here, its homologue was found in a genetic screen of *M. bovis* BCG to inhibit phagosome acidification (49), widening the functional responsibilities of this family. To date, Culp2 is the only member of the CULP family that has been attributed an intracellular function within host macrophages, but it is possible that other members of the group may also contribute to intracellular survival.

Although all but one of the CULPs have putative secretion signals, only two, Culp1 and Culp2, have been proven to be secreted, while two, Culp4 and Culp6, are cell wall associated. The homologue of Culp4 in *M. smegmatis*, which has activity as a mycobacterial phospholipase A, was secreted by *M. smegmatis*. Similar phospholipase activity was present only in the cell wall fraction of *M. tuberculosis*, suggesting that the *M. tuberculosis* Culp4 is retained in the cell wall (35). The gene encoding Culp6, Rv3802c, was one of ~800 genes identified by a genomic screen to be essential for growth of *M. tuberculosis in vitro* (18). We have demonstrated that Culp6 is not exported fully from the cell but is also retained in the cell wall. This variation in cellular location suggests divergent roles for these



**Figure 8.** Western blot analysis of *M. tuberculosis* CULPs in culture filtrate protein (CFP) and cell wall (CW) fractions. CULP-specific antibodies revealed that Culp1 and Culp2 were found only in CFPs, whereas Culp6 was entirely restricted to the CW fraction. Anti-CULP antibodies were raised against purified proteins.

homologous enzymes, and localization within the cell wall may indicate a role for Culp6 in cell wall biosynthesis and/or remodeling. Furthermore, the chromosomal location of *culp6* is directly downstream of the mycolyl transferases, *fbpA* and *fbpC1* (antigens 85A and 85C, respectively), and immediately upstream of *fadD32*, a probable fatty acid Co-A synthase, and of other key enzymes in lipid metabolism. This genomic orientation may suggest a direct role in cell wall modification or lipid metabolism for this lipase. Another possible role for CULP members with lipase activity is as scavenger enzymes using host lipids to provide a carbon source for the mycobacterium, a process that is important for mycobacterial replication within host cells (50, 51).

Analysis of the enzymatic function of the protein family revealed an unexpected diversity between them. The strong esterase activity of Culp1 distinguishes it as having a preference for short-chain fatty acids. Culp6 has a peak activity for medium-chain length fatty acids, such as myristic acid, but possesses stronger activity than Culp1 for substrates with chain lengths longer than C14, categorizing Culp6 as a lipase. It is possible that the extra amino acids found between the components of the catalytic triad in Culp6 (Fig. 1A) confer this specificity toward larger substrates. Although there is overlap in activities of these two proteins, this does not necessarily indicate enzymatic redundancy. Culp6 has been identified as a potentially essential gene from genomic mutational studies (18). In addition, Culp6 is conserved within all mycobacterial genomes examined, and its *M. leprae* ortholog, CulpL, is the only intact cutinase present in the drastically restricted genome of *M. leprae* (Fig. 1). This further supports the notion that *culp6* is essential for mycobacterial survival. Although the active site residues identified in Culp6 are conserved in other CULP members, Culp2, Culp3, Culp5, and Culp7 had little or no activity with any of the tested substrates, indicating possible divergence of substrate specificity. FLPC analysis indicated that all proteins but Culp2 were predominantly folded correctly. With Culp2 aggregating during gel filtration, we cannot rule out that the same has occurred under activity assay conditions and is one reason for perceived inactivity.

The catalytic triad of two of the CULPs, Culp2 and Culp6, differ from the other members in having additional amino acids separating the second and third residues of the triad, the majority of which were not shared between them. Culp2, which is essential for the inhibition of phagosome maturation, has an extra spacing of 6 aa compared to the others, while the extension is 19 aa in length for Culp6. Although this does not in itself point to a particular substrate, it is consistent with the notion that they have unique substrates.

All of the CULPs that displayed activity in assays for *p*-nitrophenyl ester hydrolysis were inhibited with paraoxon, suggesting they are class B esterases. The divergent sensitivity to the inhibitor between Culp1, Culp4, and Culp6 may be informative of the degree of exposure of the catalytic serine to the solvent. Our data show

that Culp1 has similar inhibitory sensitivity to both porcine liver esterase and published data for *F. solani* cutinase (40), strongly suggesting that its catalytic site is readily accessible. Our data also suggest that the active sites of Culp4 and Culp6 are not as readily accessible to a small-molecule inhibitor. This possible contrast in structure may be supported by the additional residues in the region of the catalytic site in both Culp4 and Culp6 when compared to Culp1 (Fig. 1).

This pattern of sequence homology, yet with functional diversity, has been attributed to gene duplication followed by eventual functional drift in other homologous/divergent classes of enzymes. One explanation states that when two paralogs of a bifunctional gene arise from duplication, each copy may be driven by selective pressures to specialize in one of its original functions, resulting in the creation of two essential genes (52). A more conventional view of gene duplication is that the process produces two redundant proteins, thereby relaxing selective pressures on each and so allowing the accumulation of mutations, which can eventuate in functional divergence (53). Either of these theories is consistent with the characteristics of the CULP family and may explain why *M. tuberculosis* maintains multiple CULPs without obvious redundancy. By contrast, *M. leprae* has maintained within its reduced genome only one CULP member, namely CulpL, which shows 75% amino acid homology with Culp6. This further suggests an essential role for Culp6 in survival of mycobacteria. This fact may also reflect the restriction of environmental niches and hosts permissive for each pathogen, which for *M. leprae* are primarily humans.

The CULPs are emerging as a fascinating group of mycobacterial proteins, important in numerous events during host infection. We have previously demonstrated that members of the CULPs are protective in a murine model of tuberculosis (17). We now report diversity of function and cellular location within the group and characterized the components of the active site of Culp6, which is retained within the cell wall, suggesting Culp6 performs a vital role in its biosynthesis and maintenance. Further study of these proteins may contribute to understanding the pathogenic process and potentially yield novel targets for effective antimycobacterial therapies. FJ

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