Available online at www.sciencedirect.com
SCIENCE DIRECT
Microbes and Infection 8 (2006) 747–757
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Mycobacterium tuberculosis gene expression profiling within the context of protein networks

Helmy Rachman [a,1], Michael Strong [b,1], Ulrich Schaible [a], Johannes Schuchhardt [c], Kristine Hagens [a], Hans Mollenkopf [a], David Eisenberg [b], Stefan H.E. Kaufmann [a,*]

[a] *Max Planck Institute for Infection Biology, Department of Immunology, Schumannstrasse 21-22, 10117 Berlin, Germany*
[b] *Howard Hughes Medical Institute, UCLA-DOE Institute of Genomics and Proteomics, Molecular Biology Institute, University of California, Los Angeles, CA, USA*
[c] *MicroDiscovery GmbH, Berlin, Germany*

Received 22 June 2005; accepted 16 September 2005
Available online 18 January 2006

Abstract

As one of the world's most successful intracellular pathogens, *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, is responsible for two to three million deaths annually. The pathogenicity of *M. tuberculosis* relies on its ability to survive and persist within host macrophage cells during infection. It is of central importance, therefore, to identify genes and pathways that are involved in the survival and persistence of *M. tuberculosis* within these cells. Utilizing genome-wide DNA arrays we have identified *M. tuberculosis* genes that are specifically induced during macrophage infection. To better understand the cellular context of these differentially expressed genes, we have also combined our array analyses with computational methods of protein network identification. Our combined approach reveals certain signatures of *M. tuberculosis* residing within macrophage cells, including the induction of genes involved in DNA damage repair, fatty acid degradation, iron metabolism, and cell wall metabolism.

Keywords: Tuberculosis; Protein networks; Microarray; Macrophages

1. Introduction

Numerous attempts have been made to identify genes and proteins that enable *Mycobacterium tuberculosis* to survive and persist within host macrophage cells [1–8]. At the cellular level, *M. tuberculosis* is able to evade the host immune response by residing within macrophage phagosomes [9–11]. *M. tuberculosis* is able to inhibit the fusion of macrophage phagosomes and lysosomes, thereby preventing mycobacterial degradation in the macrophage [12–16]. Genome-wide expression profiling has become a useful tool to identify genes that are differentially expressed in response to varying conditions or cellular perturbations. In many cases, these analyses have provided clues to genes that may participate in related cellular roles or pathways. In addition to providing clues to functionally related genes, global gene expression profiling also provides a useful method for identifying potential virulence factors. Virulence factors can be involved in a variety of roles including host cell invasion, survival within the host cell, long-term persistence, and manifestation of disease. Although many mycobacterial genes have been characterized as virulence factors [1–8], it is believed that there are many virulence factors yet to be identified [17]. An area of great interest, therefore, is the identification of genes and pathways involved in mycobacterial survival and persistence within macrophage cells, since the identification of such genes and pathways may facilitate new directions in anti-tuberculosis chemotherapy.

Another method of identifying functionally linked genes or proteins has been by examining the protein network connectivity among genes. Protein networks can be constructed from

* Corresponding author. Tel.: +49 30 28460500; fax: +49 30 28460501.
E-mail address: kaufmann@mpiib-berlin.mpg.de (S.H.E. Kaufmann).
[1] These authors contributed equally to this work.

1286-4579/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.micinf.2005.09.011

disparate datasets, including those generated by experimental and computational methods. Here we have examined gene expression profiles within the context of protein networks constructed using the Rosetta Stone [18], Phylogenetic Profile [19], conserved Gene Neighbor [20,21], and Operon [22,23] computational methods.

The Rosetta Stone method identifies individual genes that occur as a single fusion gene in another organism [18]. For instance the mycobacterial *gyrA* and *gyrB* genes occur as a single fusion gene in *Saccharomyces cerevisiae* (DNA Topoisomerase II). The Phylogenetic Profile method links genes that occur in a correlated manner across various genomes [19], the conserved Gene Neighbor method identifies genes that occur in close chromosomal proximity in multiple genomes [20,21], and the Operon method identifies genes likely to belong to common operons based on the intergenic distance between genes in the same orientation [22,23]. Functionally linked genes may suggest members of common pathways, complexes, or genes that serve related functions within the cell. Previously, these computational methods were used to identify putative operon structures in *M. tuberculosis* [22] as well as to identify functional modules throughout the *M. tuberculosis* genome. Here we use these methods to construct protein networks in which we examine expression profiles. This combined approach has aided in the identification of cellular pathways that may play a role in mycobacterial survival and persistence within macrophage cells, and these methods have facilitated the inference of gene function for previously uncharacterized genes.

2. Materials and methods

2.1. Primer design and PCR amplification of M. tuberculosis H37Rv ORFs for the production of filter-based DNA arrays

PCR primers were constructed to amplify the first 300 bp of each ORF described in the annotated genome sequence of *M. tuberculosis* H37Rv (http://genolist.pasteur.fr/TubercuList/). For ORFs less than 300 bp, PCR primers were designed to amplify the whole length of each ORF. Five hundred primer pairs were redesigned using PRIMER3 software to amplify corresponding ORFs in cases where the initial primer pairs did not yield a PCR product in the proper amount or in the expected size. PCR reactions were performed in a 100 μl reaction in 1× PCR buffer containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Tween 20, and 1 M Betaine (Sigma) with 200 μM dNTP mix and 0.3 M primer mix (forward and reverse) in the presence of 2 U *Taq* DNA polymerase (MBI Fermentas) and 1 ng of *M. tuberculosis* H37Rv genomic DNA. The reactions were carried out in thermal cyclers with a 96-well heating block (MJ Research). After an initial 2-min denaturation at 95 °C, 50 PCR cycles were run as follows: 95 °C for 45 s, 52 °C for 45 s, and 72 °C for 30 s. The final elongation was carried out at 72 °C for 10 min. Aliquots (1 μl) of each PCR product were run on 1.2% agarose gels to confirm the presence of a PCR product migrating at the expected size. We sent 100 PCR products, which were chosen randomly to represent each gene category, for sequencing, and all sequences match correctly to the corresponding ORFs.

2.2. Spotting of M. tuberculosis H37Rv filter-based DNA arrays

Prior to spotting, the PCR products were denatured by adding NaOH solution to a final concentration of 0.1 N. The denatured PCR products were spotted on 22×22 cm^2 positively charged nylon membranes (Amersham Biosciences) by using a customized spotting robot. Two replicates of the PCR product set were spotted on each DNA array to improve reproducibility. As a negative control, two replicates of PCR products from an *Arabidopsis thaliana* cDNA clone were also spotted on the DNA arrays. Some spot positions were kept empty and were also used as negative control.

2.3. Isolation of mycobacteria from macrophages

Murine bone marrow-derived macrophages were generated using L929 culture supernatant from bone marrow precursor cells, isolated from C57BL/6 mice. Macrophages were cultured in DMEM containing 10% FCS and 5% horse serum at 37 °C and 7% CO_2 either without treatment (resting) or upon activation with 1000 U/ml murine recombinant IFN-γ 24 h prior to infection. For infection, macrophages were incubated with *M. tuberculosis* H37Rv at an MOI of 5:1 for 2 h and washed excessively. Mycobacteria were taken from cultures growing in 7H9 medium at the logarithmic growth phase (CFU around 2×10^8/ml), washed twice in PBS/0.05% Tween 80 and resuspended in cell culture medium. Infected macrophages were cultured for three days (either resting or activated macrophages). Mycobacteria were isolated by scraping the infected macrophages in ice cold PBS/0.05% Tween 80 and lysed by putting the lysates through a fine syringe. Lysates were diluted 1:10 in PBS/0.05% Tween 80 and mycobacteria were harvested by spinning at $1800 \times g$ for 15 min. Pellets were washed in PBS/0.1% NP40 and in 0.5 M KCl/0.01% NP40 before RNA was isolated.

2.4. RNA extraction

The cell pellet from 50 ml of mid-log/stationary phase-grown cultures or from infected macrophages was resuspended in 5 ml phenol and 5 ml chloroform/methanol (3:1), and vortexed for 1 min or until the formation of an interface occurred. RNA was extracted with 4 ml RLT buffer from the RNeasy Kit (Qiagen) containing 0.5% sarcosyl and 1% β-mercaptoethanol (added prior to the use of buffer). The suspension was centrifuged to separate the aqueous phase from the organic phase. The aqueous layer was precipitated in ethanol, RNA was redissolved in 400 μl RLT buffer and further purified using RNeasy columns (Qiagen) according to the manufacturer's instructions.

2.5. RNA quantification

The size distribution and the quantity of isolated total RNA samples were determined using a high-resolution electrophoresis system, the Agilent 2100 Bioanalyzer™. This instrument separates RNA fragments through microfabricated channels with real time fluorescence detection. RNA samples were first diluted with injection buffer according to the manufacturer's instruction and then analyzed in parallel with an external RNA 6000 size ladder (Ambion).

2.6. cDNA synthesis and labeling

Five hundred nanograms of total *M. tuberculosis* H37Rv RNA from in vitro culture or 3 μg total RNA from infected macrophages was mixed with 5 pmoles mtGDP (mycobacterial genome-directed primers) [24]. The samples were heated at 70 °C for 10 min, followed by immediate cooling to 4 °C (on ice). RNA was then reverse-transcribed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) in a 20 μl reaction volume containing 200 U RevertAid™ M-MuLV reverse transcriptase, 20 U ribonuclease inhibitor, 0.5 mM each of dATP, dGTP, and dTTP, 0.5 μM dCTP, and 50 μCi of (^{33}P)-α-dCTP (Amersham Biosciences) in 1× reverse transcription buffer. The reaction was carried out at 25 °C for 10 min, and subsequently at 42 °C for 2 h. The unincorporated radioactive nucleotide was removed from the labelled cDNA by a G-50 column (Amersham Biosciences).

2.7. Hybridization of DNA arrays

DNA arrays were prehybridized at 50 °C for at least 1 h in 10 ml ULTRArray™ Hybridization Buffer (Ambion). The radioactively labelled cDNA probe was denatured at 95 °C for 10 min, cooled immediately on ice for 2 min and then transferred to the hybridization buffer. The hybridization was performed at 50 °C overnight. The DNA arrays were washed three times in washing buffer (0.5% SDS, 0.1× SSC) at 55 °C, each for 30 min. After washing, the DNA arrays were wrapped with clear food wrap film and exposed to a phosphorimaging screen for four days. Array images were scanned using a Fuji BAS 2500 phosphorimaging instrument at 50 μm pixel resolution.

2.8. Data analysis of DNA arrays

The feature extraction from the resulting image files and data analysis were carried out using the customized software 'Genespotter' (http://www.microdiscovery.com) [25,26]. Each array experiment was performed in triplicate and each RNA sample was hybridized on two DNA arrays. The raw data of signal intensities were normalized as follows. After exclusion of negative control spots (spots without DNA or containing a PCR product from an *A. thaliana* gene), the logarithmic value of each spot and the average from all these logarithmic values were calculated. The average value was then subtracted from each logarithmic value to obtain the normalized data. The normalized intensity data were used as basic values to calculate the fold of up- and downregulation of genes in each experiment. The data analysis was performed using the customized software from the bioinformatic company "Microdiscovery" [25,26].

2.9. Rosetta Stone method

Proteins were functionally linked by the Rosetta Stone method if individual proteins were found to be present as a single fused protein in another organism, as described by Marcotte et al. [18]. If individual *M. tuberculosis* proteins have significant homology to distinct regions of a single "fusion" protein in another organism then they are indicated as functionally linked by this method.

2.10. Phylogenetic Profile method

Phylogenetic profiles were used to identify proteins that occur in multiple genomes in a correlated fashion, as described by Pellegrini et al. [19]. A phylogenetic profile for each *M. tuberculosis* protein was created in the form of a bit vector, by searching for the presence or absence of homologs in each of the available sequenced genomes. The presence of an identifiable homolog in a particular genome was indicated by the integer 1 in the bit vector at the position corresponding to that genome, while the absence of a homolog was indicated by the integer 0. Phylogenetic profiles were then clustered based on the similarity of profiles.

2.11. Conserved Gene Neighbor method

Genes were linked by the conserved Gene Neighbor method if genes appeared as chromosomal gene neighbors in multiple genomes, as described by Dandekar et al. [20] and Overbeek et al. [21]. Genes that were in close proximity in multiple genomes were indicated as functionally linked.

2.12. Operon method

A series of genes were linked by the Operon method if the nucleotide distance between genes in the same orientation was less than or equal to a specified distance threshold. Multiple genes were linked if a series of genes in the same orientation all had intergenic distances less than or equal to the defined distance threshold [22,23].

2.13. Protein networks

Protein networks were constructed using functional linkages between proteins inferred by two or more computational methods. Genes identified by DNA array experiments to be upregulated during various conditions are indicated as red nodes within the networks.

2.14. Real time RT-PCR

Prior to use in the reverse transcription (RT) reaction, RNA samples were treated with DNase I (Invitrogen). After heat

inactivation of DNase I, RNA was purified using RNeasy columns (Qiagen) according to the manufacturer's instructions. The RT reaction was performed using the Superscript II Kit according to the manufacturer's instructions (Invitrogen). Each reaction contained 2 μg total RNA. Table 1 lists the primers (Proligo) used for the quantitative real time RT-PCR reactions. Real time RT-PCR was performed using the ABI PRISM 7000 Sequence Detection System™ (Applied Biosystems) in a 30 μl reaction volume with SYBR Green Kit. Each reaction was performed in triplicate. The conditions for real time PCR and the data analyses were carried out according to the manufacturer's instructions (Applied Biosystems). *sigA* was used as an internal control for the normalization of mRNA levels. The expression of this gene was relatively constant in vitro and in vivo according to our DNA array data.

3. Results

To better understand the molecular mechanisms of *M. tuberculosis* survival and persistence within host macrophage cells we collected and examined expression data from *M. tuberculosis* isolated from activated and resting macrophage cells. These studies suggest a number of key pathways that may be important for *M. tuberculosis* survival and persistence including genes involved in iron metabolism, cell wall metabolism, amino acid metabolism, lipid metabolism, DNA damage repair, transport, and regulatory functions. Figs. 1 and 2 summarize the functional classes of upregulated genes under the conditions examined.

3.1. Iron metabolism

We observed that two genes, Rv2385 (*mbtJ*) and Rv2386c (*mbtI*), which are associated with the biosynthesis of hydroxyphenyloxazoline-containing siderophore mycobactins, were upregulated upon activation of macrophages with IFN-γ. Siderophores serve primarily as transient reservoirs of ferric iron for transport into the cytoplasm. The *M. tuberculosis* genome also contains two bacterioferritin homologues, *bfrA* (Rv1876) and *bfrB* (Rv3841) [27], which provide long-term iron storage. Our DNA array result showed that the transcription of *bfrB* (Rv3841) was strongly reduced in activated macrophages. This suggests that in activated macrophages, iron is limited, reflected by the upregulation of iron-acquiring siderophores and the downregulation of long-term iron storage proteins.

A number of genes that encode proteins that require iron as their cofactor, such as oxido-reductases and oxygenases, were also downregulated during infection in activated macrophages. The decreased expression of these genes may be in response to the iron deprivation in the environment of mycobacterial cells.

To further investigate the impact of iron deprivation, in vitro cultured *M. tuberculosis* H37Rv were depleted for iron by deferoxamine. Similar to what was observed in activated macrophages, the transcription level of Rv2385 (*mbtJ*) and Rv2386c (*mbtI*) was elevated under in vitro iron-limited conditions. Correspondingly, the transcription of the long-term iron storage proteins, *brfB* (Rv3841), was repressed.

A significant proportion of genes that were differentially regulated in activated versus resting macrophages were also differentially expressed during iron depletion. Approximately 52% of the genes that were downregulated in activated versus resting macrophages were also downregulated in iron depleted versus complete medium. Approximately 22% of the genes that were upregulated in activated versus resting macrophages were also upregulated in iron depleted conditions.

3.2. Cell membrane and cell wall synthesis

Array analysis revealed significant induction of genes associated with the biosynthesis of cell wall and cell membrane components in resting and activated macrophages (Fig. 1). Among the induced genes were Rv1170, Rv2157 and Rv2482, involved in mycothiol, peptidoglycan and phospholipid biosynthesis, respectively. Genes involved in the

Table 1
List of primers for quantitative real time RT-PCR analysis

Gene name	Primer sequence	
	Forward	Reverse
Rv1064c	CTGTGCGCAATGGTTTTG	TACTCCTCCTCGGTCGATTC
Rv0467 (*icl*)	CCAAGTTCCAGAAGGAGCTG	CGTTCCTGCAGTTCGACATA
Rv1128c	TGACGGCCATGTTGAAGTAA	GTTGAGGCAGTCCATGATCC
Rv1669	GGGATACTCGAATGGACGTG	ATTGTGCCCAAGCATATTCC
Rv3628	TCGACGTGACCATCGAAAT	TCGTCACCTAGGGTGTCCTC
Rv0007	CGTTCCTCTACCTGGTGCTC	TCAGGACGATGTTGACCAAG
Rv0014c (*pknB*)	ATTGCCCTACATCGTCATGG	TGGATGATTCCGTTCTGATG
Rv0758 (*phoR*)	GCCAGAGCTATACGGCACTC	ACGGTGGTCAAGTAGCCATC
Rv2201 (*asnB*)	AGCACGTCAAAGTGGTGTTG	TGGAAACTTTTCCCATCGAC
Rv0238	TTAACGGCTACCACGAGACC	TCTGGTCGAAGTTGATGCTG
Rv3295	TCACTACTTCCCGGACAAGC	GTAGGCCATATAGGCCAGCA
Rv1276c	GACAACGTCACCACACTGCT	AACTTCTCCGAGATGCGTTC
Rv3279c (*birA*)	CGTTTGTGGTACTCGGTGTG	GGATGATCCGAGCTTCGAG
Rv1664 (*pks9*)	CGTTTCATTCGGTGTTGATG	CGCACATGTTCAACCCAATA
Rv2358	GCAATTGCGTGAATCTCAAC	GTCAGCAAGTCGGTACAGCA
Rv2703 (*sigA*)	ATCGCTGAACCCACCGAAAA	GACCTCTTCCTCGGCGTTGA

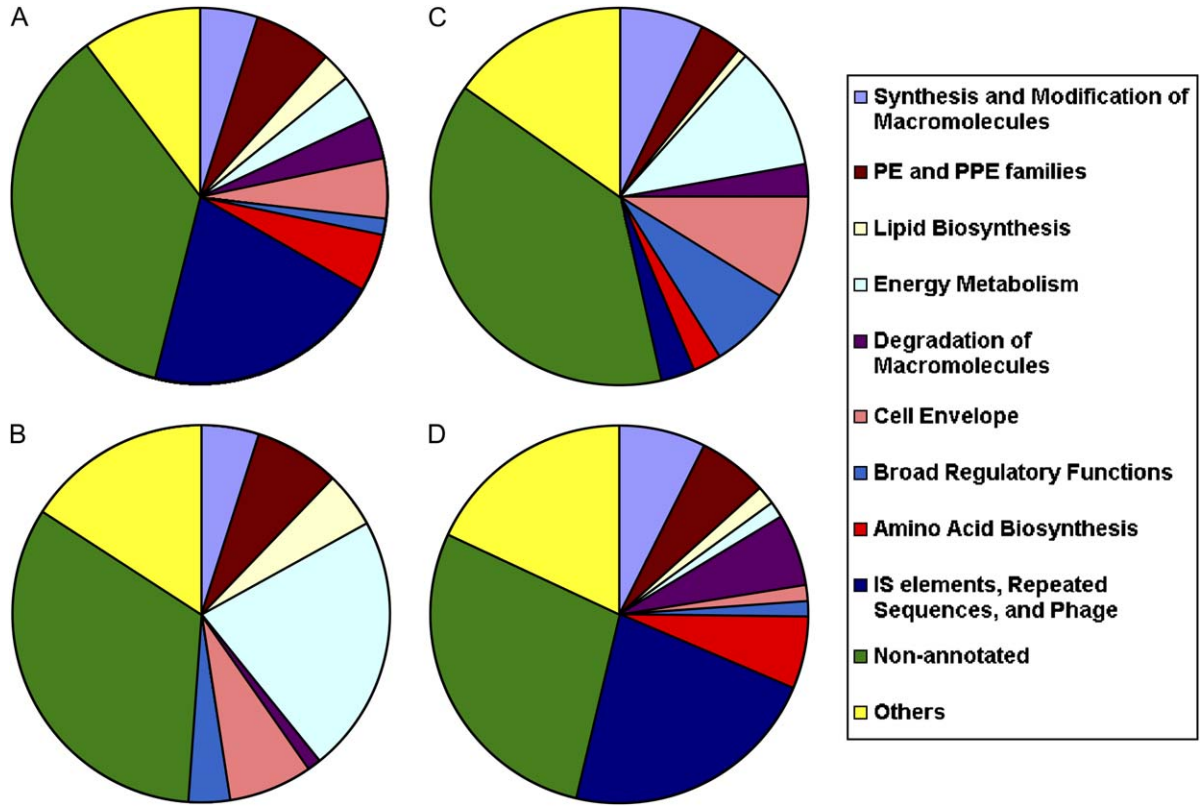

Fig. 1. Upregulated *M. tuberculosis* genes. Global gene expression analysis revealed unique signatures of *M. tuberculosis* cultures isolated from activated and resting macrophages. Genes that were upregulated by more than two-fold are indicated in the pie charts. Figures depict *M. tuberculosis* genes upregulated in (A) activated macrophages versus resting macrophages, (B) resting macrophages versus activated macrophages, (C) resting macrophages versus in vitro, and (D) in vitro versus resting macrophages.

synthesis of lipoproteins, such as *lpqV* (Rv1064c) and *lppZ* (Rv3006), were also upregulated three days after infection of activated macrophages but not in resting macrophages.

The glycosyltransferase homolog, Rv2962c, was specifically induced in activated macrophages. The expression of this gene in *Mycobacterium smegmatis* has been shown previously to increase survival rates of this avirulent strain in THP-1 macrophages [28].

pyrG (Rv1699), encoding CTP synthase, was also induced in activated macrophages. CTP is required for various metabolic pathways, including cell wall and cell membrane synthesis, and is required for the synthesis of phospholipids. The upregulation of genes involved in mycothiol, peptidoglycan, phospholipid, and other cell wall associated proteins suggests that these genes play an important role in *M. tuberculosis* protection from the external environment.

3.3. Amino acid metabolism

Certain genes that are involved in amino acid biosynthesis and transport, such as Rv0753c (valine metabolism), Rv2201 (asparagine synthesis), Rv1612 (tryptophan biosynthesis), Rv1659 (arginine biosynthesis), and Rv0346c (L-asparagine transport) were also upregulated, suggesting that the phagosomal environment may be restricted in amino acids. The high expression levels of these genes were maintained during infection of activated macrophages.

3.4. Lipid metabolism

It has been hypothesized that *M. tuberculosis* obtains nourishment from lipids as a major carbon source inside macrophages [4], mediated by the glyoxylate bypass enzyme *icl* (isocitrate lyase). Our observations are consistent with this hypothesis. Although we observed that isocitrate lyase (Rv0467) was only slightly induced during infection of resting macrophages, activation of macrophages drove the infecting *M. tuberculosis* to produce more isocitrate lyase. The elevated expression of isocitrate lyase during infection has been reported for several pathogenic microorganisms, including *M. tuberculosis* [4], *Candida albicans* [29] and the rice blast fungus *Magnaporthe grisea* [30]. In addition, a panel of genes involved in fatty acid oxidation including Rv0215c, Rv0673, Rv0852, Rv2679 and Rv3505 were induced during infection of resting and activated macrophages.

3.5. DNA damage repair, radical neutralization and transport regulation

We observed increased expression of genes responsible for DNA damage repair and neutralization of reactive radicals in macrophages, including Rv1020, Rv1329c, Rv2090, Rv2836c and *bpoB* (Rv1123c). Of the *sod* genes, only the expression level of *sodC* (Rv0432) was elevated in activated macrophages. This is consistent with the recent finding that

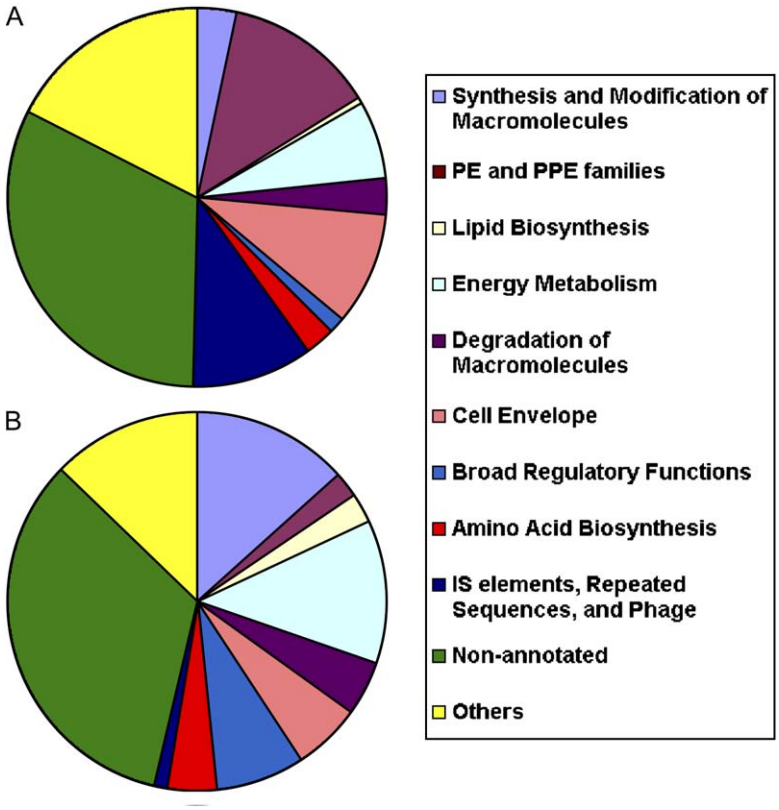

Fig. 2. *M. tuberculosis* genes upregulated in iron depleted media. In vitro cultured *M. tuberculosis* H37Rv were depleted for iron by deferoxamine. An iron deficient environment may mimic the environment *M. tuberculosis* encounters within the host macrophage cells. Figures depict *M. tuberculosis* genes upregulated in (A) iron depleted medium versus rich medium and (B) rich medium versus iron depleted medium.

sodC is essential for survival of *M. tuberculosis* in macrophages [31].

Genes associated with transport functions such as Rv1458c, Rv2691 (*ceoB*) and Rv3270 were also upregulated during infection of resting macrophages. Rv2691 (*ceoB*) encodes a protein that exhibits activity similar to that of the potassium uptake regulatory protein TrkA. Recently, it has been reported that the expression of Rv3237c, encoding a possible potassium channel protein, was strongly stimulated during *M. tuberculosis* infection of human macrophages [32]. We also observed a slight induction of this gene during macrophage infection. Moreover, it was found that *ceoA*, *B* and *C* from *M. tuberculosis* which was transformed to *Escherichia coli* conferred INH resistance in otherwise INH sensitive *E. coli*, probably by sequestering or detoxifying INH [33].

3.6. Regulatory proteins

Genes coding for regulatory proteins play vital roles in metabolic processes, including transcription, cell development and interactions with host cells. We observed that the changes in expression profile during macrophage infection were accompanied by the stimulation of a panel of genes encoding regulatory proteins. Regulatory genes that are upregulated during infection of both resting and activated macrophages include Rv0014c, Rv1534, Rv2640c, Rv2642, Rv3246, Rv3681, Rv0485, Rv0758 and Rv0903c.

Most of the bacterial eukaryotic-like protein kinases that have been characterized so far have been shown to be involved in the regulation of different developmental states of bacteria. The expression of *M. tuberculosis* Ser/Thr kinase *pknB* (Rv0014c) which belongs to the protein serine/threonine kinase (PSTK) family has been reported previously to be detected in vitro and in vivo [34]. It has been proposed that *pknB* may play a role during infection. Our DNA array results support this notion. Another member of the PSTK family, Ser/Thr kinase *pknL* (Rv2176), and a transcriptional regulatory protein belonging to LuxR-family, Rv0195, were specifically induced during infection of activated macrophages. Little is known about the function of these genes.

Another regulatory gene induced during macrophage infection was Rv3681c (*whiB4*). The *whiB* genes are essential sporulation factors in *Streptomyces coelicolor* [35]. The disruption of the *whmD* gene, the homologue of the *whiB* gene in *M. smegmatis*, was lethal. The *whmD* gene is suggested to play a role in mycobacterial septum formation and cell division [36].

3.7. Quantitative real time RT-PCR

To verify the results obtained from DNA array experiments we performed quantitative real time RT-PCR of some genes chosen randomly from different growth conditions. The results of real time RT-PCR along with the corresponding DNA array data are presented in Table 2.

Table 2
List of results of quantitative real time RT-PCR analysis

Gene name	Function of encoded protein	Gene expression ratios		Compared conditions
		Real time RT-PCR	DNA arrays	
Rv1064c	Cell envelope	8.26	14.17	Activated vs. resting macrophages
Rv0467 (*icl*)	Energy metabolism	5.01	3.38	Activated vs. resting macrophages
Rv1128c	IS elements	7.68	7.02	Activated vs. resting macrophages
Rv1669	Unknown	8.36	9.28	Activated vs. resting macrophages
Rv3628	Degradation of phosphorus compounds	4.13	2.84	Activated vs. resting macrophages
Rv0007	Unknown	6.31	4.21	Resting vs. in vitro macrophages
Rv0014c (*pknB*)	Broad regulatory functions	5.94	4.22	Resting vs. in vitro macrophages
Rv0758 (*phoR*)	Broad regulatory functions	6.19	3.64	Resting vs. in vitro macrophages
Rv2201 (*asnB*)	Amino acid biosynthesis	6.38	3.27	Resting vs. in vitro macrophages
Rv0238	Unknown	4.12	2.99	Resting vs. activated macrophages
Rv3295	Broad regulatory functions	7.21	4.06	Resting vs. activated macrophages
Rv1276c	Unknown	4.39	6.75	In vitro vs. resting macrophages
Rv3279c (*birA*)	Biosynthesis of biotin	10.11	8.50	In vitro vs. resting macrophages
Rv1664 (*pks9*)	Polyketide synthesis	4.18	3.45	Iron deficient vs. rich medium
Rv2358	Broad regulatory functions	5.48	3.62	Iron deficient vs. rich medium

3.8. Uncharacterized genes and protein linkages

The greatest percentage of genes upregulated in each condition investigated were the class labelled as non-annotated (Figs. 1 and 2). These include both conserved hypothetical and hypothetical proteins of unknown functions. Although these genes do not currently have a function assigned to them, they may play a crucial role in the survival and persistence of *M. tuberculosis* within host macrophage cells. In order to identify putative functions for these genes, genome-wide functional linkages were examined and protein networks were constructed to examine these genes within the context of all other cellular proteins. This approach facilitated the inference of protein function for previously uncharacterized *M. tuberculosis* genes. A complete list of linkages and protein networks is provided at http://www.doe-mbi.ucla.edu/~strong/skaufmann.

Fig. 3 depicts one example of assignment of function using this method. The uncharacterized gene Rv2908c was upregulated in activated macrophage cells. Although the function of Rv2908c is uncharacterized, it is functionally linked to the ribosomal protein Rv2909c, the ribosomal RNA processing protein *rimM*, and the tRNA methyltransferase protein *trmD*, by multiple computational methods (Fig. 3A). Based on these functional linkages, we hypothesize that Rv2908 may have a function associated with the ribosome. Interestingly, both Rv2908c and Rv2909c are upregulated in activated macrophage cells with an 8.25- and 8.00-fold induction, respectively. These genes are separated by only 8 bp. Together this may suggest that these genes are co-expressed as part of a common operon. An extended network, containing links to all the genes of this network is shown in Fig. 3B.

In further support of the functional inference for Rv2908c, this protein has some sequence similarity to KH domain proteins. The KH domain proteins have been implicated in RNA binding and nucleic acid binding activities. Bylund et al. [37] have shown that the *E. coli* KH domain protein *yfjA* associates with free 30S ribosomal subunits and plays a role in translation. Taken together this evidence suggests that the conserved hypothetical protein Rv2908c may play a role in translation. We provide a list of hundreds of such functionally linked genes and dozens of protein networks online at http://www.doe-mbi.ucla.edu/~strong/skaufmann.

3.9. Protein networks containing differentially expressed genes

In Fig. 4 we show a protein network containing eight genes that are upregulated in resting macrophages (*mtrA*, *prrA*, *phoR*, *pknB*, *murF*, *dnaB*, Rv1458c, and Rv1461). Upregulated genes are indicated as red nodes in the network. Protein networks shown here and on the website indicate genes which are functionally linked by two or more computational methods, linked by a combination of the Rosetta Stone, Phylogenetic Profile, conserved Gene Neighbor, and Operon methods. Most of the genes of the network in Fig. 4 are involved in signalling and transport, including the three two-component system genes (*mtrA*, *prrA*, and *phoR*), the serine-threonine protein kinase gene Rv0014c (*pknB*), and the ABC transporter gene Rv1458c. Notice that the three upregulated two-component system genes (*mtrA*, *prrA*, and *phoR*) cluster together.

Clustering of genes of related function is a common characteristic of our computationally inferred protein networks since genes that are linked by two or more computational methods are likely to share at least some functions in common [38]. Fig. 4 demonstrates four clusters in our protein network that each of which contains a number of *M. tuberculosis* genes of related function. Cluster 1 contains genes involved in two-component systems, cluster 2 contains transport and binding proteins, cluster 3 contains serine-threonine kinases and protein phosphatases, and cluster 4 contains genes involved in cell wall synthesis and protein secretion.

The conserved hypothetical protein Rv1461 was upregulated in resting macrophages versus in complete medium. Although this gene is uncharacterized, it is linked to five genes as

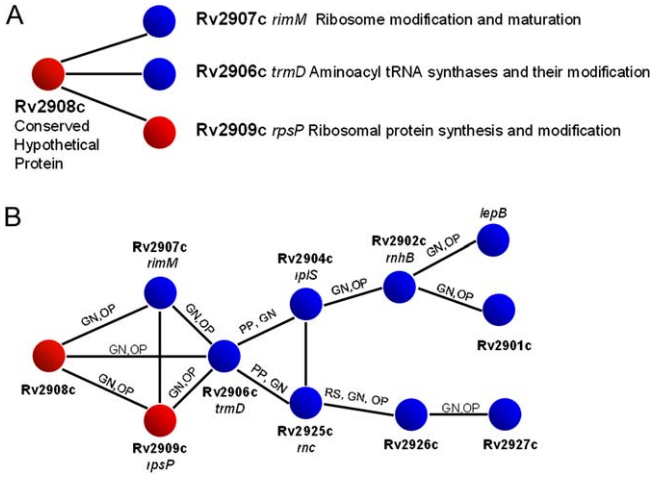

Fig. 3. Inference of protein function. The uncharacterized gene Rv2908c was upregulated in activated macrophages. (A) Although this gene is annotated as a "conserved hypothetical protein" it is linked to the ribosomal protein Rv2909c, the ribosomal RNA processing protein *rimM*, and the tRNA methyltransferase protein *trmD*, by multiple computational methods. Based on these functional linkages we hypothesize that Rv2908c may have a function associated with the ribosome. (B) The complete protein network containing the upregulated genes Rv2908c and Rv2909c. Many of the genes of this network are involved in ribosomal related functions.

shown in Fig. 4. The five functionally linked genes occur as chromosomal gene neighbors in multiple genomes, including the *M. tuberculosis* genome. While both Rv1461 and Rv1462 are annotated as conserved hypothetical proteins, they do share some sequence similarity to iron-regulated ABC transporters. Both proteins are also linked to the annotated ABC transporter Rv1463. The functionally linked gene Rv1464 (*csd*) is involved in the assembly of iron–sulfur clusters and Rv1465 is an iron–sulfur scaffolding protein. The final linked gene, Rv1460, is a transcription regulator of unknown target. The functional links in the network and sequence analysis suggest that the uncharacterized genes Rv1461 and Rv1462 may function in iron regulation or iron transport. In eukaryotes, the ABC transporter Atm1p is involved in the export of iron–sulfur clusters from the mitochondria [39], and in prokaryotes a number of ABC transporters have been shown to transport iron across the cytoplasmic membrane. We provide dozens of such protein networks and complete lists of upregulated genes at http://www.doe-mbi.ucla.edu/~strong/skaufmann. An example of other protein networks is shown in Fig. 5.

4. Discussion

We have applied a combined approach, incorporating DNA array analyses and computational methods to investigate global gene expression profiles in *M. tuberculosis*. This approach was used to investigate *M. tuberculosis* isolated from infected macrophages and *M. tuberculosis* grown under iron depletion condition. Using this combined approach we were able to infer functions of previously uncharacterized genes which were differentially expressed. Our combined approach has enabled us to identify signatures which may play critical roles in the survival of *M. tuberculosis* in vivo.

One signature we identified involves a mechanism to counteract the iron deprivation situation encountered in activated

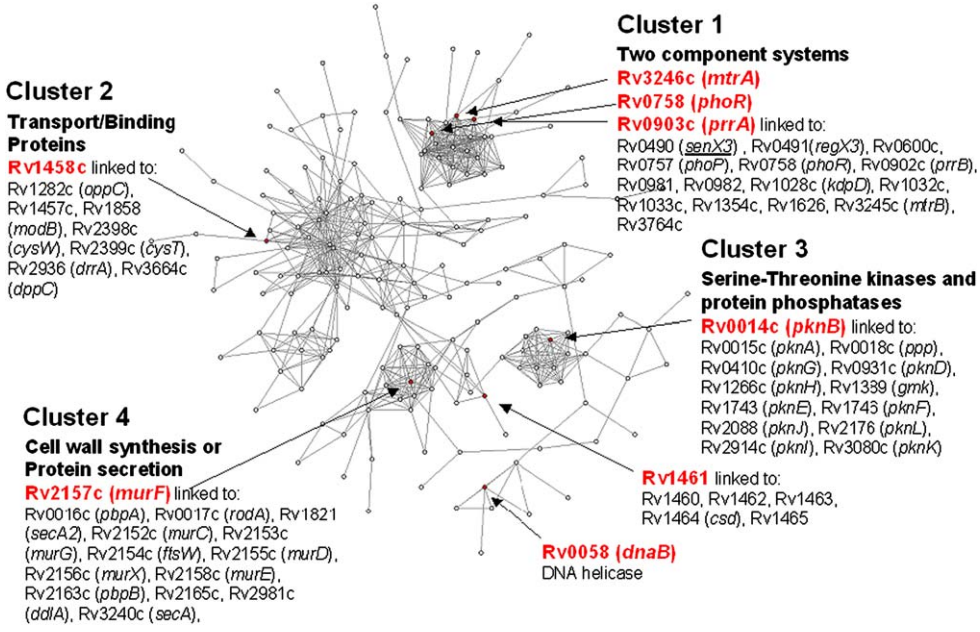

Fig. 4. A protein network containing eight differentially expressed genes. The protein network shown here represents genes that are functionally linked by two or more computational methods (Rosetta Stone, Phylogenetic Profile, conserved Gene Neighbor, and Operon methods). Upregulated genes are indicated as red nodes in the network. Upregulated genes in this network include *mtrA*, *prrA*, *phoR*, *pknB*, *murF*, *dnaB*, Rv1458c, and Rv1461. Most genes in this network are involved in signalling and transport. In cluster 1 we see a number of proteins involved in two-component systems, cluster 2 contains a number of transport and binding proteins, cluster 3 contains serine-threonine kinases and protein phosphatases, and cluster 4 contains cell wall synthesis and protein secretion proteins. Genes of related function tend to cluster together in these networks.

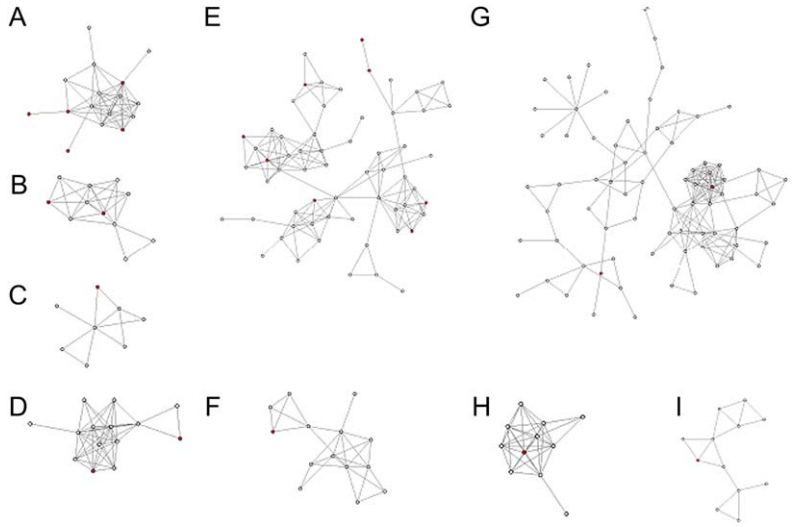

Fig. 5. Nine protein networks containing upregulated *M. tuberculosis* genes. Protein networks were constructed using computationally inferred functional linkages, and upregulated genes are indicated in red. Dozens of such networks are available at http://www.doe-mbi.ucla.edu/~strong/skaufmann.

macrophages. *M. tuberculosis* induces the expression of siderophore genes and represses genes which require iron as cofactor. We also noticed upregulation of amino acid biosynthesis genes, which may suggest a mechanism which the host employs to inhibit the growth of the tubercle bacilli in vivo. It has previously been suggested that the concentration of L-tryptophan may influence the intracellular survival of *Bordetella pertussis*, and that the host may specifically induce tryptophan degrading enzymes as a defense mechanism to inhibit survival of *B. pertussis* [40].

Another prominent signature is the immense changes in the expression of certain cell envelope components. Many of the cell wall components of *M. tuberculosis* have been associated with virulence and modulation of the host immune response [34]. Although the function of lipoproteins in *M. tuberculosis* has not been well characterized, some lipoproteins including phospholipase C have been reported to modulate the host immune response and may play an important role in virulence [27]. Similar functions have also been attributed to some exported proteins [41]. We hypothesize that the induction of a series of *M. tuberculosis* genes which are involved in the synthesis of cell wall and cell membrane components during macrophage infection may reflect the efforts of the tubercle bacilli to evade host defense by modulating the host response, and may enable the generation of a stronger peripheral fortification system.

Some genes which were upregulated during macrophage infection have been described previously as potential drug targets. For instance, *p*-aminosalicylic acid, a second-line antitubercular drug, is thought to interfere with mycobactin synthesis [42]. Drugs such as acivicin, 3-deazauridine (3-DU) and cyclopentenyl cytosine (CPEC) are known to inhibit CTP synthase [43]. K^+ transport systems have been shown to be indirect targets of some drugs with antimycobacterial properties such as B4128 (tetramethylpiperidyl (TMP)-substituted phenazine), riminophenazines, B669 and clofazimine [44]. We observed the upregulation of *ceoB* (Rv2691), which encodes a potassium uptake protein, during macrophage infection. The protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) was found to inhibit the activity of *pknB* (Rv0014c) and consequently the growth of *M. tuberculosis* [45].

Recently, efforts have been made to identify genes in the *M. tuberculosis* genome, which play an important role in survival and virulence in the host [1–8]. The results of our study revealed signatures for intraphagosomal tubercle bacilli similar to those of the previous study, such as DNA damage, nutrient starvation, etc. [1]. A set of *M. tuberculosis* genes, which are required for its survival in macrophages, has been identified recently using a unique high throughput method combining TraSH and microarray methods [2]. One striking signature, which has been observed using this method and also in our study, is the condition of phosphate limitation in macrophages. We observed the induction of genes encoding PhoR. Rv0758 (*phorR*) is a sensor part of a two-component regulatory system PhoP/PhoR. In *Bacillus subtilis* the induction of this regulon is stimulated by phosphate starvation [46]. The *phoP* mutant strain of *M. tuberculosis* shows impaired growth but is still able to survive when cultured in murine bone marrow-derived macrophages [47]. The condition of phosphate deprivation might be consistent with the induction of pyrophosphatase gene *ppa* observed in our study. The hydrolysis of pyrophosphate will provide the cells with more inorganic phosphate. The *ppa* gene is ubiquitous and the sequence is relatively conserved among different organisms. The genome sequence of *M. tuberculosis* H37Rv exhibits a 486-bp long ORF (Rv3628) with 44% sequence identity to the *Legionella pneumophila* Ppase [48]. It has been reported that Ppase is crucial for the growth in *E. coli* and yeast [49,50]. While the expression of *ppa* is constitutive in *E. coli*, it has been observed that the expression of this gene is elevated in *L. pneumophila* growing in resting macrophages [51]. The condition of phosphate limitation was also suggested in *Salmonella*-containing vacuoles during infection of this pathogen in macrophages. Since inorganic phosphate availability is closely related to the energy level in the cells, the restriction of inorganic phosphate

in phagosomes may constitute another host strategy to inhibit pathogen growth.

Together we demonstrate applications employing both microarray analyses and computational methods to investigate *M. tuberculosis* isolated from infected macrophages and under iron depletion conditions. We have constructed extensive protein networks based on the computationally assigned functional linkages, which we have used for the inference of protein function. We provide comprehensive lists of upregulated genes and protein networks at http://www.doe-mbi.ucla.edu/~strong/skaufmann. This combined approach may aid in the identification of novel pathways which are essential for virulence, persistence, and pathogenesis of *M. tuberculosis* and may suggest new targets for novel intervention strategies.

Acknowledgements

S.H.E.K. received financial support for this work from the German Ministry for Science and Technology (Competence Networks ''Pathogenomics'' and ''Structural Genomics of M. tuberculosis'') and the German Science Foundation (Priority Program ''Novel Vaccination Strategies''). H.R. thanks H. Witt and A. Saleh at AG Ruiz, H. Eickhoff, R. Reinhardt and the whole automation crew at Max Planck Institute for Molecular Genetics, Berlin, Germany. We thank H. Lehrach for his input and the company Chiron Behring for fruitful collaboration in the initial stage of this study. M.S. thanks M. Beeby, M. Pellegrini, and M.J. Thompson. M.S. is supported by a USPHS National Research Service Award GM07185. DE received financial support for this work from the US National Institutes of Health.

References

[1] D. Schnappinger, S. Ehrt, M.I. Voskuil, Y. Liu, J.A. Mangan, I.M. Monahan, et al., Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment, J. Exp. Med. 198 (2003) 693–704.

[2] J. Rengarajan, B.R. Bloom, E.J. Rubin, Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 8327–8332.

[3] K.N. Lewis, R. Liao, K.M. Guinn, M.J. Hickey, S. Smith, M.A. Behr, et al., Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette–Guerin attenuation, J. Infect. Dis. 187 (2003) 117–123.

[4] J.D. McKinney, B.K. Honer zu, E.J. Munoz-Elias, A. Miczak, B. Chen, W.T. Chan, et al., Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase, Nature 406 (2000) 735–738.

[5] T.D. Sirakova, V.S. Dubey, M.H. Cynamon, P.E. Kolattukudy, Attenuation of *Mycobacterium tuberculosis* by disruption of a mas-like gene or a chalcone synthase-like gene, which causes deficiency in dimycocerosyl phthiocerol synthesis, J. Bacteriol. 185 (2003) 2999–3008.

[6] X.Y. He, Y.H. Zhuang, X.G. Zhang, G.L. Li, Comparative proteome analysis of culture supernatant proteins of *Mycobacterium tuberculosis* H37Rv and H37Ra, Microbes Infect. 5 (2003) 851–856.

[7] J. Mattow, P.R. Jungblut, U.E. Schaible, H.J. Mollenkopf, S. Lamer, U. Zimny-Arndt, et al., Identification of proteins from *Mycobacterium tuberculosis* missing in attenuated *Mycobacterium bovis* BCG strains, Electrophoresis 22 (2001) 2936–2946.

[8] J.J. De Voss, K. Rutter, B.G. Schroeder, H. Su, Y. Zhu, C.E. Barry III, The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 1252–1257.

[9] S.H. Kaufmann, I.E. Flesch, The role of T cell–macrophage interactions in tuberculosis, Springer Semin. Immunopathol. 10 (1988) 337–358.

[10] V. Deretic, R.A. Fratti, *Mycobacterium tuberculosis* phagosome, Mol. Microbiol. 31 (1999) 1603–1609.

[11] D.L. Clemens, Characterization of the *Mycobacterium tuberculosis* phagosome, Trends Microbiol. 4 (1996) 113–118.

[12] I. Vergne, R.A. Fratti, P.J. Hill, J. Chua, J. Belisle, V. Deretic, *Mycobacterium tuberculosis* phagosome maturation arrest: mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion, Mol. Biol. Cell 15 (2004) 751–760.

[13] V. Deretic, L.E. Via, R.A. Fratti, D. Deretic, Mycobacterial phagosome maturation, rab proteins, and intracellular trafficking, Electrophoresis 18 (1997) 2542–2547.

[14] D.L. Clemens, M.A. Horwitz, Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited, J. Exp. Med. 181 (1995) 257–270.

[15] J. Pieters, Entry and survival of pathogenic mycobacteria in macrophages, Microbes Infect. 3 (2001) 249–255.

[16] S. Sturgill-Koszycki, P.H. Schlesinger, P. Chakraborty, P.L. Haddix, H.L. Collins, A.K. Fok, et al., Lack of acidification in mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase, Science 263 (1994) 678–681.

[17] J. Mehrotra, W.R. Bishai, Regulation of virulence genes in *Mycobacterium tuberculosis*, Int. J. Med. Microbiol. 291 (2001) 171–182.

[18] E.M. Marcotte, M. Pellegrini, H.L. Ng, D.W. Rice, T.O. Yeates, D. Eisenberg, Detecting protein function and protein–protein interactions from genome sequences, Science 285 (1999) 751–753.

[19] M. Pellegrini, E.M. Marcotte, M.J. Thompson, D. Eisenberg, T.O. Yeates, Assigning protein functions by comparative genome analysis: protein phylogenetic profiles, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 4285–4288.

[20] T. Dandekar, B. Snel, M. Huynen, P. Bork, Conservation of gene order: a fingerprint of proteins that physically interact, Trends Biochem. Sci. 23 (1998) 324–328.

[21] R. Overbeek, M. Fonstein, M. D'Souza, G.D. Pusch, N. Maltsev, The use of gene clusters to infer functional coupling, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 2896–2901.

[22] M. Strong, P. Mallick, M. Pellegrini, M.J. Thompson, D. Eisenberg, Inference of protein function and protein linkages in *Mycobacterium tuberculosis* based on prokaryotic genome organization: a combined computational approach, Genome Biol. 4 (2003) R59.

[23] H. Salgado, G. Moreno-Hagelsieb, T.F. Smith, J. Collado-Vides, Operons in *Escherichia coli*: genomic analyses and predictions, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 6652–6657.

[24] A.M. Talaat, P. Hunter, S.A. Johnston, Genome-directed primers for selective labeling of bacterial transcripts for DNA microarray analysis, Nat. Biotechnol. 18 (2000) 679–682.

[25] H. Eickhoff, J. Schuchhardt, I. Ivanov, S. Meier-Ewert, J. O'Brien, A. Malik, et al., Tissue gene expression analysis using arrayed normalized cDNA libraries, Genome Res. 10 (2000) 1230–1240.

[26] J. Schuchhardt, D. Beule, A. Malik, E. Wolski, H. Eickhoff, H. Lehrach, et al., Normalization strategies for cDNA microarrays, Nucleic Acids Res. 28 (2000) E47.

[27] S.T. Cole, R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, et al., Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence, Nature 393 (1998) 537–544.

[28] B.H. Miller, T.M. Shinnick, Identification of two *Mycobacterium tuberculosis* H37Rv ORFs involved in resistance to killing by human macrophages, BMC Microbiol. 1 (2001) 26.

[29] M.C. Lorenz, G.R. Fink, The glyoxylate cycle is required for fungal virulence, Nature 412 (2001) 83–86.

[30] Z.Y. Wang, C.R. Thornton, M.J. Kershaw, L. Debao, N.J. Talbot, The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*, Mol. Microbiol. 47 (2003) 1601–1612.

[31] D.L. Piddington, F.C. Fang, T. Laessig, A.M. Cooper, I.M. Orme, N.A. Buchmeier, Cu, Zn superoxide dismutase of *Mycobacterium*

tuberculosis contributes to survival in activated macrophages that are generating an oxidative burst, Infect. Immun. 69 (2001) 4980–4987.
[32] R. Ryll, Y. Kumazawa, I. Yano, Immunological properties of trehalose dimycolate (cord factor) and other mycolic acid-containing glycolipids–a review, Microbiol. Immunol. 45 (2001) 801–811.
[33] R.D. Fleischmann, D. Alland, J.A. Eisen, L. Carpenter, O. White, J. Peterson, et al., Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains, J. Bacteriol. 184 (2002) 5479–5490.
[34] Y. Av-Gay, S. Jamil, S.J. Drews, Expression and characterization of the *Mycobacterium tuberculosis* serine/threonine protein kinase PknB, Infect. Immun. 67 (1999) 5676–5682.
[35] N.K. Davis, K.F. Chater, The *Streptomyces coelicolor* whiB gene encodes a small transcription factor-like protein dispensable for growth but essential for sporulation, Mol. Gen. Genet. 232 (1992) 351–358.
[36] J.E. Gomez, W.R. Bishai, whmD is an essential mycobacterial gene required for proper septation and cell division, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 8554–8559.
[37] G.O. Bylund, B.C. Persson, L.A. Lundberg, P.M. Wikstrom, A novel ribosome-associated protein is important for efficient translation in *Escherichia coli*, J. Bacteriol. 179 (1997) 4567–4574.
[38] E.M. Marcotte, M. Pellegrini, M.J. Thompson, T.O. Yeates, D. Eisenberg, A combined algorithm for genome-wide prediction of protein function, Nature 402 (1999) 83–86.
[39] U. Muhlenhoff, R. Lill, Biogenesis of iron–sulfur proteins in eukaryotes: a novel task of mitochondria that is inherited from bacteria, Biochim. Biophys. Acta 1459 (2000) 370–382.
[40] B.P. Mahon, K.H. Mills, Interferon-gamma mediated immune effector mechanisms against *Bordetella pertussis*, Immunol. Lett. 68 (1999) 213–217.
[41] F.X. Berthet, M. Lagranderie, P. Gounon, C. Laurent-Winter, D. Ensergueix, P. Chavarot, et al., Attenuation of virulence by disruption of the *Mycobacterium tuberculosis* erp gene, Science 282 (1998) 759–762.
[42] C. Ratledge, K.A. Brown, Inhibition of mycobactin formation in *Mycobacterium smegmatis* by *p*-aminosalicylate. A new proposal for the mode of action of *p*-aminosalicylate, Am. Rev. Respir. Dis. 106 (1972) 774–776.
[43] A.A. Hindenburg, R.N. Taub, S. Grant, G. Chang, M.A. Baker, Effects of pyrimidine antagonists on sialic acid regeneration in HL-60 cells, Cancer Res. 45 (1985) 3048–3052.
[44] N.M. Matlola, H.C. Steel, R. Anderson, Antimycobacterial action of B4128, a novel tetramethylpiperidyl-substituted phenazine, J. Antimicrob. Chemother. 47 (2001) 199–202.
[45] S.J. Drews, F. Hung, Y. Av-Gay, A protein kinase inhibitor as an antimycobacterial agent, FEMS Microbiol. Lett. 205 (2001) 369–374.
[46] F.M. Hulett, J. Lee, L. Shi, G. Sun, R. Chesnut, E. Sharkova, et al., Sequential action of two-component genetic switches regulates the PHO regulon in *Bacillus subtilis*, J. Bacteriol. 176 (1994) 1348–1358.
[47] E. Perez, S. Samper, Y. Bordas, C. Guilhot, B. Gicquel, C. Martin, An essential role for phoP in *Mycobacterium tuberculosis* virulence, Mol. Microbiol. 41 (2001) 179–187.
[48] J.A. Triccas, B. Gicquel, Analysis of stress- and host cell-induced expression of the *Mycobacterium tuberculosis* inorganic pyrophosphatase, BMC Microbiol. 1 (2001) 3.
[49] J. Chen, A. Brevet, M. Fromant, F. Leveque, J.M. Schmitter, S. Blanquet, et al., Pyrophosphatase is essential for growth of *Escherichia coli*, J. Bacteriol. 172 (1990) 5686–5689.
[50] M. Lundin, H. Baltscheffsky, H. Ronne, Yeast PPA2 gene encodes a mitochondrial inorganic pyrophosphatase that is essential for mitochondrial function, J. Biol. Chem. 266 (1991) 12168–12172.
[51] K.Y. Abu, Induced expression of the *Legionella pneumophila* gene encoding a 20-kilodalton protein during intracellular infection, Infect. Immun. 66 (1998) 203–212.