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Morphological features and signature gene response elicited by inactivation of FtsI in *Mycobacterium tuberculosis*

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***Objectives*: Universally conserved events in cell division provide the opportunity for the development of novel chemotherapeutics against *Mycobacterium tuberculosis*. The aim of this study was to use the β-lactam antimicrobials cefalexin and piperacillin to inhibit FtsI and characterize the morphological changes and global transcriptional activities of genes to identify a signature response to FtsI inactivation.**

***Methods*: Cefalexin and piperacillin were used to block cell division, and microscopy was used to evaluate the effects on bacterial morphology and ultrastructure. Global transcriptional analysis was performed to determine the impact of FtsI inhibition on cell cycle processes and to identify molecular markers.**

***Results*: Inhibition of FtsI with cefalexin and piperacillin resulted in filamentous cells with multiple concentric rings and occasional branching as visualized by light and electron microscopy. Whole genome microarray-based transcriptional profiling and transcriptional mapping allowed the evaluation of cell cycle processes in response to inhibition of FtsI and characterization of transcriptional response and cell cycle processes.**

***Conclusions*: This study substantiated that FtsZ-ring constriction and septal resolution require the transpeptidase activity of FtsI, making FtsI essential for cell division in *M. tuberculosis*. Therefore, FtsI is a target for drug discovery, and these studies provided a molecular signature of FtsI inactivation that can be applied to screening strategies for novel FtsI inhibitors.**

Keywords: *M. tuberculosis*, cell division, microarray, cefalexin, piperacillin

Introduction

Despite the historical success of chemotherapy against *Mycobacterium tuberculosis*, this pathogen continues to cause high morbidity and mortality. The lengthy and complex multidrug regimens required to treat tuberculosis, the development of multidrug resistance to two or more of the front-line drugs and the increase in disease in endemic countries as a result of HIV/AIDS underscore the urgent public health need for new therapies.[1–3] Many of the current tuberculosis-specific chemotherapeutics target components of the cell wall and have proved ineffective for treating established infections.[4–6] However, analyses of the *M. tuberculosis* genome reveal a large number of essential conserved metabolic functions and numerous unexploited opportunities for drug discovery.

The cell division process, and in particular the penicillin-binding protein FtsI, represent a new target for *M. tuberculosis* drug discovery.[1,6] At the time of septation, peptidoglycan synthesis and remodelling at the site of division is coordinated with constriction of the FtsZ ring and other components of the cell wall to produce two viable daughter cells.[7] Studies in other bacteria have demonstrated that FtsI is required for peptide cross-linking of peptidoglycan at the septum and bacteria without functional FtsI proteins fail to divide, resulting in filamentous cells.[8] In addition to filamentation, treatment with the FtsI inhibitors cefalexin and piperacillin leads to the formation of concentric rings along the bacterial filament and a branching morphology.[8–11]

The limited potency of β-lactam antibiotics against *M. tuberculosis* has been attributed primarily to the presence of β-lactamase activity, and secondarily to reduced binding affinity of β-lactam antibiotics for mycobacterial penicillin-binding proteins.[12–15] Therefore, much effort has been focused on inhibition of β-lactamase activity.[16] Recently, the *M. tuberculosis* β-lactamase BlaC was crystallized and modelling has been undertaken to develop mycobacterial-specific β-lactamase inhibitors.[12] Targeting BlaC to facilitate the use of β-lactam antibiotics is substantiated by

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mutant studies that confirmed that *M. tuberculosis* resistance to β-lactam antibiotics is mediated through BlaC.[17,18]

Alternatively, the development of novel FtsI inhibitors that are not susceptible to β-lactamase activity is another promising approach. The identification of inhibitors and advancement of lead compounds involve screening drug candidates for mode of action and off-target effects in bacteria, in addition to potency and inhibition of enzymatic activity.[4–6] Accordingly, in this work, we inhibited FtsI activity and cell division with cefalexin and piperacillin, and report the corresponding alterations in morphology and response. Furthermore, characterization of these responses provides markers useful for developing appropriate drug screens to identify novel FtsI inhibitors.

Materials and methods

Bacterial growth conditions and recombinant strains

For all experiments, *M. tuberculosis* H37Rv was cultivated at 37°C in Middlebrook 7H9 liquid medium containing 0.2% glycerol, ADC (albumin, dextrose and catalase enrichment) and 0.05% Tween 80 or on Middlebrook 7H11 agar containing OADC (oleic acid, albumin, dextrose and catalase enrichment). For determination of MICs, *M. tuberculosis* was grown to an OD_{600} of ~0.5 and diluted 1:10. Cefalexin and piperacillin were added to final concentrations of 500–0.5 μM in a total volume of 0.1 mL, and tested in triplicate. The MIC was defined as the lowest concentration of drug that prevented bacterial outgrowth as monitored by OD_{600} after 7 days of incubation. For viability testing, drugs were added to 30 mL cultures. Each day, dilutions were plated on Middlebrook 7H11 agar, and viability was determined by enumeration of cfu. For microarray experiments, *M. tuberculosis* cultures (30 mL) were grown to an OD_{600} of 0.3, each drug was added at its respective MIC (20 μM cefalexin or 40 μM piperacillin) or untreated for a control, and the cultures incubated at 37°C for 5 or 24 h.

The *FtsI* open reading frame was amplified from *M. tuberculosis* H37Rv genomic DNA (TB Vaccine Testing and Research Material Contract HHSN266200400091c) using Accuprime *pfx* DNA polymerase with *ftsI-5′*: *atg agc cgc gcc gcc* and *ftsI-3′*: *cta ggt ggc ctg caa gac c* including engineered asymmetric *Nde*I and *Hin*dIII restriction sites for insertion into the mycobacterial-inducible shuttle vector *p*VV16ap. The *p*VV16ap vector contains an acetamidase promoter region that provides enhanced expression in the presence of 2% acetamide.

Ultrastructural analysis by scanning electron microscopy (SEM)

Bacteria were collected by centrifugation and washed three times in PBS, pH 7.4, and fixed with 2.5% gluteraldehyde in buffer A [0.1 M potassium phosphate (pH 7.4), 1 mM $CaCl_2$ and 1 mM $MgCl_2$] at 4°C for 48 h. The fixed bacterial cells were collected by centrifugation, washed three times in buffer A and treated with 1% OsO_4 in buffer A for 30 min at 4°C. Again cells were washed three times with buffer A, and prepared for SEM with a graded series of ethanol treatments (20% to 100%). Ultrastructural examination was performed using a JOEL JEM-100CX electron microscope.

Microarray processing and data analysis

The *M. tuberculosis* microarrays were obtained through the TB Vaccine Testing and Research Materials Contract (HHSN266200400091c) at Colorado State University. Treated and control bacterial cells were suspended in TRIzol and physically disrupted with 0.1 mm zirconium beads.[1] Total RNA was purified using an RNeasy Kit (Qiagen). Approximately, 8 μg of total RNA from each treatment was converted into cDNA in the presence of either Cy5- or Cy3-labelled nucleotides as previously described.[1] Hybridization was performed at 42°C for 12 h. Slides were scanned using a VersArray Chipreader Pro. Data reduction and global normalization were performed on the raw fluorescent intensities. The normalized intensity values of treated and control cultures were used to generate ratio and $\log_2$ expression values for each gene. The final microarray dataset used for transcriptional mapping resulted from combining the two biologically independent replicates of 24 h cefalexin and piperacillin treatments and four controls. For defining and evaluating the molecular markers of FtsI inhibition, independent biological replicates of 5 h cefalexin and piperacillin treatments and four 24 h cefalexin and piperacillin treatments were used.

Quantitative real-time PCR

Quantitative real-time PCR was performed on selected genes to verify differential gene expression observed through microarray data analysis. Quantitative real-time PCR was performed using SYBR-green (Invitrogen). PCR amplification was performed with a thermocycling programme 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 of *M. tuberculosis* genomic DNA. The total number of targets (n) was calculated by the equation $n = a + b\log(x)$, where a is the intercept, b the slope of the standard curve and x the threshold cycle obtained by amplifying n targets. All reactions were performed in triplicate on two independent RNA preparations from *M. tuberculosis* treated with cefalexin or piperacillin using primers as described.[1]

Results

Inhibition of FtsI results in bacterial filaments and branching and budding

The susceptibility and viability of *M. tuberculosis* to cefalexin and piperacillin were determined by microbroth dilution assay and plating.[1,19] The MICs of cefalexin and piperacillin were found to be 20 and 40 μM, respectively, for wild-type bacteria. Treatment with cefalexin or piperacillin at the MIC for 3 days reduced bacterial viability by 4 and 5 logs, respectively (Figure 1), and there was no bacterial growth after exposure to either drug for 5 days. Overexpression of *ftsI* resulted in a 6-fold increase in the MIC values of each drug (125 and 250 μM for cefalexin and piperacillin, respectively), which corresponds to the relative increase in the level of *ftsI* expression in the *ftsI* merodiploid strain as determined by quantitative real-time PCR.

When visualized by acid-fast staining, piperacillin- and cefalexin-treated bacteria possessed dark staining regions at equal intervals (Figure 2a and b). SEM allowed for length determinations of piperacillin- and cefalexin-treated cells to be performed. Treatment for 3 days with piperacillin or cefalexin resulted in a mean length of 7.1 ± 1.6 and 7.2 ± 1.3 μm, respectively, which is in sharp contrast to the 3.3 ± 1.0 μm length of untreated cells ($P < 0.05$). Closer examination using electron microscopy revealed the presence of concentric rings in addition to filamentation (Figure 2c and d). The observation of

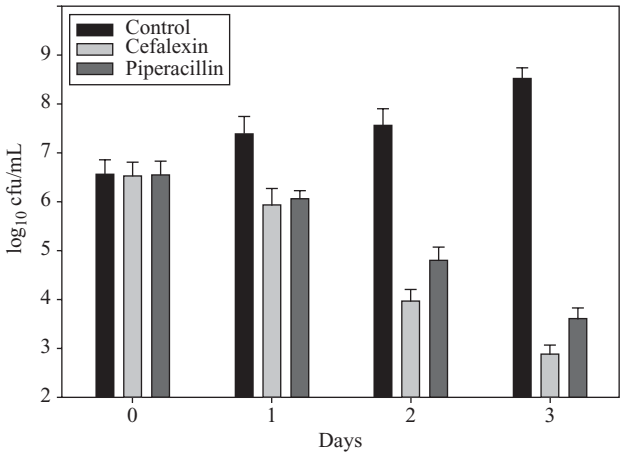


Figure 1. Bacterial growth in the presence of FtsI inhibitors. Bacterial cfu after treatment with 20 μM cefalexin or 40 μM piperacillin at designated timepoints.

clearly visible concentric rings indicative of septa was consistent with our previous observations and those from other organisms treated with FtsI inhibitors.[1,20–22] Bacteria with prolonged exposure to cefalexin also developed clearly visible budding and branching; this, however, was not observed as frequently for piperacillin treatment (Figure 2e and f). Together, these data confirm that FtsI is essential for cell division and overall bacteria viability.

Transcriptional mapping and identification of a signature profile for FtsI inactivation

To evaluate the overall effect of FtsI inactivation on cellular processes, such as DNA replication, cell division and macromolecular synthesis, the global transcriptional response of *M. tuberculosis* to cefalexin and piperacillin treatment was assessed through DNA microarray analysis. When the overall expression profiles of cells treated for 24 h with cefalexin and piperacillin were compared [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)], there was a 78% concordance in the transcriptional response between these treatments, indicating that FtsI inhibition was similar in the mode of action for both these β-lactam drugs. The transcriptionally active open reading frames were subjected to self-organizing mapping (SOM) analyses. Sets of discriminant genes representing the major cell cycle processes of DNA initiation, replication and chromosome segregation; cell division; peptidoglycan synthesis; lipoarabinomannan synthesis; arabinogalactan; and mycolic acid synthesis were selected to provide a more concise picture of how genes involved in cell division are grouped relative to other cell cycle processes (Figure 3). Overall, cell cycle processes were distributed in an overlapping fashion consistent with their execution in cellular replication. Inhibition of cell division via FtsI resulted in repression of DNA initiation, replication and segregation of discriminant genes. Genes encoding cell division components were distributed in a bimodal fashion. Specifically, genes involved in septum resolution were induced and genes involved in formation of the septum were repressed. Discriminant genes encoding for pathways for cell wall synthesis were largely distributed between those involved in cell division and DNA initiation. Gene

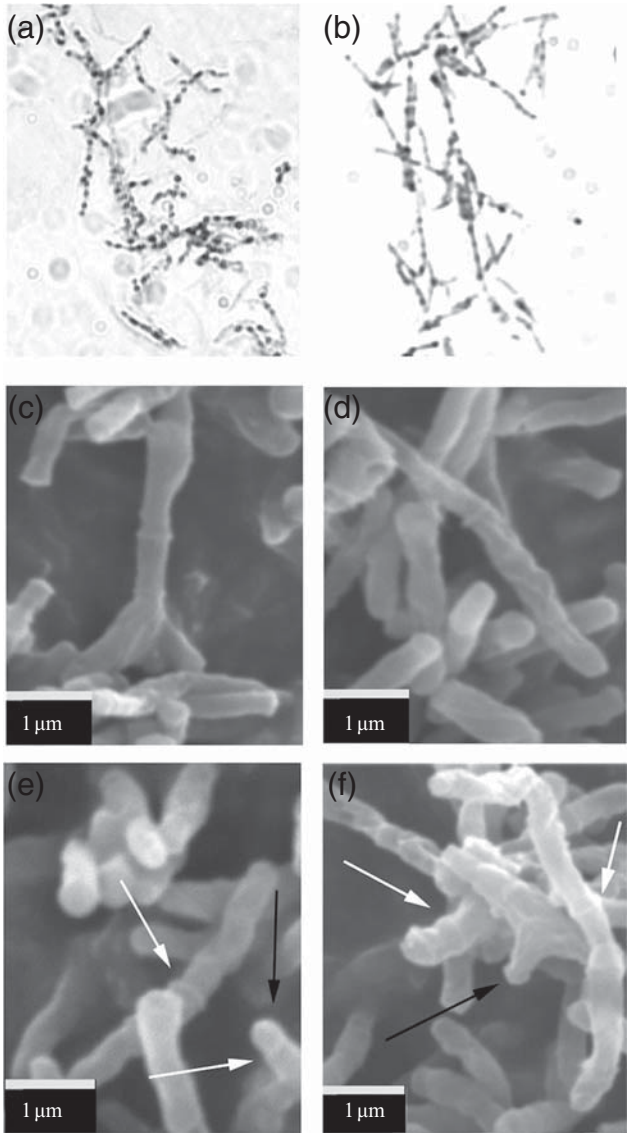


Figure 2. Bacterial morphology of *M. tuberculosis* treated with the cell division inhibitors. Bacteria treated with piperacillin or cefalexin were visualized by acid-fast staining (a and b) and SEM (c–f). Inhibition of FtsI with 40 μM piperacillin (c) or with 20 μM cefalexin (d) for 3 days, and with 40 μM piperacillin (a and e) or with 20 μM cefalexin (b and f) for 5 days. White arrows highlight the concentric rings and black arrows highlight budding and branching.

expression levels observed by microarray analysis were confirmed by quantitative real-time PCR (Figure 4). The arrangement of cell cycle processes is consistent with inactivation of FtsI and inhibition of the final step in cell division. The results were also similar to reports on other bacteria of temporal regulation of genes encoding septum formation or septum resolution.[23,24]

Molecular markers of FtsI inhibition

Based on transcriptional activity, cell cycle-associated genes segregate into two defined groups, those induced and those repressed in response to FtsI inhibition (Figure 5a). The

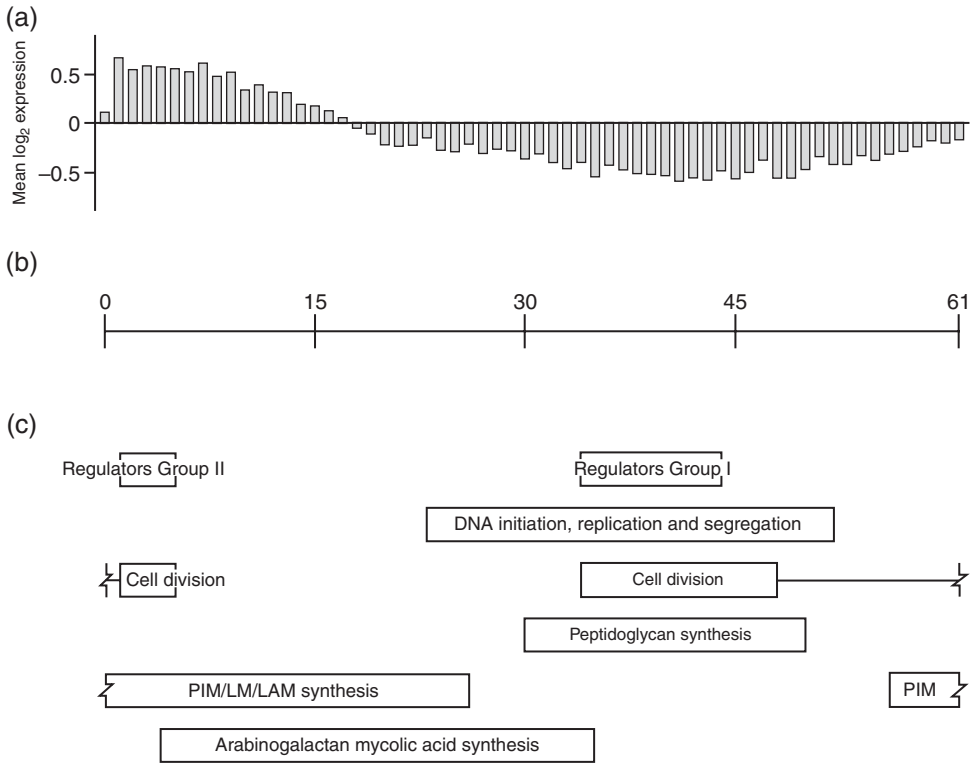


Figure 3. Transcriptional map of cell cycle process in *M. tuberculosis*. (a) Mean expression activity of each SOM group. (b) Coordinates of the transcriptional map used to map cell cycle processes as determined by SOM analysis. (c) Bacterial cell cycle processes mapped by SOM analysis. A set of (discriminant) genes representing chromosome replication, chromosome segregation, cell division and macromolecular biosynthesis (cell wall components) were chosen based on annotation and experimental information. PIM, phosphatidylinositol mannosides; LM, lipomannan; LAM, lipoarabinomannan.

transcriptional response was further evaluated using independent experiments of cefalexin and piperacillin treatments at 5 and 24 h to identify a subset of genes that can serve as molecular markers for FtsI inhibition. Hierarchal clustering analysis of the transcriptional response of cell cycle discriminant genes assigned the independent treatment experiments into two groups that correspond to either 5 or 24 h exposures (Figure 5b) and revealed five separate groups of genes with high correlations of transcriptional activities (Figure 5b). Based on this analysis and the overall trend in transcriptional activity upon inhibition of FtsI, genes from Groups Ia and VIa were selected for further evaluation as to whether they were highly predictive of FtsI inhibition for high throughput screening (Figure 5c and d). Upon analysis of the experimental expression profiles, the transcriptional response of signature genes had a 96% correlation at both the 5 and 24 h exposure times, demonstrating that this set of genes could discern inactivation of FtsI independent of exposure time. The mean $\log_2$ expression of each FtsI inactivation signature gene was consistently expressed over six experimental treatments, thus providing a quantifiable set of molecular markers. To assess whether the consensus response profile for FtsI inactivation was similar to the transcriptional response to treatment with drugs with other modes of action, they were compared with previously reported expression profiles of drug treatment and with inhibition of FtsZ.[1,6,25] This inspection did not identify overlaps in transcriptional responses of FtsI inhibition with other treatments, thus adding confidence that the observed response of the consensus genes was due to inhibition of FtsI.

Discussion

One of the most challenging tasks in developing chemotherapeutics against a specific target is the identification of robust whole cell molecular markers that are informative of the mode of action and can discern off-target activity.[4–6,25] This is largely because of a lack of tools to assess the suitability of a specific cellular process as a target for drug development. A requirement of such screening markers is the ability to differentiate with confidence between compounds with either an unrelated or closely related mode of action and those with the desired mode of action.[5] Accordingly, morphological analyses and global transcriptional profiling of *M. tuberculosis* treated with the β-lactams cefalexin and piperacillin were undertaken to identify morphological characteristics and define molecular markers of FtsI inhibition that offer the potential for the development of facile whole cell screens.

Visualization of *M. tuberculosis* treated with cefalexin and piperacillin revealed that similar to the prevention of septum formation, inhibition of FtsI resulted in filamentation.[9] However, an important distinction between the filaments resulting from cefalexin and piperacillin treatment and those observed when *M. tuberculosis* was treated with FtsZ inhibitors was that FtsI inhibition leads to the development of concentric rings at even intervals along the filament.[1] Concentric rings are indicative of septa, and their presence and persistence indicate that FtsI activity is essential for completion of cell division in *M. tuberculosis*. It also indicates that the initiation of FtsZ

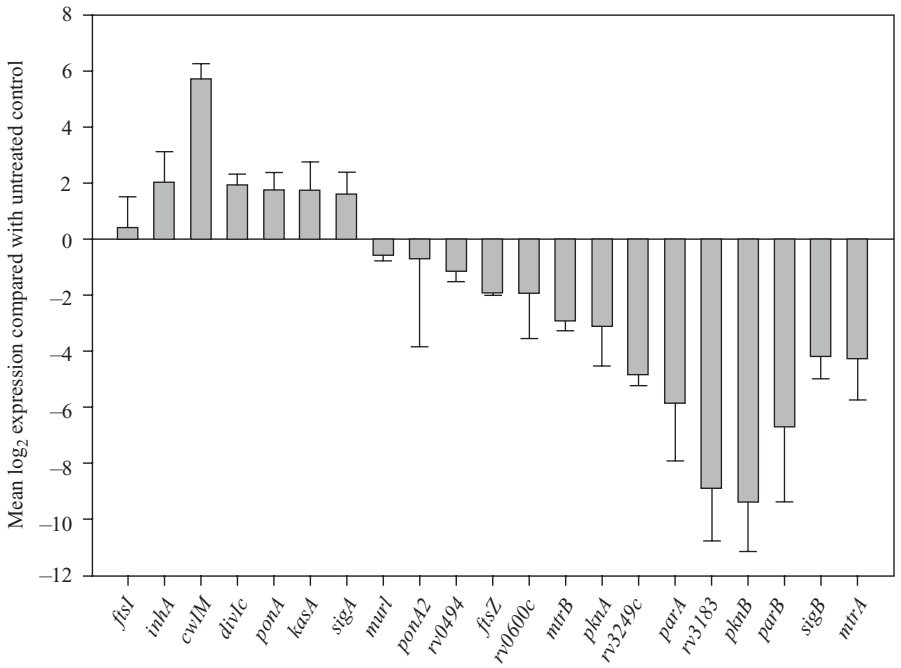


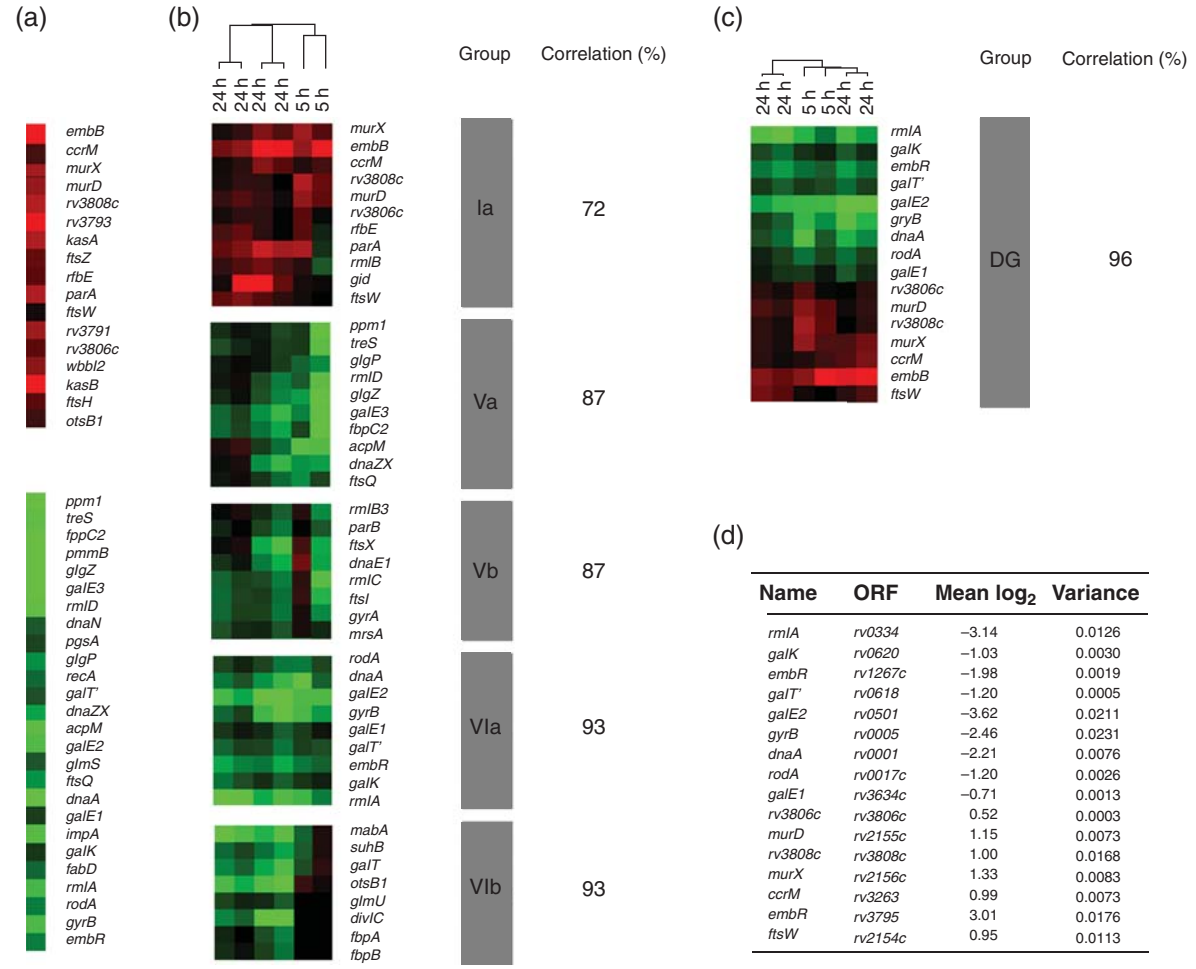
Figure 4. Quantitative real-time PCR analysis of discriminant cell cycle genes. Data are means ($\pm$SD) of $\log_2$ expression from at least two biological independent samples. Ratios were calculated using the total number of gene targets from treated bacteria compared with paired untreated control.

assembly at other sites is not negatively regulated with respect to the completion of the previous round of cell division. This observation agrees with previous conclusions that FtsI activity is not required for assembly of FtsZ rings at future sites,[9] and substantiates the notion that regulation of cell division in *M. tuberculosis* occurs at the level of FtsZ polymerization, despite the general lack of annotated FtsZ regulatory elements. Importantly, initiation of cell division is uncoupled to completion of the previous round of cell division in mycobacterium. This is further supported by the presence of occasional budding and branching as a result of FtsI inhibition caused by an imbalance in the regulation and activity of septal proteins such that asymmetric cell division events occur.[11,26]

An area of interest regarding the development of new chemotherapeutics against novel or underexploited molecular targets is the ability to discern the mode of action during the screening process. The identification of a signature transcriptional pattern of genes allows for the development of whole cell-based mode of action screens. Transcriptional mapping organized genes into groups consistent with cell cycle processes and it was found that genes encoding proteins involved with cell cycle processes that precede the completion of cell division were repressed and those involved in resolution of the septum and cell wall synthesis were induced. This general expression profile was in contrast to the transcriptional response to inhibition of septum formation previously reported.[1,6] Notably, these trends in the transcriptional response highlight the temporal nature of cell cycle events and the dynamic regulation between early and late cell cycle processes. Together, these studies demonstrated that morphological and transcriptional profiling can be used to identify features predictive of FtsI inhibition and substantiated that these genes can be used as a tool to discern the mode of action in the evaluation of new drug candidates.

The availability of a transcriptional response-based screening tool capable of discerning the mode of action for high throughput screening of compound libraries provides an additional level of information about cellular processes. Such gene responses reflect metabolic reactions to a specific deficit and have been successfully translated to report a specific pathway, thus revealing the mode of action.[1,6,25,27] Further analysis of the transcriptional response of cell cycle discriminant genes led to the identification of molecular markers suitable for high throughput screening of anti-FtsI candidates. When these molecular markers were evaluated using independent treatments of FtsI inhibitors, it was found that in each case they were capable of reporting the mode of action with a 96% correlation. Identification of these molecular markers based on transcriptional mapping established that a limited number of genes could be used to discern FtsI inhibition based solely on non-subjective criteria, and inclusion of the distinct morphological features resulting from FtsI inhibition provides additional evidence of a compound's mode of action.

Importantly, with regard to the development of novel chemotherapeutics that target cell division, the identification of high content molecular features capable of reporting the inhibition of a molecular target with high predictability affords the opportunity to screen compounds for FtsI mode of action. Not only can a single feature be chosen to design a recombinant strain for high throughput screening, an approach that has proved useful for the identification of novel compounds with a specific mode of action,[28] multiple features can be used together as a platform for determining the mode of action and potentially identifying off-target effects. Notably, the signature pattern of FtsI inhibition and the set of molecular markers identified in this study were capable of categorizing known FtsI inhibitors, and therefore can be used to predict the mode of action of FtsI inhibition.



Name	ORF	Mean $\log_2$	Variance
rmlA	*rv0334*	–3.14	0.0126
galK	*rv0620*	–1.03	0.0030
embR	*rv1267c*	–1.98	0.0019
galT'	*rv0618*	–1.20	0.0005
galE2	*rv0501*	–3.62	0.0211
gyrB	*rv0005*	–2.46	0.0231
dnaA	*rv0001*	–2.21	0.0076
rodA	*rv0017c*	–1.20	0.0026
galE1	*rv3634c*	–0.71	0.0013
rv3806c	*rv3806c*	0.52	0.0003
murD	*rv2155c*	1.15	0.0073
rv3808c	*rv3808c*	1.00	0.0168
murX	*rv2156c*	1.33	0.0083
ccrM	*rv3263*	0.99	0.0073
embR	*rv3795*	3.01	0.0176
ftsW	*rv2154c*	0.95	0.0113

Figure 5. Transcriptional response of cell cycle discriminant genes and signature transcriptional pattern of FtsI inhibition. (a) Transcriptional response of cell cycle discriminant genes determined by whole genome microarray analysis. (b) Hierarchal clustering analysis of cell cycle discriminant genes in response to FtsI inhibition for 5 or 24 h. (c) Transcriptional response of the discriminant group (DG) genes, and hierarchal clustering analysis of FtsI inhibition for 5 or 24 h. (d) Mean $\log_2$ expression values of FtsI signature response genes.

This provides a molecular tool for the development of reporter strains and high content screening platforms capable of identifying potential FtsI inhibitors and discerning potential off-target effects.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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