Identification of cell cycle regulators in *Mycobacterium tuberculosis* by inhibition of septum formation and global transcriptional analysis

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In *Mycobacterium tuberculosis* the mechanism of septum formation and regulation of cell division remains undefined. In other bacterial species FtsZ polymerization and septum formation are influenced through protein interactions in addition to transcriptional regulation, and the combination of these provides tight regulation of this process. However, homologues of proteins known to affect FtsZ assembly have not been identified and substantiated in *M. tuberculosis*. This suggests that *M. tuberculosis* may possess unique processes for regulation of septum formation. To begin to address this poorly understood aspect of *M. tuberculosis* physiology, FtsZ inhibitors were used to block cell division and the effects on bacterial morphology and the transcriptional response were examined. Inhibition of septum formation prevented cell division and led to bacterial filamentation. Microarray-based transcriptional profiling allowed the evaluation of multiple metabolic processes in response to inhibition of septum formation and when coupled with bioinformatics provided a means to identify regulatory elements and other gene products that probably influence septum formation.

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INTRODUCTION

Cell division at the molecular level has been well studied in a number of bacteria and it is recognized that septum formation is the first committed step of this event (Harry *et al.*, 1999). In general, inhibition of bacterial division at the point of septum formation is characterized by normal chromosome replication and segregation that is not followed by cellular division leading to a filamentous phenotype (Goehring & Beckwith, 2005). Septum formation is dependent on the assembly of FtsZ into the contractile Z-ring at the site of division (Den Blaauwen *et al.*, 1999; Romberg & Levin, 2003) and additional proteins recruited to the septum site in a sequential fashion, resulting in constriction and cell division. In spite of this biochemical information, the current understanding of regulatory elements and the precise nature of the signals driving the coordination of cell division with other cell cycle processes is largely unknown, especially in *Mycobacterium tuberculosis*.

Many of the proteins involved in cell division, such as FtsZ, are conserved across taxa, while others appear limited to

one or a few bacterial groups (Goehring & Beckwith, 2005; Margolin, 2000). These include proteins known to interact with FtsZ and that modulate Z-ring assembly (Goehring & Beckwith, 2005; Romberg & Levin, 2003). The Z-ring is highly dynamic, and proper assembly of this structure and cell division are strongly influenced by direct interactions of FtsZ with ZipA, ZapA, FtsA, MinD, EzrA and SulA (Harry *et al.*, 1999; Harry, 2001; Migocki *et al.*, 2004; Romberg & Levin, 2003). These factors together with transcriptional regulators form a regulatory network that orchestrates the spatial and temporal control of cell division with other cell cycle processes. However, the underlying molecular mechanisms are only partially understood in model organisms and remain largely unknown for many unrelated organisms.

Analysis of the *M. tuberculosis* genome sequence revealed that homologues of the FtsZ-interacting proteins are not present (Table 1). It is likely that *M. tuberculosis* encodes orthologues of the FtsZ-binding proteins that fulfil these functions, but they are not evident. An alternative global approach is needed to identify putative regulators and potential FtsZ-interacting proteins such that networks controlling cell division in *M. tuberculosis* can be elucidated. In this current work we report inhibition of septum formation, morphological changes consistent with prevention

Abbreviation: SOM, self-organizing map.

Supplementary tables of data are available with the online version of this paper at <http://mic.sgmjournals.org>

Table 1. Cell-division-associated proteins of *M. tuberculosis*

Cell division in *M. tuberculosis* (Mtb) is a complex process that requires proteins to partition the replicating chromosome to the poles, select the septal site for Z-ring assembly and complete division. No proteins associated with modulation of Z-ring assembly have been identified in *M. tuberculosis*.

Cell cycle role	Protein	Mtb	Function	Orthologue
DNA replication	XerC/D	+	Recombinase	
Chromosome segregation	SeqA	–	Chromosome segregation	Par A/B
	MukB/E/F	–	Chromosome maintenance	Smc
Site selection	MinC/D	–	FtsZ destabilizing factor and oscillator	
	MinE	–	Topological regulator	
Z-Ring formation	ZapA	–	FtsZ assembly factor	
	FtsZ	+	Z-Ring septum matrix	
	ZipA	–	Stabilizes Z-ring	
	FtsA	–	Stabilizes Z-ring membrane	
Septum formation	FtsE	+	Transporter – unknown septum component	
	FtsH	+	Proteolytic activity	
	FtsK	+	Chromosome positioning	
	FtsQ	+	Unknown	
	FtsW	+	Unknown	
	FtsX	+	Unknown	
	FtsY	+	Unknown	
	FtsI	+	Transpeptidase	
Z-Ring formation regulation	EzrA	–	FtsZ destabilizing factor	
	SulA	–	FtsZ destabilizing factor	

of septum formation and the transcriptional responses associated with this process. This allowed the assignment of potential regulatory elements and FtsZ-interacting proteins in *M. tuberculosis*.

METHODS

Bacterial culture and drug treatment conditions. For all experiments *M. tuberculosis* strain H37Rv was cultivated at 37 °C in Middlebrook 7H9 liquid medium containing 0.2% glycerol, ADC and 0.05% Tween 80, or on Middlebrook 7H11 agar containing OADC (oleic acid, albumin, dextrose and catalase enrichment; Difco). For MIC determinations *M. tuberculosis* was grown to an OD₆₀₀ of ~0.5 and diluted 1:10. Drugs were prepared in DMSO (500–0.5 µM in a twofold serial dilution in 100 µl total volume of <0.05% DMSO), tested in triplicate and MIC values were determined as the lowest concentration of drug that prevented bacterial outgrowth after 7 days incubation. Untreated paired control bacteria were grown under identical conditions to treated bacteria with the exception that no drug was added. For viability testing, drugs were added to 30 ml cultures and incubated for 7 days, and on each day an aliquot was plated on Middlebrook 7H11 agar medium. After continued incubation for 3 weeks, viability of the treated bacteria was determined by enumeration of c.f.u.

Ultrastructural analysis of *M. tuberculosis*. For electron microscopy a 50 µl aliquot of bacteria was mixed with an equal volume of 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2), 5 mM CaCl₂ and 5 mM MgCl₂, and incubated at room temperature (RT) for 1 h. The fixed bacterial cells were collected by centrifugation, washed twice and fixed overnight at 4 °C with 2.5% glutaraldehyde. Final preparation for SEM was achieved by post-fixing with 1%

OsO₄ in sodium cacodylate buffer for 1 h and dehydrating in a graded series of acetone (25–100%). Ultrastructural examination was performed using a JOEL JEM-100CX electron microscope.

Microarray processing. The *M. tuberculosis* microarrays were obtained through the TB Research Materials and Vaccine Testing Contract (N01 AI-75320) at Colorado State University. These arrays contained probes representing the ORFs of *M. tuberculosis* strains H37Rv (<http://genolist.pasteur.fr/TubercuList>) and CDC1551 (www.tigr.org). Oligonucleotides were printed at ~40 µM with SMP-3 pins producing ~100 µm spots. Slides were post-processed using succinic anhydride as described by Brown and colleagues (<http://cmgm.stanford.edu/pbrown/protocols/index.html>). *M. tuberculosis* cultures (30 ml) were grown to an OD₆₀₀ of 0.3 with shaking at 150 r.p.m., each drug was added at its respective MIC (60 µM albendazole, 80 µM thiabendazole), or 0.01% DMSO for the untreated control, and the cultures were grown for 5 h (~0.25 of a generation). Bacterial cells were harvested by centrifugation and suspended in 1 ml TRIzol. Following physical disruption with 0.1 mm zirconium beads, RNA was purified using an RNeasy kit (Qiagen). Approximately 8 µg total RNA was converted to cDNA in the presence of either Cy5- or Cy3-labelled nucleotides as described by Brown and colleagues (<http://cmgm.stanford.edu/pbrown/protocols/index.html>). Hybridization was performed at 42 °C for 12 h. Slides were scanned using a Bio-Rad Chipreader Pro.

Data reduction and analysis. The final microarray dataset resulted from combining two independent replicates of albendazole and thiabendazole treatments, thus four biological replicates of septum formation inhibition. Data reduction and global normalization were performed on the raw fluorescent intensities. Low intensity spots were eliminated based on a 15% mean channel intensity cut-off. The normalized intensity values of treated and control cultures were used to generate ratio and log₂ expression values for each gene. Self-organizing map (SOM) analysis (Xiao *et al.*, 2003)

was performed on the entire dataset. SOM is an unsupervised neural network model that effectively categorizes and clusters genes based on similarities in the gene expression profiles among treatment groups.

Quantitative real-time PCR verification. Quantitative RT-PCR was performed on selected genes to verify the differential gene expression observed through microarray data analysis. Quantitative RT-PCR primers were designed and analyses were performed according to Manganelli *et al.* (2001a). PCR amplification was performed with a thermocycling program of 55 °C for 5 min, then 95 °C for 2 min and 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. The relative number of transcripts for each gene was determined based on linear regression analysis of 100, 10 and 1 ng *M. tuberculosis* genomic DNA (see Table S1 for primer sequences, and the intercept and slope constants for each primer pair; available with the online version of this paper at <http://mic.sgmjournals.org>). All reactions were performed in triplicate on two independent RNA preparations from *M. tuberculosis* treated for 5 h with albendazole or thiabendazole.

Bioinformatic analysis. To identify putative orthologues for genes that influence Z-ring formation, a reciprocal best hits (RBH) dataset was generated comparing the *M. tuberculosis* ORFs using FASTA (Pearson & Lipman, 1988) against ORFs from 20 actinobacterial genomes and from the genomes of *Ralstonia solanacearum*, *Erwinia carotovora* subsp. *atroseptica*, *Escherichia coli* and *Xanthomonas campestris*. Any gene selected for the RBH database aligned with the query over at least 80 % of its length to > 30 % identity as described elsewhere (Bell *et al.*, 2004). The resulting RBH dataset was mined to find putative orthologues of genes that influence Z-ring formation (FtsA, ZapA, ZipA, EzrA, MinD and SulA). BLASTX, BLASTP, TBLASTN analysis against searchable databases prepared from prototype protein datasets of each FtsZ assembly gene were also used. The resulting datasets of encoded FtsZ assembly proteins were analysed using POLYDOT (EMBOSS package; Rice *et al.*, 2000) to confirm the similarity. In all BLAST searches, the percentage identity and bit-score were optimized to yield putative *M. tuberculosis* FtsZ-related proteins. Candidate proteins were further analysed using InterPro release 11 for motifs or domains and compared with those seen in the prototype FtsZ assembly protein datasets (Mulder *et al.*, 2005).

RESULTS

Effect of FtsZ inhibition on bacterial viability and cell morphology

Albendazole and thiabendazole are known inhibitors of tubulin polymerization, and the only known prokaryotic mechanism analogous to tubulin polymerization is FtsZ assembly (Desai & Mitchison, 1998; Downing, 2000). Moreover, the three-dimensional structures of bacterial FtsZ orthologues show striking similarity to the structure of α - and β -tubulin and both FtsZ and tubulin possess GTPase activity (Lowe & Amos, 1998; Nogales *et al.*, 1998). FtsZ of *M. tuberculosis* is similar to other prokaryotic FtsZ genes in sequence and structure, and possesses GTPase activity (White *et al.*, 2000).

Based on the ability of benzimidazole and structurally related compounds to inhibit the GTPase activity of the *M. tuberculosis* FtsZ we hypothesized that albendazole and thiabendazole inhibit septum formation (Huang *et al.*, 2006; Margalit *et al.*, 2004). The MICs for albendazole and

thiabendazole as determined by the microbroth dilution assay (Slayden *et al.*, 2000) were found to be 60–120 μ M and 80–160 μ M, respectively. Treatment of *M. tuberculosis* with 60 μ M albendazole or 80 μ M thiabendazole for 3 days led to ~ 4 and ~ 3 logs reduction in bacterial viability, respectively (Fig. 1). These treatments also significantly altered bacterial cell length, as observed and measured by scanning electron microscopy. Maximum filamentation was achieved by the third day of drug exposure. Albendazole- and thiabendazole-treated cells displayed mean lengths of 6.2 ± 1.4 μ m and 9.6 ± 1.9 μ m, respectively. These observed lengths are in sharp contrast to the length of untreated cells 2.3 ± 0.97 μ m (Fig. 2).

In addition to cell elongation, ultrastructural features indicative of septum formation were not seen (Fig. 3A–D) and this was consistent with the inhibition of Z-ring formation (Goehring & Beckwith, 2005; Huang *et al.*, 2006). As a control, *M. tuberculosis* cultures were also treated with 20 μ M cephalixin and 40 μ M piperacillin, two known inhibitors of FtsI (Pogliano *et al.*, 1997). FtsI is a penicillin-binding protein required in the completion of cell division and acts post-septum formation. As expected the FtsI-treated cells were filamentous but, in contrast to FtsZ-inhibited cells, possessed clearly visible concentric rings indicative of septa (Fig. 3E, F) (Romberg & Levin, 2003).

Whole-genome expression profiling

The global transcriptional response of *M. tuberculosis* to inhibition of FtsZ was assessed through DNA microarray analysis. *M. tuberculosis* was treated with 60 μ M albendazole or 80 μ M thiabendazole for 5 h. The complete dataset of normalized mean log₂ expression and SOM is provided in

Fig. 1. Bacterial growth in the presence of FtsZ inhibitors. Bacterial growth (c.f.u.) is shown after treatment with 60 μ M albendazole (grey bars) or 80 μ M thiabendazole (white bars) at designated time points; black bars, control. Inhibitors were added to exponentially growing cells diluted to an OD₆₀₀ of 0.1 and allowed to grow at 37 °C. Data are mean values $\pm$ SD of three independent experiments plated in triplicate as described in Methods.

Fig. 2. Length distribution of *M. tuberculosis* from albendazole- and thiabendazole-treated cultures. Cultures were treated for 3 days with 60 μ M albendazole (grey bars) or 80 μ M thiabendazole (white bars); black bars, control. The lengths of the bacterial cells were calculated from the co-ordinates of both ends as measured with an electron microscope. Multiple fields were examined and values were calculated in 0.5 μ m increments from greater than 100 bacterial cells per field.

Table S2 (available with the online version of this paper at <http://mic.sgmjournals.org>). In comparison to untreated cells, a total of 250 genes displayed a 1.5-fold or greater change in expression (P values < 0.05) when treated with either albendazole or thiabendazole (Table 2). This represents an altered expression of 6% of the *M. tuberculosis* ORFs, a value consistent with global effects on regulatory cascades and compensatory responses of gene expression involved in multiple cell processes. It was noted that when the expression profiles of albendazole- and thiabendazole-treated cells were compared, there was a $> 70\%$ ($n = 1722$) concordance in the transcriptional response between these treatments, indicating a similar mode of action for both these Z-ring inhibitors. Furthermore, when compared to previously reported expression profiles obtained during inhibition of basic metabolic processes such as transcription and protein synthesis (Boshoff *et al.*, 2004), there was no overlap with the expression profiles induced by albendazole and thiabendazole treatment, thus adding confidence that the observed transcriptional response is due to inhibition of septum formation.

The microarray data were validated by quantitative RT-PCR analysis of selected genes involved in regulation and the cell cycle (Fig. 4). This assessment revealed a strong concordance in gene expression trends as measured by RT-PCR and microarray analyses. Specifically, inhibition of FtsZ polymerization led to an average induction of genes associated with DNA replication and septum formation, and an average repression of genes involved in resolution of the septum and cell wall synthesis.

Fig. 3. Bacterial morphology. Inhibition of FtsZ with 60 μ M albendazole for (A) 3 days and (B) 5 days or with 80 μ M thiabendazole for (C) 3 days and (D) 5 days; inhibition of FtsI with 20 μ M cephalexin for (E) 3 days or with 40 μ M piperacillin for (F) 3 days. Drugs were added to exponentially growing cells diluted to an OD_{600} of 0.1 and allowed to grow at 37 °C.

Construction of a transcriptional map

SOM analysis (Garrigues *et al.*, 2005; Wang *et al.*, 2002; Xiao *et al.*, 2003) was performed on the complete dataset of 3182 transcriptionally active genes to construct a transcriptional map. SOM analysis organized the 3182 genes into 62 groups (0–61), with the number of genes in each group ranging from 23 to 337. Sets of discriminant genes representing the major cell cycle processes of DNA initiation and replication, chromosome segregation, cell division, peptidoglycan synthesis, LAM synthesis, arabinogalactan (AG) synthesis, and mycolic acid and related lipoglycan synthesis were selected to provide a more concise picture of how genes involved in cell division grouped relative to other cell cycle

Table 2. Summary of differentially regulated genes according to functional classification during inhibition of FtsZ polymerization

Functional classification*	Total in genome	Total subjected to analysis	FtsZ inhibition analysis†	
			Up	Down
Whole genome	3924	3182	105	145
Cell wall and cell processes	515	418	16	22
Conserved hypotheticals	911	756	25	31
Information pathways	206	173	4	14
Insertion sequences and phages	137	108	3	1
Intermediary metabolism and respiration	877	705	20	23
Lipid metabolism	225	180	4	12
PE/PPE	167	131	6	3
Regulatory proteins	188	151	6	8
Unknown	607	483	20	19
Virulence, detoxification, adaptation	91	77	1	12

*Functional classification as annotated in Cole *et al.* (1998) or <http://genolist.pasteur.fr/TubercuList/>

†1.5-fold or greater; $P < 0.05$.

processes (Table S3, available with the online version of this paper at <http://mic.sgmjournals.org>). The discriminant gene sets were identified based on experimental work in *M. tuberculosis* and other bacteria (Crick *et al.*, 2001, 2004; Laub *et al.*, 2000, 2002; Mahapatra *et al.*, 2005; Ryan & Shapiro, 2003; Slayden *et al.*, 2003). The relative SOM grouping of genes discriminant for each cell process is displayed as the mean SOM group for all discriminant genes of a single cellular process (Fig. 5).

Several features arose from visualizing the relative placement of discriminant genes based on their SOM assignments. First, as shown in Fig. 5, discriminant genes of DNA

initiation and replication (*dnaA* and *dnaE1*, *dnaN* and *dnaZX*) fell within the SOM range of 9 ± 5 (Greendyke *et al.*, 2002; Salazar *et al.*, 2003), and discriminant genes of DNA segregation (*parA*, *parB* and *ftsK*) were also distributed across a relatively narrow set of SOM groups (SOM mean 15 ± 2) (Goehring & Beckwith, 2005). This SOM clustering is consistent with many of these genes being organized in a regulon structure. Moreover, the SOM assignments reflect transcriptional induction in response to albendazole and thiabendazole treatment, consistent with inhibition of septum formation.

In contrast to the SOM clustering of DNA replication and segregation genes, the genes encoding cell division components were distributed in a bimodal fashion (SOM 15 ± 5 and 53 ± 2) (Fig. 5). This major separation of genes was associated with the up-regulation of one set of genes (SOM 15 ± 5) and down-regulation of another (SOM 53 ± 2) in response to septum inhibition. Notably, the up-regulated genes (*ftsZ*, *ftsW*, *ftsQ*, *ftsH* and *ftsE*) are those involved in septum formation, whereas repressed expression occurred with genes involved in septum resolution (*divIC* and *ftsI*). This is consistent with the observed inhibition of septum formation and reports in other bacteria of temporal regulation of genes encoding septum formation and genes encoding septum resolution (Goehring *et al.*, 2005; Katis *et al.*, 1997).

Interestingly, regardless of genome location the genes encoding peptidoglycan synthesis demonstrated a transcriptional profile that overlapped with the genes for septum formation (Fig. 5). Correlated transcriptional activity of genes involved in septum formation and peptidoglycan synthesis with DNA replication and segregation is observed in other bacteria (Romberg & Levin, 2003). Thus, the transcriptional profiles for these cellular events support our hypothesis that inhibition of septum formation and subsequent interrogation of gene

Fig. 4. Real-time PCR analysis of select cell cycle genes. Mean log₂ expression ratios are presented for cell cycle genes identified by DNA microarray analysis. Data are mean values $\pm$ SD from at least two independent biological samples. Ratios were calculated using the total number of gene targets from treated bacteria compared to a paired untreated control.

Fig. 5. Transcriptional map of cell cycle process in *M. tuberculosis*. (a) Mean expression activity of each SOM group. (b) Coordinates of the transcriptional map used to map cell cycle processes as determined by SOM analysis. (c) Bacterial cell cycle processes mapped by SOM analysis. A set of (discriminant) genes representing chromosome replication, chromosome segregation, cell division and macromolecule biosynthesis (cell wall components) were chosen based on annotation and experimental information.

expression patterns will enable the identification of unknown gene products that participate in or regulate cell division.

It is important to note that genes encoding the synthesis of other mycobacterial cell envelope products (LAM, AG and mycolic acids) do not yield tight clusters based on expression profile analysis (Fig. 5). Together, the transcriptional response and organization of genes involved in cell wall biosynthesis suggest that these cell cycle processes are not linked to septum formation.

Regulatory elements and putative FtsZ-interacting proteins

The transcriptional profile that emerged in response to inhibition of septum formation was coupled with bioinformatic analyses to identify other genes that potentially participated in septum formation and cell division. Specifically, we targeted the identification of transcriptional regulators and proteins that would substitute for septum-associated products (ZipA, ZapA, FtsA, MinD, ErzA and SulA) that were not previously annotated in the *M. tuberculosis* genome. This process led to the identification of 14 genes encoding regulatory proteins. These could be grouped as regulators induced by inhibition of septum formation (Group I in Fig. 5 and Table 3) and those that were repressed by inhibition of septum formation (Group II in Fig. 5 and Table 3).

The Group I regulators included the *mtrA* and *mtrB* two-component system (Ausmees & Jacobs-Wagner, 2003; Moker *et al.*, 2004; Zahrt & Deretic, 2000), TetR-family transcriptional regulator, a Lpr-family transcriptional regulator and

three putative transcriptional regulators. The regulators with repressed expression (Group II) included GntR family regulators, two TetR like regulators, an alternative sigma factor and two putative transcriptional regulators. The differential expression of a large number of transcriptional regulators was consistent with the significant change in gene expression profiles and the wide number of metabolic processes that were altered as a result of cell division inhibition. As discussed below several of these regulators have already been associated with the control of bacterial growth and cell division.

The genes that encode proteins associated with septum formation were narrowly spread between SOM groups 7–18. Thus, to identify genes encoding products that potentially interact with FtsZ and influence Z ring formation we applied detailed bioinformatics focusing on genes in SOM groups within this range. This limitation reduced the number of gene products to be analysed to 553, and when analysed for orthologues of ZipA, ZapA, FtsA, MinD, ErzA and SulA, four ORFs were identified. Two of these ORFs encode putative ZipA orthologues and two encode putative MinD orthologues as determined by bioinformatic analysis. The ZipA-like proteins, Rv2345 and Rv3835, are annotated as conserved hypothetical proteins. The MinD-like proteins are encoded by Rv1708 and Rv3660c, and are annotated as a putative initiation inhibition protein and a putative septum-site-determining protein, respectively. These genes have BLASTP bit scores of 30–100 to their respective orthologue. Thus, the use of gene expression profiling not only provided further confirmation of the involvement of already annotated gene products, such as FtsZ and FtsI, in cell division, but when coupled with bioinformatic approaches

Table 3. Analysis of regulatory elements

ORFs used as discriminant genes for cell cycle processes. Gene names, functional class and gene function are as annotated in Cole *et al.* (1998) or <http://genolist.pasteur.fr/TubercuList/>

Group	ORF	Gene	Functional class	Cellular function	SOM group	Expression (log ₂)	<i>p</i> val
I	<i>rv3183</i>		Regulatory proteins	Putative transcriptional regulator	4	1.22	0.06
	<i>rv1675C</i>		Regulatory proteins	Putative transcriptional regulator	6	0.71	0.04
	<i>rv3249C</i>		Regulatory proteins	Transcriptional regulator (TetR/AcrR family)	8	1.50	0.01
	<i>rv3291C</i>		Regulatory proteins	Transcriptional regulator (Lrp/AsnC family)	9	1.21	0.05
	<i>rv3246C</i>	<i>mtrA</i>	Regulatory proteins	Two-component response regulator	10	1.37	0.05
	<i>rv3245C</i>	<i>mtrB</i>	Regulatory proteins	Sensor histidine kinase	11	1.41	0.02
	<i>rv1151C</i>		Regulatory proteins	Putative transcriptional regulator	13	0.68	0.02
II	<i>rv0238</i>		Regulatory proteins	Transcriptional regulator (TetR/AcrR family)	50	-0.81	0.02
	<i>rv1534</i>		Regulatory proteins	Transcriptional regulator (TetR/AcrR family)	50	-0.66	0.01
	<i>rv0182C</i>	<i>sigG</i>	Information pathways	Sigma-70 factors ECF subfamily	51	-0.72	0.04
	<i>rv0894</i>		Regulatory proteins	Putative transcriptional regulator	51	-0.66	0.01
	<i>rv0494</i>		Regulatory proteins	Transcriptional regulator (GntR family)	52	-0.99	0.04
	<i>rv2250C</i>		Regulatory proteins	Putative transcriptional regulator	52	-0.69	0.01
	<i>rv0792C</i>		Regulatory proteins	Transcriptional regulator (GntR family)	53	-0.87	0.04
	<i>rv0600C</i>		Regulatory proteins	Sensor histidine kinase	54	-1.33	0.04

allowed for the identification of putative FtsZ-interacting gene products.

DISCUSSION

Microarray analyses have proven effective in identifying genes of *M. tuberculosis* that respond to environmental stimuli, including drug treatments (Betts *et al.*, 2002, 2003; Boshoff *et al.*, 2004; Manganelli *et al.*, 2001b, 2002; Sherman *et al.*, 2001; Wilson *et al.*, 1999). Thus, we employed microarray analyses to evaluate our hypothesis that inhibition of septum formation would increase the expression of cell division genes and genes involved in upstream processes such as DNA replication and segregation, and would repress expression of genes involved in cell division events downstream of septum formation. It was also anticipated that this approach would allow for the identification of unassigned mycobacterial gene products involved in septum formation and regulation of cell division.

Albendazole and thiabendazole are known inhibitors of tubulin polymerization and, recently, additional benzimidazole and structurally related compounds were shown to inhibit the GTPase activity of *M. tuberculosis* FtsZ (Margalit *et al.*, 2004). Albendazole and thiabendazole are relatively insoluble and have proven difficult to use with *in vitro* assays (White *et al.*, 2000). Nevertheless, as we demonstrated in this work, concentrations within the soluble range of these drugs inhibited the growth of *M. tuberculosis*. It is well established in other organisms and in *M. tuberculosis* that inhibition of FtsZ leads to smooth filamentous cells while, in contrast, inhibition of FtsI produces filamentous cells with distinct septi (Goehring & Beckwith, 2005; Huang *et al.*, 2006; Pogliano *et al.*, 1997; Romberg & Levin, 2003). Analysis of

M. tuberculosis treated with the benzimidazole compounds (FtsZ inhibitors) or known FtsI inhibitors (cephalexin and piperacillin) revealed cellular morphologies fully consistent with observations in other bacteria (Romberg & Levin, 2003). Moreover, when gene expression profiles were evaluated it was found that genes encoding septum formation in *M. tuberculosis* and events that proceed this process were up-regulated upon inhibition of Z-ring formation, and those genes associated with the resolution of septi were down-regulated. Thus, both the morphological characteristics and transcription profiles of *M. tuberculosis* treated with albendazole or thiabendazole demonstrate that septum formation was inhibited through the inhibition of FtsZ polymerization.

Bioinformatic analyses have led to the identification of several *M. tuberculosis* gene products that are involved in septum assembly and cell division. However, this same analysis failed to identify homologues to other cell-division-associated proteins (Table 1). The wealth of transcriptional profiling data obtained in our current studies when coupled with bioinformatic analysis led to the identification of potential orthologues of these otherwise unassigned proteins. With this two-pronged approach, multiple annotated regulatory proteins were defined. Of particular interest was the two-component system encoded by *mtrA* and *mtrB*. In *Caulobacter crescentus* the homologue of this regulatory module is also expressed at the transition of DNA replication and Z-ring formation (Ausmees & Jacobs-Wagner, 2003). Although attempts to knockout *mtrA* in *M. tuberculosis* have failed, similar studies performed in *C. glutamicum* revealed that MtrA-MtrB directly regulates cell morphology and cell wall macrostructure (Moker *et al.*, 2004; Zahrt & Deretic, 2000). It is also of interest to note that

the *TetR*-like transcriptional regulator (Rv3249c) located adjacent to *mtrA/B* is also upregulated. However, it is unknown whether this transcriptional regulator participates in cell division. Other regulatory elements that are of interest include an Lpr-like transcriptional regulator (*rv3291c*) and SigG. In *M. tuberculosis*, *rv3291* expression is inversely related to bacterial growth rate and has been linked to a decrease in cell division, suggesting that Lpr may regulate multiple cellular processes (Betts *et al.*, 2002; Landgraf *et al.*, 1996). SigG is one of 13 sigma factors encoded in this bacterium and elucidating the role of these gene products has been a major focus (Manganelli *et al.*, 2004). Previous expression profiling studies have reported that *sigG* is expressed under exponential growth in aerated conditions (Voskuil *et al.*, 2004). The biological function of this sigma factor and the significance of its repression due to inhibition of Z-ring formation are not known. However, SOM analysis grouped SigG with genes involved with completion of cell division, suggesting that this sigma factor may be a regulatory 'checkpoint' in the *M. tuberculosis* cell cycle. Moreover, it is intriguing that this was the only sigma factor with consistently altered expression in these studies.

Beyond transcriptional control, regulation can be fine-tuned through post-translational mechanisms, including protein interactions. Bioinformatic and transcriptional mapping identified ORFs that encode putative ZipA and MinD orthologues. ZipA and MinD act as FtsZ assembly factors; specifically influencing the assembly or disassembly of FtsZ, respectively (Goehring & Beckwith, 2005). The transcriptional activity and resulting SOM assignments of the putative *zipA* and *minD* ORFs were consistent with a role in cell division.

Clearly, further experimentation is required to elucidate the precise role of the identified regulators and validate the involvement of the ZipA/MinD orthologues in mycobacterial cell division. Nevertheless, these studies now provide a foundation and rationale to target these gene products for further studies. Association of regulatory elements and identification of ORFs that encode proteins with previously undefined roles in Z-ring assembly will help unravel the regulatory circuitry controlling growth. Continued research in this area will help define regulatory cascades used by *M. tuberculosis* to emerge from a long-term persistent infection.

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