Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages

Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages

Jyothi Rengarajan, Barry R. Bloom, and Eric J. Rubin*

Department of Immunology and Infectious Disease, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115

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Macrophages are central to host defense against microbes, but intracellular pathogens have evolved to evade their antimicrobial functions. *Mycobacterium tuberculosis* (MTB) has successfully exploited macrophages as its primary niche *in vivo*, but the bacterial genome-wide requirements that promote its intracellular survival remain undefined. Here we comprehensively identify the MTB genes required for survival by screening for transposon mutants that fail to grow within primary macrophages. We identify mutants showing decreased growth in macrophage environments that model stages of the host immune response. By systematically analyzing several biologically relevant data sets, we have been able to identify putative pathways that could not be predicted by genome organization alone. In one example, phosphate transport, requiring physically unlinked genes, was found to be critical for MTB growth in macrophages and important for establishing persistent infection in lungs. Remarkably, the majority of MTB genes found by this analysis to be required for survival are constitutively expressed rather than regulated by macrophages, revealing the host-adapted lifestyle of an evolutionarily selected intracellular pathogen.

mutagenesis

Up to one-third of humans world-wide harbor *Mycobacterium tuberculosis* (MTB) in a latent asymptomatic state and are thus at risk for tuberculosis when immune-compromised (1). Macrophages are critical both for permitting the survival of MTB and in linking innate and adaptive immunity in the host (2). They promote T cell activation and recruitment, crucial for containing MTB within granulomas in the lung. Virulent MTB can replicate within the hostile environment of macrophages, sequestered in poorly acidified phagosomes that fail to fuse with lysosomes (3–5). Although phagocytosis by IFN-γ-activated murine macrophages results in some bacterial killing, in part via nitric oxide-dependent mechanisms (6), MTB can evade macrophage bactericidal function by inhibiting IFN-γ-mediated signaling (7). MTB is also reported to interfere with antigen presentation, multiple signaling pathways, and transcriptional responses within the macrophage (2). However, the mycobacterial genes involved are largely unknown.

We sought to systematically identify the MTB genes required for growth in macrophages. We used transposon site hybridization (TraSH) (8), a microarray-based technique that comprehensively identifies genes from large pools of transposon mutants that are essential for growth under different conditions (described in Fig. 1*a*). We devised screens to identify MTB mutants that fail to survive prolonged infection of primary murine macrophages and are thus attenuated for growth. We have identified 126 genes as necessary for survival in macrophages under conditions that model the immune response. Comparative analyses with biologically relevant data sets allow association of genes into functional groups, provide insights into gene regulation in mycobacteria, and highlight key gene products for further detailed study. We show that one such group of genes encoding a phosphate transport apparatus is important for growth in macrophages and lungs of mice.

Methods

Macrophage Screens and TraSH to Identify Genes Required for Survival in Macrophages. A transposon mutant library was made in MTB strain H37Rv by using the MycoMarT7 phage, as described (8). Macrophages were derived from bone marrow precursors of C57BL/6 mice, grown in DMEM/F12 medium with 10% FCS/2 mM glutamine/20% L cell conditioned medium/2 ng/ml of rIL-3 for 8 days of differentiation. Cells were 99% CD11b+ and 90–95% F480+ by FACS. Macrophages (10^7) were plated onto T_{150} flasks and preactivated with 100 units/ml of IFN-γ for 16 h before or after 8 h of infection followed by washing. Adherent monolayers were infected with 10^7 mutants [multiplicity of infection (moi) 1:1]. Bacteria were resuspended in DMEM/F12 and sonicated for 5 sec 2× before addition. After 8 h, monolayers were replaced with medium containing 200 μg/ml of amikacin for 1 h to kill extracellular bacteria and washed 4× with PBS. We determined by microscopy and plating that the effective moi after infection was one bacterium per 5–10 macrophages. On day 7, macrophages were lysed with PBS plus 0.05% Triton X-100 and the lysate pelleted. The bacterial pellet was resuspended in 1 ml of 7H9 medium and titered. Colony-forming units (cfu, 2×10^5) were plated onto 7H10-kanamycin (20 μg/ml) plates to harvest surviving mutants. The *in vitro* pool was generated by replating 2×10^5 cfu of library. Bacteria from the first round of infection were used to similarly reinfect a fresh pool of macrophages. Genomic DNA was isolated from each pool, and Cy5- or Cy3-labeled TraSH probes were generated and hybridized to the microarray, as described (8). Each experimental condition (macrophage condition, reinfection) was performed in duplicate; probes from each pool were generated twice and analyzed on duplicate microarrays. Data were collected by using GENEPIX software (Axon Instruments, Union City, CA) and analyzed by using GENESPRING software (Silicon Genetics, Redwood City, CA).

Data Analysis. Data for each experiment were averaged and filtered to include only those features with control or raw signal >300 whose ratios were significantly different from 1.0 ($P < 0.05$ by *t* test). For most of the analyses presented, we excluded mutants with ratios that differed from the median by <2.5-fold. Thus mutants with *in vitro*/in macrophage ratios <0.4 on a log scale of normalized intensities (lowest intensity-dependent normalization) were defined as attenuated for growth in macrophages. Hierarchical clustering was performed by using standard correlation. Based on these clusters, 10-cluster *k* means clustering was performed by using GENESPRING software (Silicon Genetics).

Isolation of Transposon Mutants, Construction of Plasmids, and Bacterial Strains. The *pstA1*::*Tn* strain was obtained by sequencing mutants from the library by using arbitrary primers ARB1:

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Abbreviations: TraSH, transposon site hybridization; MTB, *Mycobacterium tuberculosis*; moi, multiplicity of infection.

*To whom correspondence should be addressed. E-mail: erubin@hsph.harvard.edu.

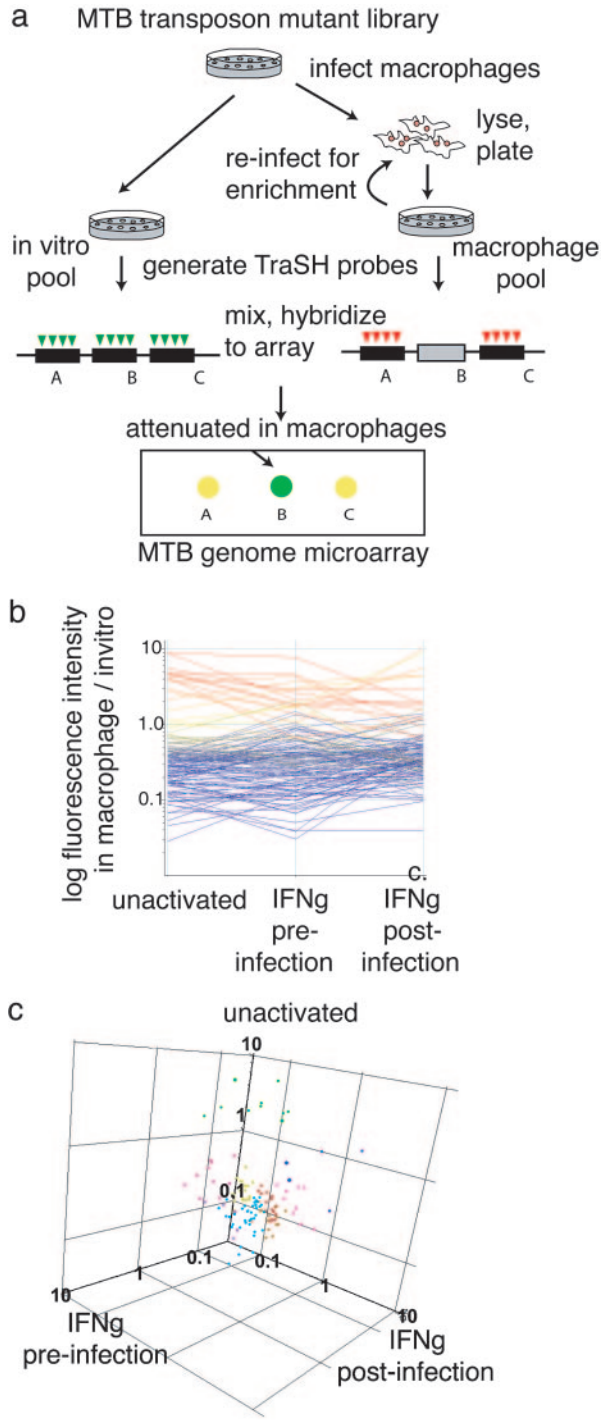

Fig. 1. Comprehensive screen identifies genes required for surviving within macrophages under different activation conditions. (*a*) TraSH in primary murine macrophages. We infected pools of macrophages derived from bone marrows of C57BL/6 mice with 10^7 MTB *mariner* transposon mutants, moi of 1:1. To identify MTB genes required for survival within macrophages, we lysed macrophages after 7 days and plated surviving bacteria. Mutants that failed to survive this macrophage selection (macrophage pool) are absent in the recovered bacteria. These mutants are present when the library is directly plated (*in vitro* pool). To map transposon insertion sites in each pool, genomic DNA from the macrophage and *in vitro* pools was isolated and TraSH probe generated that was complementary to the chromosomal sequence flanking insertion sites in each pool. We compared mutants from the macrophage pool to the *in vitro* pool by labeling the probe from each pool with a different colored fluorophore, mixing and hybridizing them to a spotted MTB genome microarray. Spots that hybridized to the probe from the *in vitro* pool but not the macrophage pool correspond to genes required for growth in macrophages. To enrich for such mutants, we reinfected a fresh pool of macrophages with bacteria that survived the first round of infection and performed TraSH. Data from the enrichment were used in subsequent analyses. (*b*) Genes required for survival in macrophages. A vertical line corresponds to each experimental condition; macrophages that are unactivated or activated with IFN-γ pre- or postinfection. The *y* axis corresponds to average ratios of in macrophage/*in vitro* growth, and mutants with ratios <0.4 or >4 ($P < 0.05$) are represented. Each line indicates the growth of the mutant in macrophages/*in vitro* across the three conditions of macrophage culture. Values <1.0 (blue) represent mutants that grow poorly in macrophages, whereas ratios >1.0 (red) are mutants that show enhanced intracellular growth. (*c*) *k* means clustering of MTB genes required for survival in any of the three macrophage conditions. Genes with ratios differing from the median by 2.5-fold or less ($P < 0.05$) were clustered into 10 clusters and represented in a 3D scatter plot when each axis corresponds to the indicated condition. Each color corresponds to a different cluster.

5′-ggccacgcgtcgactagtacnnnnnnnnnngatat-3′ and Tnout2.2: 5′-cgcttcctcgtgctttacggtatcg-3′ followed by ARB2: 5′-ggccacgcgtcgactagtac-3′ and TnPCRout2.2: 5′-cgccttcttgacgagttcttctgag-3′. *phoT*::*Tn* was isolated from arrayed individual mutants by screening with primers 5′-gctctacgtgggagtcggacaatgttg-3′ and 5′-gggacttatcagccaacctgtta-3′. Insertion sites were confirmed upon sequencing by using the T7 primer. The *pstA1* gene was amplified from MTB genomic DNA with primers 5′-gctctggccatcgccggcaggaagg-3′(MscI site) and 5′-ccgagtggggatcctcacgtcataac-3′ (BamHI site) and cloned into the BalI and BamHI sites of the episomal vector pMV261(hyg), downstream of the hsp60 promoter. The *phoT* gene was cloned into the above vector by amplifying with primers 5′-ggagcttaaggatccaagcggttgg-3′ (BamHI site) and 5′-ccgcggtgacgcaagcttggccatc-3′ (HindIII site). Constructs were transformed into H37Rv, or mutants *pstA1*::*Tn* and *phoT*::*Tn* and transformants selected on 7H10 plates containing 50 μg/ml of hygromycin.

***In Vitro* Growth Assays in Low-Phosphate Medium.** Strains were grown to OD_{600} of 0.6 at 37°C in Middlebrook 7H9 medium (Difco) containing OADC enrichment and 0.05% Tween 80 pelleted, and washed 3× in Sauton's medium minus phosphate (0.5 g of $MgSo_4$·7 H_2O, 2 g of citric acid, 0.05 g of ferric ammonium sulfate, 60 ml of glycerol, 4.0 g of asparagine in 1,000 ml of H_2O). Each strain was inoculated into 5 ml of Sauton's medium containing varying concentrations of phosphate (KH_2PO_4) to a final OD_{600} 0.05 for each strain. Strains were grown at 37°C under agitation for 10 days. Data are represented as fold increase over growth in zero-phosphate medium, as determined by plating for viable bacteria, and are representative of three independent experiments.

Macrophage Survival Assays. To assess growth of wild-type, *pstA1*::*Tn*, and *phoT*::*Tn* mutants and complemented strains over time in macrophages, cells were plated onto 24-well plates (2×10^5 per well). Activation was performed as described above. Each strain was used for infection (in triplicate per time point) at moi 1:1, passed through a 5-μm filter, and sonicated before infection. On days 0 (6 h after infection), 2, 5, and 7 postinfection, monolayers were lysed in 0.5% Triton in PBS and serial dilutions plated onto 7H10 and 7H10 kanamycin plates for colony-forming units.

Mouse Infection. C57BL/6J mice (The Jackson Laboratory) were infected by tail-vein injection with $\approx 2 \times 10^6$ colony-forming units of each strain (three mice per time point). On day 1, weeks 2, 4, and 11 after infection, mice were killed and liver, spleen, and lungs of each mouse harvested, homogenized, and serial dilutions plated onto 7H10 and 7H10 kanamycin plates, as described (9).

Results

To identify the MTB genes required for survival in macrophages, we devised screens to identify MTB mutants that fail to survive prolonged infection of primary murine macrophages and are thus attenuated for growth (described in Fig. 1*a*). The infecting pool of mutants represents transposon insertions in almost all of the genes in the MTB genome except those mutations that are lethal for growth *in vitro* (8). To enhance selectivity, fresh pools of macrophages were reinfected with bacteria from the first round of infection and TraSH analysis performed on each passage. This enrichment improved sensitivity and reliability in detecting attenuated mutants. Each mutated gene in the infecting pool is represented by ≈25 different strains, each harboring a transposon insertion in that gene.

We performed screens using macrophage culture conditions that simulate three pivotal stages of tuberculosis progression. Unactivated macrophages likely model initial or latent infection, infection of macrophages preactivated with IFN-γ models the ongoing immune response, and activating macrophages with IFN-γ postinfection models the ability of MTB to modulate IFN-γ-mediated signaling (7). To measure the ratio of growth in macrophages versus the growth *in vitro* for each mutant (see *Methods* and Fig. 1*a*), we determined the ratios of fluorescence intensity of TraSH probes generated under these two conditions (Fig. 1*b*). Mutants attenuated for growth in macrophages have ratios significantly <1.0, whereas those that grow better in macrophages than *in vitro* have ratios >1.0 (Data Set 1, which is published as supporting information on the PNAS web site). Fig. 1*b* depicts the subset of genes with log ratios significantly <0.4 or >4.0 after enrichment. By these criteria, 126 MTB genes were identified that are required for growth in macrophages, under any one of the three experimental conditions (Data Set 2, which is published as supporting information on the PNAS web site). Although the majority of mutants have attenuating phenotypes, some mutations lead to enhanced growth in macrophages relative to *in vitro* growth. These include mutations in *sseA* and *moeA*, encoding a putative thiosulfate sulfurtransferase and a molybdopterin biosynthesis protein respectively. Production of these enzymes might carry a genetic cost during *in vitro* growth in culture medium.

TraSH Defines Functional Subsets of Genes Required for Survival in Macrophages. To determine how mutant phenotypes can be informative about MTB functions during the host immune response, we adapted clustering tools developed for microarray expression analyses that group genes according to similarities in gene expression, thus providing clues to their functions (10). Cluster analysis of data from the three different conditions using hierarchical and supervised methods uncovered genes whose functions depend on the macrophage environment. *k* means clustering of the subset of 126 genes from unactivated IFN-γ pre- and postinfection conditions is shown in Fig. 1*c*, with each cluster represented by a different color. Although some mutants appear to be strongly attenuated under all three conditions, other genes are seen to be critical for surviving within a specific environment (Fig. 1*c*).

Genes within a tight cluster appear to have shared functions. A significant proportion of the genes within one large cluster correspond to members of putative operons. Products encoded by genes within operons often interact with one another or participate in common pathways (11). We find that individual members of several putative operons are each required for macrophage survival (listed in Table 1). For example, genes of the *fadE28* locus appear to be critical (Table 1). These genes have been proposed to be involved in lipid transport and degradation and likely function in assimilating exogenous lipids from host cell membranes (12, 13). Notably, mutants in most of the ≈100 genes annotated as involved in β-oxidation and fatty acid degradation (12) do not show similar phenotypes. MTB also requires the *mce1* locus to survive within macrophages under all three conditions. The four *mce* gene clusters (*mce1–4*) are 12-gene operons, each with similar gene organization, which may assemble into an apparatus for transport of molecules between bacteria and host. Genes of the *mce1* and *mce4* loci were previously reported to be important for growth in mice, whereas those of the mce1 locus appear to play a role in modulating macrophage environments.

Table 1. List of putative operons identified by the TraSH screen

Gene group	Putative function
mce1 0170 0171 0172 lprK 0174 0175 0176 0177 0178	Transport apparatus
sugC sugB sugA lpqY	Disaccharide transporter
pstA1 pstC2 pstS3 *phoT*	Inorganic phosphate transporter
3864 3868 3870 3871 3877	Components of ESX-1 secretion apparatus
3569c 3570c	Unknown
3552 3551 echA20	β-oxidation of fatty acids, lipid degradation
3545c ***fadE28*** *fadE29* ***3542c 3541c*** *ltp2*	β-oxidation of fatty acids, lipid degradation

Encoded genes found to be required under any of the three macrophage conditions are in bold.

Comparative Analysis of Genes Required for Growth in Macrophages and *in Vivo*. The data derived from macrophage experiments serve to illuminate results from our previous experiments performed in mice (14). Hierarchical clustering of genes required for survival in macrophages under any condition with genes required for *in vivo* growth in mouse spleens revealed three main categories. The first includes genes that have cell-autonomous functions, represented by those required for both macrophage and *in vivo* survival (Fig. 2). Some of these appear to play a role in early stages of infection when innate immunity operates. For example, genes of the *sugABC-lpqY* system, thought to encode an ABC-type disaccharide importer, are essential for surviving unactivated macrophages and are important for growth *in vivo* as early as 1 week after infection. Thus, although lipid metabolism *in vivo* is a major energy source for MTB (13), we find evidence that carbohydrate acquisition is also necessary for intracellular growth. Other genes correlate with specific temporal patterns of *in vivo* infection, and we can infer functions related to adaptive immunity. For example, *Rv2808* is important for growth in macrophages that are preactivated with IFN-γ and those activated postinfection (see Data Sets 1 and 2). The corresponding mutant is compromised for growth only after 2 weeks of *in vivo* infection, which coincides with the initiation of the T cell immune response and remodeling of the host environment. The mutant recovers its ability to grow at 4 and 8 weeks (14), when there is probably less immune flux in the host. In contrast, *Rv3805c* is required to resist killing by macrophages treated with IFN-γ postinfection at late stages in infection (8 weeks) (14) and may be involved in maintaining persistence in the host.

A second set of genes is necessary for *in vivo* infection but dispensable for survival in macrophages, suggesting that they function independently of intracellular replication, for example by promoting dissemination within the host. A third cluster comprises genes that are crucial for survival in macrophages but not identified in the *in vivo* study. These genes could play important roles in intracellular adaptation but might not be

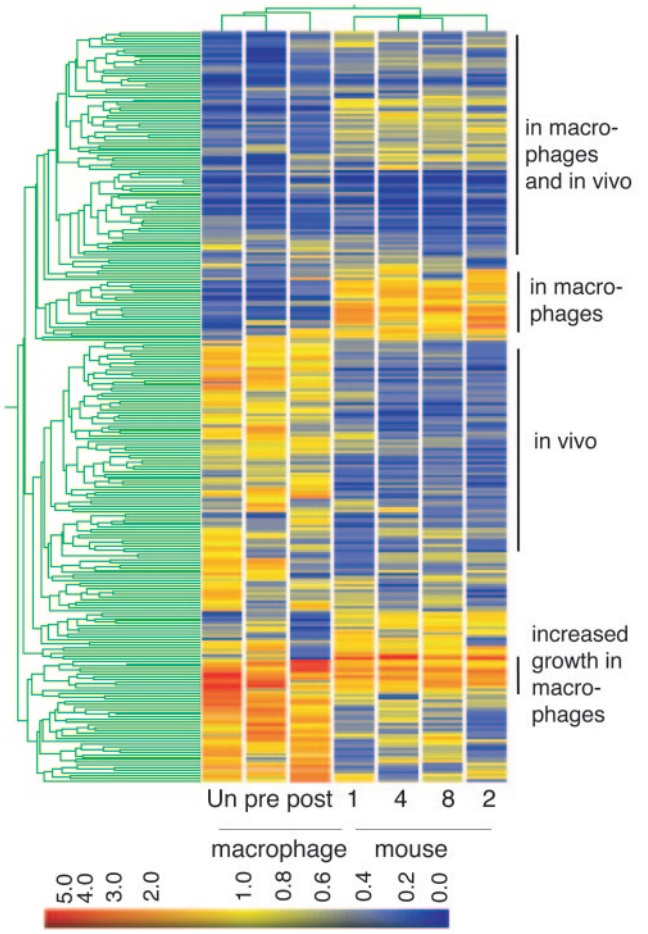

Fig. 2. Hierarchical clustering of genes required for survival in macrophages (unactivated, IFN-γ preinfection, and IFN-γ postinfection) and *in vivo* in mouse spleens (1, 2, 4, and 8 weeks postinfection). Blue indicates decreased survival of mutant in macrophages relative to growth *in vitro*. Red indicates increased survival of mutant in macrophages relative to *in vitro*

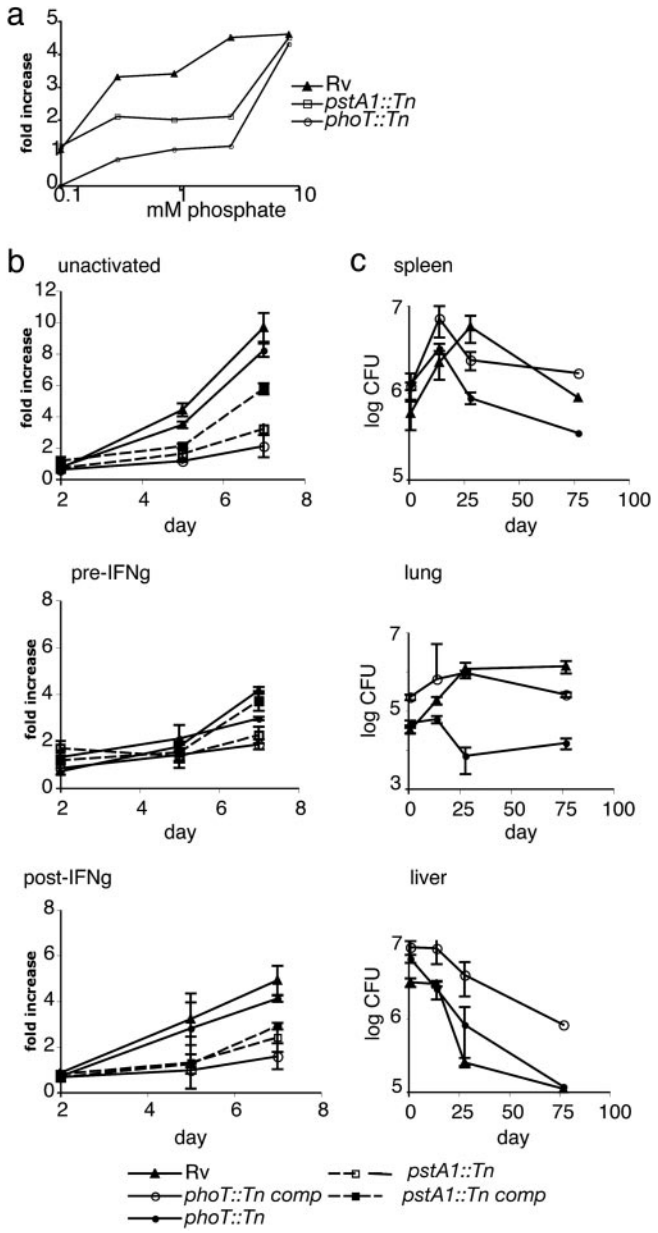

Fig. 3. *pstA1* and *phoT*, components of a phosphate transport apparatus, are essential for MTB growth in phosphate starved conditions *in vitro* (*a*), in macrophages (unactivated or activated with IFN-γ preinfection or IFN-γ postinfection) (*b*), and *phoT* is required for growth in lungs of mice (*c*).

modeled in the murine model. The *in vivo* infection model studied growth of bacteria in the spleen, whereas bacteria are largely confined to the lung in human disease (7). Thus, some of the macrophage-specific genes we identified might be required for the initial interaction with alveolar macrophages or continued intracellular growth of bacteria in the lung.

Phosphate Transport Genes Are Physically Unlinked but Functionally Associated. Using data clustered from multiple experiments permits some functional predictions about genes, even those not physically linked. *pstA1*, *pstC2*, and *pstS3*, members of a putative operon, are each essential for growing in macrophages under all three conditions. The encoded proteins show homology to three of four components of ABC transporters that are thought to import inorganic phosphate during phosphate starvation (15). Upon clustering our macrophage data, we found that these genes share a cluster with *phoT*, located elsewhere in the genome and encoding a protein with homology to nucleotide-binding domain proteins of phosphate transporters. Guinea pigs and possums are less susceptible to *Mycobacterium bovis phoT* mutants than to wild type (16). We hypothesized that the phagosome is limiting in phosphate, which is acquired via a transport system encoded by these four unlinked genes.

We isolated two transposon mutants, *pstA1*::*Tn* and *phoT*::*Tn*, and found both to be more sensitive to phosphate limitation in their growth media than wild type (Fig. 3*a*). Both mutants grew poorly in resting and activated macrophages, and we could partially complement this phenotype by overexpressing the wild-type gene in trans (Fig. 3*b*). Interestingly, a second *pst* operon (*pstB*, *pstS1*, *pstC1*, and *pstA2*) present in MTB appears not to be required for virulence in either macrophages or mice and is unable to substitute functionally. Parenthetically, this second *pst* locus encodes pseudogenes in the related pathogen *Mycobacterium leprae* (17), whose genome has undergone reductive evolution. To test *in vivo* requirements for phosphate transport, we assessed the growth of wild-type, *phoT*::*Tn* and the complemented strain in mice (Fig. 3*c*). *phoT* appears to be critical for growth in mouse lungs, suggesting that alveolar macrophages represent a phosphate-starved environment, and transport of phosphate is critical for establishing infection. *phoT*

was not found to play a role in growth in the splenic infection model (14), indicating that many mutants identified as important in the macrophage screen may have lung-specific phenotypes during *in vivo* infection.

Gene Expression Analysis Is Poorly Predictive of Requirements for Survival in Macrophages. The expression of many genes important for virulence is regulated during infection (18). To test whether this is true for genes required for MTB growth in macrophages, we compared our TraSH survival data with expression profiling performed in infected macrophages (19). Surprisingly, we see little correlation between gene expression in macrophages and requirements for survival (Fig. 4). Although some genes are found in both analyses (e.g., *Rv0790* and genes of the *mce1* operon), the majority of genes necessary for growth in macrophages are not regulated. For example, many of the MTB genes that are highly induced in macrophages and in response to NO stress and hypoxia, including genes controlled by the regulator dosR (19–21), appear not to be required for intracellular growth under comparable conditions. In contrast, a significant number of MTB genes that are essential for growth in macrophages are constitutively expressed. Unlike pathogens with environmental reservoirs like *Salmonella typhi* or *Vibrio cholerae* (22, 23), MTB never resides outside of the human host and depends exclusively on transmission between humans for its continued survival (1). Constitutive expression of genes required for survival may be a necessary adaptation to a complex lifestyle in the host milieu. For example, expression of the *pst* operon in MTB is not induced upon macrophage infection (19), unlike in *Salmonella typhimurium* (24). In contrast to the related actinomycete *Corynebacterium glutamicum* (25), these genes were not induced upon phosphate starvation *in vitro* but were constitutively expressed in both phosphate-sufficient and limiting conditions, as assessed by quantitative PCR (data not shown). Regulation may occur at the protein level, because secretion of PstS3 protein is induced upon *in vitro* phosphate starvation in *M. bovis* bacillus Calmette–Guérin (26). Regulation at the protein level would ensure rapid mobilization of protein complexes and may be widely used in MTB.

Discussion

MTB is a human pathogen that has successfully exploited macrophages as its primary niche *in vivo*. To understand how MTB interferes with macrophage function, we comprehensively identified the MTB genes required for intracellular survival. By screening for transposon mutants that fail to grow within primary macrophages, we identified mutants with decreased growth within macrophage environments. We are able to associate these genes into functional groups using clustering tools and find that individual members of several putative operons are each required for macrophage survival (Table 1). These data lend support to many operon predictions and indicate shared biochemical or regulatory functions. This also provides functional phenotypes for mutants in uncharacterized genes. For example, *glnB* mutants are attenuated mostly in the IFN-γ preinfection condition, implying that *glnB*, encoding a putative nitrogen regulatory protein, is important for resisting the IFN-γ-activated macrophage milieu.

Comparison of macrophage survival data with those from mouse experiments leads to several predictions of gene function, providing insights into MTB's intracellular lifestyle. For example, we implicate genes in defense against innate or adaptive immunity (*Rv2808* and *Rv3805*). We also highlight physically unlinked genes important in phosphate transport, as required for survival within macrophages. Thus, *pstA1*, *pstC2*, and *pstS3*, members of a putative operon encoding phosphate-transport proteins, are likely to associate with *phoT*, which is found within the same functional cluster. *phoT* appears to be critical for growth in mouse lungs, suggesting that lung macrophages represent a phosphate-starved environment, and transport of inorganic phosphate is critical for establishing infection.

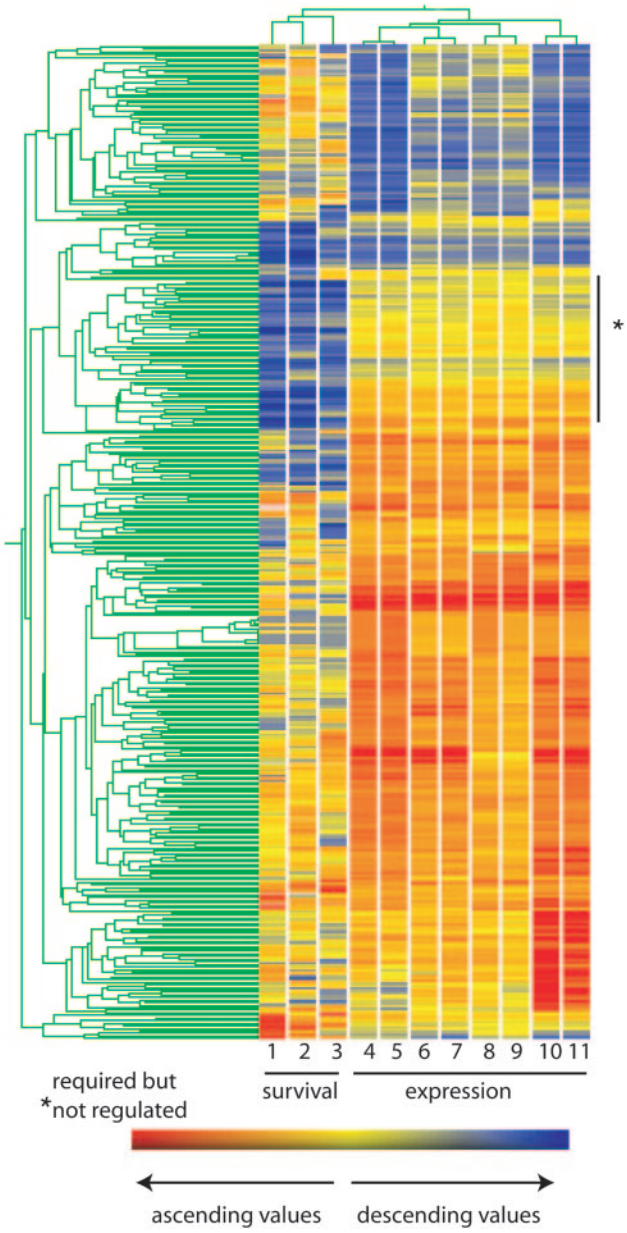

Fig. 4. Hierarchical clustering of genes required for survival in macrophages (lane 1, unactivated; lane 2, IFN-γ preinfection; lane 3, IFN-γ postinfection) with genes induced in infected wild-type (C57BL/6) macrophages 4, 24, and 48 h after infection in unactivated (lane 6, 24 h; lane 7, 48 h; lane 9, 4 h) or pre-IFN-γ-activated macrophages (lane 8, 4 h; lane 10, 24 h; lane 11, 48 h) or in NOS2−/− macrophages unactivated (lane 4, 24 h) or pre-IFN-γ-activated (lane 5, 24 h). Blue indicates decreased survival of mutant in macrophages relative to *in vitro* (survival data) or down-regulation of genes after infection of macrophages, relative to expression *in vitro* (expression data). Red indicates increased survival of mutant in macrophages relative to *in vitro* or up-regulation of genes after infection in macrophages. Shades of color correspond to numerical ratios and are indicated by the color key.

One striking unexpected result was the finding of little or no correlation between how essential a gene is for survival and its expression. Bacterial virulence factors are commonly held to be highly regulated, and many of the methods designed to identify them rely on their up-regulation upon infection. Although this appears true for important genes in other pathogens like *Salmonella*, we show this is not universally true. Our findings emphasize the complementary nature of the two

experimental approaches and reveal the complexity of pathogen–host interactions. Studying gene expression is powerful for gauging bacterial responses to the host but may reflect short-term adaptations to changing host environments. Microarray expression analyses measure transcript abundance, which for some essential genes may be only briefly altered and thus missed by the sampling intervals chosen. In contrast, the survival we measure by TraSH is the cumulative effect of a mutation over time during prolonged intracellular growth. Still, those mutants that are complemented in trans by either bacterial or host factors might not be detected. Our systematic comparison of gene essentiality and expression and other recent observations suggest that expression screens may have limited value for identifying virulence genes in host-dependent and adapted pathogens like MTB (27).

Conclusion

We have shown that combining data from complementary genome-wide approaches, coupled with the use of analytic clustering tools, can lead to new insights into adaptations unique to intracellular pathogens. Further, using a tractable *ex vivo* model of macrophage infection allows more detailed functional analysis of genes than can be performed in whole-animal experiments alone.

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1. Bloom, B. R. & Murray, C. J. (1992) *Science* **257,** 1055–1064.
2. Flynn, J. L. & Chan, J. (2003) *Curr. Opin. Immunol.* **15,** 450–455.
3. Mwandumba, H. C., Russell, D. G., Nyirenda, M. H., Anderson, J., White, S. A., Molyneux, M. E. & Squire, S. B. (2004) *J. Immunol.* **172,** 4592–4598.
4. Brown, C. A., Draper, P. & Hart, P. D. (1969) *Nature* **221,** 658–660.
5. Russell, D. G. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 569–577.
6. Chan, J., Xing, Y., Magliozzo, R. S. & Bloom, B. R. (1992) *J. Exp. Med.* **175,** 1111–1122.
7. Flynn, J. L. (2004) *Tuberculosis* **84,** 93–101.
8. Sassetti, C., Boyd, D. H. & Rubin, E. J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 12712–12717.
9. Hondalus, M. K., Bardarov, S., Russell, R., Chan, J., Jacobs, W. R., Jr., & Bloom, B. R. (2000) *Infect. Immun.* **68,** 2888–2898.
10. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14863–14868.
11. Lawrence, J. G. (2003) *Annu. Rev. Microbiol.* **57,** 419–440.
12. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, *et al.* (1998) *Nature* **393,** 537–544.
13. McKinney, J. D., Honer zu Bentrup, K., Munoz-Elias, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr., *et al.* (2000) *Nature* **406,** 735–738.
14. Sassetti, C. M. & Rubin, E. J. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 12989–12994.
15. Braibant, M., Gilot, P. & Content, J. (2000) *FEMS Microbiol. Rev.* **24,** 449–467.
16. Collins, D. M., Kawakami, R. P., Buddle, B. M., Wards, B. J. & de Lisle, G. W. (2003) *Microbiology* **149,** 3203–3212.
17. Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honore, N., Garnier, T., Churcher, C., Harris, D., *et al.* (2001) *Nature* **409,** 1007–1011.
18. Falkow, S. (1997) *J. Clin. Invest.* **100,** 239–243.
19. Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D., Nathan, C., *et al.* (2003) *J. Exp. Med.* **198,** 693–704.
20. Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R. & Schoolnik, G. K. (2003) *J. Exp. Med.* **198,** 705–713.
21. Sherman, D. R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M. I. & Schoolnik, G. K. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 7534–7539.
22. Santamaria, J. & Toranzos, G. A. (2003) *Int. Microbiol.* **6,** 5–9.
23. Colwell, R. R. & Huq, A. (1994) *Ann. N.Y. Acad. Sci.* **740,** 44–54.
24. Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. (2003) *Mol. Microbiol.* **47,** 103–118.
25. Ishige, T., Krause, M., Bott, M., Wendisch, V. F. & Sahm, H. (2003) *J. Bacteriol.* **185,** 4519–4529.
26. Lefevre, P., Braibant, M., de Wit, L., Kalai, M., Roeper, D., Grotzinger, J., Delville, J. P., Peirs, P., Ooms, J., Huygen, K. & Content, J. (1997) *J. Bacteriol.* **179,** 2900–2906.
27. Kendall, S. L., Rison, S. C., Movahedzadeh, F., Frita, R. & Stoker, N. G. (2004) *Trends Microbiol.* **12,** 537–544.