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Evaluation of Transcript Labeling Techniques and Development of a Membrane-based Parallel Gene Expression Assay

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Abstract: An inexpensive alternative to micro arrays was developed to examine the expression of small sets of genes in parallel and used to compare direct and indirect transcript labeling methods. Psoralen-biotin direct labeling was ~10-fold less sensitive than reverse transcriptase-biotin-dUTP labeling, but the enzymatic method generated higher levels of background noise in miniarray hybridizations. The reproducibility of hybridization intensities was the same for the two labeling methods, although differences in signal intensities for several genes were observed. The miniarray hybridization assay was validated using the known responses of mycobacteria to heat shock, exposure to isoniazid and growth phase. Expression profiles were generated for 14 *Mycobacterium smegmatis* and 26 *M. tuberculosis* genes. The transcriptional response to isoniazid and peculiar regulation of *acr* in different growth phases were confirmed and a potential role of oxidative stress enzymes in the heat shock response was revealed. The miniarray system was also used to demonstrate that an RNA stabilizing reagent, RNALater™, was effective in inhibiting both RNA degradation and transcription in mycobacteria, which may prove useful when significant manipulation of the bacteria are required prior to RNA extraction such as in experimental infections of cell cultures or animals.

Key words: Direct Labeling of RNA, RNA Stabilizer

INTRODUCTION

High-density micro array analysis has become a driving force in gene expression studies [reviewed in 1, 2]. Because of the high cost and specialized equipment needed for micro arrays, a less expensive alternative suitable for routine use in any molecular biology laboratory would be useful, particularly when only a relatively few genes are to be studied. For our studies to identify potential virulence factors, we developed such an array-based gene expression technique to evaluate differential gene expression of a small set of genes in *Mycobacterium tuberculosis*.

The typical method of transcript analysis by micro array uses cDNA produced by incorporation of labeled nucleotides during reverse transcription (RT) reactions. There are several potential disadvantages of this system, including possible enzymatic problems (inhibition, reproducibility, sensitivity) and problems associated with the use of random primers for synthesis of cDNA from bacterial RNA [1-3]. Recently however, a commercial labeling kit utilizing the nucleic acid intercalating agent psoralen coupled to biotin was used in a micro array application to directly label bacterial RNA [3]. This method proved more sensitive and reproducible than an enzymatic system in a single-color fluorescence micro array system. Because micro arrays and fluorescence detection systems are too expensive for many laboratories, a membrane-based hybridization assay utilizing single-color chemiluminescent detection of psoralen-hapten-labeled RNA was developed to identify differences in transcript levels between samples. This direct labeling method was compared with an enzymatic labeling system based on RT-mediated incorporation of biotin-dUTP into nascent cDNA. The membrane-based "miniarray" assay was utilized to examine gene expression in mycobacteria under several different conditions.

MATERIALS AND METHODS

Bacteria and Growth Conditions: Cultures of *M. tuberculosis* (H37Rv-TMC102, CDC1227 and CDC1551) and *M. smegmatis* (LR222) were routinely grown in Middlebrook 7H9 broth (7H9-T) (Difco, Detroit, MI) supplemented with 10% (v/v) albumin-dextrose-catalase (ADC, Difco) and 0.05% (v/v) Tween 80 (Sigma, St. Louis, MO) at 37°C. *M. tuberculosis* cultures were incubated in 250 mL nephelometer flasks held either stationary or on a rotating platform at 50 rpm and OD_{600} readings were taken daily or every other day with a spectrophotometer to monitor growth.

Isoniazid Treatment: *M. tuberculosis* bacteria were grown at 37°C with shaking to early log phase (OD_{600}=0.26). The culture was split into two portions, isoniazid (Sigma) was added to one sample to a final

concentration of 1 μg mL ^{1} and both cultures were incubated at 37°C for 4 h.

Heat Shock Experiments: *M. smegmatis* bacteria were grown at 30°C in 7H9-T to early log phase (OD_{600} 0.3). The culture was split into three aliquots and each was exposed to a different condition: no shock (30°C, 30 min), 15-min heat shock (15 min at 30°C, then 15 min at 42°C) and 30-min heat shock (30 min at 42°C). Cultures of *M. tuberculosis* H37Rv bacteria were grown at 37°C to mid-log phase (A_{600} 0.6) and split into two portions. The samples were incubated for 15 min at either 37°C (control) or 45°C (heat shock).

RNA Later™ Evaluation: To assess inhibition of RNA degradation, replicate log-phase cultures of mycobacteria were treated as follows:

a. No heat shock (37°C, 15 min)
b. Heat shock (45°C, 15 min)
c. Fifteen min heat shock at 45°C followed by addition of an equal volume of RNALater™ (Ambion, Austin, TX) and incubation at 37°C for 40 min.
d. Fifteen min heat shock at 45°C followed by addition of an equal volume of 7H9-T medium and incubation at 37°C for 40 min.

To measure inhibition of transcription, *M. smegmatis* cultures were subjected to:

a. No heat shock (30°C, 15 min)
b. Heat shock (42°C, 15 min) in the presence of RNALater™ (1:1 v/v)
c. Heat shock (42°C, 15 min) in the presence of 7H9-T medium (1:1 v/v).

RNA Isolation: RNA was harvested by a modification of the method of DesJardin [4]. Upon completion of a treatment, bacteria were immediately harvested by centrifugation (1 min, 25000 x g, 8°C) and transferred to Fast Prep tubes (Bio 101, Vista, CA) containing Trizol (Life Technologies, Gaithersburg, MD). In some cases, cultures were mixed with an equal volume of RNALater™ prior to centrifugation. Mycobacteria were mechanically disrupted in a Fast Prep apparatus (Bio 101) [5]. The aqueous phase was recovered, treated with Cleanascite (CPG, Lincoln Park, NJ) and extracted with chloroform-isoamyl alcohol (24:1 v/v). Nucleic acids were ethanol precipitated. DNaseI (Ambion) treatment to digest contaminating DNA was performed in the presence of Prime RNase inhibitor (5'-3', Boulder, CO). The absence of residual DNA was confirmed by the lack of a product after 25 cycles of PCR using primers specific for the *hspX/acr* gene for *M. tuberculosis* RNA samples or the *ahpC* gene for *M. smegmatis* samples. RNA integrity was monitored by agarose gel electrophoresis and purity and concentration were determined by spectrophotometry.

Total RNA Labeling: Direct labeling of total mycobacterial RNA (0.5-2 μg) was performed with either psoralen-biotin or psoralen-fluorescein (0.4 nmol label μg ^{1} RNA, Schleicher & Schuell, Keene, NH) by UV irradiation (365 nm) for 20-40 minutes at 4°C in diethyl pyrocarbonate-treated (DEPC) water (Ambion). Enzymatic labeling was performed by reverse transcription of mycobacterial RNA with Superscript II reverse transcriptase (Life Technologies) in the presence of biotin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) using "arbitrary" decamers to prime cDNA synthesis. Arbitrary decamers were produced by pooling three oligonucleotide syntheses in which an A, G, C, or T residue was randomly incorporated at each position, except that a G or C residue was randomly incorporated at every third position and the first biased G or C substitution fell in either the first, second, or third position of the oligonucleotide in one of the three syntheses. This design takes into account the high overall G + C content of *M. tuberculosis* and the strong G/C bias in the third codon position of some ORFs [6-8]. RNA was heat denatured in the presence of 2 μg decamers per μg RNA and added to reverse transcription mixes containing 1x first strand RT buffer (Life Technologies), 10 mM dithiothreitol, dNTPs (0.56 mM dATP, dGTP and dCTP and 0.23 mM dTTP) and 180 U reverse transcriptase. Biotin-dUTP was added (1.75 nmol) and RT reactions were incubated at room temperature for 5 min then at 42°C for 2.5 h. Prior to purification by Centrisep spin column chromatography (Princeton Separations, Adelphia, NJ), the volumes of both direct and enzymatic labeling reactions were adjusted to 99 μL in dH_2O (DEPC-treated for RNA samples) and 1 μL sheared salmon sperm DNA (10 mg mL ^{1}) was added as carrier.

Miniarray Hybridizations: Miniarrays for the dot blot hybridization experiments were prepared by spotting 100 ng of purified PCR products (33 ng μL ^{1}) onto discrete locations of at least two nylon membranes (Hybond N+, Amersham Biosciences, Piscataway, NJ,) and allowed to air dry. Double-stranded DNA was denatured with 0.5 N NaOH and cross-linked to the membrane using a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Arrays were rinsed in 5x SSC (750 mM NaCl, 75 mM sodium citrate) and prehybridized for 30 min at 42°C with ECL Gold stringent hybridization solution (Amersham Biosciences). Each array was hybridized with a labeled total RNA (0.5-2 μg) or cDNA (equivalent to 0.1-0.5 μg of initial RNA mass) sample in 1.5 mL stringent hybridization solution for 14-16 h at 42°C with rotation, then washed in 0.1x SSC + 0.4% SDS at 55°C and 2x SSC at 25°C. After washing, the arrays were blocked with 1% Amersham ECL blocking agent in TBS (100 mM Tris pH7.5, 150 mM NaCl), reacted with streptavidin-horseradish peroxidase (HRP, Amersham

Biosciences) or anti-fluorescein-HRP antibody (Amersham Biosciences) diluted 1:1000 in blocking solution, washed in TBS + 0.1% Tween 20 and detected on X-ray film (Eastman Kodak, Rochester, NY) by enhanced chemiluminescence (Amersham Biosciences). Differential expression was determined by quantifying the density of each spot on the arrays with the AlphaImager 2000 image analysis system (Alpha Innotech, San Leandro, CA) and normalizing the value by the densities of control genomic DNA and/or 16s rDNA spots. The mean density of the control spots for the set of arrays being compared was adjusted to a common density value. The factor used to obtain this value was multiplied by the densities of the other spots on each array to give normalized density values. Normalized values were compared between blots to obtain a ratio of the relative amount of mRNA present for a given gene.

RESULTS

Comparison of Direct Chemical with Enzymatic Labeling: Equal amounts of total RNA from *M. smegmatis* bacteria were directly labeled with psoralen-biotin or psoralen-fluorescein or enzymatically labeled with biotin-dUTP RT reactions. Tenfold dilutions of the purified probes were made and equal volumes spotted onto nylon membranes. The nucleic acids were UV cross-linked and reacted with either streptavidin-HRP for detection of biotin or anti-fluorescein-HRP antibody for detection of fluorescein. Based on signal intensities from the dilutions, the limit of detection was ~1 ng for the psoralen-fluorescein-labeled RNA, ~100 pg for the psoralen-biotin-labeled RNA and 10 pg for the RT-labeled RNA (data not shown). In preliminary miniarray hybridizations, use of the RT-labeled sample led to higher levels of non-specific signal across the membrane than either of the psoralen-labeled samples (data not shown). Because of its 10-fold higher labeling, the psoralen-biotin-labeling method was used as the direct-labeling method for the miniarray experiments described below.

Reproducibility: To assess reproducibility of the labeling reactions, RNA samples from three replicate *M. smegmatis* cultures were labeled by direct psoralen-biotin incorporation or by enzymatic incorporation of biotin-dUTP into cDNA. The RNA samples were hybridized under stringent conditions to miniarrays containing denatured PCR products representing 13 *M. smegmatis* genes (Table 1) plus control DNA (genomic DNA and 16s rDNA). Coefficients of variation (CVs) were determined for each gene on data normalized among arrays by control spot intensities. For both methods, CV values ranged from 1% to 18% for individual genes. The mean CVs for all genes on the arrays were 6.1% for the psoralen-labeled samples and 8.2% for the RT-labeled samples (Table 2).

Table 1: *M. smegmatis* Genes used in Miniarray Experiments

Gene	Product
ahpC	Alkyl hydroperoxide reductase
ami	Acetamidase
fxbA	Ferric exochelin biosynthesis enzyme
gltA	Citrate synthase
gyrB	DNA gyrase subunit B
hisD	Histidinol dehydrogenase
hsp60	65kDa heat shock protein
IS1549	Insertion element
IS6120	Insertion element
katG	Catalase-peroxidase
lon	ATP-dependent protease
mysA	Primary sigma factor
recA	Recombinase
secA	Preprotein translocase

Table 2: Reproducibility of Transcript Labeling Methods in Miniarray Analyses

Gene	Psoralen*	RT*
ahpC	6.20%	6.75%
ami	4.81%	17.62%
fxbA	7.05%	11.16%
gltA	18.39%	3.08%
gyrB	5.60%	6.58%
hisD	7.62%	12.76%
hsp60	5.07%	7.41%
IS1549	6.37%	17.01%
IS6120	4.45%	9.81%
lon	5.64%	2.63%
mysA	2.05%	7.70%
recA	0.99%	3.85%
secA	4.57%	0.51%
mean CV	6.06%	8.22%

*Values expressed as percent coefficient of variation (CV)

Preferential Labeling: To determine if the direct-labeling method produced the same distribution of labeled products as the RT-labeling method, identical arrays (Table 3) were hybridized with biotin-labeled samples produced by psoralen-labeling or RT-labeling of RNA isolated from the same pair of *M. tuberculosis* heat shock samples and examined for signal intensity from each gene. Most spots but not all (e.g., *acr*), gave similar intensities with either labeling method (e.g., *glnA*, Fig. 1). In addition, although most of the genes, including *hsp60*, showed similar levels of differential expression, some genes displayed considerably different expression ratios when comparing psoralen with RT labeling methods. For example, *sigF* gene induction was calculated to be 1.3-fold in the psoralen-labeled samples but 2.2-fold in the RT-labeled samples; *phoP* gene induction was calculated to be 1.8-fold in the psoralen-labeled samples but 1.0-fold in the RT-labeled samples (data not shown).

Table 3: *M. tuberculosis* Genes used in Miniarray Experiments

TB gene	Rv number†	Product
aceA/icl	Rv0467	isocitrate lyase
acr/hspX	Rv2031c	16kDa -crystallin homolog, Hsp20 family
*ahpC**	Rv2428	alkyl hydroperoxide reductase
asd	Rv3708c	L-aspartic-beta-semialdehyde dehydrogenase
dnaA	Rv0001	replication initiator protein
dnaN	Rv0002	DNA polymerase III -chain
efpA	Rv2846c	putative efflux pump
fadE24	Rv3139	acyl-CoA dehydrogenase
fas	Rv2524c	type-1 fatty acid synthetase
gap	Rv1436	glyceraldehyde 3-phosphate dehydrogenase
glnA1	Rv2220	probable glutamine synthetase A
hsp60	Rv0440	65kDa heat shock protein, GroEL homolog
inhA	Rv1484	enoyl-ACP reductase
iniA	Rv0342	unknown protein-induced by isoniazid
IS6110		insertion element
kasA	Rv2245	beta-ketoacyl-ACP synthase
katG	Rv1908c	catalase-peroxidase
phoP	Rv0757	transcriptional regulator
plcB	Rv2350c	phospholipase C
rpoB	Rv0667	RNA polymerase b-subunit
rv0365c		unknown protein-allows increased survival of *M. smegmatis* in macrophages
rv2235		unknown protein-allows increased survival of *M. smegmatis* in macrophages
rv2958c		putative glycosyltransferase-allows increased survival of *M. smegmatis* in macrophages
rv2962c		putative glycosyltransferase-allows increased survival of *M. smegmatis* in macrophages
sigF	Rv3286c	alternate sigma factor
sodA	Rv3846	superoxide dismutase

† Rv numbers are as annotated in Cole *et al.* [8]
* *ahpC* targets on miniarrays were from *M. smegmatis*. This ORF is 81% identical to the *M. tuberculosis* gene

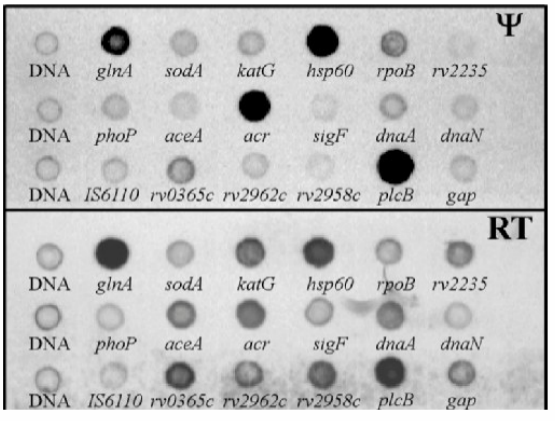

Fig. 1: Different Signal Intensities are Obtained with Direct and Enzymatic Labeling. Identical aliquots of *M. tuberculosis* RNA were Labeled with Psoralen-biotin ( ) or Biotin-dUTP during Reverse Transcription (RT) and used to Probe Miniarrays of Selected *M. tuberculosis* Genes

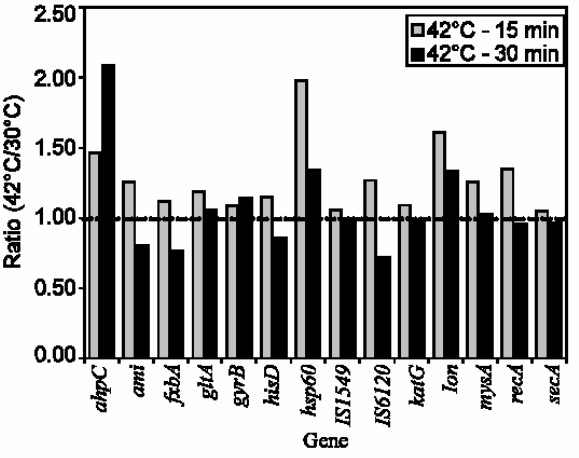

Fig. 2: Differential Gene Expression in *M. smegmatis* after Heat Shocks. Aliquots of RNA from *M. smegmatis* Cultures Subjected to Heat Shock (42°C) or Maintained at 30°C were Labeled with Psoralen-biotin and Hybridized to Replicate Miniarrays of Selected *M. smegmatis* Genes. Ratios were Obtained by Dividing the Density of a given Spot on an Array Probed with RNA from a Shocked Culture by the Density of the Corresponding Spot on an Array Probed with RNA from a Culture Maintained at 30°C. Ratios Greater than One Indicate Larger Numbers of Transcripts for a Gene after the Indicated Heat Shock

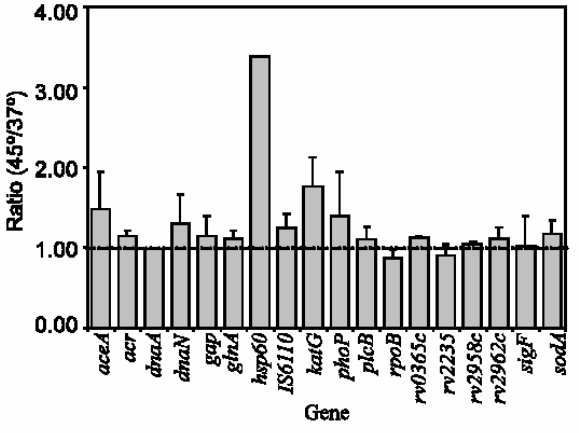

Fig. 3: Differential Gene Expression in M. *tuberculosis* after Heat Shock. Aliquots of RNA from M. *tuberculosis* Cultures Either Subjected to Heat Shock (45°C) or Maintained at 37°C were Labeled with Psoralen-biotin and Hybridized to Replicate Miniarrays of Selected M. *tuberculosis* Genes. Ratios were Obtained by Dividing the Density of a given Spot on an Array Probed with RNA from M. *tuberculosis* Shocked at 45°C for 15 min by the Density of the Corresponding Spot on an Array Probed with RNA from a Culture Maintained at 37°C. Data are given as the mean ratios ±SD and Ratios Greater than One Indicate Larger Numbers of Transcripts for a Gene after Heat Shock

Gene Expression Studies

Heat Shock with *M. smegmatis*: To evaluate the performance of the miniarray system, RNA samples from heat-shocked *M. smegmatis* cultures were labeled with psoralen-biotin and hybridized to arrays containing 14 *M. smegmatis* genes (Table 1) and control spots.

The RNA samples were isolated from aliquots of a culture which had been left at 30°C for 30 min (control) or shocked at 42°C for 15 min or 30 min. Most genes, including three putative housekeeping genes (*secA, mysA and gyrB*), showed little change after heat shock, although some showed slightly reduced expression after the longer heat shock (Fig. 2). As expected for the *hsp60* gene, the signal in the 15-min heat-shocked sample was nearly 2-fold greater than that in the control sample, but the signal in the 30-min heat-shocked sample was only slight greater than that of the control sample (Fig. 2). The *ahpC* gene, which encodes an alkyl hydroperoxide reductase, displayed a slight induction over time at 42°C; *lon*, a homolog of a gene encoding a heat-induced protease in *Escherichia coli* [9] and other bacteria, was slightly induced after both heat shocks.

Heat Shock with *M. tuberculosis*: To evaluate the transcriptional response of *M. tuberculosis* to heat shock, RNA samples from cultures shocked for 15 min at 45°C or maintained at 37°C (control) were labeled with psoralen-biotin and hybridized to arrays containing 18 *M. tuberculosis* genes as well as 16s rDNA and genomic DNA control spots (Table 3). Most of the genes evaluated showed little or no differential expression after the temperature shift (Fig. 3). The most notable exception was *hsp60*, which displayed greater than three-fold induction. In addition, the catalase/peroxidase gene *katG* was induced approximately 1.8-fold after heat shock. The genes encoding a putative two-component system response regulator (*phoP*) and an isocitrate lyase (*aceA/icl*) were both induced slightly (approximately 1.4-fold and 1.5-fold, respectively), but the induction levels were more variable than those seen with either *hsp60* or *katG* (Fig. 3).

Effects of Isoniazid Treatment: Psoralen-labeled RNA from cultures of *M. tuberculosis* H37Rv bacteria exposed to the anti-tuberculosis drug isoniazid (INH) and from unexposed control cultures were hybridized to miniarrays (Table 3) containing eight genes whose transcription had previously been examined after INH treatment [10, 11] plus control spots (Table 3). Six of the eight genes behaved essentially as reported in the literature (Fig. 4). In contrast, the *ahpC* gene was repressed approximately 2.4-fold in the miniarray analysis but induced 2.8-fold in the competitive fluorescence microarray analysis and the *fadE24* gene was nearly unchanged in the miniarray analysis but induced 3.2-fold by fluorescence microarray analysis [11].

Growth Phase Studies: Gene expression in three *M. tuberculosis* strains (H37Rv, CDC1227 and CDC1551) was examined by comparing expression in cultures grown for 6 days (log phase) with that in cultures grown for 39 days (stationary phase) using miniarrays containing 18 genes (Table 3). Most genes showed less

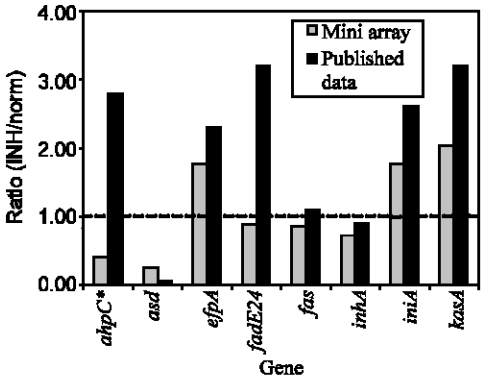

Fig. 4: Differential Gene Expression in *M. tuberculosis* after Treatment with Isoniazid. Aliquots of RNA from *M. tuberculosis* Cultures Either Treated with Isoniazid (1 g mL $^{-1}$) or Untreated were Labeled with Psoralen-biotin and Hybridized to Replicate Miniarrays of Selected *M. tuberculosis* Genes. Ratios were Obtained by Dividing the Density of a Given Spot on an Array Probed with RNA from Cultures Treated with Isoniazid by the Density of the Corresponding Spot on Arrays Probed with RNA from Untreated Cultures. Data are Given as the Ratio Obtained by Miniarray Compared with Published Data [10, 11] and Ratios Greater than One Indicate Larger Numbers of Transcripts for a Gene after Isoniazid Treatment. Asterisk Indicates that the *ahpC* Gene Target was Amplified from *M. smegmatis* rather than *M. tuberculosis*

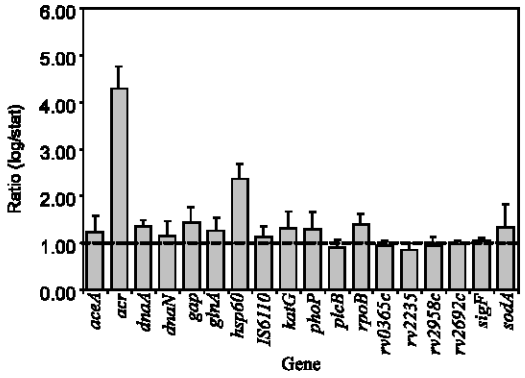

Fig. 5: Differential Gene Expression in *M. tuberculosis* in Log vs. Stationary Phase. Aliquots of RNA from Three *M. tuberculosis* Isolates (H37Rv, CDC1227 and CDC1551) either in Log Phase or Stationary Phase were Labeled with Psoralen-biotin and Hybridized to Replicate Miniarrays of Selected *M. tuberculosis* Genes. Ratios were Obtained by Dividing the Density of a Given Spot on an Array Probed with RNA from Cultures in Log Phase (6 d) by the Density of the Corresponding Spot on Arrays Probed with RNA from Stationary-phase Cultures (39 d). Data are Given as the Mean Ratios ± SD from all Three Isolates and Ratios Greater than One Indicate Larger Numbers of Transcripts for a Gene in Log Phase

than 1.5-fold induction or repression, but two genes, *acr* and *hsp60*, were significantly repressed at stationary phase compared with log phase in all three strains (Fig. 5). Compared with log-phase cultures, the *hsp60* gene in the stationary cultures was repressed an average of 2.4-fold with a range of 2.1- to 2.7-fold among the three isolates and the *acr* gene was repressed an average of 4.3-fold (range of 3.8- to 4.8-fold) in stationary phase.

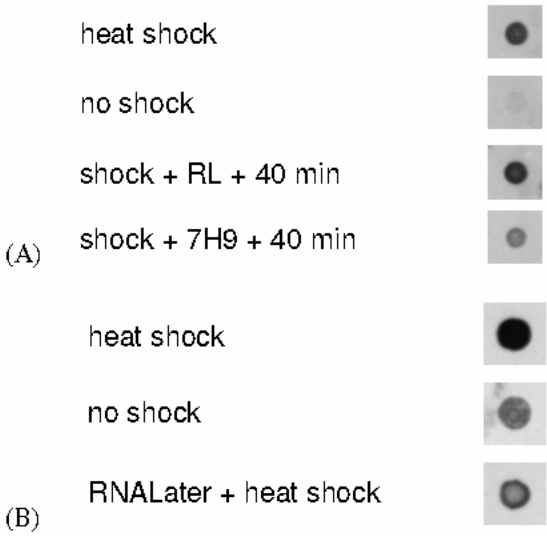

Fig. 6: RNALater™ Stabilizes and Inhibits Transcription of *hsp60* mRNA. A) Aliquots of RNA from *M. tuberculosis* Cultures Subjected to Heat Shock (45°C), Maintained at 37°C, or Subjected to Heat Shock, Mixed with RNALater™ or 7H9-T Broth and Incubated for 40 min at 37°C (to allow a decline in the level of *hsp60* mRNA) were Labeled with Psoralen-biotin and Hybridized to Replicate Miniarrays of Selected *M. tuberculosis* Genes. Array Spots Revealing *hsp60* Expression Levels after each Treatment are shown. B) Aliquots of RNA from *M. smegmatis* Cultures Subjected to Heat Shock (42°C), Maintained at 30°C, or Mixed with RNALater™ Prior to Heat Shock were Labeled with Psoralen-biotin and Hybridized to Replicate Miniarrays of Selected *M. smegmatis* Genes. Array Spots Revealing *hsp60* Expression Levels after each Treatment are Shown

Evaluation of an RNA Stabilizing Solution in Mycobacteria: Differential expression of the *hsp60* gene in heat shock experiments was used to assess the ability of RNALater™, an aqueous RNA stabilizing reagent, to stabilize RNA in mycobacteria, an application for which its effectiveness had not been demonstrated. To measure the effect on RNA degradation, a culture was heat shocked, mixed with an equal volume of fresh 7H9-T or RNALater™ and incubated for 40 min and RNA levels assessed by miniarray (Fig. 6a). As expected, the 7H9-T sample had substantially lower levels of *hsp60* transcript than a sample that was heat shocked and immediately processed for RNA. The *hsp60* transcript levels were essentially the same in the sample that was heat shocked and immediately processed for RNA extraction and in the sample to which RNALater™ was added and incubated for an additional 40 min. RNALater™ also appears to inhibit transcription in mycobacteria: a culture mixed with an equal volume of RNALater™ prior to a 15-min heat shock showed essentially the same level of *hsp60* transcript as a culture which was not heat shocked (Fig. 6b).

DISCUSSION

The safe manipulation of viable *M. tuberculosis* cultures under the required biosafety level 3 conditions is necessarily deliberate, cumbersome and time consuming. To address concerns that such lengthy manipulations might influence the quality and reliability of an RNA preparation, RNALater™ was evaluated for RNA-stabilizing activity in mycobacteria. Using the differential expression of *hsp60* as an indicator, it was determined that RNALater™ was effective in inhibiting both RNA degradation and transcription. These results suggest that this reagent should allow the maintenance of transcriptome as it was at the time of RNALater™ treatment. This, in turn, should facilitate studies that might require a lengthy separation procedure such as for experimental infections of cell cultures or animals in which differential centrifugation would be used to recover bacteria from the eukaryotic cell background prior to extracting RNA.

In the studies reported here, a psoralen-biotin direct labeling method was used because initial studies revealed that the psoralen-biotin labeling method was at least 10-fold more sensitive than a psoralen-fluorescein system and that the latter gave poor signals on miniarray hybridizations. Miniarray hybridizations were then used to compare the psoralen-biotin system with enzymatic incorporation of biotin-dUTP into cDNA. Although the RT-labeling system was at least 10-fold more sensitive than the psoralen-biotin labeling, the enzymatic method gave considerably higher levels of background signal in hybridization experiments. Reproducibility of the hybridization intensities for replicate samples was essentially the same for the two labeling methods. However, differences in intensities of hybridization signal from several genes were observed when comparing psoralen-biotin with biotin-dUTP labeling, which might affect the calculated expression ratios of some transcripts. Additional studies are needed to determine which system gave the correct representation of the transcription profile for these discrepant results.

Gene expression studies using previously characterized environmental challenges were used to assess the performance of the miniarray system. Because this system, as well as high-density micro array hybridization systems, actually measures differences in steady state levels of mRNA, 'induction' is an increase in a steady state level which may correspond to increased transcription, decreased degradation, or a change in apparent mRNA levels due to a change in the steady state level of rRNA. In heat shock experiments with both *M. smegmatis* and *M. tuberculosis*, the differential expression of the *hsp60* gene observed in miniarrays was consistent with previous observations [12, 13], with *hsp60* expression increasing 2- to 3-fold after heat shock. The detoxification enzymes *ahpC* and

katG were also induced by heat shock in the mycobacteria. The induction of *ahpC* by heat shock has been demonstrated in *Bacillus subtilis* [14] and oxidative stress has been suggested as a major cause of heat-related cell death in *Saccharomyces cerevisiae* [15, 16]. The *M. tuberculosis phoP* and aceA/*icl* genes were also slightly induced by heat shock, but considering the variability in expression levels, these may not be significant.

The miniarray assay was also used to examine the expression of genes previously shown to be either stably (*fas, inhA*) or differentially (*ahpC, asd, efpA, fadE24, iniA, kasA*) expressed upon exposure to the anti-tuberculosis drug isoniazid [10, 11]. Although the miniarray system did not produce expression ratios identical to those previously published, six of the eight genes behaved in the same manner with respect to change of expression, i.e., induced, repressed, or unchanged. The differences in the behavior of the two discrepant genes, *ahpC* and *fadE24*, could be a result of differences in RT-labeling (published studies) and direct labeling (this study), strain differences, or other minor variations in experimental protocols.

Miniarrays were also used to examine the differential expression of several *M. tuberculosis* genes in log-phase and stationary phase. As might be expected because of the metabolic slowdown in stationary phase, most genes showed somewhat higher levels of expression in log phase than in stationary phase. Two genes in particular, *hsp60* and *acr/hspX*, showed considerably higher levels of transcript in the log-phase samples. The decreased expression of the *hsp60* gene in stationary phase might reflect less chaperone activity being required because of the overall reduced level of protein synthesis in stationary phase [17]. Decreased expression of *acr/hspX* in stationary phase was initially surprising: the *M. tuberculosis acr/hspX* gene product, an alpha-crystallin-like small heat shock protein (Acr), had been reported to be produced in stationary phase but not in log phase [17] and the expression of *acr/hspX* had been reported to be induced by low oxygen concentrations [18]. The growth of our cultures without aeration might have generated a low oxygen condition that could account for *acr/hspX* expression in our log-phase cultures. With respect to decreased expression in stationary phase, Hu and Coates [19] demonstrated that transcription of *acr/hspX* is inversely related to Acr protein concentration and that higher levels of mRNA are present in log-phase cultures than in cultures grown in a microaerobic condition, which may induce a stationary-phase-like state in the bacteria [20, 21]. Because recent work using cultures grown under slightly different microaerobic conditions has disputed these findings [22], further study is needed to resolve this issue.

Overall, the data demonstrate that the miniarray analysis system is a useful method for detecting differential gene expression in mycobacteria. It is effective for examining the relative expression of multiple genes in parallel and thus is an efficient method with respect to both time and quantity of RNA used per gene. Direct labeling of RNA is a suitable alternative to enzymatic labeling of RNA, but further work is needed to characterize the differences observed in relative hybridization intensities for some genes. RNALater™ is an effective RNA stabilizing solution in mycobacteria, which may prove useful in future studies of gene expression in models of infection.

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