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Mycobacterium tuberculosis Genes Induced during Infection of Human Macrophages†

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We identified *Mycobacterium tuberculosis* genes preferentially expressed during infection of human macrophages using a promoter trap adapted for this pathogen. *inhA* encodes an enoyl-acyl carrier protein reductase that is required for mycolic acid biosynthesis (A. Quemard et al., Biochemistry 34:8235-8241, 1995) and is a major target for isoniazid (INH) in mycobacterial species (A. Banerjee et al., Science 263:227-230, 1994). Since overexpression of *inhA* confers INH resistance in *Mycobacterium smegmatis* (Banerjee et al., Science 263:227-230, 1994), we designed a promoter trap based on this gene. A library of clones, containing small fragments of *M. tuberculosis* DNA cloned upstream of *inhA* in a plasmid vector, was electroporated into *M. tuberculosis*, and the resulting culture was used to infect the human monocytic THP-1 cell line. Selection was made for clones surviving INH treatment during infection but retaining INH sensitivity on plates. The DNA upstream of *inhA* was sequenced in each clone to identify the promoter driving *inhA* expression. Thirteen genes identified by this method were analyzed by quantitative reverse transcription-PCR (R. Manganelli et al., Mol. Microbiol. 31:715-724, 1999), and eight of them were found to be differentially expressed from cultures grown in macrophages compared with broth-grown cultures. Several of these genes are presumed to be involved in fatty acid metabolism; one potentially codes for a unique DNA binding protein, one codes for a possible potassium channel protein, and the others code for proteins of unknown function. Genes which are induced during infection are likely to be significant for survival and growth of the pathogen; our results lend support to the view that fatty acid metabolism is essential for the virulence of *M. tuberculosis*.

Virulence genes are generally defined as genes that are necessary for survival of the pathogen in a host and are involved in pathogenicity but are not necessary for growth in culture medium. Several virulence genes of *Mycobacterium tuberculosis* have been identified by comparing the pathogenicity of strains with mutations in the genes to the pathogenicity of isogenic strains carrying the wild-type alleles. These genes include *katG*, encoding a catalase peroxidase (35), *hspX* (*acr*), encoding a homologue of α-crystallin, (36), and *erp*, encoding an uncharacterized exported protein (2). Specific modifications of mycolic acids are essential for virulence since strains carrying disruptions in *hma*, required for the biosynthesis of oxygenated mycolic acids (8), and in *pcaA*, a gene coding for a mycolic acid cyclopropane synthetase (11), are attenuated in mice. Synthesis of the exported phthiocerol dimycocerosate (3, 6) is also required for virulence in mice. Fatty acid degradation is implicated in pathogenicity since a strain carrying a mutated *aceA* gene, which is required for synthesis of isocitrate lyase, was attenuated in mice (23).

Mahan et al. (19, 20) were the first workers to develop a promoter trap, known as an in vivo expression technology (IVET) system, to select for genes of *Salmonella enterica* serovar Typhimurium specifically induced in host tissues. This method has also been used to study pathogenesis in *Staphylococcus aureus*, *Vibrio cholerae*, and other bacteria (4). Using the same approach, we created an IVET method for *M. tuberculosis* to select for genes specifically upregulated during growth in macrophages derived from the human monocytic cell line THP-1. Our goal was to identify genes expressed during infection of macrophages as a means of analyzing the environmental conditions faced by the pathogen during infection, as well as to identify targets for diagnosis and treatment of tuberculosis. Selection was based upon increased expression of *inhA*, driven by promoters which are upregulated specifically during infection. *inhA* codes for an enoyl-ACP reductase, which is required for mycolic acid biosynthesis (26) and is a major target for isoniazid (INH) in *M. tuberculosis*. Overproduction of this enzyme confers resistance to INH in *Mycobacterium smegmatis* (1) and in *M. tuberculosis* (this communication). Specific induction of several candidate genes during growth in macrophages was confirmed by using real-time reverse transcription (RT)-PCR with molecular beacons (mbRT-PCR) (21).

MATERIALS AND METHODS

Construction of the promoter trap. The plasmid promoter trap vector pJD32 was constructed by cloning a promoterless PCR-amplified *M. smegmatis inhA* gene containing an INH resistance mutation (1) into pYUB378, an *Escherichia coli-Mycobacterium* shuttle vector conferring kanamycin resistance in both organisms (9). This vector has a unique *Bam*HI site upstream of *inhA*, and a derivative pJD33 plasmid was constructed with the *hsp60* promoter from *Mycobacterium bovis* BCG cloned into this site. A library of small DNA fragments from *M. tuberculosis* was made by partial *Sau*IIIA digestion and cloned into the *Bam*HI site of pJD32. The plasmid library consisted of 1×10^6 clones in *E. coli*, and the minimum insertion frequency, measured by PCR, was 62%. Assuming

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that the average insertion size was 300 bp, as estimated by PCR, our library consisted of 192 Mb (6.4×10^5 clones $\times$ 0.3 kb) of DNA from *M. tuberculosis*. Since the size of the genome was 4.4 Mb, this library represented the entire genome reiterated about 43 times. We electroporated the plasmid library into *M. tuberculosis* H37Rv and selected for kanamycin resistance in broth culture. An aliquot of the transformation mixture was plated onto solid medium containing kanamycin, and a total of about 9×10^4 *M. tuberculosis* clones were obtained. With an insertion frequency of 62% and an average insertion size of 300 bp, this represented about 16.7 Mb of the 4.4-Mb genome and thus represented the entire genome about four times. A small percentage (0.01%) of the clones from the broth culture grew on plates containing INH (1 μg/ml), and these clones were presumed to contain a promoter upstream of *inhA* which was active when *M. tuberculosis* was grown on plates. The low incidence of INH-resistant clones in the library was presumably due to the low probability of a promoter fragment being inserted in the correct orientation upstream of *inhA*. Most of the small DNA inserts were expected to be internal fragments of various genes or sequences oriented in the wrong direction for transcription, and therefore the frequency of INH-resistant clones was expected to be low. In addition, since we plated the *M. tuberculosis* library on plates containing 1 μg of INH per ml, a rather high concentration, our estimate of the frequency of INH-resistant clones may have been low.

Growth of strains. Cultures of *M. tuberculosis* H37Rv obtained from Barry Kreiswirth (TB Center, Public Health Research Institute) were grown in 7H9 broth supplemented with albumin, dextrose, and NaCl (ADN) and Tween 80, as previously described (17). Kanamycin was added when appropriate at a concentration of 10 μg/ml, and INH was added at a concentration of 0.5 μg/ml. Cultures in the exponential phase of growth were obtained by diluting logarithmically growing cultures at least 20-fold, followed by incubation on a rotator at 37°C.

Selection for INH resistance during infection of THP-1 cells. THP-1 (= ATCC TIB-202) is a monocytic human cell line which can be induced with phorbol esters to a macrophage-like state (32). THP-1 cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal calf serum, 0.45% glucose, 0.15% sodium pyruvate, and 4 mM L-glutamine. Cultures were maintained at concentrations of 1×10^5 to 5×10^5 cells/ml, and the cells were induced to the macrophage-like state by 24 h of treatment with 40 nM 12-*O*-tetradecanoylphorbol-13-acetate (PMA) (32). The cells were infected with the *M. tuberculosis* library for 4 h at a multiplicity of infection of about 1; this was followed by washing with phosphate-buffered saline and replacement of the medium with fresh medium. The number of CFU was estimated based on the optical density of the culture, as previously determined experimentally. After 1 day, the medium was replaced with medium containing 0.5 μg of INH per ml, and the infected cultures were incubated five more days, with changing of the medium after 4 days. Most cells remained adherent throughout the infection. Intracellular bacteria were isolated after lysis of the macrophages with 0.05% sodium dodecyl sulfate (SDS), collection of the bacteria by centrifugation, and resuspension in fresh broth for reinfection of THP-1 cells. An aliquot of the culture was plated on plates containing kanamycin.

PCR primers and molecular beacons. PCR primers were designed to anneal to their targets at the same temperature (60°C) and to amplify DNA fragments internal to the coding sequences of the genes. The molecular beacons, synthesized as previously described, were designed to hybridize to the relevant PCR products (33). The primer pair and the molecular beacon for *sigA* have been described previously (21), and the sequences of all primers and beacons are shown in Table 1.

Preparation of RNA from *M. tuberculosis* growing in THP-1 cells . THP-1 cells were differentiated with PMA as described above and seeded in 24-well tissue culture plates at a density of 7.5×10^5cells/well. After 24 h of PMA treatment, cells were infected with an exponentially growing broth culture of *M. tuberculosis* H37Rv at a multiplicity of infection of 0.5 to 1 CFU/macrophage. In several experiments larger volumes of macrophages were seeded into either 75- or 175-cm^2 flasks pretreated with 0.2% gelatin overnight at 4°C. These macrophages were incubated with RPMI 1640 medium containing 20% fetal calf serum instead of 10% fetal calf serum. These conditions improved the ability of the differentiated THP-1 cells to adhere to the flasks (27). After 2 h of infection, the cells were washed twice with phosphate-buffered saline and incubated with fresh RPMI 1640 medium containing 50 μg of gentamicin per ml; at different time points the medium was removed, and each monolayer was lysed with an appropriate volume of TRI reagent (Molecular Research Center) mixed with polyacrylamide carrier provided by the manufacturer. The lysate was immediately transferred to a 2-ml screw-cap microcentrifuge tube with O-rings containing 0.5 ml of zirconia-silica beads (diameter, 0.1 mm; Biospec Products, Inc.), frozen in dry ice, and stored at −80°C. Aliquots of the rolling culture used for infection and bacteria incubated for 2 h in RPMI 1640 medium were collected by centrifugation, resuspended in 1 ml of TRI reagent with glass beads, and frozen as described above. The viability of intracellular bacteria was assayed in separate wells, infected as described above, by plating for CFU after lysis of the macrophages with 0.05% SDS. *M. tuberculosis* cells in the frozen samples were disrupted by two 1-min pulses in a miniBeadBeater; the samples were kept on ice for 2 min between the pulses. The liquid was removed from the beads, incubated at room temperature for 10 min, and centrifuged for 10 min at 12,000 $\times$ *g*. The supernatant was transferred to a clean tube, 100-μl portions of BCP reagent (Molecular Research Center) were added to 1-ml samples in TRI reagent, and then the tubes were shaken vigorously for 15 s, incubated for 10 min at room temperature, and then centrifuged for 15 min at 12,000 $\times$ *g*. The supernatant was recovered and precipitated with 600 μl of isopropanol. After the RNA was washed with 75% ethanol, it was resuspended in 30 to 100 μl of diethyl pyrocarbonate (DEPC)-treated H_2O, 300 to 1,000 μl of TRI reagent containing polyacrylamide carrier was added, and the extraction procedure was repeated. The RNA was dissolved in 30 μl of DEPC-treated H_2O, treated with RNase-free DNase (Ambion) as recommended by the manufacturer, precipitated again with 95% ethanol, washed with 75% ethanol, dissolved in 30 μl of DEPC-treated H_2O, and stored at −80°C.

mbRT-PCR. For RT, 2 μl of RNA was added to 1.2 μl of 10× PCR buffer II (Perkin-Elmer), 2.4 μl of $MgCl_2$ (25 mM), 0.24 μl of dimethyl sulfoxide, and enough water to bring the volume to 6 μl. After denaturation at 95°C for 1.5 min, annealing between the RNA and the antisense primers was carried out for 3 min at 65°C and then for 5 min at 57°C. Subsequently, 5 μl of the annealing mixture was added to 2.5 μl of 10× PCR buffer II (Perkin-Elmer), 5 μl of $MgCl_2$ (25 mM), 1 μl of a deoxynucleoside triphosphate mixture (25 mM each), 1.5 μl (6 U) of C. therm RT polymerase (Roche), 1.3 μl of dimethyl sulfoxide, 1.3 μl of dithiothreitol (100 mM), and enough water to bring the volume to 20 μl. Samples were incubated for 1 h at 60°C, heated to 95°C for 1 min, and then chilled on ice. Control samples that were not treated with C. therm RT polymerase were also prepared. These template samples for PCRs were then diluted with 70 μl of H_2O and stored at −20°C. The PCR conditions were identical for all reactions. Each 25-μl reaction mixture consisted of 1× DNA polymerase buffer, 4 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.25 mM, 1.25 U of AmpliTaq Gold polymerase (Perkin-Elmer), each primer at a concentration of 1 μM, 50 ng of the appropriate molecular beacon, and 10 μl of template. After incubation for 10 min at 94°C to activate the DNA polymerase, the first set of 10 cycles was run with an annealing temperature of 65°C. The denaturation step was 30 s at 94°C, and the extension step was 30 s at 72°C. In the second set of 30 cycles, denaturation was at 94°C for 30 s, annealing was at 60°C for 30 s, and extension was at 72°C for 30 s. The reactions were carried out in sealed tubes in an Applied Biosystems 7700 Prism spectrofluorometric thermal cycler (Perkin-Elmer). Fluorescence was measured during the annealing step of the second set of cycles and was plotted automatically for each sample. Quantitative analysis of the data was performed as previously described (21). In order to obtain standard curves for the mbRT-PCR, a PCR was performed with each primer-beacon set using different amounts of H37Rv chromosomal DNA, and these reactions were performed at the same time as the mbRT-PCR. The standard curves (data not shown) were used to calculate the amount of cDNA for each gene present in the different samples. All values were normalized to the amount of *sigA* mRNA. The values obtained with the RNA sample from the bacteria incubated in 7H9 medium were used as arbitrary standards for the calculations. RNAs from bacterial cultures initially grown in 7H9 medium and then incubated for 2 h in RPMI 1640 medium were also analyzed by mbRT-PCR.

RESULTS

Construction of a promoter trap for *M. tuberculosis*. Drug resistance can result from a decreased ratio of drug to target (7), and this mechanism explains the observation that overexpression of *inhA* confers INH resistance to *M. smegmatis* (1). InhA, in the presence of NADH, binds INH (25), consistent with its role as a major target for this antibiotic. These considerations led us to predict that *inhA* would be suitable for construction of a promoter trap which could allow selection (INH resistance) for promoters driving *inhA* expression in *M. tuberculosis*. We cloned the *inhA* gene from *M. smegmatis* (1) without its promoter into the shuttle vector pYUB378 (9) to form the vector pJD32. To test if increased expression of *inhA* could confer INH resistance in *M. tuberculosis*, we cloned the

TABLE 1. Sequences of primers and beacons[a]

Primer or beacon	Sequence
Rv1171.rt	5′GTCAGTAGCCACGCCCACAG3′
Rv1171.up	5′TGTCCTGGCCCTGTTGA3′
Rv1171.down	5′GGCACTGATCGGAAACGGAG3′
Rv1171.beacon	5′**GCAGCC** CGGTGAGGCATTGCTGT **GGCTGC**3′
Rv3237c.rt	5′AAACAAGCTACCCGCCCGGATG3′
Rv3237c.up	5′TGTATGGCCGCGATGA3′
Rv3237c.down	5′GGCGCACCCAGAATCTGAG3′
Rv3237c.beacon	5′**GCCACC** GTGAGCCGCAAACCG **GGTGGC**3′
Rv3321c.rt	5′TTCGATACCGCTCCAC3′
Rv3321c.up	5′GCGGGTGAAATCCTGTC3′
Rv3321c.down	5′CCATGAAAGGCGTCCTC3′
Rv3321c.beacon	5′**GCAGCC** ACCAACAGAATCCA **GGCTGC**3′
nirA.rt	5′TGGCGTAGATGTTTTCG3′
nirA.up	5′GAAGGAGAACCCCCAAT3′
nirA.down	5′CCTTCTTCAGCTCTTCGT3′
nirA.beacon	5′**GCAGCC** AAATGAGGGCCAGTGG **GGCTGC**3′
Rv0977.rt	5′TATCGCCGTCGTCCGTTTC3′
Rv0977.up	5′CCTCTTCCCCGAGTTTTCTGA3′
Rv0977.down	5′CATCAACCGGGTGTCGAAGGT3′
Rv0977.beacon	5′**GCAGCC** GTCGGCGGCGTCGGATCTGG **GGCTGC**3′
Rv2224c.rt	5′AGACCTCGACGGCTTTGG3′
Rv2224c.up	5′CGGGTGCGGGCAATGA3′
Rv2224c.down	5′AGTCGGCGGCAATAGTTGT3′
Rv2224c.beacon	5′**GCAGCC** GGCATTGGGGTCGACG **GGCTGC**3′
echA19.rt	5′ATGCCGAACTTCGCACTTTCACCG3′
echA19.up	5′ATCCGACATCCGTTGCT3′
echA19.down	5′GCTGCCGTCCTTGAAAGA3′
echA19.beacon	5′**GCAGCC** GCAACCCAGAAACCGCCG **GGCTGC**3′
Rv1774c.rt	5′GGCGAGGCAAGGGAGATGA3′
Rv1774c.up	5′AGGGCGGATACGGCTGGAA3′
Rv1774c.down	5′CGGCGTGATTGTCTGCGTC3′
Rv1774c.beacon	5′**GCAGCC** CAATCACGCTCTCGCAC **GGCTGC**3′
fadA4.rt	5′GCACCGTCGGAGATCTGT3′
fadA4.up	5′TTTGGACCACATGGCCTACG3′
fadA4.down	5′CTTTTGGTGGGACGCAGCC3′
fadA4.beacon	5′**GCAGCC** CAACGACGTCGACATGTTCACC **GGCTGC**3′
aceA.rt	5′CGCACCTGCTGGACGGCCA3′
aceA.up	5′GCGGAGCAGATCCAGCAGGT3′
aceA.down	5′GCGGCGGGCCAGCGTGTGCTC3′
aceA.beacon	5′**GCGAGG** AGGACGTCACCCGCACC **CCTCGC**3′
fadA5.rt	5′CGAACGCCTCGTTGATCT3′
fadA5.up	5′GATCGTCGCCCAGGCACT3′
fadA5.down	5′GATGTCGCCGATCTTCATGC3′
fadA5.beacon	5′**GCAGCC** CCTACTACCACCTGGACGGCC **GGCTGC**3′
pckA.up	5′ATACCGCGCCGACGAATCAC3′
pckA.down	5′ATCGCAGAGCCGCTGGAACTC3′
pckA.beacon	5′**GGACGC** GGGTGGTCTTCACTGACGG **GCGTCC**3′
Rv2520c.up	5′GGCTCCAACGTCCCGATCA3′
Rv2520c.down	5′CGCACCGCACCCACTCGTC3′
Rv2520c.beacon	5′**CGGTCG** GGGAAGGCCGCGAACA **CGACCG**3′
hspX.up	5′CCGAGCGCACCGAGCAGAAG3′
hspX.down	5′GGTGGCCTTAATGTCGTCCTCGTC3′
hspX.beacon	5′**GGCTCC** CCTTCGTTCGCACGGTGTC **GGAGCC**3′
sigA.rt	5′CGGACGAGACCATGGTGCGGC3′
sigA.up	5′GGCCAGCCGCGCACCCTTGAC3′
sigA.down	5′GTCCAGGTAGTCGCGCAGGACC3′
sigA.beacon	5′**CCTCGC** GTCGAAGTTGCGCCATCCGA **GCGAGG**3′

[a] rt, RT primer; up, 5′ primer; down, 3′ primer. Where an RT primer is not indicated, the 3′ primer was used for RT. Beacon generic stems are indicated by boldface type. All beacons were synthesized with tetrachlorofluorescein attached to the 5′ end and dabcyl (quencher) attached at the 3′ end.

hsp60 promoter (*Phsp60*) (30) upstream of *inhA* in pJD32 to form pJD33. This promoter is expressed constitutively at high levels when it is present on a multicopy plasmid (30). The *inhA* promoterless plasmid (pJD32) and the plasmid containing *Phsp60* driving *inhA* (pJD33) were electroporated into *M. tuberculosis* H37Rv, and the resulting strains were tested for INH resistance during growth in broth and during infection of human monocytic cell line THP-1 (32) after differentiation into macrophage-like cells with phorbol ester. The strain with *Phsp60* driving *inhA* expression conferred INH resistance under both growth conditions, whereas the strain carrying the promoterless *inhA* gene remained relatively sensitive to INH (Fig. 1). These results show that as previously reported for *M. smegmatis* (1), overproduction of InhA confers INH resistance to *M. tuberculosis.* We concluded that a promoter trap based upon INH resistance caused by overexpression of *inhA* should be suitable for identification of *M. tuberculosis* promoters induced during infection. We should note that *M. tuberculosis* carrying the plasmid with no promoter driving *inhA* is more sensitive to INH in broth culture (Fig. 1A) than in THP-1 cells (Fig. 1B). This could be due to lower actual levels of INH in the macrophages than in broth or to decreased INH sensitivity of the macrophage-grown *M. tuberculosis*. Thus, it is possible that clones with a weak, constitutively expressed promoter might appear to be INH resistant during infection of THP-1 cells while remaining INH sensitive in broth. Therefore, all clones identified by this system must be further evaluated to eliminate false positives (see below).

Selection of *M. tuberculosis* INH-resistant clones during infection of THP-1 cells. We constructed a library using small (100- to 500-bp) *M. tuberculosis* DNA fragments cloned upstream of *inhA* in the pJD32 vector. This plasmid library was electroporated into *M. tuberculosis*, with selection for kanamycin resistance; 0.01% of the clones resulting from the electroporation procedure were INH resistant prior to further selection. This low frequency was presumably due to the fact that most clones contained small DNA fragments with no promoter activity at all; pos sibly there were also some promoters that conferred resistance to levels of INH lower than that used in the original selection. A culture from the library was used to infect THP-1 cells that had been differentiated into macrophage-like cells by the addition of phorbol esters. Treatment with INH was imposed after 1 day of infection, and clones that survived a 5-day treatment with 0.5 μg of INH per ml were selected. Surviving clones were isolated from the macrophages, diluted into broth, and used to reinfect THP-1 cells, again with treatment with INH for 5 days. Passaging of the library in this manner was repeated four times. Each time, an aliquot of the culture containing survivors of the INH treatment was plated on medium with no INH, and single colonies were picked and tested individually for INH resistance by streaking on plates containing INH (0.5 μg/ml). Clones completely sensitive to this level of INH were analyzed by PCR for the presence of a cloned insert by using primers for *inhA* and vector sequences that flanked the insert.

Clones were analyzed for the frequency of inserts and the frequency of INH resistance before and after selection in macrophages in order to determine if INH selection was working during growth in macrophages. It was expected that the frequency of clones resistant to INH on plates, as well as the frequency of inserts in INH-sensitive clones, would increase with each selection round. The presence of an insert was determined by PCR analysis with heat-killed bacteria by using primers flanking the cloning site, and INH resistance was assayed by streaking on plates with 0.5 μg of INH per ml. The enrichment of the frequency of INH-resistant clones and INH-

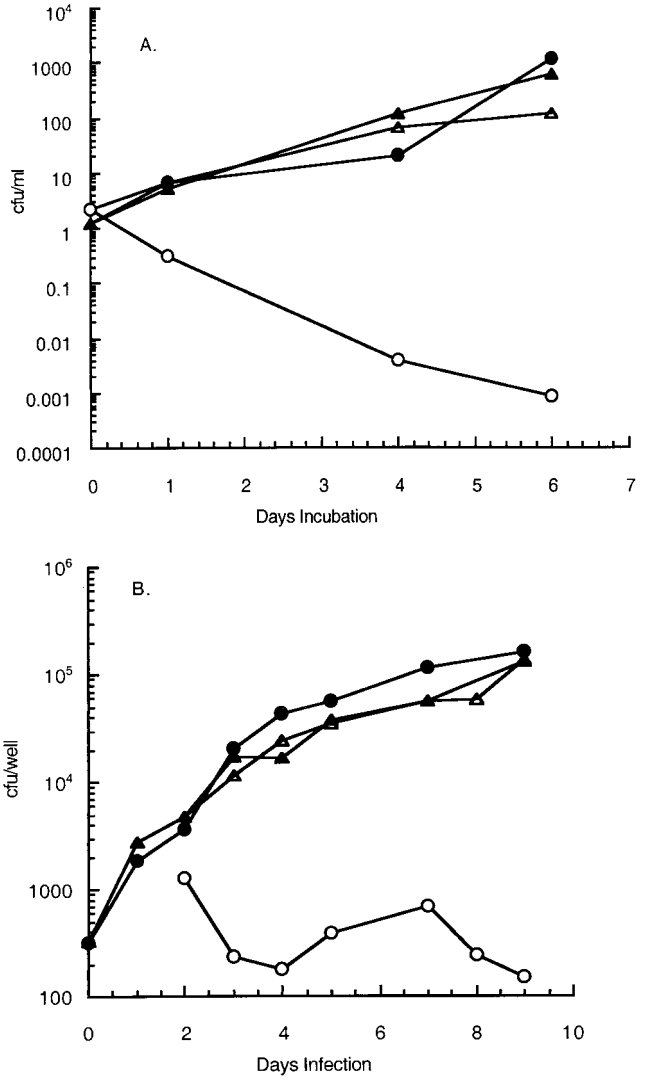

FIG. 1. INH sensitivity of *M. tuberculosis* H37Rv carrying pJD32 (promoterless *inhA*) or pJD33 (*inhA* driven by *Phsp60*). (A) Growth in broth culture. Exponentially growing cultures of *M. tuberculosis* H37Rv were diluted to a concentration of about 10^6 CFU/ml, and INH (0.5 μg/ml) was added to one sample of each strain. The cultures were incubated in a 24-well plate at 37°C, and samples were assayed to determine the number of CFU per milliliter at timed intervals. (B) Growth in THP-1 cells. Exponentially growing cultures of *M. tuberculosis* H37Rv were diluted to a concentration of about 5×10^3 CFU/ml with tissue culture medium, and 0.1-ml portions of the diluted cultures (5×10^2 CFU per well) were used to infect differentiated THP-1 macrophages (2×10^4 cells per well) in 96-well plates. After 1 day of incubation, the medium was replaced with fresh medium with or without 0.5 μg of INH per ml. Extracellular and intracellular bacteria were assayed to determine the number of CFU at different times. Symbols: ○, H37Rv(pJD32) with INH; ●, H37Rv(pJD32) without INH; △, H37Rv(pJD33) with INH; ▲, H37Rv(pJD33) without INH.

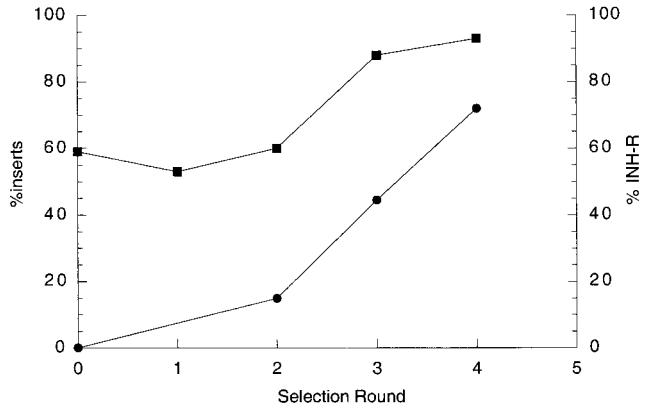

FIG. 2. Enrichment for INH-resistant (INH-R) clones and clones containing DNA inserts after multiple rounds of selection in THP-1 cells. Individual colonies surviving the infection were picked from plates with kanamycin and tested for INH resistance by streaking on plates with and without INH. Colonies were also tested for the presence of a DNA insert by PCR analysis of heat-killed bacteria using primers flanking the site of insertion. Symbols: ●, percentage of INH-resistant clones; ■, percentage of clones with inserts.

sensitive clones containing inserts is shown in Fig. 2. Only 59% of the INH-sensitive clones from the original library had inserts, whereas after four passages with selection with INH in macrophages, 93% of the INH-sensitive clones had inserts and 72% were INH resistant.

Identification of promoters driving *inhA* in clones surviving INH treatment during macrophage infection. Plasmid DNAs from individual *M. tuberculosis* clones that survived INH treatment during growth in THP-1 cells, that were INH sensitive on plates, and that carried DNA inserts upstream of *inhA*, as determined by direct PCR analysis of the *M. tuberculosis* colonies, were isolated from *M. tuberculosis* cultures and used to transform *E. coli*. Plasmid DNAs were then prepared from the *E. coli* kanamycin-resistant transformants, and the sequences of the inserts upstream of *inhA* were determined by using a primer specific for the adjacent 5′ region of *M. smegmatis inhA*. The DNA sequences of the inserts were then compared to the *M. tuberculosis* H37Rv genome database (5). Whereas only 24% (4 of 17) of the INH-sensitive clones from the second passage contained sequences 5′ to an open reading frame in the correct orientation for transcription of *inhA*, 41% (12 of 41) of the clones from the third passage and 69% (45 of 65) of the comparable clones from the fourth passage contained such sequences. Our initial conclusion was that these sequences contained promoters that drove expression of *inhA* during *M. tuberculosis* infection of macrophages, resulting in overexpression of InhA and conferring INH resistance during infection. A list of the open reading frames found downstream from these putative promoter sequences is shown in Table 2.

Differential expression of selected genes measured by RT-PCR. Since some of the promoters identified by our system could be false positives, selected on the basis of artifactual increased expression from a multicopy plasmid or because they were low-level constitutive promoters, as discussed above, we set out to verify whether they were indeed induced during growth of wild-type *M. tuberculosis* in macrophages. We determined the ratio of specific mRNAs from *M. tuberculosis* H37Rv grown in broth to specific mRNAs found in bacteria infecting THP-1 cells by using mbRT-PCR (21). Normalization is necessary for quantitative analysis by RT-PCR, and since initial experiments had shown that the *sigA* mRNA/total bacterial RNA ratio was the same in log-phase broth-grown and macrophage-grown cultures (data not shown), we performed an experiment to verify that *sigA* mRNA could be used for normalization of the mRNAs of the genes identified by the promoter trap selection procedure. We monitored the relative levels of *sigA* DNA by PCR and the levels of *sigA* mRNA by RT-PCR with samples prepared during growth of *M. tuberculosis* in THP-1 cells. At the same time, we measured the CFU. The levels of *sigA* mRNA increase in parallel with the levels of *sigA* DNA and the CFU (Fig. 3). This shows that as expected for a housekeeping gene, *sigA* mRNA accumulates in proportion to DNA levels and bacterial replication under conditions of growth in THP-1 macrophages, validating the use of *sigA* mRNA to normalize the mRNA levels of the genes analyzed.

Next, we tested if our methods would work for a gene whose expression was known to be induced in *M. tuberculosis* during growth in macrophages. *hspX*, coding for the α-crystallin-like protein, had previously been shown to be highly induced (36), as was the protein itself (24). Therefore, we measured the relative levels of *hspX* mRNA during infection and during growth in broth. As expected, *hspX* was induced very dramatically (maximum, 366-fold) in bacteria grown in THP-1 cells after only 6 h of incubation (Fig. 4). This result validated the mbRT-PCR method for quantitating mRNA levels in bacteria grown in macrophages. We chose 13 of the 43 genes identified by the selection system for validation by mbRT-PCR (21). We made the selection based upon genes which could be involved in a variety of functions. Some of these genes encoded proteins postulated to be involved in fatty acid degradation, others could be involved in export or import, others with DNA binding motifs are potential transcriptional activators, and others encoded proteins whose functions are completely unknown. We determined the ratios of the mRNAs of these genes in broth-grown and THP-1-grown cultures of *M. tuberculosis*. In order to be sure that our RNA preparations were not contaminated with RNA from a small population of extracellular bacteria which had not been removed by washing, we added gentamicin (50 μg/ml) to the medium after the infected monolayer was washed, and the antibiotic was present throughout the infection. This treatment did not significantly affect the yield of intracellular bacteria but was effective in killing extracellular bacteria (data not shown). In an earlier series of experiments, we infected the cells in the absence of gentamicin and obtained the same results with mbRT-PCR (R. Manganelli, E. Dubnau, and I. Smith, unpublished data). Eight of the 13 genes tested (Rv2224c, Rv3237c, Rv3321c, Rv2520c, *pckA*, *echA19*, *fadA4*, and *aceA*) were upregulated more than twofold during growth in THP-1 cells relative to growth in broth culture (Table 3). Similar data were obtained with two separate infections and, in the case of Rv2520c and *aceA*, with three separate infections. Therefore, 61% (8 of 13) of the genes tested were found to be actually induced during growth in THP-1 cells. In order to control for differential expression of bacterial genes in response to the tissue culture medium (RPMI 1640 medium) during the 2 h of incubation allowed for

TABLE 2. Genes identified by INH selection in macrophages

Category	Gene	Rv no.[a]	Comment
Fatty acid degradation	*aceA*	Rv0467	Isocitrate lyase
	fadA4	Rv1323	Acetyl-coenzyme A acetyltransferase
	echA19	Rv3516	Enoyl-coenzyme A hydratase
	fadA5	Rv3546	Acetyl-coenzyme A acetyltransferase
Possible fatty acid metabolism	*ephF*	Rv0134	Epoxide hydrolase
		Rv0610c	Monooxygenase
		Rv1144	Alcohol dehydrogenase
		Rv1774	Oxidoreductase
Cell envelope		Rv0102	Membrane
		Rv1171	Hydrophobic protein
		Rv2120c	Membrane
	lppM	Rv2171	Lipoprotein
		Rv3237c	Potassium channel
		Rv3524	Membrane, sensor
		Rv3717	*N*-Acetyl-muramoyl-*L*-alanine amidase
Intermediary metabolism	*pckA*	Rv0211	Phosphoenol carboxykinase
	eno	Rv1023	Enolase
PPE/PEPGRS		Rv0977	PE/PGRS
		Rv1361c	PPE
		Rv1840c	PE/PGRS
Putative transcriptional regulators		Rv0549c	Helix-turn-helix motif
		Rv2009	Helix-turn-helix motif
		Rv3321c	Helix-turn-helix motif
Miscellaneous	*fusA2*	Rv0120c	Elongation factor G
	proC	Rv0500	Proline biosynthesis
	uvrC	Rv1420	Exonuclease ABC
	infC	Rv1641	Initiation factor 3
		Rv2224c	Exported protease
	nirA	Rv2391	Nitrite reductase
		Rv2520c	Transmembrane domain
	dut	Rv2697c	Deoxyuridine triphosphatase
		Rv3225c	Aminoglycoside 3′ phosphotransferase
Unknown		Rv0036c, Rv0406c, Rv0811c, Rv1778c, Rv2273, Rv2468c, Rv2632c, Rv2717c, Rv3427c, Rv3493c, Rv3717	

[a] The Rv number is the number assigned to the open reading frame, and the genes are annotated as described by the Pasteur Institute at the TUBERCULIST website (http://genolist.pasteur.fr/TubercuList).

uptake of the bacteria, we also compared the levels of mRNAs from bacteria incubated in RPMI 1640 medium for 2 h with the levels of RNAs from bacteria grown in broth culture. Most of the genes showed no significant differences in RNA levels, but *aceA* and *echA19* were induced by this treatment (9.5- and 4.2-fold, respectively), albeit at lower levels than during growth in THP-1 cells (85.4- and 25-fold, respectively) (Table 3). This induction may have been due to the high levels of fetal calf serum (20%) added to the RPMI 1640 medium. We expected that this component would be very rich in fatty acids and could cause induction of genes postulated to be involved in fatty acid metabolism. We are currently investigating the induction patterns of these genes in various media.

Several genes identified by our selection system, (Rv0977, Rv1774, Rv1171, *fadA5*, and *nirA*) were not upregulated, and in fact, two of these genes (Rv1171 and Rv1774) seemed to be actually repressed in macrophages, suggesting that our system does identify some false positives. *nirA* appears to be downregulated both in RPMI 1640 medium and in macrophages. Therefore, the other genes listed in Table 2 must be considered candidate genes prior to further validation.

DISCUSSION

The virulence of any pathogen is determined by its ability to adapt to the host environment. Our approach to describe adaptation by *M. tuberculosis* to the macrophage environment, in which a promoter trap screen was used, resulted in identification of 43 genes, 9 of which may code for proteins involved directly or indirectly in fatty acid metabolism: *aceA*, *echA19*, *fadA4*, *pcKA*, *ephF*, Rv0610c, Rv1144, *fadA5*, and Rv1774. It is expected that microarray analysis of gene expression in macrophages should produce a far more extensive list of genes that are transcriptionally upregulated during growth in macrophages, and several laboratories, as well as our laboratory, are currently doing this sort of analysis. Our survey was certainly

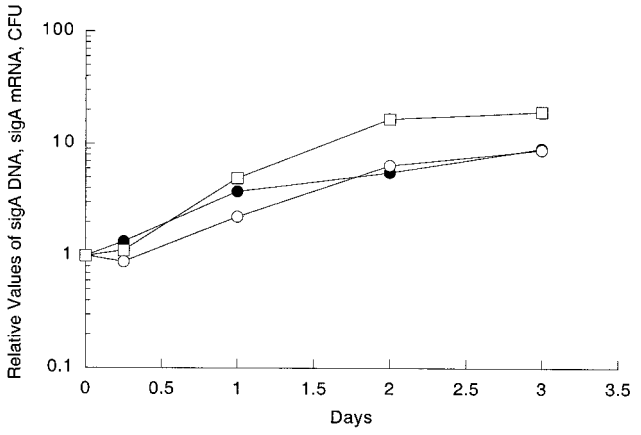

FIG. 3. Levels of *sigA* DNA and *sigA* mRNA and growth of *M. tuberculosis* H37Rv in THP-1 cells. The numbers of CFU (□), the levels of *sigA* mRNA (○), and the levels of *sigA* DNA (●) during growth of *M. tuberculosis* H37Rv in THP-1 cells were determined. The values were normalized to the values obtained after 1 h of infection in THP-1 cells.

not extensive enough to detect all *M. tuberculosis* genes upregulated in macrophages since various genes, including iron-regulated genes, *sigE*, *sigH*, *hspX*, and *fbpB*, known to be upregulated in macrophages (12, 18, 28, 36), were not identified by our screening procedure. None of the genes identified as upregulated during growth in the presence of INH (34), several of which are also involved in fatty acid metabolism, were identified by our method. Obviously, the main reason for this is the fact that we selected against this group of genes by choosing only clones which are sensitive to INH on plates. Promoters induced by INH would confer resistance to INH.

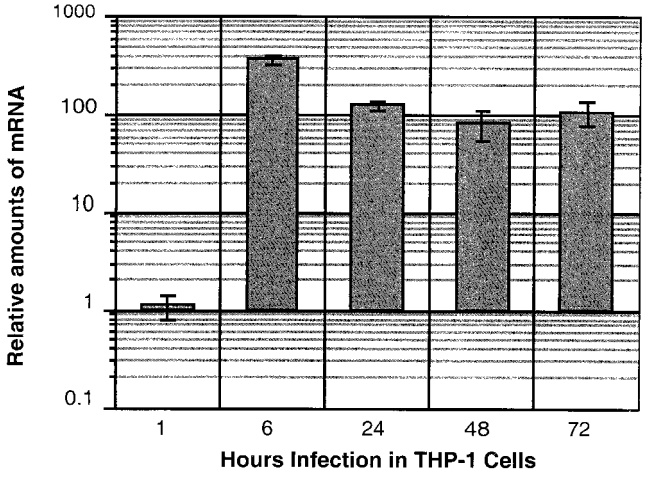

FIG. 4. Changes in *hspX* mRNA levels during growth in THP-1 cells. The results are expressed as the ratios of the numbers of RNA copies detected in RNA samples taken at various times (shown on the *x* axis and expressed in hours) from cultures of *M. tuberculosis* H37Rv to the numbers of RNA copies detected in samples obtained from bacteria growing in 7H9 medium. The values were normalized to the *sigA* RNA value. Each measurement was obtained at least twice using independent RNA preparations.

TABLE 3. *M. tuberculosis* genes induced during macrophage infection

Gene	Induction ratios[a]		
	RPMI 1640 medium[b]	7H9 medium	
		24 h after infection[c]	72 h after infection[c]
Induced genes			
Rv2224c	0.9 ± 0.1	4.0 ± 0.8	3.5 ± 0.7
Rv3237c	0.6 ± 0.0	3.6 ± 1.8	4.0 ± 1.2
Rv3321c	1.3 ± 0.2	2.3 ± 0.0	2.0 ± 0.2
echA19	4.2 ± 0.4	25.0 ± 14.5	35.0 ± 13.5
aceA	9.5 ± 1.3	85.4 ± 3.1	88.4 ± 8.6
fadA4	1.0 ± 0.3	2.6 ± 1.0	4.0 ± 1.5
pckA	2.2 ± 0.1	7.0 ± 2.0	6.1 ± 0.9
Rv2520c	1.1 ± 0.1	2.2 ± 1.3	0.9 ± 0.5
Genes not induced			
fadA5	1.5 ± 0.4	0.3 ± 0.1	0.5 ± 0.3
Rv1171	1.0 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Rv0977	1.0 ± 0.0	0.5 ± 0.1	1.5 ± 0.3
Rv1774	0.4 ± 0.1	0.1 ± 0.2	0.7 ± 0.0
nirA	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1

[a] The values are means ± standard errors based on triplicate samples.
[b] The values are the relative amounts of *M. tuberculosis* mRNA obtained after 2 h of incubation in RPMI 1640 medium compared to the amounts of mRNA obtained after growth in broth. The values are normalized to *sigA* mRNA.
[c] The values are the relative amounts of *M. tuberculosis* mRNA obtained 24 and 72 h after infection in THP-1 cells compared to the amounts of mRNA obtained after growth in broth. The values are normalized to *sigA* mRNA.

Thirteen genes were selected for quantitative analysis by mbRT-PCR from RNAs prepared from broth-grown cultures and THP-1-grown cultures. It is important to emphasize that this analysis was done with wild-type *M. tuberculosis* H37Rv and not with strains carrying plasmids containing *inhA* under the control of cloned promoters. Thus, the measurements of mRNA were based upon transcription from the native promoters of the genes in their normal chromosomal location. *hspX* was highly induced under our conditions, validating our methods, since it has been shown previously that this gene is upregulated in macrophages (24, 36). Some workers reported high levels of *hspX* mRNA in log-phase cultures of *M. tuberculosis* (16), but we found this molecule to be present at very low levels in such cultures and we assume that the different results arose from differences in bacterial growth conditions.

Expression of eight genes was shown to be induced during growth in THP-1 cells. Five genes were not found to be induced, and therefore it is important to stress that the genetic screening method does pick up false positives. There are several different sources of false positives: (i) promoter activity due to multicopy effect, (ii) low-activity promoters leading to survival in vivo and sensitivity in vitro, (iii) lower sensitivity to INH in vivo because of lower levels of INH in the macrophages, decreased levels of catalase, or other unknown reasons, and (iv) induced expression which is below the detection limit of the RT-PCR. Although most of the genes which we identified coded for unknown functions, 9 of the 43 genes are predicted to be involved in fatty acid metabolism. The genome of *M. tuberculosis* has approximately 250 open reading frames which are annotated to be involved in fatty acid metabolism, out of a total of about 4,000 open reading frames. The probability of detecting 9 of these 250 open reading frames in 43

genes simply by chance is very low ($P < 0.001$, as determined by chi-square test).

The genes identified by our selection procedure include *fadA4* and *echA19*, which are annotated as genes involved in β-oxidation of fatty acids, as well as *aceA* (*icl*) coding for isocitrate lyase, an enzyme of the glyoxylate shunt pathway. This pathway is required to replenish substrates for the Krebs cycle during growth on fatty acids. The fact that *aceA* appeared among the selected genes validated our approach since it was shown previously that *aceA* was upregulated during infection of macrophages (13, 23) and that the levels of isocitrate lyase increase during infection (15, 31). Significantly, this gene is necessary for virulence in mice (23). *pckA*, although not directly involved in fatty acid metabolism, is required to produce phosphoenolpyruvate from the tricarboxylic acid cycle during metabolism of acetate, the product of fatty acid β-oxidation. Other genes identified by our selection procedure which may be involved in fatty acid metabolism include *ephF*, encoding an annotated epoxide hydrolase, Rv0610c, encoding an annotated monooxygenase, and Rv1144, encoding an annotated short-chain alcohol dehydrogenase. This group of genes has not yet been validated. Compared with the 50 genes in *E. coli*, the number of genes presumed to be involved in β-oxidation of fatty acids in *M. tuberculosis* is astounding (5). There are 36 *fadD*, 36 *fadE*, 21 *echA*, 5 *fadB*, and 6 *fadA* paralogs in *M. tuberculosis*, whereas there is only one copy of each of these genes in *E. coli*. This, together with biochemical data on the differences in metabolism of *M. tuberculosis* growing in mouse lungs and in broth (29), has led to a consensus that fatty acid degradation may provide a major source of energy during infection.

It is interesting that *pckA*, *aceA*, and seven *fad* genes are also upregulated in *M. tuberculosis* by treatment with SDS (22), a stress condition which may cause cell envelope damage and require remodeling of the cell envelope. *S. enterica* serovar Typhimurium modifies the structure of lipid A during growth in macrophages; the modifications require *phoP-phoQ*, a two-component system activated in macrophages, and are postulated to function by attenuating the host cell innate immune response (10, 14). It is possible that *M. tuberculosis* also remodels its cell envelope upon entry into macrophages, a process that would require degradation followed by resynthesis of various lipids in the cell envelope. Consistent with this hypothesis is the fact that our search identified Rv3717, encoding *N*-acetyl-muramyl-L-alanine amidase.

There are several other interesting genes which are also induced in THP-1 macrophages. Rv3321c is a member of a subfamily of genes with helix-turn-helix motifs typical of DNA binding proteins (http://cbcsrv.watson.imb.com/servlets/Utility); all of these motifs are upstream of a family of open reading frames with some similarity to one another. Rv3237c is annotated as a possible potassium channel protein gene.

Our findings highlight the significance of fatty acid metabolism for *M. tuberculosis* during growth in macrophages. It is interesting that *fadB*, coding for an enzyme required for β-oxidation of fatty acids, was upregulated in *S. enterica* serovar Typhimurium during infection of mice, and this in vivo induction was thought to be due to the high concentration of fatty acids encountered by the pathogen during infection (19). Lipids have long been postulated to be an energy source for *M. tuberculosis* during infection, but it is also possible that lipid biosynthesis and degradation may be important for the remodeling of the cell envelope upon entry into the macrophage. Ultimately, we are interested in defining new *M. tuberculosis* drug targets, and we are currently conducting experiments to determine if any of the genes identified by our approach are required for virulence. This is being done by assaying the virulence in macrophages and mice of mutant strains inactivated for the various genes.

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