Expression analysis of putative small regulatory RNAs in *Mycobacterium tuberculosis:* effects of growth phase and oxidative stress

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KEY MESSAGE

Results from northern blotting and RT-PCR can reveal which putative genes are authentic small RNA-coding elements and provide preliminary data on the induction characteristics of these important genetic regulators, thereby extending the number of small RNAs in MTB that are experimentally detectable at a transcriptional level, and fostering future research to delineate their regulatory role in this pathogen.

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Introduction

Mycobacterium tuberculosis (MTB) is responsible for about 2.9 million deaths per year, and one third of the world's population are infected with MTB.[1] As an intracellular pathogen, MTB occupies different environmental niches and endures different growth conditions during the infection process. The bacterium responds to changing growth conditions by eliciting a series of adaptive responses that often entail spatial and temporal coordinated regulation of gene expression events that enable MTB to colonise the host, escape immune defence, scavenge for nutrients, and finally survive and proliferate in macrophages.[2] Thus, the pathogenicity of MTB is closely related to its ability to adjust gene expression patterns in the ever-changing environment in the host. Emerging evidence has revealed that small RNA molecules are the key gene expression regulators of adaptive stress responses in a wide range of bacterial pathogens.[3] These genetic elements exert their regulatory function by acting directly on target mRNAs by an antisense mechanism or act indirectly by interacting with regulatory proteins, thereby mediating the translation and/or the stability of mRNA transcripts that encode proteins with virulent functions, or proteins with important roles in adaptive responses during the infection process. In silico analysis of the genome sequence of MTB CDC 1551 has suggested a complex array of 47 putative small RNA-coding genes in this organism, highlighting the importance of these genetic elements in the bacterial adaptive responses during MTB infection.[4] Nevertheless, experimental evidence of the existence of small RNAs in this pathogen is scarce. This study aimed to analyse the possible occurrence of small RNAs in MTB. The presence and relative abundance of RNA transcripts of each of the putative small RNA-coding genes in MTB CDC 1551 was examined using northern blotting and RT-PCR. MTB cells were collected from cultures at different growth phases or after being subjected to hydrogen peroxide treatment for detection of small RNAs. Results from these experiments can reveal which putative genes are authentic small RNA-coding elements and provide preliminary data on the induction characteristics of these important genetic regulators, thereby extending the number of small RNAs in MTB that are experimentally detectable at a transcriptional level, and fostering future research to delineate their regulatory role in this pathogen.

Methods

This study was conducted from February 2010 to July 2011. The MTB clinical reference strain CDC 1551 was grown in Middlebrook 7H9 broth at 37°C. Exponentially grown CDC 1551 cultures were harvested at OD600 between 0.4 and 0.6; stationary cultures were harvested 1 week after OD600 had reached 0.8.

A total of 47 putative MTB CDC 1551 small RNAs were identified by the sRNAPredict2 program.[4] These putative small RNAs are located in the chromosomal intergenic regions of the MTB CDC 1551 genome, and their length ranged from 60 to 500 nucleotides.

MTB cells at different growth stages (exponential/stationary) or after being subjected to H_2O_2 challenge were harvested by centrifugation. Total RNA was purified from the cell pellets with Trizol (Invitrogen, CA, USA). To enrich the small RNAs in the preparation, the 16S and 23S rRNA species were removed from the total RNA using the MICROBExpress kit (Ambion, USA). For northern blot analysis, RNA samples were fractionated on denaturing gel. The relative abundance of 5S rRNA was used to standardise the loading amounts

between samples. After electrophoresis, RNA was transferred to Hybond-N+ membranes (Amersham Life Science, UK) by electroblotting. Expression of small RNAs was detected by hybridising the membranes with specific probes that were PCR amplified from the MTB CDC 1551 genomic DNA using oligonucleotide primer pairs covering entirely each of the putative small RNA-coding genes. The labelling of the probes with digoxigenin and the chemiluminescence detection of the hybridised small RNAs were performed using a commercially available kit (Roche Diagnostics, Switzerland). As a control, the expression of a constitutively expressed MTB gene, sigA,[5] was also detected in parallel.

Total RNA prepared by the Trizol method was treated with RNase-free DNase to remove potential DNA contamination. The purified RNA was reverse-transcribed using random hexamers in the SuperScript III Reverse Transcription kits (Invitrogen). PCR was performed using reagents from Qiagen (Germany). Reactions for the MTB housekeeping gene, sigA, were also run in parallel as control.

Cells of MTB CDC 1551 at the exponential phase were first harvested by centrifugation and re-suspended in a minimal volume of Middlebrook 7H9 broth. Ten times the volume of Middlebrook 7H9 broth with 20 mM H_2O_2 was then added to the cell suspension and incubated at 37°C for 16 hours. The isolation of total RNA and the detection of small RNA expression were conducted as described above.

Results

Detection of the expression of putative small RNAs in MTB CDC 1551 at different growth phases

Total RNA purified from exponential and stationary phase cultures of CDC 1551 was used for the detection of the 47 putative small RNAs. Using northern blotting and RT-PCR, the small RNA transcripts accumulated in these two growth phases were examined. Signals corresponding to the transcripts of five putative small RNAs (MTBsRNA-

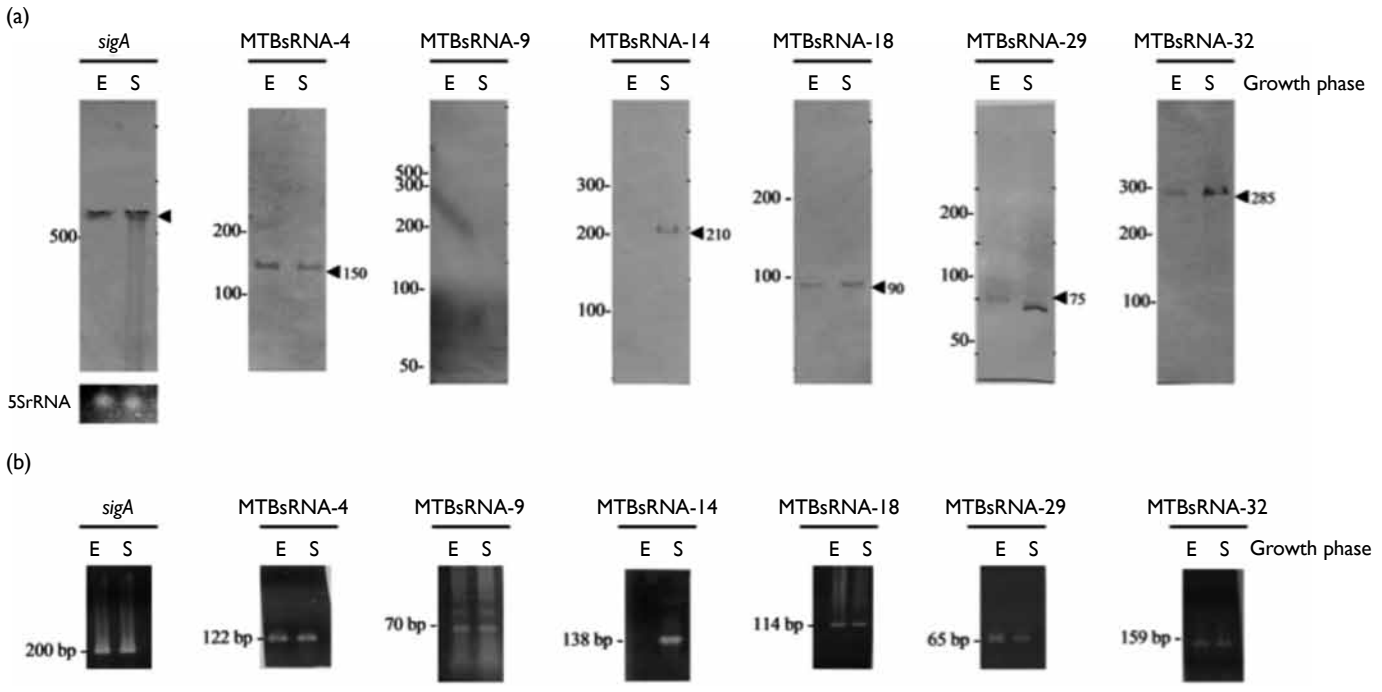

FIG 1. Experimental verification of the predicted small RNAs in different phases of *Mycobacterium tuberculosis* (MTB) growth: MTB cultures grown to exponential (E) or stationary (S) phases were used in this experiment. (a) Northern blots hybridised with digoxigenin-labelled DNA probes. Total RNA samples depleted with rRNAs were separated by denaturing polyacrylamide gel electrophoresis and electro-transferred on to Hybond-N+ membrane, followed by hybridisation with the probes at 42°C overnight. After washing with 2X SSC, the blots were developed for slightly varied time periods; thus the relative intensities of the signals may not accurately reflect the relative abundance of each small RNA transcript. 5S rRNA, which was stained with SYBR Green dye on the gel, was used as a control for RNA loading. The MTB housekeeping gene, sig A, was used as an internal control for monitoring the RNA integrity. Hybridisation bands in the blots are indicated by arrows. The approximate positions of the size standards shown on the left of the blots were obtained by calibrating the blots to a RNA ladder run on a separate gel. The transcript sizes of the small RNAs on the right were estimated by comparing to these standards. For sig A, as the hybridisation band lay outside the range of the standards, its transcript size was not determined. (b) The same batch of RNA samples analysed by northern blotting was used in this RT-PCR analysis. After reverse transcription, the cDNA samples were PCR amplified using the appropriate set of oligonucleotide pairs. After PCR, the reactions were assessed on an agarose gel and stained with SYBR Green dye. The sizes of the reaction products are indicated on the left. The presence of the MTBsRNA-9 transcript was detectable only after RT-PCR amplification.

4, -14, -18, -29, and -32) were detectable by northern analysis (Fig 1a). On the blots, each small RNA was visible as one distinct hybridisation band, suggesting the presence of only one form of transcript for each small RNA under the two growth phases. A comparison of the relative transcript levels of the small RNAs in the two growth phases revealed variation in their expression pattern. For two small RNAs, MTBsRNA-4 and -18, expression appeared to be constitutive as the abundance of their transcripts did not change significantly between the two growth phases. In contrast, up-regulation of expression in the stationary phase was observed for three small RNAs (MTBsRNA-14, 29, and -32). For MTBsRNA-29 and -32, expression in this growth phase was moderately increased. For the small RNA MTBsRNA-14, expression was exclusively induced in stationary phase. As small RNAs are important gene regulators in bacteria, these findings imply a regulatory role of the three differentially expressed MTB small RNAs in mediating gene expression in response to entry into the stationary phase.

The expression of the 47 putative small RNAs in exponential and stationary phases of MTB growth was further examined using RT-PCR (Fig 1b). Judging from the size of the RT-PCR products, transcripts of five small RNAs (MTBsRNA-4, -14, -18, -29, and -32) whose expression had been confirmed by northern blotting were detectable. Moreover, the expression of an additional small RNA (MTBsRNA-9) was also recognised by RT-PCR. Transcripts of this small RNA appeared at an extremely low level in both growth phases as their RT-PCR only showed very faint bands. Detailed analysis of the results from northern blotting and RT-PCR revealed that, for detecting highly expressed small RNAs (eg MTBsRNA-4, -14, -18, -29, and -32), the results from both methods were consistent. Transcripts of those small RNAs detectable in northern blotting were equally detectable by RT-PCR. Nevertheless, for small RNAs expressed at a low level (eg MTBsRNA-9), northern blotting might not be sensitive enough to detect a low level of the transcript. On the other hand, to detect the differential expression of highly expressed small RNA by RT-PCR, owing to its robust amplification efficiency as well as the fact that detection was performed in a background containing a considerable level of the small RNA transcript, RT-PCR may fail to discriminate the expression differences (compare the RT-PCRs of MTBsRNA-14 with MTBsRNA-29 and -32 in Fig 1). Such findings highlight the need to use two complementary methods for a thorough assessment of the expression of the small RNAs.

In summary, this experiment demonstrated the expression of six small RNAs (MTBsRNA-4, -9, -14, -18, -29, and -32). Three of them (MTBsRNA-14, -29, and -32) were shown to express differentially between the exponential and stationary phases of MTB growth and two (MTBsRNA-4 and -18) were shown to express constitutively in both growth phases. The relative expression of MTBsRNA-9 in both growth phases was not clear (Table).

Examination of the effects of oxidative stress on the expression of putative small RNAs in MTB CDC 1551

Bacterial small RNAs are often stress-induced. To investigate how the expression of the 47 putative MTB small RNAs responded to stress similar to that in the host body during the infection process, exponential cultures of CDC 1551 were subjected to oxidative stress (H_2O_2 treatment). Total RNA was isolated and the expression of the 47 small RNA putative small RNAs was examined as described above. For each small RNA, the expression upon stress was compared

TABLE. Expression characteristics of *Mycobacterium tuberculosis* (MTB) CDC 1551 small RNAs identified*

Small RNA	Predicted length (nt)	Approximate observed lengths (nt)	Growth phases				Oxidative stress (H_2O_2)			
			Northern blotting		RT-PCR		Northern blotting		RT-PCR	
			Exponential phase	Stationary phase	Exponential phase	Stationary phase	-	+	-	+
MTBsRNA-4	122	150	√	√	√	√	√	√	√	√
MTBsRNA-9	70	100	×	×	√	√	×	√	√	√
MTBsRNA-14	138	210, 280	×	√	×	√	×	√	×	√
MTBsRNA-18	114	90	√	√	√	√	√	√	√	√
MTBsRNA-24	121	120	×	×	×	×	×	√	×	√
MTBsRNA-29	65	75	√	√√	√	√	√	×	√	×
MTBsRNA-32	159	285	√	√√	√	√	√	√	√	√

* √ and × denote presence and absence of detectable expression, respectively, √√ denotes a higher level of expression, and + and - denote with and without H_2O_2 treatment, respectively

with an unstressed control. Transcripts for a total of seven small RNAs were detectable by both northern blotting and RT-PCR (Fig 2). These included the six small RNAs (MTBsRNA-4, -9, -14, -18, -29, and -32) identified previously and a new small RNA (MTBsRNA-24). It was apparent that oxidative stress induced marked variation in the expression profile of these small RNAs; some remained unchanged (MTBsRNA-4, -18, and -32) and some displayed up-regulation upon stress (MTBsRNA-9, -14, and -24). For the small RNA MTBsRNA-29, expression was down-regulated upon stress. Of particular interest was the small RNA MTBsRNA-14, as over-expression of which was associated with the appearance of a novel transcript (~280 nucleotides), which was not observed in the previous experiment (Fig 1). This finding suggests the presence of two differentially processed transcripts of MTBsRNA-14. Taken together, this experiment further validated the presence of six small RNAs (MTBsRNA-4, -9, -14, -18, -29, and -32) at the transcriptional level as identified in the growth phase experiments, and confirmed the specific expression of an additional small RNA (MTBsRNA-24) under oxidative stress conditions (Table).

Discussion

In this study, up to seven small RNAs were differentially expressed in MTB according to changes in environmental conditions. Production of such sub-cellular elements was up-regulated under adverse conditions such as oxidative stress and those leading to the onset of stationary phase. Identification of small RNAs in MTB and the existence of small RNA-mediated regulatory functions in MTB have been reported,[6,7] but none of the small RNAs identified matched any of the seven MTB small RNAs identified in our study. The number and pattern of actively transcribed small RNA genes in MTB vary between different pathogenic strains and according to changes in environmental conditions. Our work has contributed to a database on the prevalence as well as strain- and condition-specific features of the expression of small RNA genes in MTB. Such database should facilitate future selection of the key elements that play a significant regulatory role in various physiological parameters of this pathogen.

Comparison of the northern blot results and computational predictions of the small RNAs showed inconsistencies in the size of the transcripts, as the actual sizes measurable in our experiments differed by 15-79% from the predicted sizes (Table 1). This may partly be due to estimation of the lengths of small RNAs by northern blotting that may have more than 20% error, as the strong secondary structures can lead to alteration in gel mobility. In addition, the use of the sRNA predicative program, sRNAPredict2, on the GC-rich genome sequence of MTB may result in false predication of the transcriptional endpoints of the putative small RNAs.[4] Taking these factors into consideration, the predicted length and the observed

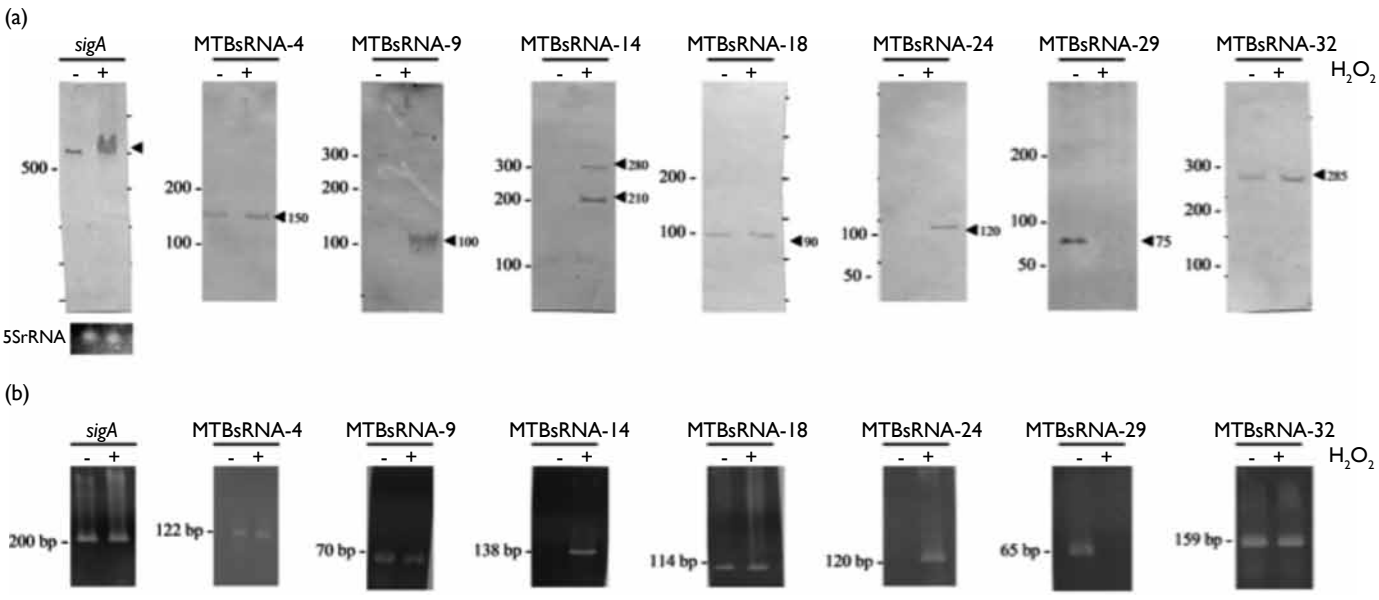

FIG 2. Experimental verification of predicted *Mycobacterium tuberculosis* (MTB) small RNAs after oxidative stress: MTB cells from exponentially grown cultures were collected by centrifugation. After re-suspension in a minimal volume of 7H9 medium, the cells were divided in half. To one half of the cells, ten times the volume of 7H9 medium containing 20 mM of H_2O_2 was added. For the other half, only 7H9 medium was added. After incubating at 37°C for 16 hours, total RNA was isolated from the cells. (a) Northern blot and (b) RT-PCR analyses of RNA samples from the untreated (-) and H_2O_2- treated (+) cells.

length of the small RNAs were within an acceptable range of error in most cases. It is also highly possible that the raw small RNA transcripts are subjected to varying degrees of processing to fine tune their regulatory functions, so that different sizes of small RNA may be generated under different conditions. Future studies should aim to determine the actual size of the transcripts through genome walking, cloning, and nucleotide sequencing, as these experimental approaches can reveal their full genetic sequences. Although sRNAPredict2 predicated correctly 60% of sRNAs for *Pseudomonas*,[7] the rate of predication for MTB was significantly lower, transcripts were detectable only for 7 of the 47 putative sRNAs (about 15%). As sRNAPredict2 predicates sRNAs by searching for co-localisation of genetic structures such as consensus sequences, Rho-independent terminators and putative promoters that are associated with sRNAs in other bacteria, we speculate that sRNAPredict2 may not recognise these genetic structures in the GC-rich genome sequence of MTB. Apart from the difference between the theoretical and measurable size of the small RNA transcripts, a comparison of the results from northern blotting and RT-PCR also revealed discrepancies in these two methods in terms of qualitative difference, level of expression and even product size of such transcripts. For small RNAs whose expression was detectable by northern blotting, the expression was equally recognisable by RT-PCR (eg MTBsRNA-4, -14, -18, -29 and -32). Nonetheless, the reverse was not always true (eg MTBsRNA-9). This phenomenon may be due to the relatively low detection sensitivity of northern blotting. When the expression level of a small RNA is extremely low, such as that of the MTBsRNA-9, the amount of transcript may be well below the detection limit of northern blotting, and hence undetectable. For this reason the expression level of MTBsRNA-9 in both growth phases could not be determined in this study. In addition, for small RNAs expressed at high level (eg MTBsRNA-29 and -32), the RT-PCR reactions appeared to be saturated and failed to discriminate any expression differences. In future studies, we suggest using real-time RT-PCR, which has a higher discriminatory power to test whether the transcription level of specific small RNAs varies according to changes in the level of stress. Furthermore, RT-PCR does not truly reflect the actual size of the transcripts, as the size of the RT-PCR product is defined by the primers, which were designed according to the data generated by theoretical predictions. Hence the size of the transcripts that were measurable in northern blot studies should more accurately reflect the real size of such elements. This explains why the sizes of the transcripts as revealed by northern blotting are mostly larger than the corresponding RT-PCR products.

The small RNA data identified in this study may represent a signature regulatory pattern at the transcriptional level. It is desirable to examine the small RNA expression profile in strains with different virulence levels to assess the correlation between small RNA expression profile and pathogenicity of clinical MTB strains. Together with information on the expression of other virulence factors and proteins responsible for stress response functions, the small RNA data will provide important insight into the basic mechanisms by which MTB maintains long-term viability in the human host and causes infection.

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