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Responses of *Mycobacterium tuberculosis* to Growth in the Mouse Lung

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Using a promoter trap, we have identified 56 *Mycobacterium tuberculosis* genes preferentially expressed in the mouse lung. Quantitative real-time PCR showed that RNA levels of several genes were higher from bacteria growing in mouse lungs than from broth cultures. These results support the current hypothesis that *Mycobacterium tuberculosis* utilizes fatty acids as a carbon source in the mouse lung.

In one of the earliest experiments comparing the physiology of *Mycobacterium tuberculosis* growing in broth culture with that of bacteria isolated from the mouse lung, Segal and Bloch (16) showed that bacteria isolated from lungs only responded to substrates containing fatty acids, whereas broth-grown bacilli responded to a variety of substrates, including carbohydrates. This was the first indication that the physiological state of this important pathogen was altered during infection of a mammalian host and that *M. tuberculosis* growing in the host obtains its energy mainly from degradation of fatty acids rather than from carbohydrates. Consistent with this idea, the annotated DNA sequence (http://genolist.pasteur.fr/TubercuList/) of *M. tuberculosis* (2) contains more than 250 genes involved in fatty acid metabolism, compared with an estimated 50 in *Escherichia coli*. Genetic experiments have shown that *icl*, coding for isocitrate lyase, is required for persistence of *M. tuberculosis* in the mouse lung (9). This enzyme is part of the glyoxylate cycle, an anapleurotic pathway required for growth on acetate, the end product of fatty acid oxidation. Results from several laboratories indicate that numerous genes probably involved in lipid metabolism are transcriptionally upregulated during growth in the mammalian macrophage (4, 15) and the mouse lung (15, 20). Genes required for iron uptake, various stress responses, and other processes are also upregulated during growth in the host (4, 15, 17, 20). This regulation may reflect the requirements of *M. tuberculosis* for survival and/or growth during infection, and such information may be useful in the design of new antibiotics or vaccines.

Classic promoter trap technology, which predates the modern use of DNA microarrays for genomic transcriptional analyses, is still useful under conditions where transcriptional profiling is impractical. We have described a promoter trap for *M. tuberculosis*, based upon overexpression of *inhA*, coding for an enoyl-ACP-reductase, a protein required for mycolic acid biosynthesis and the major target of isoniazid (INH) (1). Since overexpression of *inhA* confers resistance to INH (1), a promoter trap using this INH resistance was used to identify *M. tuberculosis* genes expressed during infection of human macrophages but not during growth in broth (4). We have now applied this technology to identify genes specifically expressed during mouse infection and have identified 56 promoters that drive *inhA* expression at higher levels during infection in the mouse lung than during growth in laboratory medium. The differential expression of seven of these genes has been validated by quantitative reverse transcription-PCR (RT-PCR) comparing *M. tuberculosis* RNA prepared from broth cultures with RNA from infected mouse lungs.

An *M. tuberculosis* strain expressing *inhA* from the *hsp60* promoter confers resistance to INH in mouse lungs. We infected mice intravenously with 10^5 to 10^6 CFU of *M. tuberculosis* H37Rv carrying plasmid pJD32 (the promoter trap vector with no promoter driving *inhA*) or plasmid pJD33 (same as pJD32, with the *hsp60* promoter driving *inhA*) (4). One group of mice was treated with INH (0.1 g/liter of drinking water) (14) 24 h postinfection, and the other group was not given the antibiotic, and at various times after infection, the bacterial load was measured in the lung. As expected, the strain carrying pJD32 was sensitive to INH and the one carrying pJD33 was resistant (Fig. 1). This result indicated that the promoter trap selection could work in mice, since a difference of approximately 100-fold was evident between treated and untreated INH-sensitive (INH-s) strains after 35 days. We then infected mice with a library of strains carrying DNA fragments of *M. tuberculosis* cloned upstream of *inhA* in the pJD32 vector (4). The mice were provided INH in the drinking water and sacrificed after 5 weeks or 3 months. Whereas only 0.01% of the original library of *M. tuberculosis* clones were INH resistant (0.5 μg/ml INH) in vitro, after 5 weeks of selection in INH-treated mice, 5% surviving in the lungs were INH resistant in vitro (108 clones were tested), and after 3 months of selection in INH-treated mice, 55% surviving in the lungs were resistant when 1,122 clones were picked and tested by streaking them on plates containing 0.5 μg/ml INH. These results show that the selection worked in the mice since INH-sensitive clones were eliminated with increased selection time. The clones resistant on plates carry constitutively active cloned promoters, whereas the clones remaining sensitive on plates presumably carry promoters active in the mouse lung, but not in vitro. One hundred fifty-five clones isolated from the 3-month sample that were INH sensitive on plates were picked for determination of the

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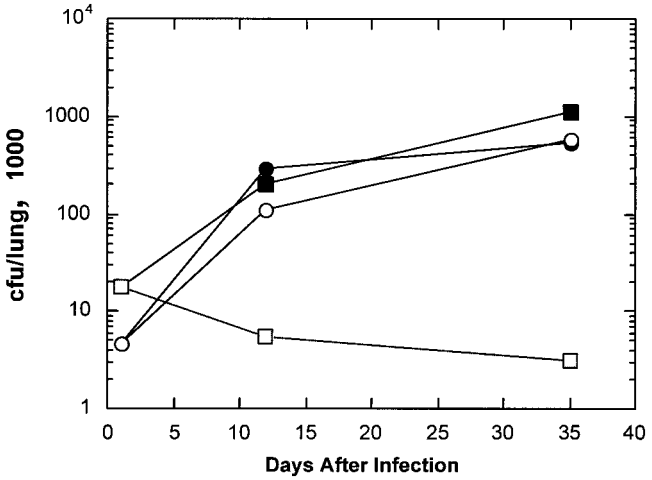

FIG. 1. INH sensitivity of strains in mouse lungs. *M. tuberculosis* H37Rv(pJD32) or *M. tuberculosis* H37Rv(pJD33) was used to infect C57BL/6 mice by tail vein injection. One group of mice was treated with INH, and the other group was not treated. Lungs were removed at various times after the infection, and the CFU were measured. pJD32 − INH, ■; pJD32 + INH, □; pJD33 − INH, ●; pJD33 + INH, ○.

DNA sequence cloned upstream of *inhA* in pJD32. Such clones are presumed to carry promoter sequences driving *inhA* expression specifically in the mouse lungs but not on laboratory medium.

Sequencing DNA inserts in selected clones. Colonies of *M. tuberculosis* obtained from mouse lungs which were INH-s on plates were selected, the inserts were amplified by PCR, and the amplicons were sequenced as described previously (4). A total of 75/155 clones contained sequences corresponding to DNA regions 5′ to a known open reading frame in the *M. tuberculosis* H37Rv genome database (2) in the correct orientation to drive *inhA* expression, whereas the others carried internal fragments of open reading frames or fragments oriented in the wrong orientation. These inserts could contain sequences which function artifactually as promoters in the host. A total of 56/75 sequences were unique, and the genes driven by these promoters are shown in Table 1. Three (Rv0036c, Rv0406c, and Rv1323) had been identified as upregulated in THP-1 macrophages using the same promoter library (4).

Differential expression of selected genes measured by RT-PCR. Mice were infected with the wild-type *M. tuberculosis* H37Rv by tail vein injection, and RNA was prepared from lungs 3 weeks after infection as well as from exponentially growing broth cultures. It should be emphasized that these measurements of RNA levels were done with a plasmid-free wild-type strain. Infected mouse lungs were removed from mice, snap-frozen in liquid nitrogen, and then maintained at −80°C. A frozen lung was placed in a petri dish and quickly cut into four pieces with a scalpel. Each piece was placed into a bead beater tube containing 0.5 ml of an equal mixture of 1- and 0.1-mm-diameter glass beads (Biospec products) and 1 ml Trizol. The samples were disrupted using the bead beater set at maximum speed for 1 min and then cooled on ice for 2 min. This procedure was repeated seven times, and the RNA was purified as previously described (4). RNA was also prepared from 40-ml broth cultures growing exponentially (optical density, 0.3 to 0.6) in Middlebrook 7H9 medium. Reverse transcription and PCR using molecular beacons were done as described previously (4). RNA was prepared from lungs of three infected mice, as well as from two different exponentially grow-

TABLE 1. Genes identified by promoter trap in mouse lungs[a]

Rv no.	Gene annotation listing and possible function
Rv0036c	Unknown
Rv0079	Unknown
Rv0158	Transcriptional regulator
Rv0243	*fadA2;* fatty acid oxidation
Rv0270	*fadD2*; fatty acid oxidation
Rv0276	Unknown
Rv0288	*esxH;* ESAT-6 family of antigens
Rv0315	Unknown
Rv0406c	Unknown
Rv0471c	Unknown
Rv0486	*mshA;* mycothiol biosynthesis
Rv0628c	Unknown
Rv0812	*pabC;* amino acid transferase
Rv0896	*gltA2;* citrate synthase
Rv0945	Short-chain dehydrogenase/reductase
Rv1056	Unknown
Rv1151c	Transcriptional regulator
Rv1323	*fadA4;* fatty acid oxidation
Rv1419	Unknown
Rv1508c	Membrane protein
Rv1541c	*lprl;* lipoprotein
Rv1731	*gabD2* succinate semialdehyde dehydrogenase
Rv1779c	Membrane protein
Rv1931c	Transcriptional regulatory protein
Rv1963c	*mce3R* transcriptional regulator of *mce3* operon
Rv1997	*ctpF;* metal cation transport ATPase, membrane protein
Rv2011c	Unknown
Rv2024c	Unknown
Rv2141c	Unknown
Rv2144c	Transmembrane protein
Rv2266	*cyp124:* cytochrome P450 family, oxidation of fatty acids
Rv2336	Unknown
Rv2346c	*esXO;* ESAT-6 family of antigens
Rv2376c	*cfp2;* low-molecular-weight antigen
Rv2405	Unknown
Rv2510c	Unknown
Rv2673	Membrane protein
Rv2713	*sthA;* soluble pyridine nucleotide transhydrogenase
Rv2738c	Unknown
Rv2799	Membrane protein
Rv2854	Unknown
Rv2989	Transcriptional regulator
Rv3206c	*moeB1;* molybdopterin biosynthesis
Rv3230c	Oxido-reductase
Rv3248c	*sahH;* adenosylhomocysteinase
Rv3260c	*whiB2;* transcriptional regulator
Rv3266c	*rmlD;* dTDP L-rhamnose biosynthesis
Rv3323c	*moaX;* molybdopterin biosynthesis
Rv3371	Unknown
Rv3404c	Methionyl-tRNA formyl transferase
Rv3619c	*esxV; esat-6* family of antigens
Rv3683	Unknown
Rv3732	Unknown
Rv3801c	*fadD32;* fatty acid oxidation
Rv3831	Unknown
Rv3883c	*mycP1;* membrane-anchored serine protease
ValV	tRNA valine

[a] The genes were identified by sequencing the DNA upstream of *inhA* in *M. tuberculosis* clones surviving INH treatment in mice which were INH-s on plates.

TABLE 2. Ratios of expression of selected genes in mouse lungs versus broth[a]

Gene	Rv no.	Lung/broth gene expression ratio			Avg
		Lung 1	Lung 2	Lung 3	
esxH[b]	Rv0288	49	25.3	20.6	31.6
whiB2[b]	Rv3260c	5.5	1.8	2.5	3.3
moeB1[b]	Rv3206c	2.38	0.76	5	2.7
sthA[b]	Rv2713	7.1	17.9	15.7	13.6
Unknown[b]	Rv3230c	0.29	4.9	4.9	3.4
Unknown[b]	Rv2854	ND[g]	8.7	11.8	10.25
fadA4[b,c]	Rv1323	6.6	11.9	ND	9.25
echA19[c]	Rv3516	56	69.4	27.6	51
fadA5[c]	Rv3546	6.1	2.4	ND	4.25
iclA[d]	Rv0467	57.5	ND	ND	57.5
choD[e]	Rv03409c	7.3	10.7	11.3	9.8
cyp124[b]	Rv2266	0.25	0.73	0.51	0.5
lprl[b]	Rv1541c	0.51	2	ND	1.26
fadD2[b]	Rv0270	0.128	0.318	2.9	1.1
bfrB[f]	Rv3841	1.2	0.35	0.36	0.64
desA3[e]	Rv3229c	0.165	0.8	ND	0.48

[a] Mice were infected with *M. tuberculosis* H37Rv by tail vein injection, and the lungs were harvested 3 weeks after the infection when the bacteria are still growing. Gene ratios (lung/broth) are expressed as the number of cDNA copies for a particular gene/0.1 μl RNA isolated from infected mouse lungs, determined by mbRT-PCR, normalized to the number of cDNA copies for 16S RNA, compared with the same number determined for RNA isolated from broth cultures. The results shown are from three separate mice.

[b] Genes identified by promoter trap in mice.

[c] Genes identified by promoter trap in macrophages (4).

[d] *iclA* was published as upregulated in mice (20); therefore, the analysis was only done with one lung.

[e] Genes annotated as involved in lipid metabolism (2).

[f] Induced by high iron (13).

[g] ND, not determined.

ing broth cultures. The values obtained for each gene are normalized to 16S RNA levels, which do not change during mouse lung infection (17). RNA levels of seven genes (*esxH*, *whiB2*, *moeB1*, *sthA*, Rv2854, Rv3230c, and *fadA4*) identified by the promoter trap selection in this paper and two (*echA19* and *fadA5*^) identified previously in THP-1 cells (4) were all induced in mouse lungs (Table 2). *echA19* and several other genes involved in fatty acid metabolism have been shown to be upregulated in murine bone marrow macrophages and mice (15). Three genes identified by the promoter trap described in this paper were probably not induced in mouse lungs (*fadD2*, *cyp124*, and *lprl*), although they may be induced at later times of infection. The differential expression of a few genes not identified by the promoter trap but predicted to code for proteins involved in lipid metabolism was also tested. As previously reported (9), *iclA* was upregulated. *choD* coding for cholesterol oxidase, a protein known to be required for virulence in *Rhodococcus equi* (10), was upregulated, but *desA3*, coding for a desaturase, was not. *bfrB*, coding for a bacteriferritin, which is induced under conditions of high iron (13), was not expressed at higher levels in the mouse lung. The *bfrB* results are consistent with the finding that *mbtB*, induced by low iron (5, 13), is upregulated in mouse lungs and that *bfrA*, also requiring high iron for its expression, was not induced in mouse lungs (20). These results support the hypothesis that *M. tuberculosis* faces a low-iron environment in mouse lungs.

Although we identified *fadA4* in this screen as well as the one in macrophages, (4), there was no other overlap in the genes identified by the two screens. We should emphasize that neither screen should be considered as saturated for all genes upregulated during infection. The promoter trap technology can result in artifacts because we used a multicopy plasmid in which gene expression does not necessarily reflect normal gene expression from the chromosome. Also, the readout for gene expression is resistance to INH in the host, and the dose received in the lung may be variable in different areas of the lung and at different times. In addition, we selected clones which were completely sensitive to INH in vitro. This selection would therefore eliminate many genes which are differentially expressed but which have some level of expression in vitro.

We identified three members of the ESAT-6 family, *esxH*, *esXO*, and *esxV*, as upregulated during growth in mouse lungs. ESAT-6 and TB10.4 are members of a large family of small secreted proteins. These two proteins are also immunodominant antigens in tuberculosis patients (12, 18). TB10.4 is coded for by Rv0288, one of the genes identified by our promoter trap and validated by reverse transcription-PCR using molecular beacons. ESAT-6 was recognized as of possible importance for virulence since it is one of the genes present in the RD1 region of the *M. tuberculosis* chromosome, which is deleted in the avirulent vaccine strain, *Mycobacterium bovis* BCG (8). Since the host responds vigorously to this group of proteins, there are several promising strategies for vaccine production involving the expression of ESAT-6 either as a recombinant protein fused to other antigens (11) or as a DNA vaccine (6, 7).

Molybdopterin is a cofactor required for nitrate reductase and other enzymes involved in anaerobic metabolism. *M. tuberculosis* dedicates 21 genes to the biosynthesis of this cofactor (2), and we identified two of them (*moaX* and *moeB1*) as upregulated in mouse lungs. Seven orthologues of the *Streptomyces whi* genes are found in the *M. tuberculosis* chromosome (2), and we identified *whiB2* as upregulated in mouse lungs. In *Streptomyces*, they code for small transcriptional regulators, but, with the exception of *whiB3*, their function is unknown in *M. tuberculosis*. *whiB3* codes for a small DNA binding protein which interacts with SigA, the housekeeping sigma factor (19). Specific mutations in *sigA* cause attenuation of *M. bovis* (3) due to the inability of the mutant SigA to interact with WhiB3 (19).

Four new genes involved in lipid metabolism were shown to be upregulated during growth in mouse lungs, lending further support to the idea that *M. tuberculosis* utilizes fatty acids during infection of the host. Some of the genes may be indispensable for growth and/or persistence in the host, and we are currently constructing strains carrying disruptions in the genes identified in this report. Preliminary experiments have shown that a mutation in *fadA5* causes attenuation (Fontan et al., unpublished observations).

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