The catalase-peroxidase of *Mycobacterium intracellulare*: nucleotide sequence analysis and expression in *Escherichia coli*

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The activation of catalase genes in response to oxidative stress may contribute to the intracellular survival of mycobacteria. In this report, the nucleotide sequence of a mycobacterial catalase gene is described. The deduced protein sequence of this *Mycobacterium intracellulare* gene (MI85) was 60% identical to the *Escherichia coli* hydroperoxidase I (HPI) protein, 59% identical to the *Salmonella typhimurium* (HPI) catalase, and 47% identical to a *Bacillus stearothermophilus* peroxidase. The MI85 protein, expressed in *E. coli*, has also been shown to have peroxidase and catalase activities. Furthermore, Southern blot hybridizations, which demonstrated that a MI85 gene probe hybridizes with chromosomal DNA from thirteen different strains of mycobacteria, suggest that this catalase-peroxidase gene is prevalent in the mycobacterial genus. The availability of catalase gene probes should permit an evaluation, at the molecular level, of the role of catalase in mycobacterial pathogenesis.

Introduction

One hundred years after the pioneering studies of Robert Koch on *Mycobacterium tuberculosis*, mycobacterial disease remains an enormous international public health problem. About 1·7 billion people, one-third of the world's population, are infected with *Mycobacterium tuberculosis*. Annually, three million deaths – 26% of preventable deaths worldwide – result from disease caused by *M. tuberculosis* (Kochi, 1991). Ten to fifteen million people suffer with leprosy, the complex chronic infectious disease caused by *M. leprae* (Bloom & Godal, 1983). More than 25% of patients with acquired immunodeficiency syndrome (AIDS) in the United States develop *M. avium*, *M. intracellulare* complex (MAC) disease (Horsbaugh, 1991). MAC bacilli are the most common bacterial isolate and the most frequent cause of systemic bacterial infections in American AIDS patients (Young, 1988).

Efforts to improve diagnosis and treatment of mycobacterial diseases have been impeded by a lack of progress in understanding the mechanisms of mycobacterial pathogenesis and factors which contribute to the

virulence of mycobacteria. Since mycobacteria proliferate inside macrophages, it has been speculated that catalases may protect acid-fast bacilli from the deleterious effects of peroxide and, therefore, may play a crucial role in the *in vivo* survival of mycobacteria. The virulence of two other intracellular pathogens, *Nocardia asteroides* and *Leishmania donovani*, has been related to their catalase content (Beaman & Beaman, 1984). Catalases probably enhance the pathogenicity of these microorganisms by metabolizing hydrogen peroxide, a toxic oxygen metabolite which is released by phagocytes in response to bacterial challenge. There is extensive evidence which suggests that peroxide and its associated toxic oxygen metabolites are responsible, in part, for the anti-mycobacterial activity of macrophages (Jackett *et al.* 1981*a, b*; Lowrie, 1983). The most direct evidence implicating catalases as mycobacterial virulence factors is derived from studies demonstrating the protective effect of exogenous catalase. In these experiments, exogenous catalase protected against the killing of *M. microti* by lymphokine-activated murine macrophages (Walker & Lowrie, 1981). More recent studies have shown, however, that the resistance of *M. intracellulare* strains to peroxide does not correlate with their catalase content and that the susceptibility of *M. tuberculosis* to killing by activated macrophages is not related to peroxide susceptibility (Gangadharam & Pratt, 1984; O'Brien *et al.*, 1991). The role of catalase in mycobac-

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terial virulence, therefore, may be very complex and has not been clearly delineated.

In this report, we describe the initial cloning, nucleotide sequence analysis, and expression of a mycobacterial gene encoding a catalase-peroxidase. We also demonstrate that this gene encodes a protein which is 60% identical to the *E. coli* hydroperoxidase I (HPI) enzyme. Finally, we discuss how molecular studies with catalase gene probes may clarify the role of catalases in mycobacterial pathogenesis.

Methods

Cloning and nucleotide sequence analysis of the MI85 gene. The identification and isolation of the bacteriophage from a *M. intracellulare* λ gt11 gene expression library which expresses the MI85 protein has been previously described (Morris *et al.*, 1988). Recombinant bacteriophage DNA was isolated using the LambdaSorb reagent (Promega) and protocols provided by the manufacturer. The 4.8 kb mycobacterial DNA insert was removed from the bacteriophage by digestion with *Eco*RI and passage through a low-melting-point agarose gel. This insert was then cloned into the pGEMEX vector (Promega). Deletions of the insert fragment were prepared in the pGEMEX vector using exonuclease gene deletion protocols. The pGEMEX recombinants containing the deleted gene fragments were subsequently expressed as gene 10 fusion proteins. The sizes of the resultant fusion proteins were evaluated using SDS-PAGE. Plasmids containing fragments greater than 2.3 kb expressed nearly full-length fusion proteins, indicating that most of the protein coding sequence was located within a 2.3 kb *Eco*RI/*Sma*I restriction fragment. This 2.3 kb fragment was then cloned into the Bluescript KS vector (Stratagene). To facilitate nucleotide sequence analysis, plasmids containing deletions of the *Eco*RI/*Sma*I fragment were generated by exonuclease III digestions. Dideoxynucleotide sequence analysis was performed by Lark Sequencing (Houston) and by our laboratory using *Bst*II polymerase protocols (Bio-Rad Laboratories). Because the *Eco*RI/*Sma*I fragment did not include DNA encoding the amino terminus of the MI85 protein, overlapping clones were identified using nucleic acid hybridizations. A 338 bp *Eco*RI/*Sph*I fragment from the 5' region of the 2.3 kb fragment was nick-translated (Bethesda Research Laboratories) with α -³²P-dCTP (NEN/DuPont) and served as the probe for screening the *M. intracellulare* library. Plaque hybridizations were performed using standard protocols (Sambrook *et al.*, 1989). *Eco*RI insert fragments from hybridization-positive clones were cloned into Bluescript vectors. The nucleotide sequencing of the gene was then completed using primers designed from the 5' end of the 2.3 kb fragment.

Expression of the MI85 protein and characterization of the peroxidase and catalase enzymic activities. The isolation and characterization of λ gt11 bacteriophages expressing recombinant nontuberculous mycobacterial antigens has been reported (Morris *et al.*, 1988, 1990; Rouse *et al.*, 1991). DNA insert fragments from recombinant bacteriophages expressing MI22, MK35, and MI85 mycobacterial antigens were cloned into the pGEMEX vector system (Promega) and then transformed into the *E. coli* strain BL21 (*pLysS*). As a control, the pGEMEX plasmid, which expresses only the T7 gene 10 protein, was also transformed into the same *E. coli* host. Expression of the gene 10 fusion proteins and the wild-type gene 10 protein was performed as described by the manufacturer. The level and integrity of expression was evaluated by SDS-PAGE and by immunoblot analyses using

specific monoclonal antibodies and absorbed hyperimmune anti-*M. intracellulare* burro sera.

The peroxidase activity of 10 μ g of each cell lysate was evaluated by assaying for the metabolism of the peroxidase substrates [2,2'-azino bis-(2-ethylbenzthiazoline 6-sulphonic acid)] (ABTS) and *o*-dianisidine, using protocols provided by the manufacturer (Sigma). Both substrates produce soluble end products that can be measured as A_{405} . The catalase activity of the cell lysates was determined by the method of Beers & Sizer (1952), which monitors the decomposition of hydrogen peroxide as A_{240} .

Southern blot hybridizations using MI85 gene probes. The following mycobacterial strains were obtained from the American Type Culture Collection (Rockville, MD): *M. asiaticum* (25274), *M. avium* (35714), *M. bovis* BCG (27291), *M. bovis* (35720), *M. fortuitum* (6841), *M. intracellulare* (13950), *M. kansasii* (12480), *M. phlei* (11758), *M. scrofulaceum* (19981), *M. smegmatis* (14468), *M. terrae* (15755), *M. tuberculosis* H37Ra (25177), and *M. tuberculosis* H37Rv (27294). These strains were grown in Long's synthetic medium (with 1% glucose, w/v) until mid-exponential phase. Total DNA was isolated as described previously (Rouse *et al.*, 1991).

Approximately 3 μ g of each preparation of total DNA was digested with *Eco*RI for 2–3 h precipitated with ethanol, separated on a 1% (w/v) agarose gel, and transferred to nylon membranes by standard procedures. The original 4.8 kb insert cloned into pGEMEX (1 μ g) was biotinylated with the BioNick Labelling System as described by the manufacturer (Bethesda Research Laboratories). The Southern blot was hybridized to this probe using reagents and protocols provided in the PHOTOGENE Detection System (BRL). After hybridization at 42 °C for 18 h, the Southern blot was washed twice, 5 min per wash, in 5 $\times$ SSC/0.5% SDS at 65 °C and then twice, 20 min per wash, in 0.1 $\times$ SSC/1.0% SDS at 52 °C. The membrane was washed for 5 min in 2 $\times$ SSC followed by a brief wash in TBS [100 mM-Tris (pH 7.4), 150 mM-NaCl] supplemented with 0.05% Tween 20 (TTBS). After blocking with 3% (w/v) bovine serum albumin (Sigma) in TTBS at 65 °C for 1 h, the blot was incubated with streptavidin-alkaline phosphatase conjugate for 10 min at room temperature, and washed twice with TTBS (15 min per wash). A final wash for 1 h at room temperature was performed using a solution provided by the manufacturer. The membrane was then air-dried and incubated with the detection reagent for 3.5 h. Kodak XAR-2 film was exposed to the Southern blot for 10 min and then developed.

Results

Nucleotide sequence analysis of the MI85 gene

The isolation and characterization of a recombinant bacteriophage from a *M. intracellulare* λ gt11 gene expression library which expresses a recombinant fusion protein derived from a 85 kDa mycobacterial antigen (MI85) has been previously described (Morris *et al.*, 1988). Restriction digestions and gene deletion analyses indicated that most of the MI85 coding sequence was encoded by a 2.3 kb *Eco*RI/*Sma*I fragment within the original 4.8 kb mycobacterial DNA insert fragment. The subcloning of this 2.3 kb fragment, the identification of overlapping λ gt11 bacteriophages with nucleic acid hybridization procedures, and the sequencing protocols are described in Methods. The complete nucleotide sequencing strategy is summarized in Fig. 1.

Fig. 1. Sequencing strategy for the MI 85 gene. The orientation of the overlapping insert fragments from λ gt11 bacteriophages 85-1 and 85-2 is shown at the top. Hatched boxes designate the coding sequence of the MI85 protein. Below is the nucleotide sequencing strategy. The chromosomal sequence from the *Pst*I site (85-2) to the *Sma*I site (85-1) was sequenced as described in Methods. The arrows indicate the direction and extent of elongation in the sequencing reactions.

AGCTACAGCGTCTAGATACACAGCCGGTGATCGACGGCGATTACCCACCGCTCGA	120
AGCAAGAGCGAAAGCGAAAACCGACCAATCCCTTACCGAACCGGAAGGCACATGCCCGGTGACCAACCGGAGCTGGTGCCCGACCAKPGTCGTQPDGSGTAT	240
CUGCTGTCCACCCGCTCGGGACGATTCGATACCGCGGGCACTTCGCAAGCTCGACCTCGAGCGCTCAAGCGCCACATGATCTCGTGTGACCCAGCTCGCGAGGCTGGTGGCG	360
CGCGATACCGCGGCTCTTCATTCGGGATGAGCTCGACACCGCGGGACCATACCGCATACCGACCGCGGGCGCGCGAGGATCGAGCGCTTCGCCCGCGTG	480
AACAGCTGCGCGGACACCGCGAGCTTGGACAGCGCGCGCGCTGCTGTGGCGCATCAGAAGAAGTACCGCAACAGATCTCCTGGCGGACCTGATACCTACCGGGCAACGTGGCC	600
CTGGAGTGTGCGGTTCAGAGCTTTCGGTTCGGCTTCGGCGCGGACGAGCTTCGGAGCGCAACAGATGCTCTGGGAGAAGAGGAAGTAAGCTGGGACCGACCAAGCGCTACTCG	720
GGTGAAGCGGAGCTCGGCGCGCTACGGCGGACGACCTGGCGCTGATCTACCTCAACCGCAAGCGCGCGGAGGCAAGCCGACCGGCTCGCGCGGCTCGCATCTCCGCGAGAG	840
TTGCGCGGATGGGATCAAGCAGAGGAGAGACCGCGCTGATGCTCGCGCGGACAGCTTCGGCAAGCGCAAGCGCGGCTGAGCGGACCTGGTGGCGCGCGAGCGCGCGCG	960
CGATCGAACAGCAGCGGCTCGGCTTGAAGAGCTGTGATCGGACCGCTTCGGGCAAGGATGCGATCACCACCGCGCTCGAGGTGCTTGGACGCCACCGCGAGAGTGGGACAAACGC	1080
TTCTTGGAAGCGCTGTACGGCTTACCAATGCGAGTCAACCAAGAGCTCGCGCGGGCTGTGACCTTACGCAAGCGCGGGCTTGGGAGCATCCCCAGCGCTTCGGCGGTGCC	1200
GGGCGGCGCGGAGTGTGTGACCGACATCTCGTTCGGGAGCTCCCGATCTACCGCGACATACCGCGGGCTGGTGGACAGCGCGGAGAGTGGCGACGGCTTCGCCACAGCG	1320
TGTATCAAGCTGTTCGACCGACATACCGCGGATGACCGGCTACTTGGCGCGCTGGTTCGGCAGCGCGAGCTTGGAGAGCCGCTCCCGCGGCTCGACACAGCTGGTTCGACGAC	1440
AACGACGCTCGCGCGCTTGAAGAAGAGCTGCTCGACTCCGGGTTCGATCCCGCAGTTGGTCAAGACGGCTGGTTCGGCGCGCGGAGCTATCGCAACACCGACAAGCGCGCGGAGCG	1560
AACGCTGGCGCGCTCGGCTTCGCGCGCGAGCGAGCTGGAGCTCAACGAGCTTCGAGCTCGCAAGGCTTCCGCGCTGTGAAAGAAGTACAGGACGATCAACGCTTCGCGATCC	1680
GGTGCAGAGAAGATCTCGCTCGGACCATGATGCTGTGGCGGCTTCGGCGCGCTCGAGAAGCGCGCCCAAGGACAGGCTACGAGATCTCGGTGCCTTCGCGCGCGCGCCACCG	1800
GCTTCGAGAGAGTACCGACCGAGCTGCTTCGCGGCTCGCAACCGCGCGCGGACGATTCGCAAGCTTACATTCGCGCGGTGAGAAGCGCGCGCTCGAGACCGTACTGATTCGACGCT	1920
CGCTACTCTGCTCGGCTGACCGCTCGGAGATGAGCGTGTCTCGCGGCTCTCGGTGCCCTCGTTCGCAACCGCAGCAGCAGCGCGGCTCTTACCGACAGCGCGCGCGCGGTG	2040
ACCACGACTTCTGCTCAACTCTCGACATGGCGACCGGTGAAGCGCTCGGAGACCGCGGAGAAGCTCTACGAGGACCGCAGCGGCTTCGCGCGCTTCAAGTGACCGCGGACT	2160
CGCAAGCACTTCGCTTCGCTCCCAACTCGGTGCTGGTGGCTGGTTCGAGGCTACCGCGCAGGATACGCCACCGGAAGTCTGTCGAGGACTCTGTCGCGCGGTGGGTGAAGTCAT	2280
AACAGCGACCGGTTCGACCTCAATTAACAGTCGGATAGCGAGTACCGCGCGCGCGGCTTCGCGCGGTTCGCTTCGGATCAGCGCGGACATCCATTTGCTCGTAGTCCGG	2400

Fig. 2. Sequence of a 2452 bp fragment from the *M. intracellulare* genome containing the MI85 gene. The ORF commences at base 66 and terminates at 2304. The predicted amino acid sequence is indicated below the DNA sequence. A potential ribosome binding site, 5 bp upstream of the ORF, is underlined. The arrows designate an inverted repeat sequence 15 bp downstream of the termination codon.

The nucleotide sequence and the deduced protein sequence of MI85 are shown in Fig. 2. A 2238 bp open reading frame (ORF) initiating at GTG (base pair 66)

and terminating at TAA (base pair 2306) was identified within this sequence. The ORF predicts a 745 amino acid residue protein with a molecular mass of 83.5 kDa. This

molecular mass is consistent with our initial immunoblot size estimations. Five base pairs upstream from the initiation codon is a putative Shine-Dalgarno site, the ribosomal binding sequence AAGGA (Gold *et al.*, 1981). A potential rho-independent termination sequence containing a 12 bp hyphenated dyad was found 15 base pairs downstream from the termination codon. Inverted repeat sequences have been identified downstream from several genes encoding mycobacterial proteins and may be a general mechanism for transcriptional termination in mycobacteria (Shinnick *et al.*, 1989; Collins *et al.*, 1990; Nair *et al.*, 1992). The 70% G+C content of the MI85 gene is consistent with the high percentage of G+C base pairs found in genomic DNA from all mycobacterial species. The G+C content increases to 94% in the third base pair of the amino acid codons. Similar elevated G+C ratios at the third codon base position have been reported for other mycobacterial genes (Shinnick, 1987; Matsuo *et al.*, 1988; Nair *et al.*, 1992).

The MI85 gene encodes a mycobacterial catalase-peroxidase

Computer-aided comparisons (Devereux *et al.*, 1984) of the MI85 nucleic acid and deduced amino acid sequences revealed that MI85 encodes a mycobacterial catalase-peroxidase. As seen in Fig. 3, the deduced MI85 protein sequence is 60% identical and 72% similar (identical and conserved amino acid residues) to the *E. coli* peroxidase-catalase HPI (Triggs-Raine *et al.*, 1988). Furthermore, the deduced *M. intracellulare* protein sequence is 59% identical and 71% similar to a *Salmonella typhimurium* hydroperoxidase I (Loewen & Stauffer, 1990) and 47% identical and 62% similar to a peroxidase from *Bacillus stearothermophilus* (Loprasert *et al.*, 1989). In highly conserved regions of the protein, the sequence identity exceeds 75%. The region from Trp95 to Pro199 of MI85 is 82% and 81% identical to the corresponding residues of the respective *E. coli* HPI enzyme and the *S. typhimurium* catalase. The carboxyl terminal portion of the MI85 protein from Asp701 to Leu746 is 83% identical to the *E. coli* HPI protein, 85% identical to the *S. typhimurium* protein, and 75% identical to the *B. stearothermophilus* peroxidase-catalase.

Southern blot hybridizations with MI85 gene probes

To evaluate the prevalence of this catalase gene within the mycobacterial genus, a nucleic acid probe constructed from the original 4.8 kb insert fragment containing the MI85 coding sequence was hybridized to *Eco*RI-digested DNA extracted from thirteen different strains of mycobacteria. As seen in Fig. 4, the MI85 gene probe

MI85	VSSDSSSRFPQPSDGTASKSESENPAIPSPKKAHAPLTNRDWWPDQVD	50
Ecol1	M TSDDIHNTATG CPHQGG...HDQS G GT T	N LR 42
Salty	MSTTDDTHN L TGKCPFHQ.GGHDRS G GTAS	N LR 42
Bacst	MENQNRQNAQQC FHE VTNQSSNRT K	N LN 37
MI85	VSSLHPHSPLSNPLGDDFDYAAEFALDVEALKADMISLMTTSQDWWPAD	100
Ecol1	DL NQ NR E RK S YYG K LKA L E P	92
Salty	DL NQ NR E RK S YYS G LKA L D P	92
Bacst	L I Q DRKT HDEE N E Q YW E LRK E	87
MI85	YGHYGLFIRMSWHAAGTYRIHGRGGAGQGMQRFAPLNSWPDNASLDKA	150
Ecol1	W S A A G SI R Q	V 142
Salty	W S V A G SI R Q	TV 142
Bacst	P A S G ST T	N 137
MI85	RRLWPICKKYGNKISWADLITYAGNVALESMGFKTFGFGREDVWEPE	200
Ecol1	Q Q FIL NS R A	D 192
Salty	Q Q FIL NS R A	D 192
Bacst	CYGRS RNT T . LGPICFWRAMS LNRWEKRLDASAGPLTSGIR	186
MI85	.EILWGEELGLTDRKYSGERLAQPYGATTMLIYVNPPEGKPDPI	249
Ecol1	LDVN D KA THRH...P ALAKA L E	DHSGE L 239
Salty	LDVN D KA THRH...P ALAKA L E D T	NHSGE L 239
Bacst	KKTFIGDRKKS. SPLNAIPVIASSKTRSPRAN VNLQ RRAGRQAGSK	235
MI85	AAADIRETFGRMAMNDEETAALIVGHSFGKTHGAGDADLVGPEEAP	299
Ecol1	S AA A NG V A TL	PTSN D 289
Salty	S AA A NG V A TL	PAA SH AD 289
Bacst	SRG SA. R G V A T A RG P TH	284
MI85	IEQQGLGWKSSYGTGSGKDAITSGLEVVWTPPTKWDNSFLETLYGEWE	349
Ecol1	E A T S V A	Q S Y F N FK V 339
Salty	A A S V A	Q S Y F N FK V 339
Bacst	A I K K S T I GA	Q T YFDM F D W 334
MI85	LTKSPAGAWQFTAKDGAGAGTIPDPFGGA. GRAPTMIV. TDISLRESPIYA	398
Ecol1	Q R I E V .APEI DPSKK K	. LT FD EFE 387
Salty	Q R I E V .APEI DPSKK K	. LT FD EFE 387
Bacst	WM V PDEKDLA AEDPSKK.V MMT LA FD EE 385	
MI85	DITRRWLDHPEELADAFKAWYKLLHRMGPIISRYLGPWVAEQPL.WQDP	447
Ecol1	K S F ND QAFNE R F T K	I E PKED I 437
Salty	K S F ND QAFNE R F T K	KA I E PKED I 437
Bacst	K A FHQN F E R F T	KT E PKEDFI 434
MI85	VPAVDHVELDDNDVAALKKKVLDGLSIPQLVKTAWSAASVYRNTDKRGG	497
Ecol1	L OPIYN.PTEQ IID FAIA VSE SV AS STF GG	486
Salty	L OPLYQPTQE. IIN AAIAA SEM SV AS STF GG	486
Bacst	I E Y TEA.EIEEI A IN TVSE AS R.SA RISAA	482
MI85	ANGGRLRLQFQRSWEVNEPSELDKVLVLEKIQDQDFNASASGGKKISLAD	547
Ecol1	A A M D D AAA.VRA KE.....SG A	527
Salty	A A A D D AVA..AR E KTT A	527
Bacst	T R I A KD ER A S R.....GHP RTAE SKHRR	526
MI85	LIVLAGSAAVEKAAKDAGYEISVHF...APGRDASQESTDVESF....	589
Ecol1	VVG SA LS H P... V R DQ I M	569
Salty	I VVG I Q AA RVS H P... P V RHDQ I M	570
Bacst	DR G TLRWKRQPATPALMSKC SLA AM HKSKPM KALPCWNRSQM	576
MI85	.AVLEPRADGFRNYIRPGEKAPLEQLLIERAYLLGVTPGMTVLVGLLR	637
Ecol1	.EL I RARLDVSTT S DK QQ TL A A M	617
Salty	.SL I RARLDVSTT S DK QQ TL A M	618
Bacst	AS TIKSKSTR RKSCSST PS.....SSADR RNDG SWRFA	615
MI85	ALGANHGSSKHGVFTRDRPGALTNDFVNLDMGTWAKSETAENVYEGRD	687
Ecol1	V G FDG N V V S RY TDESKELF	667
Salty	V T FDG QN K V ST A RY PTDD NELF	668
Bacst	RV P YRHLR I V NY VP..DSGI I	663
MI85	RASGALKWTATANDLVFGSNVLRGLVEVYQAQDAHGKFVDFVAWVKV	737
Ecol1	ET EV F SRA AVA SS E K	718
Salty	LT EV Y RA A A CS E K	718
Bacst	KT EVR RV I I SYA F NQE R IN	713
MI85	MNSDRFDLK* 747	
Ecol1	L* 727	
Salty	L Q* 728	
Bacst	A VKKARESVTA* 732	

Fig. 3. Comparison of the predicted amino acid sequence of the MI85 protein with the sequences of *E. coli* (Ecol1), *S. typhimurium* (Salty) and *B. stearothermophilus* (Bacst) peroxidase-catalases. The differences in the peroxidase protein sequences can be identified by the substitution of another amino acid below the MI85 sequence. Regions of protein sequence identity are designated by blank spaces. Dots represent amino acid residues that are not present within a specific predicted protein sequence.

hybridized to genomic DNA from all species of mycobacteria tested. Subsequent experiments using an amino terminus 840 bp *Eco*RI/*Nco*I probe and with a 2.3 kb *Eco*RI/*Sma*I probe have shown identical hybridization patterns. Although the banding patterns differ among several of the strains, the MI85 gene probe reacts

Fig. 4. Non-radioactive Southern blot hybridization of *Eco*RI-digested mycobacterial total DNA against MI85 gene probe. The PhotoGene chemiluminescence hybridization protocols are described in Methods. Mycobacterial DNA was applied to the following lanes: 1, *M. asiaticum*; 2, *M. avium*; 3, *M. bovis* BCG; 4, *M. bovis*; 5, *M. fortuitum*; 6, *M. intracellulare*; 7, *M. kansasii*; 8, *M. phlei*; 9, *M. scrofulaceum*; 10, *M. smegmatis*; 11, *M. terrae*; 12, *M. tuberculosis* H37Ra, and 13, *M. tuberculosis* H37Rv. Lane M contains biotinylated lambda/*Hind*III DNA molecular mass markers.

with *M. asiaticum*, *M. avium*, *M. bovis*, *M. bovis* BCG, *M. fortuitum*, *M. kansasii*, *M. smegmatis*, *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv, and to homologous *M. intracellulare* DNA. The hybridization of the MI85 probe to *M. phlei*, *M. scrofulaceum*, and *M. terrae* DNA is weak but readily apparent after longer exposures (data not shown).

Expression of the MI85 catalase-peroxidase in *E. coli*

To evaluate the enzymic activity of the MI85 recombinant proteins, lysates containing the MI85-gene 10 fusion constructs were assayed for peroxidase and catalase activity. Similar assays were done with control lysates containing overexpressed gene 10 protein and two other mycobacterial antigens expressed in *E. coli* – the MI22-gene 10 fusion protein and the MK35-gene 10 fusion protein. The MI22 gene encodes the *M. intracellulare* homologue of the *M. tuberculosis* 19 kDa antigen (Nair *et al.*, 1992) and the MK35 gene encodes an antigen derived from a 27 kDa *M. kansasii* protein (S. Morris, unpublished data). Coomassie-blue-stained SDS-PAGE separations of these lysates are shown in Fig. 5(a). The apparent molecular masses for the fusion proteins (as determined by SDS-PAGE) are 107 kDa for MI85-gene 10, 60 kDa for MK35-gene 10, and 45 kDa for MI22-gene 10. It is apparent that the relative amounts of fusion

proteins in each of these lysates is nearly identical. Two common peroxidase substrates, *o*-dianisidine and ABTS, were chosen to evaluate the peroxidase activity in each of these lysates. The lysates containing the MI85-gene 10 fusion protein were significantly active against both *o*-dianisidine and ABTS (Fig. 5b, c). In contrast, none of the control lysates metabolize these peroxidase substrates. The capacity of these lysates to utilize hydrogen peroxide as a substrate was evaluated using the catalase assay developed by Beers and Sizer (1952). This method assays the decomposition of peroxide in a phosphate buffer solution by measuring decreases in A_{240} . Catalatic activity was detected in only the MI85 and MK35 lysates (Fig. 5d). Moreover, the MI85 lysate showed a greater capacity to metabolize hydrogen peroxide [30 units (mg total protein)⁻¹] than MK35 [9 units (mg total protein)⁻¹] during this time period.

Discussion

The immunopathogenic mechanisms of mycobacteria are not well understood, and mycobacterial virulence has not been clearly defined. Because mycobacteria are intracellular parasites which proliferate in an environment with high concentrations of toxic oxygen molecules, it has been suggested that catalases may play a role

Fig. 5. Demonstration of peroxidase and catalase enzyme activities in a cell lysate containing an overexpressed MI85-gene 10 recombinant fusion protein. (a) SDS-PAGE and Coomassie blue staining of cell lysates containing overexpressed mycobacterial gene 10 fusion proteins. Lysates containing overexpressed gene 10 (lane 1), MI85 gene 10 (lane 2), MK35 gene 10 (lane 3), and MI22 gene 10 (lane 4) were applied to the gel. (b, c) Assays of the peroxidase activities of cell lysates containing the MI85 ($\square$), MI22 ($\blacksquare$), and MK35 ($\diamond$) gene 10 fusion proteins and the gene 10 control ($\bullet$), using ABTS (b) and *o*-dianisidine (c) as substrates. Peroxidase activity was measured as changes in A_{405} as described in Methods. (d) Assays of the catalatic activity of the recombinant gene 10 cell lysates. Catalatic activity was evaluated by measuring the decomposition of hydrogen peroxide as described in Methods. The results are plotted as a percentage of control A_{240} values. Symbols as Fig. 5(b).

in the intracellular survival of these acid-fast bacilli (Jackett *et al.* 1981a, b; Walker & Lowrie, 1981). However, more recent experiments examining the importance of mycobacterial catalases have yielded conflicting results (Gangadharam & Pratt, 1984; O'Brien *et al.*, 1991). We have initiated genetic studies aimed at better defining the role of catalase in mycobacterial virulence. The nucleotide sequence of a mycobacterial catalase-peroxidase has been obtained, and active mycobacterial catalase expressed in *E. coli*. The deduced *M. intracellulare* catalase protein sequence is 60% identical to the *E. coli* HPI enzyme (Triggs-Raine *et al.*, 1988), 59% identical to the *S. typhimurium* HPI catalase

(Loewen & Stauffer, 1990), and 47% identical to a *B. stearotheophilus* peroxidase-catalase (Loprasert *et al.*, 1989).

Two catalases have been extensively characterized in *E. coli*. HPI (encoded by *katG*) is a prokaryotic broad-spectrum bifunctional peroxidase-catalase inducible by hydrogen peroxide (Loewen *et al.*, 1985). The *E. coli* catalase HPII (encoded by *katE*) is a monofunctional enzyme that has sequence similarities to eukaryotic catalases (von Ossowski *et al.*, 1991). Biochemical and serological characterizations of mycobacterial lysates have also identified two mycobacterial catalases (Wayne & Diaz, 1982). The mycobacterial T catalases, which

have been identified in most mycobacterial species, have substrate specificities similar to the *E. coli* HPI peroxidase-catalases. The mycobacterial M catalase is a monofunctional HPII-like catalase which has a limited distribution within the mycobacterial genus. The broad-spectrum substrate specificity of the overexpressed MI85 fusion protein suggests that we have cloned and expressed the gene encoding the *M. intracellulare* T catalase-peroxidase. Our Southern blot hybridization results are consistent with this prediction, since a MI85 gene probe hybridizes to total DNA isolated from all strains of mycobacteria that we have tested. Our nucleotide sequence analysis predicts that MI85 has 84 kDa subunits. However, the molecular mass of the native *M. intracellulare* T catalase has been estimated to be 335 kDa (Gruft & Gaafar, 1974). These results suggest that the *M. intracellulare* T catalase is probably a tetramer, like the *E. coli* HPI enzyme. In contrast, the native molecular mass of the *M. tuberculosis* catalase has been estimated to be 160 kDa (Diaz & Wayne, 1974). Therefore, the analogous *M. tuberculosis* protein may be a dimer, similar to the *B. stearothermophilus* peroxidase-catalase. Experiments designed to clone, sequence and characterize the gene encoding the homologous *M. tuberculosis* catalase-peroxidase are currently in progress.

The identification of the gene encoding a mycobacterial T catalase-peroxidase provides a probe to study, at the molecular level, the function of this catalase in mycobacterial pathogenesis. The availability of this gene probe and new methods for isolating mRNA from mycobacteria should permit evaluation of the transcription activation of catalase genes in response to oxidative stress (Patel *et al.*, 1991). Cloning and expression of this gene in *E. coli* should enable biochemical characterization of protein structure and function. Of particular importance, the development of new systems for cloning and expression in mycobacteria should permit gene replacement and substitution experiments with the catalase gene in mycobacteria (Stover *et al.*, 1991). Studies assessing the relative virulence of recombinant mycobacteria having mutated or substituted catalase genes may elucidate the importance of catalase in mycobacterial pathogenesis. Furthermore, the catalase gene may be a useful probe for examining gene regulation in mycobacteria. The expression of the *E. coli* and *Salmonella* HPI peroxidase-catalases and at least seven other proteins, in response to oxidative stress, is mediated by the *oxyR* gene. (Christman *et al.*, 1985; Greenberg & Demple, 1989). A comparison of the sequences upstream from the putative MI85 initiation codon shows similarity to the putative binding domain of the *oxyR* trans-activating protein in the *katG* gene of *E. coli* (Tartagila *et al.*, 1989). Experiments are in progress to examine the presence and function of an *oxyR*-like

regulon in mycobacteria and to ascertain the importance of *oxyR*-regulated proteins in mycobacterial virulence. Finally, resistance to isoniazid, the primary drug used in the treatment of tuberculosis, often correlates with loss of catalase-peroxidase activity (Gangadharam, 1984). A catalase gene probe should allow a genetic examination of the association between catalase activity and isoniazid resistance in drug-resistant *M. tuberculosis* strains.

Note added in proof. The cloning of the *M. tuberculosis* catalase-peroxidase and the role of this enzyme in isoniazid resistance has recently been described (Zhang *et al.*, 1992).

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