Characterization and Transcriptome Analysis of *Mycobacterium tuberculosis* Persisters

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ABSTRACT Tuberculosis continues to be a major public health problem in many parts of the world. Significant obstacles in controlling the epidemic are the length of treatment and the large reservoir of latently infected people. Bacteria form dormant, drug-tolerant persister cells, which may be responsible for the difficulty in treating both acute and latent infections. We find that in *Mycobacterium tuberculosis*, low numbers of drug-tolerant persisters are present in lag and early exponential phases, increasing sharply at late exponential and stationary phases to make up ~1% of the population. This suggests that persister formation is governed by both stochastic and deterministic mechanisms. In order to isolate persisters, an exponentially growing population was treated with D-cycloserine, and cells surviving lysis were collected by centrifugation. A transcriptome of persisters was obtained by using hybridization to an Affymetrix array. The transcriptome shows downregulation of metabolic and biosynthetic pathways, consistent with a certain degree of dormancy. A set of genes was upregulated in persisters, and these are likely involved in persister formation and maintenance. A comparison of the persister transcriptome with transcriptomes obtained for several *in vitro* dormancy models identified a small number of genes upregulated in all cases, which may represent a core dormancy response.

IMPORTANCE It is estimated that every third person on the planet is infected with *Mycobacterium tuberculosis*. The two major problems in controlling *M. tuberculosis* are the length of the treatment and the large reservoir of latently infected people. Dormant persister cells may be responsible for both problems. We find that *M. tuberculosis* produces persisters *in vitro* in a growth phase-dependent manner. Persisters were isolated from an exponentially growing population, and their transcriptome shows a distinct pattern of dormancy. These results give the first insight into *M. tuberculosis* persisters and point to possible mechanisms responsible for their formation.

Tuberculosis remains a major worldwide public health problem in spite of the availability of antibiotics that show excellent activity against the pathogen *in vitro* (1, 2). The World Health Organization estimates that a third of the world population is infected with *Mycobacterium tuberculosis* (3). In 2009, approximately 1.7 million people died from tuberculosis (2). Two of the main problems in *M. tuberculosis* control are the lengthy treatment and the enormous reservoir of latent carriers. The current "short-course" chemotherapy includes a combination of four antibiotics (isoniazid, rifampin, pyrazinamide, and ethambutol) that needs to be taken for 2 months, followed by isoniazid and rifampin for an additional 4 months. An even longer treatment course is required for curing the latent form, up to 9 months (4). Because a persistent population of pathogens remains until late in treatment, incomplete therapy is common and has resulted in the rise of multidrug-resistant and extensively drug-resistant tuberculosis (5–7). A common explanation for the need for lengthy treatment is that all or part of the population enters a nonreplicating persistent state characterized by some degree of dormancy,

with the bacilli becoming tolerant to antibiotics (8–10). Some lines of evidence suggest that persistent bacteria might reside in the granuloma, a lesion of immune cells that creates a microenvironment for the residing *M. tuberculosis* cells (11, 12). The granuloma environment imposes several stressful conditions on the resident bacilli, including low pH, NO, hypoxia, and limited nutrients (8, 10, 13). All of these factors have been shown to produce a nonreplicating state *in vitro* (10, 14–17). In these model experiments with stress factors, the entire population ceased to grow, which is probably typical of the pathogen in the latent state of infection. However, it remains unclear why it is so difficult to eradicate the pathogen from a growing population in an acute infection.

All pathogens examined so far produce dormant persister cells in growing exponential cultures (18), although virtually nothing is known about these cells in *M. tuberculosis*. Persisters are highly tolerant to antibiotics, and their presence may very well explain the recalcitrance of tuberculosis to therapy in both acute and latent infections. Importantly, persisters are not mutants but are

phenotypic variants of the wild type (19, 20). Persisters of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* make up a small fraction of an exponential population, ~10^{-5}, suggesting that their formation is due to stochastic expression of persister genes (20). Experiments based on time-lapse microscopy showed that nongrowing persister cells form before the addition of antibiotics (21). Sorting of an *E. coli* population expressing degradable green fluorescent protein under the control of a ribosomal promoter revealed the presence of a small subpopulation of dim cells tolerant to antibiotics (22). This suggested that persisters have limited translation and are dormant. The transcriptome of isolated persisters indeed showed downregulation of biosynthetic functions and pointed to a possible class of genes that can induce dormancy. These are toxin/antitoxin (TA) modules, which are widely spread in bacteria. A TA system consists of a stable toxin, which inhibits an essential cellular function, and a labile antitoxin. In *E. coli*, gain-of-function mutations in the HipA toxin generate high-persistence (*hip*) mutants (23, 24). HipA is a protein kinase which phosphorylates EF-Tu, inhibiting translation (25, 26). The RelE toxin inhibits translation by a different mechanism; it is an mRNA endonuclease. Ectopic overexpression of RelE produces drug-tolerant persisters (27). Similarly, overexpression of another *E. coli* mRNA endonuclease, the MazF toxin, also leads to the formation of persisters (28). Ectopic expression of three *M. tuberculosis* RelE homologs also produces drug-tolerant cells (29). TA modules are highly redundant: there are more than 20 of them in *E. coli* (30–32) and more than 65 in *M. tuberculosis* (TADB; http://bioinfo-mml.sjtu.edu.cn/TADB/browse.php). Consistent with this observation, screens of *E. coli* and knockout libraries did not identify a single strain lacking persisters (33, 34).

While persisters form stochastically, their numbers rise sharply as population density increases, reaching 1% in stationary-phase cultures (20). This indicates a deterministic component of persister formation. The nature of this density-dependent increase in persisters is unknown but is likely to be caused by nutrient depletion. A deterministic component was recently identified in a study of persister formation under conditions of DNA damage (35, 36). Fluoroquinolone antibiotics induce the SOS response, and the LexA repressor, which controls repair functions, was also shown to regulate transcription of the TisAB TA module (37). A knockout mutant of TisB had a sharply reduced level of persisters, showing that they are mainly produced in a TisB-dependent manner when the SOS response is activated. TisB is an antimicrobial peptide that apparently forms an ion channel in the membrane, leading to a drop in proton motive force (PMF) and ATP levels (7), which would shut down important functions and lead to a dormant state (35).

Knowledge of persisters obtained from studies of other species forms a good basis for investigating this subject in *M. tuberculosis*. Here we describe *M. tuberculosis* persisters and their growth-phase-dependent formation, isolation, and transcriptome analysis.

RESULTS

Characterizing *M. tuberculosis* persisters. A biphasic pattern of killing in a time-dependent experiment indicates the presence of drug-tolerant persisters in a bacterial population (38). Four antibiotics with different mechanisms of action were chosen to probe the presence of persisters in a growing culture of *M. tuberculosis* (Fig. 1). Streptomycin is a translational inhibitor (Fig. 1A), isoni-

azid is a prodrug which interferes with mycolic acid and nucleic acid biosynthesis (Fig. 1B), ciprofloxacin is a gyrase inhibitor (Fig. 1C), and rifampin is a transcriptional inhibitor (Fig. 1B). The killing generally follows a biphasic pattern—rapid death of the bulk of the population followed by a diminishing killing rate of a more tolerant subpopulation. In all cases tested, the killing rate declined by day 7. These results indicate that *M. tuberculosis*, like all other bacterial species tested, produces a subpopulation of drug-tolerant persister cells. Antibiotics require active targets in order to kill (18), and relatively good survival of *M. tuberculosis* persisters suggests that they are dormant. This would agree well with a recent report of isoniazid having no effect on a dormant population adapted to microaerophilic conditions of the Wayne model (39).

Growth phase dependence. We next investigated persister formation at different phases of growth. In order to track the level of persisters, a sample of the growing culture was withdrawn at each of the designated time points. The sample was split into three tubes, and the three antibiotics (streptomycin, ciprofloxacin, and D-cycloserine [DCS]) were added, one to each tube. The exposed samples were incubated (at 37°C) for 7 days in the presence of antibiotic to kill the nonpersister cells (Fig. 1). After the challenge, the cultures were washed and sonicated and the surviving persisters were quantified by plating for colony counts. The dynamics of persister formation were similar for all three antibiotics (Fig. 2). A low level of persisters was observed at early exponential phases, followed by a sharp increase in mid-exponential phase and then leveling off in stationary phase. Streptomycin, which can kill nongrowing cells, retained killing ability well into stationary phase (Fig. 2A), but eventually the population became completely tolerant (see time point at 18 days). In the case of ciprofloxacin and DCS, the entire population became tolerant by day 14 (Fig. 2B and C). The rapid increase in *M. tuberculosis* persisters is similar to that seen in other bacteria (20) and suggests the presence of a program which is activated at high cell density. The rate of persister formation is not directly proportional to growth rate. At early time points (*t* = 1, 3 and 5 days), the growth rates are similar, while the level of persisters changes by orders of magnitude. In early stationary phase (5 to 12 days), the population is not growing but the level of persisters is increasing. This indicates that growth rate alone cannot explain the dynamics of persister formation and that other factors must be involved. The increase in persister levels is not due to an increase in resistance, as the number of survivors decreased or stayed unchanged when the culture was incubated for an additional week, unlike the expected growth in the case of resistance (data not shown).

Persister isolation and transcription profiling. We previously reported that persisters could be isolated by lysing a growing culture of *E. coli* with ampicillin and collecting surviving cells by centrifugation (27). DCS was reported to effectively lyse *M. tuberculosis*, and we used this antibiotic for persister isolation (40). DCS was tested by adding the antibiotics to a growing population of H37Rv at several concentrations in order to identify optimal conditions for persister isolation. A concentration-dependent increase in killing leveled off at ~100 μg/ml, suggesting that this was an appropriate concentration for persister isolation (Fig. 3A). The population treated with DCS rapidly lysed, as determined using optical density (OD) measurements (Fig. 3B).

The next step was the isolation of total RNA from DCS-treated culture. An exponential-phase culture of *M. tuberculosis* was chal-
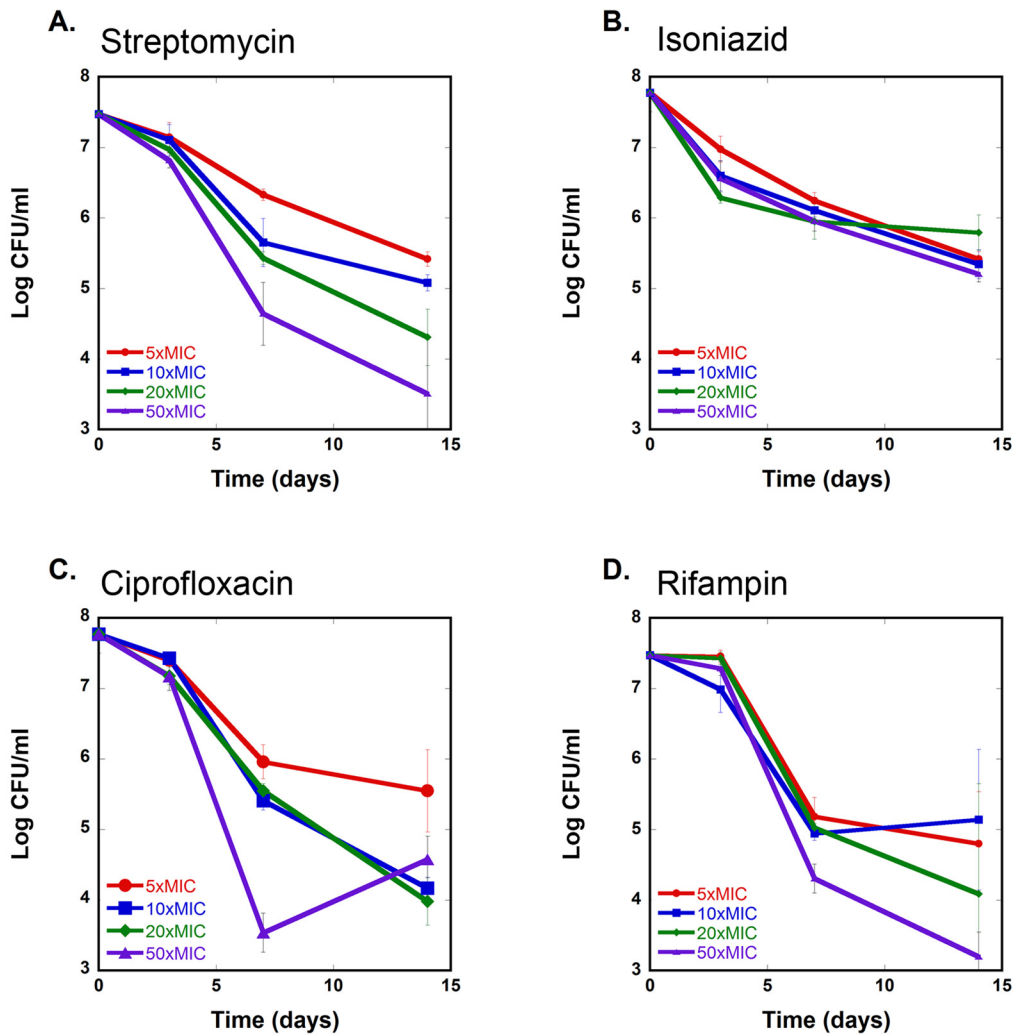
FIG 1 Killing of *M. tuberculosis* by antibiotics. An exponentially growing population of *M. tuberculosis* was exposed to antibiotics at 5, 10, 20, and 50 times the MIC. (A) Streptomycin (MIC = 0.625 μg/ml); (B) isoniazid (MIC = 0.135 μg/ml); (C) ciprofloxacin (MIC = 0.625 μg/ml); (D) rifampin (MIC = 0.1 μg/ml).

lenged with 100 μg/ml DCS, and RNA was collected at five time points, i.e., before DCS addition (t0) and at 1, 3, 7, and 14 days (t1, t3, t7, and t14, respectively). In conjunction with the RNA extraction, we determined the OD of the culture and the colony count of the surviving persisters (Fig. 3B and C). The culture was washed, sonicated, serially diluted, and plated on 7H10 plates, and colonies were counted after 3 weeks of incubation (at 37°C). From Fig. 3C, it is evident that regular cells are dying at a much higher rate (1 log/day, days 0 to 3) than persisters (about one-half log/week, days 7 to 14) and that days 7 and 14 are appropriate for isolating persisters. The RNA isolated from samples collected at four time points was amplified using the MessageAmp II-Bacteria kit for prokaryotic RNA amplification (Ambion). The amplified RNA (aRNA) was hybridized to an Affymetrix array designed for this project at the Broad Institute. It is based on the 49-5241 Affymetrix chip and contains 8,871 features, including probes for all protein-coding genes, newly annotated genes (e.g., toxin/antitoxin genes), rRNAs, tRNAs, Rfam domains, and tiled intergenic regions (forward and reverse strands).

The RNA isolated from the sample collected at day 3 was of poor quality and unsuitable for use, even though the sample had 10 times more cells than those collected at later time points. This is likely due to the large numbers of dead and dying cells at this time point and the consequent degradation of RNA. For this reason, only RNA from time points 0, 1, 7, and 14 was used for hybridization.

Lysing the culture to obtain persisters for transcriptome analysis may be problematic, since the antibiotic will induce changes in gene expression. We therefore applied statistical analysis to see whether global expression in samples containing persisters (days 7 and 14) was distinct from that in samples from the earlier time points. According to hierarchical clustering (MATLAB), one can see that expression patterns at t0 and t1 cluster together, as do those at t7 and t14 (Fig. 4B). This is even more apparent in the principal-component analysis (Fig. 4A), where the expression profiles from the initial two time points (t0 in red and t1 in green) cluster together and are distinct from those of the last two time points (t7 in blue and t14 in yellow), which also cluster together. The transcriptomes of t0 and t1 are less similar to each other than those of t7 and t14, since t1 represents the transcriptome in re-
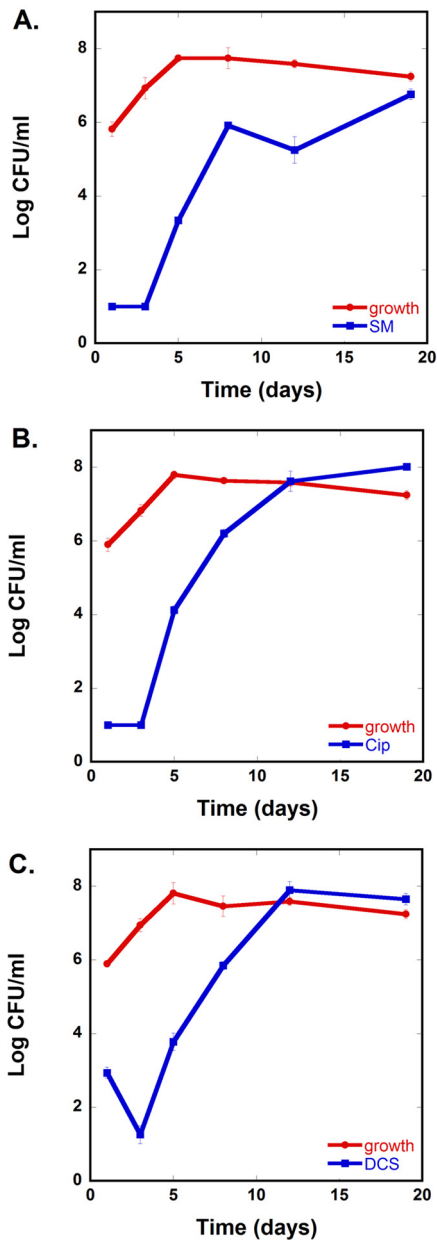
FIG 2 Growth stage dependence of persister formation in *M. tuberculosis*. A stationary-phase culture of *M. tuberculosis* was diluted 1:100, and growth and persister levels were followed over time. Persister levels were determined after 1 week of challenge with streptomycin (SM) (40 µg/ml) (A), ciprofloxacin (Cip) (5 µg/ml) (B), and D-cycloserine (DCS) (100 µg/ml) (C).

FIG 3 Killing of *M. tuberculosis* by D-cycloserine. An exponentially growing population of *M. tuberculosis* was exposed to D-cycloserine at 10, 20, 100, and 400 µg/ml (A) and at 100 µg/ml (B and C). (B) Optical density (OD$_{600}$) and (C) viable count (CFU/ml) results are shown. Samples for RNA isolation were collected at the time points indicated by arrows.

sponse to antibiotics. This means that the addition of antibiotic does not preclude obtaining meaningful data from transcriptome analysis of persisters obtained by lysis.

Transcriptome analysis. Analyzing RNA of persisters at t14 relative to cells prior to antibiotic addition (t0) shows a considerable decrease in expression. Two hundred eighty-two and 68 genes were upregulated >2- and >4-fold, respectively (*P* value, <0.01), while 1,408 and 628 genes were downregulated >2- and >4-fold, respectively (see Table S1 in the supplemental material). The shutdown of expression is massive, especially compared to the rela-
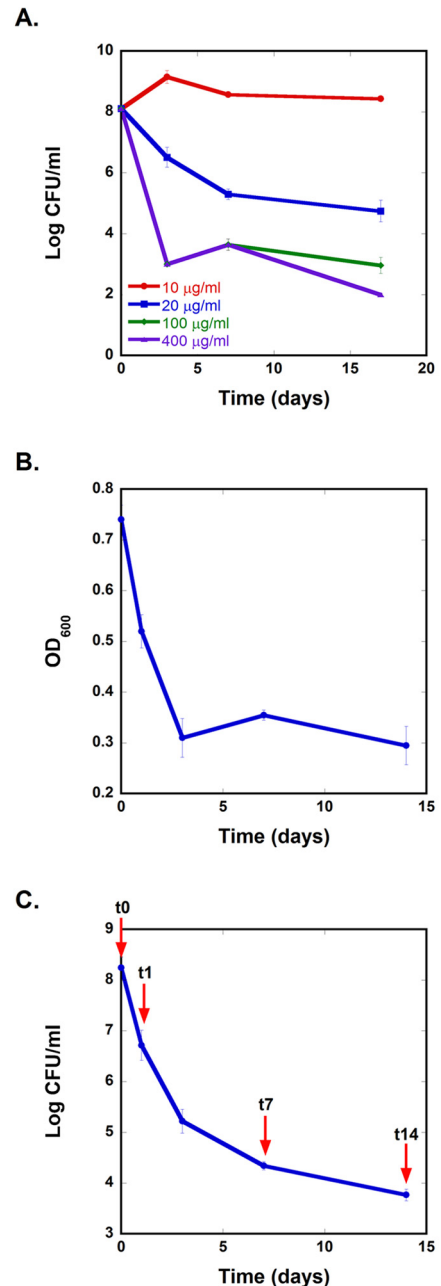
tively modest number of genes with increased expression. This finding is consistent with results obtained for *E. coli* persisters (22). Many of the energy and metabolism pathways (41) were considerably downregulated (Fig. 5). Data from stationary-phase and low-oxygen adaptations reveal strong downregulation of respiration, glycolysis, electron transport (about 30% of the genes were downregulated in response to low oxygen), and oxidative phosphorylation, similarly to what we have previously shown for *E. coli* persisters (22, 27). Ribosomal proteins were almost exclu-
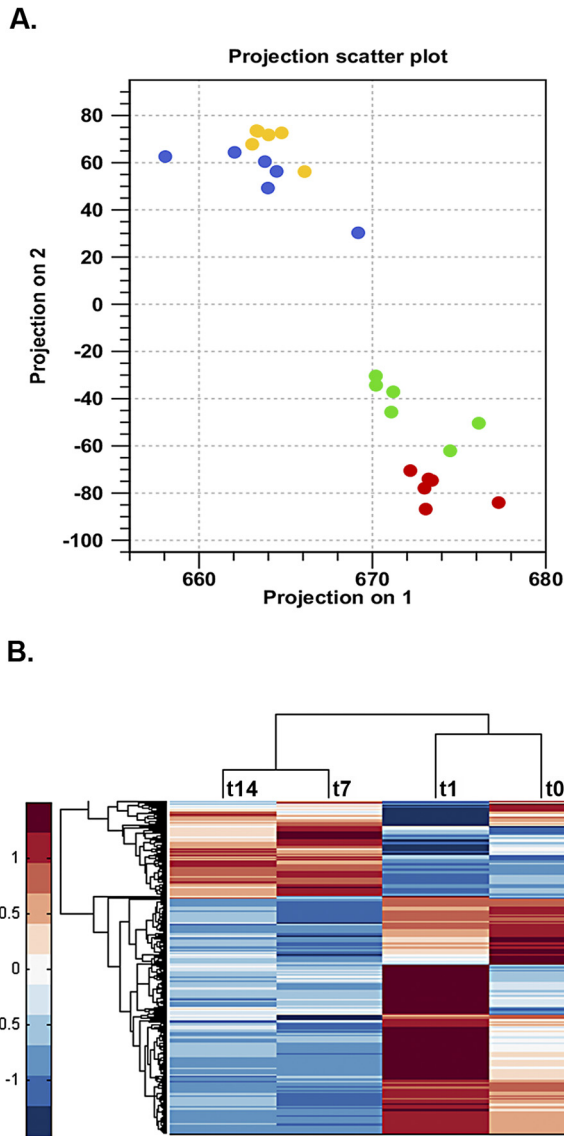
A.

B.

FIG 4 Principal-component analysis and hierarchical clustering of the transcriptome over time. (A) Principal-component analysis of the 24 arrays, performed using the CLC Genomics Workbench. Red circles, t0; green circles, t1; blue circles, t7; yellow circles, t14. Each point in the figure represents one array. (B) Hierarchical clustering of all genes (rows) and at all time points (columns), performed in MatLab. Each row represents a gene; each column represents a time point.

sively downregulated, with the exception of encoded by a single operon (*rpsR2-rpsN2-rpmG1-rpmB2*). Note that proteins encoded by this operon have not been shown to be part of the ribosome (42). Ribosomal proteins were reported to be downregulated in other dormancy models, including starvation (16), stationary state, and the Wayne model (15). Somewhat unexpectedly, glyoxylate metabolism, which is important for survival in hypoxic conditions (43) and for persistence in mice (43, 44), was also downregulated in persisters. Together, these results indicate a metabolic downshift consistent with some degree of dormancy.

Of special interest is the expression status of the large number of *M. tuberculosis* TA modules (32, 45). Ten TA modules were overexpressed in persisters (see Fig. S1 in the supplemental material), including Rv0549c/Rv0550c, Rv2021c/Rv2022c, Rv1989c/Rv1990c, Rv2865/Rv2866, Rv0918/Rv0919, Rv3180c/Rv3181c, Rv1955/Rv1956, Rv3188/Rv3189, and Rv2034/Rv2035. Rv1955/Rv1956, Rv2034/Rv2035, and Rv2022c (toxin) were also upregulated during adaptation to starvation (16) and the enduring hypoxic response (EHR) (14). Both the toxin and the antitoxin of Rv1989c/Rv1990c, Rv2865/Rv2866, and Rv2021c/Rv2022c were also upregulated in EHR. We have shown that TA modules play a role in *E. coli* persisters (22, 25, 35), and it is logical to assume a similar function in *M. tuberculosis* persistence.

A comparison of expression profiles at t7 and t14 with that at t1 indicates genes that are specifically upregulated in persisters independent of the presence of antibiotic. However, a considerable number of genes induced after the addition of antibiotic (t1) continue to be expressed in largely dormant persisters at t7 and t14, suggesting that these genes may also be important for persister formation and maintenance. We therefore analyzed both groups, as detailed below.

In order to identify genes specifically expressed in persisters, we performed hierarchical clustering of the profiles of the 68 genes that were >4-fold overexpressed at t14 (Fig. 6). Cluster analysis of the profiles was based on similar patterns of gene expression over time. Each cluster groups genes with similar behaviors over time, and each line represents a single gene. Clusters 3, 4, and 9 show genes specifically upregulated in persisters. In these three clusters are genes with little or no change in expression between the first two time points (t0 and t1) and with significant increases in expression at the last two time points (t7 and t14). The 15 genes in these three clusters are listed in Table 1. Seven of these genes encode either hypothetical proteins, conserved hypothetical proteins, or conserved membrane proteins. One of the genes in cluster 4, which codes for the universal stress protein (UspA, Rv2623), was previously shown to be important for establishing a chronic infection in mice (46). The alternative stationary-phase sigma factor sigma F and its cognate anti-sigma factor RsbW (also known as UsfX) are both members of cluster 3. The error-prone DNA polymerase gene *dnaE2* is a member of cluster 4. A similar analysis was done using all genes that were >2-fold overexpressed (282), which clustered into 16 clusters (Fig. S2). This produced five interesting clusters with 56 persister-specific genes (see Table S1).

There are several *in vitro* models that are thought to mimic some aspects of the *in vivo* conditions during chronic and latent infections, including models of starvation (16) and hypoxia (10, 14, 15). We compared our results with published data from these models, and included all genes upregulated at t14 (in persisters). We used the genes that are upregulated >2-fold in the following models: (i) nutrient starvation of an exponential-phase culture that was starved in phosphate-buffered saline (PBS) for 96 h (16); (ii) the enduring hypoxic response (EHR) at 7 days (14), in which hypoxia was generated by a continuous flow of low oxygen; (iii) the Wayne model, in which restriction of headspace in the culture vial leads to nonreplicating persistence (10, 15); and (iv) the 49 genes of the Dos regulon (14). This comparison is similar to the one performed by Sherman and colleagues (14), with the important addition of the persister transcriptome. Only eight genes of the Dos regulon were overexpressed in persisters, although this set did not include the master regulator *dosR* (see Fig. S3 in the supplemental material). This suggests that the Dos regulon is not involved in the formation of
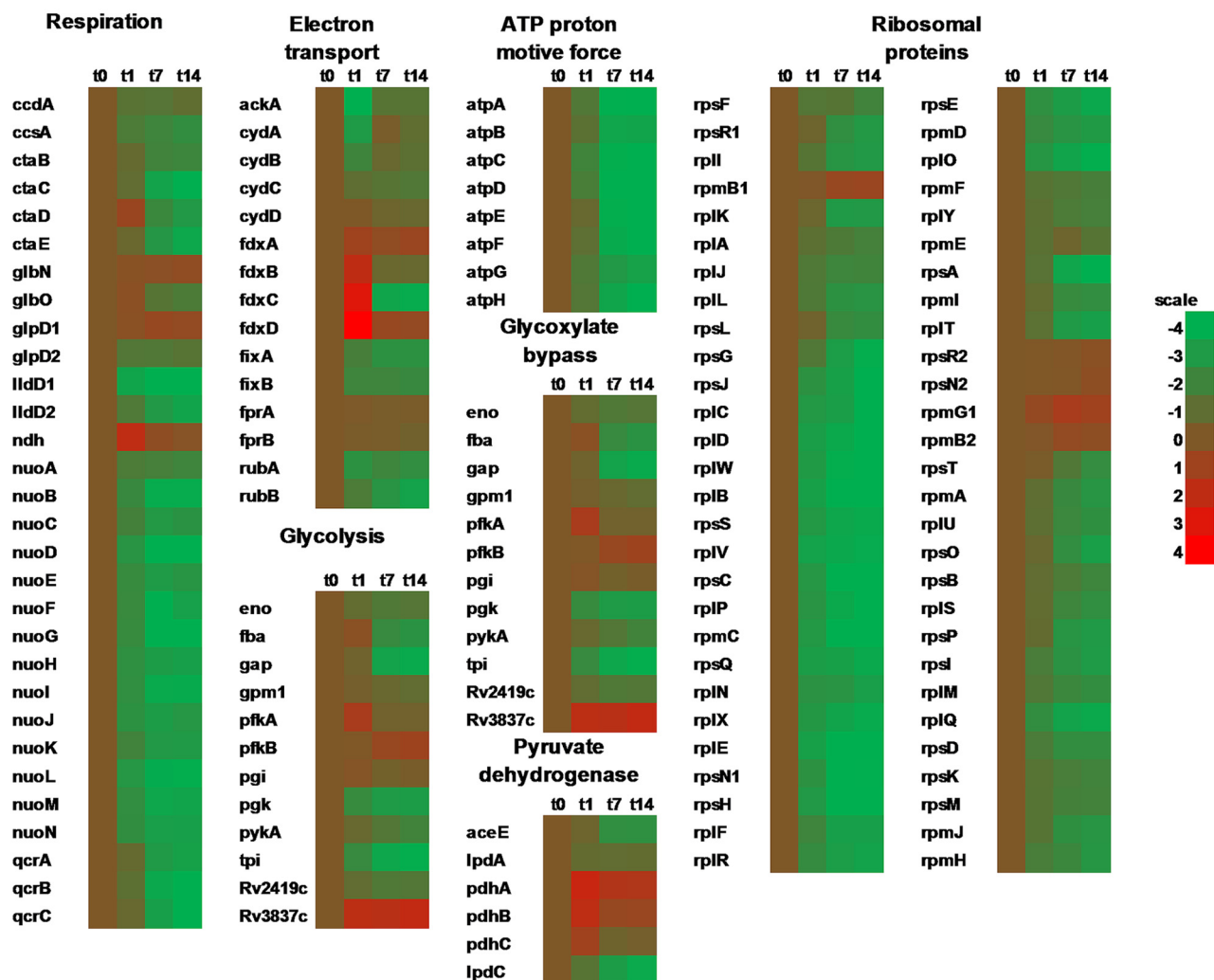
FIG 5 Heat maps of energy metabolism and biosynthetic pathways. The relative mean log$_2$ expression of genes in several energy metabolism and biosynthetic pathways was calculated and visualized over time using Excel.

persisters, and therefore the Dos regulon genes were not included in further analysis.

A four-dimensional Venn diagram (Fig. 7) summarizes the differences and commonalities of the four models, persisters, starvation, EHR, and nonreplicating persistence. There are five genes that are common to all models and 55 genes that are common to persisters and at least 2 other models (Table S2). Of special interest are the five genes that are overexpressed in all models. These genes are Rv0251c (*acr2*), encoding an α-crystallin heat shock protein, Rv1152, a transcriptional regulator of the *gntR* family, Rv2497c (*pdhA*), encoding a probable pyruvate dehydrogenase component, Rv2517c, encoding a hypothetical protein, and Rv3290c (*lat*), encoding an L-lysine-epsilon-aminotransferase. Two of these genes were also upregulated in the stationary phase, Rv3290c and Rv1805c.

We have previously suggested that drug tolerance results from inactivation of the targets of antibiotics caused by dormancy (18). It was interesting to see whether reduction of expression of the antibiotic targets plays a role in persisters. We found that ribosomal proteins are significantly downregulated (Fig. 5), which

may contribute to streptomycin tolerance. *gyrA* and *gyrB* were both downregulated (ratios of 0.12 and 0.35, respectively), possibly contributing to ciprofloxacin tolerance. *katG* was upregulated in persisters (ratio of 1.8), which could result in more isoniazid activation, but the target *inhA* was downregulated (ratio of 0.15). Expression of *rpoB*, the target of rifampin, did not change significantly (ratio of 1.28). The protein level of RpoB, however, is most likely reduced as a result of the shutdown in ribosomal protein expression. D-alanine:D-alanine lygase (*ddlA*) and alanine racemase (*alr*), the DCS targets, were both slightly downregulated (ratio of 0.6).

DISCUSSION

Bacteria employ numerous mechanisms, such as stress responses and antibiotic resistance, that help them survive in a hostile environment. A very different and particularly effective strategy is to produce specialized survivor cells, persisters. This is a hedge-betting strategy, whereby a small portion of cells enters into dormancy and becomes highly tolerant to killing by antibiotics (18).
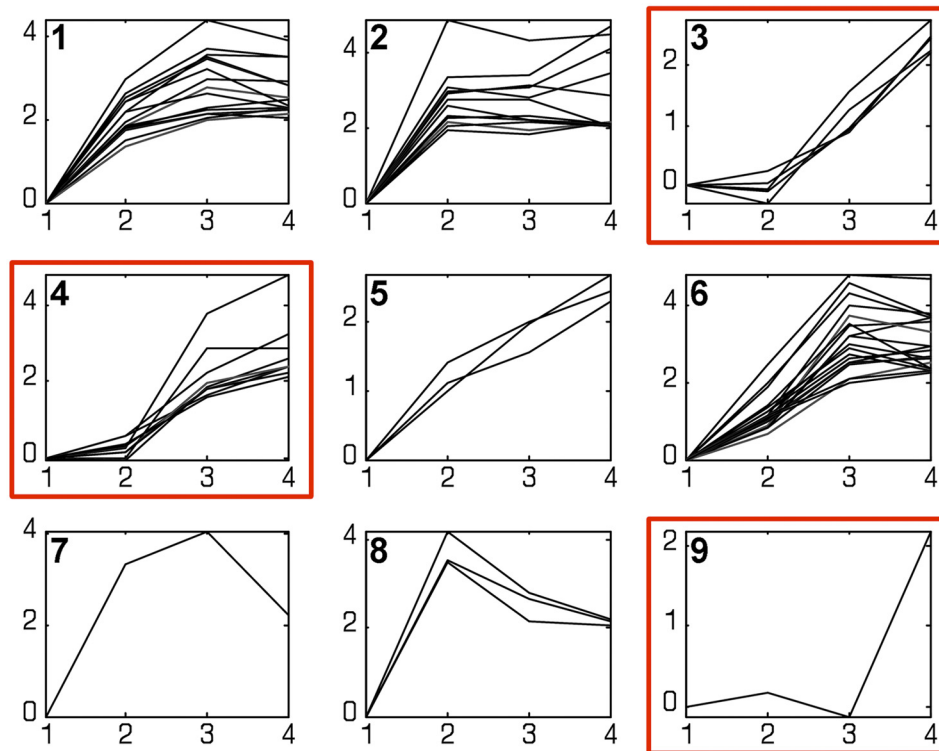
FIG 6 Hierarchical clustering of profiles of genes overexpressed in persisters. The profiles of genes that were overexpressed >4-fold at t14 relative to t0 (*P* value, <0.01) were hierarchically clustered using MATLAB. Each line in the clusters represents the profile of one gene. The numbers 1, 2, 3, and 4 correspond to t0, t1, t7, and t14, respectively. Clusters 3, 4, and 9 (outlined in red) represent genes that are specifically overexpressed in persisters, as their expression is unchanged at the first two time points but is upregulated in persisters.

The mechanisms leading to dormancy are starting to be unraveled in *E. coli*.

Dormancy was implicated more than 50 years ago (47) in the pathogenesis of tuberculosis. Latent, asymptomatic carriers can develop an acute disease decades after infection, which points to a dormant form of the pathogen (48). The Wayne model of *M. tuberculosis* dormancy is based on gradual transfer of the population into a low-oxygen environment and has been extensively studied. A number of genes were found to be upregulated in the Wayne model (15). At the same time, an execution mechanism that governs dormancy in the Wayne model and others has not been identified. One possibility is that such a mechanism does not operate in these *in vitro* dormancy models, where the entire population is forced into a nonreplicating form by external conditions. In contrast, persisters can form in a growing population and there is little doubt that there must be a dormancy program, which sends them down this distinct developmental pathway. Given the prominent role of persisters in recalcitrant infections caused by a variety of pathogens (18, 20, 49), it is surprising that so little is known about these cells in *M. tuberculosis*. This study aimed to document persister formation and obtain the persister transcriptome.

As with other pathogens studied, we found that unrelated antibiotics produce a characteristic biphasic pattern of killing *M. tuberculosis* cells in a time-dependent manner. This is a clear indication of the presence of drug-tolerant persisters. Similar behavior can be seen in published results for *M. tuberculosis* killing *in vivo* in the mouse model, guinea pig model, and human sputum (43, 50–52). The level of persisters rises sharply as the population den-

sity increases, which is also similar to what we have reported previously with other bacterial species. This is in contrast to a recent

TABLE 1 Genes that are upregulated in persisters >4-fold

Gene cluster	Locus tag	Gene	Function[a]
3	Rv2667	*clpC2*	Possible ATP-dependent protease ATP-binding subunit
	Rv3287c	*rsbW*	Anti-sigma factor (also *usfX*)
	Rv3286c	*sigF*	Alternate RNA polymerase sigma factor
	Rv2651c	*Rv2651c*	Possible phiRv2 prophage protease
	Rv0840c	*pip*	Probable proline iminopeptidase
4	Rv2662	*Rv2662*	HP
	Rv2666	*Rv2666*	Probable transposase for insertion sequence elementIS*1081*
	Rv0792c	*Rv0792c*	Probable transcriptional regulatory protein (gntr family)
	Rv0142	*Rv0142*	cHP
	Rv0837c	*Rv0837c*	HP
	Rv2661c	*Rv2661c*	HP
	Rv0849	*Rv0849*	Probable conserved integral membrane transport protein
	Rv3370c	*dnaE2*	Probable DNA polymerase III
	Rv2623	*TB31.7*	Universal stress protein (UspA like)
9	Rv1707	*Rv1707*	Probable conserved transmembrane protein

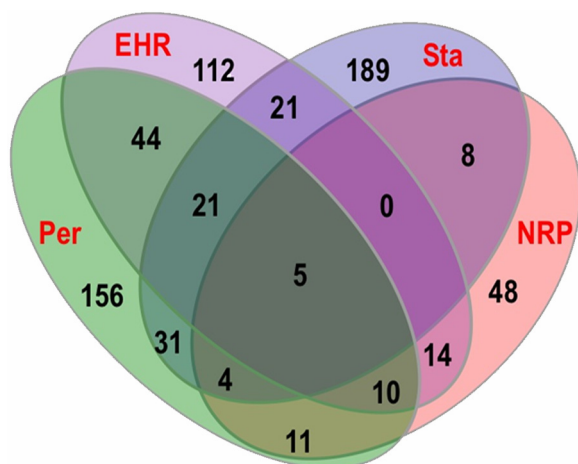[a] HP, hypothetical protein; cHP, conserved hypothetical protein.

FIG 7 Venn diagram comparing four models of dormancy. Per, persisters (representing our data); EHR, enduring hypoxic response (hypoxia by continuous flow of low oxygen); Sta, starvation (starving exponential-phase culture in PBS); NRP, nonreplicating persistence (the Wayne model of hypoxia by restriction of headspace).

publication suggesting that there is no density dependence of persister levels in *M. tuberculosis* (29). In that study, however, samples from the growing culture were diluted in fresh medium when challenged with antibiotic, which was likely to reduce persister levels. Other studies (53, 54) have shown an increase in antibiotic tolerance in stationary phase, as we report.

Survival of persisters in a population treated with an antibiotic suggests a simple method of their isolation. DCS lyses regular cells of *M. tuberculosis*, and persisters can be simply collected by centrifugation. These cells were then used to obtain a transcription profile of persisters using an Affymetrix array.

There were five times more downregulated genes in persisters than upregulated ones (1,408 versus 282). The growth and energy metabolism pathways are overwhelmingly downregulated. *M. tuberculosis* persisters appear to have shut down growth and energy metabolism, consistent with dormancy. This result also matches our finding that *E. coli* persisters show downregulation of biosynthetic and energy-producing functions (22).

The *M. tuberculosis* transcriptome also pointed to a number of genes whose expression was upregulated. We were particularly interested to see if any TA modules are expressed in *M. tuberculosis* persisters. The *E. coli* persister transcriptome led to the identification of a number of TA modules as a dormancy mechanism. Among these are mRNA endonucleases RelE (27) and MazF (28); HipA, the kinase that phosphorylates and inhibits Ef-Tu (25); and TisB (35), which forms an ion channel, causing dormancy by decreasing PMF and ATP. Currently, there are 65 TA modules listed for *M. tuberculosis* in the TA database (TADB; http://bioinfo-mml .sjtu.edu.cn/TADB/browse.php). Of these, 10 were overexpressed in *M. tuberculosis* persisters. One of these, Rv2866, is a *relE* homologue and was recently shown to increase drug tolerance when overexpressed (29).

We used cluster analysis to identify 15 genes specifically induced in persisters. Some of these genes have been shown to play a role in persistent infection in mice. UspA is required for long-term survival and establishment of chronic infection in mice (46). It is also one of the Dos regulon genes (55) and was overexpressed

in the nonreplicating persistence model *in vitro* (15). SigF, the alternative sigma factor, is overexpressed in stationary phase (15) and during starvation (16). A *sigF* deletion strain is attenuated in monocytes and in the murine model of infection (56). The error-prone DNA polymerase gene *dnaE2* has been shown to be upregulated in response to DNA-damaging agents and to contribute to *in vivo* survival (57).

Of special interest are the five genes that are overexpressed in all of the models we compared. The gene *acr2*, which encodes an α-crystallin heat shock protein, is a homologue of *acr1* (*hspX*), one of the most overexpressed genes in the hypoxia model (58). The *lat* gene is part of the *sigG* regulon (59), and *sigG* is upregulated in persisters (see Table S1 in the supplemental material). Additionally, *lat* is upregulated in stationary phase (15), another non-growth model.

Genes that are overexpressed in persisters could be used as reporters to capture naive persisters, prior to the addition of antibiotic. Placing a fluorescent protein under the control of a promoter of such an overexpressed gene will enable isolation of persisters by cell sorting. A transcriptome of such naive persisters will show how much (or how little) the expression profile is affected by the addition of an antibiotic.

Our results indicate that persisters isolated from a growing culture of *M. tuberculosis* are in a state of dormancy and express a small set of genes. Since persisters form in both growing and stationary-phase cultures *in vitro*, they must be produced *in vivo* as well. Importantly, *M. tuberculosis* resides in a granuloma, protected from the immune system. Persisters that will survive exposure to an antibiotic will then be able to repopulate the infection (Fig. 8), necessitating a lengthy course of therapy.

The persister signature transcription profile identified in this study will enable comparisons with dormant cells *in vivo*. Such comparisons will test the persister hypothesis of tuberculosis dormancy. This study will also enable testing of candidate genes, such as TA modules expressed in persisters, for their role in executing dormancy.

MATERIALS AND METHODS

Strains and culture conditions. *Mycobacterium tuberculosis* H37Rv was cultured at 37°C in 7H9 medium supplemented with OADC (oleic acid-albumin-dextrose-catalast), glycerol, and Tween 80. 7H10 medium supplemented with OADC and glycerol was used as the solid medium.

Antibiotic killing experiments. Freezer stocks of H37Rv were diluted 1:100 into 7H9 medium and cultured for 2 weeks. The 2-week-old culture was diluted 1:100 and grown to mid-exponential phase (OD_{600}, 0.6 to 0.8). Antibiotics were added at the desired concentration, and the culture was returned to the 37°C shaker for up to 14 days. At the designated time points, a sample was removed, washed, sonicated to break up clumps (XL2020; Misonix), serially diluted, and spot plated for CFU count. All experiments were performed with three biological replicates.

Growth phase dependence. A 2-week-old culture was diluted 1:100 into 7H9 medium. At the designated time point, a sample was removed and divided in two. One portion was sonicated (XL2020;Misonix), serially diluted, and spot plated for CFU counts. The other portion was challenged with antibiotics for 1 week as described above.

Persister isolation and RNA extraction. A 2-week-old culture was diluted 1:100 into 7H9 medium and cultured until it reached mid-exponential phase (OD_{600}, 0.75; ~1×10^8 CFU/ml). DCS was added to the culture at a concentration of 100 µg/ml. At the designated time points, a sample was removed and divided into three portions for determining the CFU count and OD_{600} and for RNA isolation. Two volumes of RNA Protect (Qiagen) was added to the cell suspension, which was then cen-
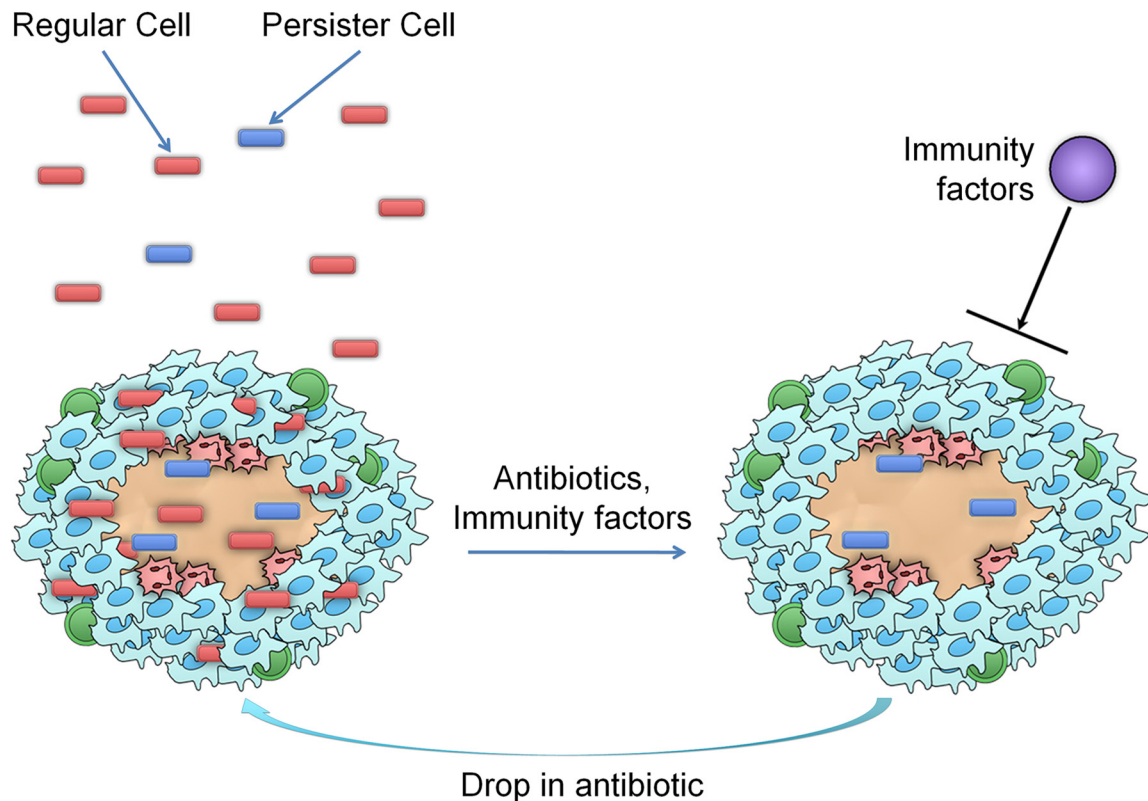
FIG 8 Model explaining the need for lengthy antibiotic treatment of *M. tuberculosis* infection.

trifuged (4,000 rpm, 8 min). The pellet was resuspended in 1 ml RNA Pro (MP Biochemicals), transferred into bead beater tubes (lysing matrix B; MP Biochemicals), and disrupted in the bead beater (FastPrep-24; MP Biochemicals) for 45 s at maximum power (6.5). The samples were centrifuged for 1 min at 13,000 rpm to sediment the beads and cell debris. The supernatant was transferred into a tube containing 300 μl chloroform. The contents were mixed vigorously by inverting the tube for 1 min and occasionally inverting it for an additional 5 min. The tubes were centrifuged for 15 min at 13,000 rpm. The aqueous phase was transferred to another tube, and 800 μl of isopropanol was added. The RNA was precipitated overnight at -80°C and then pelleted by centrifugation for 10 min at 13,000 rpm. The pellet was washed with 70% ethanol, air-dried, and resuspended in water. The isolated RNA was cleaned by running it through an RNeasy column (Qiagen).

Microarray analysis. Expression data were obtained using an Affymetrix *M. tuberculosis* chip in accordance with the manufacturer's protocol. Briefly, the total RNA from the time series experiments was normalized to 25 ng/μl and amplified using the MessageAmp II-Bacteria kit for prokaryotic RNA amplification (Life Technologies). The aRNA was labeled, fragmented, and hybridized to the custom-designed GeneChip MTbH37Rva520730F (PN520730). Washing and staining were performed on the GeneChip fluidics station, and the GeneChip Scanner 3000 was used for scanning the arrays. Affymetrix.cel files were processesed and converted into .txt files by the RMA (robust multiarray analysis) method using the Affy package in R (60). Hierarchical clustering of the arrays was performed in MatLab, and principal-component analysis was performed in the CLC Genomics Workbench. The time course gene clustering was done in MatLab, and the heat maps were generated in Excel.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00100-11/-/DCSupplemental.

Figure S1, TIF file, 0.598 MB.
Figure S2, TIF file, 0.563 MB.
Figure S3, TIF file, 0.260 MB.
Table S1, DOCX file, 1.028 MB.
Table S2, DOCX file, 0.693 MB.

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