

# Mutation in the Transcriptional Regulator PhoP Contributes to Avirulence of *Mycobacterium tuberculosis* H37Ra Strain

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## SUMMARY

Attenuated strains of mycobacteria can be exploited to determine genes essential for their pathogenesis and persistence. To this goal, we sequenced the genome of H37Ra, an attenuated variant of *Mycobacterium tuberculosis* H37Rv strain. Comparison with H37Rv revealed three unique coding region polymorphisms. One polymorphism was located in the DNA-binding domain of the transcriptional regulator PhoP, causing the protein's diminished DNA-binding capacity. Temporal gene expression profiles showed that several genes with reduced expression in H37Ra were also repressed in an H37Rv *phoP* knockout strain. At later time points, genes of the dormancy regulon, typically expressed in a state of nonreplicating persistence, were upregulated in H37Ra. Complementation of H37Ra with H37Rv *phoP* partially restored its persistence in a murine macrophage infection model. Our approach demonstrates the feasibility of identifying minute but distinct differences between isogenic strains and illustrates the consequences of single point mutations on the survival stratagem of *M. tuberculosis*.

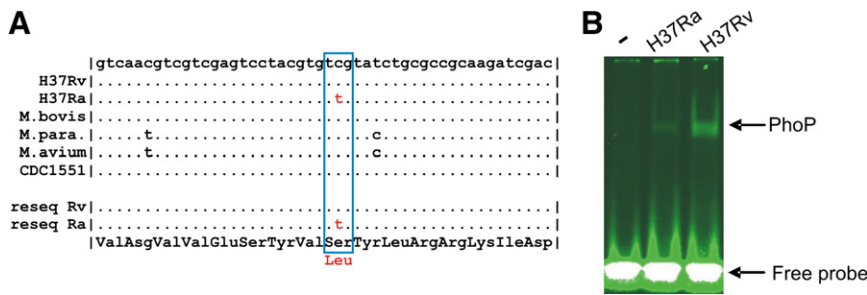
## INTRODUCTION

Tuberculosis remains a significant health problem with high morbidity and mortality. One-third of the world's population is infected with *Mycobacterium tuberculosis*, and up to 10 million new cases develop annually, 20% of which lead to death (World Health Organization, 2007). In spite of global research efforts, mechanisms underlying pathogenesis, virulence, and persistence of *M. tuberculosis* infection remain poorly understood (Kaufmann, 2006).

Attenuated strains of mycobacteria allow determination of genes essential for pathogenesis and persistence. The best studied laboratory strain of *M. tuberculosis* H37Rv has an avirulent counterpart in H37Ra, which was recognized as early as 1934 (Steenken et al., 1934). Though infectious, it does not replicate in macrophages (McDonough et al., 1993) and thus resembles the dormancy of *M. tuberculosis* during latent infection. H37Ra does not undergo the typical cord formation of *M. tuberculosis* (Bloch, 1950; Gao et al., 2004) and differs in colony morphology. However, reasons for the decreased virulence remain unknown (Sharma and Tyagi, 2007).

The best understood genomic difference among mycobacteria related to attenuation is the absence of the RD1 region of *M. bovis* BCG, which contains several genes essential for virulence, including *esat6* and *cfp10* (Behr et al., 1999; Pym et al., 2002). The H37Ra genome, however, comprises the intact RD1 region (Mostowy et al., 2004), and microarray analysis rules out deletions larger than 350 bp (Kato-Maeda et al., 2001). Even though single-nucleotide changes can have a strong impact on phenotypic traits (Wilmes-Riesenberg et al., 1997), a restriction enzyme polymorphism identified earlier in H37Ra has not been associated with loss of virulence (Brosch et al., 1999; Pascopella et al., 1994).

Irrespective of genomic differences between H37Ra and H37Rv, other studies investigated the phenotypic consequences and determined changes in gene expression. This led to the identification of the *dev* genes by subtractive hybridization (Kinger and Tyagi, 1993), and a differential display approach revealed additional genes that were downregulated in H37Ra as compared to H37Rv (Rindi et al., 1999). More recently, Gao et al. (2004) performed a genome-wide approach using microarrays to compare the transcriptomes of H37Rv and H37Ra. Many genes whose expression was repressed in H37Ra were discovered, although the genes described in earlier studies could not be confirmed. The aforementioned studies focused on exponentially growing bacteria. Comparisons of the expression profiles of H37Rv and H37Ra in long-term cultures have not been



**Figure 1. Mutation in *phoP***

(A) The mutation in *phoP* of H37Ra results in an amino acid change from serine to leucine.

(B) Gel shift of a fluorescently labeled oligonucleotide containing the PhoP-binding site that was incubated with overexpressed and purified PhoP proteins of H37Ra and H37Rv. Similar amounts of the two proteins were used as determined by western blotting.

attempted but may provide insights into the behavior of these strains under dormancy- or starvation-like conditions.

Major changes in gene expression profiles could result from differences in transcriptional regulation, and consequently numerous studies have addressed the regulatory machinery in *M. tuberculosis* with respect to virulence. An important class of regulatory proteins is found in two-component systems in which several proteins have been implicated in mycobacterial virulence (Parish et al., 2003). Prominent and recently identified members of these protein families in pathogenic mycobacteria include DosR, which mediates dormancy, and PhoP, which positively regulates polyketide-derived lipid biosynthesis (Gonzalo et al., 2006; Martin et al., 2006; Park et al., 2003; Perez et al., 2001). Since these genes are of importance to virulence, microarray studies of knockout (KO) strains have been performed to identify potential PhoP target genes (Walters et al., 2006). Interestingly, mycobacterial PhoP, although sharing the same name, is not formally orthologous to PhoP from Salmonella, which is also essential for virulence (see Figure S1 available online). Therefore, transfer of experimental information on PhoP proteins between species should be interpreted with great caution.

In this work, we extend upon previous genomic and transcriptomic approaches by comparing the genomic sequence of H37Ra with several virulent strains at the single nucleotide level, discovering mutations in *phoP* (*Rv0757*), *Rv0101*, and *Rv0637*. The mutation in PhoP compromised the functionality of the protein, and expression analysis revealed a significant overlap between genes deregulated in H37Ra and a PhoP KO strain. The introduction of the original *phoP* gene into H37Ra enhanced the bacterial persistence in murine bone marrow-derived macrophages when compared to H37Ra. Our findings suggest that the *phoP* mutation in H37Ra resulted in the partial loss of regulatory function and that this contributed to reduced virulence of H37Ra.

## RESULTS

### Differences in the Genome

The genome of H37Ra was sequenced, and 1.4 M reads were produced (Margulies et al., 2005). The reads were compared to the H37Rv reference sequence (Cole et al., 1998), and the regions matching >95% to genomic regions were assembled into 183 contigs. We focused on high-quality regions covering 95.66% of the genome. A total of 75 nucleotide polymorphisms were detected between H37Ra and H37Rv genomes (Table S1). Sequencing of H37Ra confirmed an additional deleted region in H37Rv following position 1,987,700 previously reported as *RvD2* (Lari et al., 2001).

Of the 75 polymorphisms identified, 53 loci were found to diverge from the published H37Rv genome sequence but were

identical to the corresponding loci in the published genomes of *M. tuberculosis* CDC1551, a recent clinical isolate (Fleischmann et al., 2002), and *M. bovis* (Garnier et al., 2003). These 53 polymorphisms could represent sequencing errors in the original sequence. A potential sequencing error reported in the literature (Dubey et al., 2002), which would fuse the reading frames of *Rv1180* (*pk3*) and *Rv1181* (*pk4*), was not found in this H37Rv strain by resequencing. However, CDC1551, *M. bovis*, and H37Ra apparently code for the fusion protein. Twenty-two polymorphisms could be confirmed by resequencing. There were 19 cases in which the H37Rv sequence peculiarly diverges from the consensus built from *M. bovis*, CDC1551, and H37Ra; these regions were therefore unlikely to be related to virulence. Only three polymorphisms, located in *Rv0101*, *Rv0637*, and *phoP*, were unique to H37Ra and are therefore most likely responsible for its phenotype.

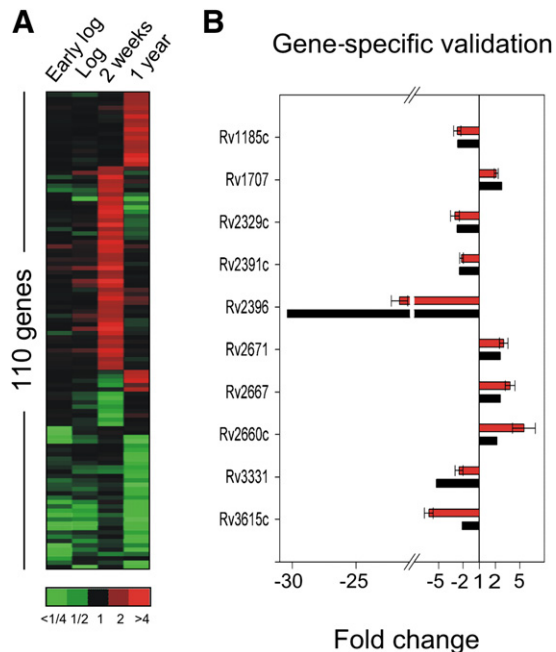
### A Mutation in the Response Regulator PhoP of H37Ra

Among the three mutations the most significant effect on attenuation of H37Ra was likely located in the *phoP* gene (Figure 1A), which encodes the response regulator of the PhoP/PhoR two-component system and is essential for virulence (Perez et al., 2001). The mutation in H37Ra described here leads to an amino acid change located in the DNA-binding domain of PhoP replacing a serine by a leucine (see Figure S2 for a structural model).

To investigate whether the mutation in the DNA-binding domain interfered with its functionality, we performed gel shift assays using overexpressed proteins (Figure 1B). The only described interaction between mycobacterial PhoP and DNA is the binding to its own promoter, where it interacts with a 9-mer consensus motif containing the sequence ACT/GT/GT/GYARC (Gupta et al., 2006). The intensity of the shifted band was higher when the H37Rv version of the protein was incubated with DNA containing the PhoP-binding motif. Therefore, we conclude that the PhoP from H37Ra has a reduced DNA-binding capability.

### Transcriptional Differences between H37Rv and H37Ra

To unravel differentially regulated genes, we determined the RNA expression profile of H37Ra and H37Rv in a longitudinal analysis. Bacteria were harvested at the time points indicated in Figure 2A. We detected 110 genes of H37Rv that were differentially expressed in at least one time point (Tables S2 and S3). A substantial number of these genes were located adjacent to each other: 11 of the 21 upregulated genes in H37Ra at the 1-year time point reside between *Rv2660c* and *Rv2672*, and 19 of the 47 genes coexpressed during the stationary phase were adjacent to each other, indicating that they could be part of operons. Due to the spacing of individual spots across the array, it



**Figure 2. Gene Expression Comparison of H37Ra and H37Rv**

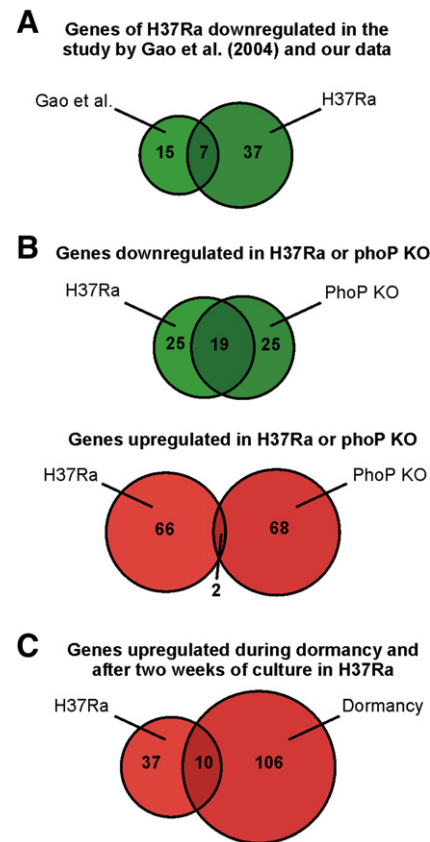
(A) Heat map of significantly regulated genes in H37Ra and H37Rv at the indicated time points. Red corresponds to elevated gene expression in H37Ra; green represents reduced gene expression.

(B) Confirmation of expression levels using qRT-PCR. Fold-change differences between gene expression in H37Ra and H37Rv were calculated using the  $\Delta\Delta C_t$  method (Pfaffl, 2001). *SigA* (*Rv2703*) expression was chosen to normalize cDNA amounts because it did not change significantly in expression during the time course study. Validation is exemplified here using unamplified RNA harvested at the 1-year time point (see Supplemental Experimental Procedures). All significant changes in gene expression that were observed by microarray analyses could be confirmed by qRT-PCR qualitatively for the 1-year sample. Error bars represent standard deviation.

is unlikely that this is the result of technical error. To validate our data and the accuracy of our amplification protocol, we performed gene-specific quantitative real-time PCR (qRT-PCR) on nonamplified cDNA prepared after 1 year of culture (Figure 2B). Gene repression or activation was confirmed qualitatively in all cases analyzed for the 1-year time point (Figure S3 contains additional validation experiments with comparable results for other sampling points of the time course).

### Comparison with Earlier Studies of the H37Ra Transcriptome and a *PhoP* KO Strain

Similar to previous studies, we exclusively observed downregulated genes in H37Ra during the first two time points, whereas most of the upregulated genes appeared at the later culture growth stages (Gao et al., 2004). Gao et al. determined the gene expression profiles of H37Ra and H37Rv at a single time point and found 22 genes that were consistently repressed in the avirulent mycobacterial strain during exponential growth. In our results, 20 of these genes were repressed in H37Ra at early time points, while the other two genes remained virtually unchanged in expression. Due to the stringent cutoff values applied, only 7 of these 20 genes qualified as significantly downregulated in our study. This nevertheless resulted in a set of genes



**Figure 3. Comparison with Previous Studies Interrogating Gene Expression Levels in *M. tuberculosis* Strains**

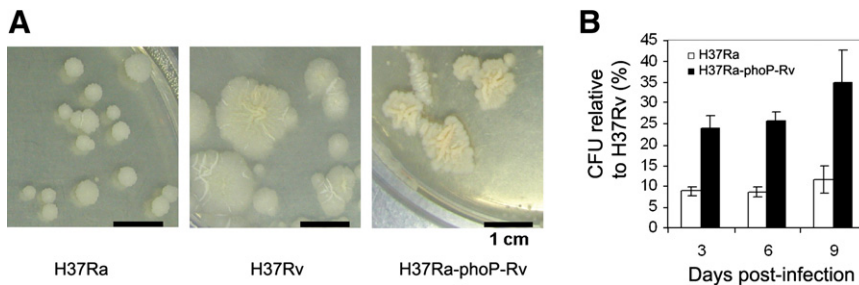
(A) Gao et al. (2004) investigated genes differentially expressed in cording and noncording mycobacteria. Seven of these genes were also repressed in our study at early time points.

(B) Overlap of expression data of a *phoP* KO strain as compared to gene expression of H37Rv (Walters et al., 2006). Of the 44 genes, which were significantly downregulated in H37Ra versus H37Rv, 19 were also assigned to the repressed category in a *phoP* KO strain. In contrast, overlap among activated genes of the two studies was confined to only two genes.

(C) Voskuil et al. (2003, 2004) described the induced genes constituting the dormancy regulon. Among the 47 genes upregulated in H37Ra after 2 weeks of culture, 10 belonged to this group.

that overlapped remarkably (Figure 3A and Table S2). The *p* value for overlap using the Fisher exact test was  $<0.001$ .

Another survey compared the expression patterns of the wild-type strain H37Rv with a strain in which the *phoP* gene had been ablated (Walters et al., 2006). Given that the mutation of *phoP* in H37Ra influences the functionality of the protein, the pattern of differentially expressed genes in H37Ra and the *phoP* KO strain should show a substantial overlap. Indeed, of the 44 genes repressed in the *phoP* KO mutant, 19 were also significantly downregulated in H37Ra ( $p < 0.001$ ). Walters et al. (2006) also described 70 genes that were more actively transcribed in the *phoP* mutant than in H37Rv during logarithmic growth. We did not observe upregulation of any gene at the two early sampling points, in concordance with the results of Gao et al. (2004). At later time points, two genes were upregulated in H37Ra that were induced in the *phoP* KO mutant (Figure 3B). Thus, the transcriptional activation potential of PhoP appears strongly



**Figure 4. Complementation of H37Ra with PhoP of H37Rv**

(A) H37Ra forms typical small-sized colonies. The complementation of H37Ra with the *phoP* gene of H37Rv reveals a colony morphology resembling H37Rv.

(B) Time course of the intracellular bacterial load of H37-derived strains in murine bone marrow-derived macrophages. CFU were counted at the indicated time points postinfection and are depicted as percent of bacterial load following infection with H37Rv. CFU values for all data points collected, including initial infection (day 0), can be found in Figure S5. Error bars represent standard deviation.

diminished in H37Ra, whereas additional repressive features of this protein seem to remain intact.

### The Transcriptome under Hypoxic Conditions and Starvation

Comparing H37Rv under hypoxic conditions in the stationary phase with exponential growth, Voskuil et al. (2003, 2004) observed the expression of a unique set of genes referred to as the dormancy regulon in H37Rv. The two-component system DosR/DosS (DevR/DevS, Rv3133c/Rv3132c) is pivotal for the transcriptional gene activation in models of dormancy (Boon et al., 2001; Sherman et al., 2001; Voskuil et al., 2003, 2004). We were interested in whether genes induced under hypoxic conditions in H37Rv were similarly activated in H37Ra because attenuation of H37Ra could be caused by expression changes in the dormancy regulon of the mycobacterium. At the 2-week time point, when conditions became hypoxic, we noticed upregulation (1.8-fold,  $p$  value  $3 \times 10^{-14}$ ) of *dosR* transcripts in H37Ra. Other studies also documented the upregulation of *dosR* under nonreplicating persistence (NRP) conditions in H37Rv when expression was compared to exponentially growing bacteria (Voskuil et al., 2004). We directly compared expression patterns of H37Rv and H37Ra and found that *dosR* is induced even higher in the latter. Interestingly, this activation also resulted in transcriptional induction of several genes of the dormancy regulon in the avirulent strain. Of the 116 transcripts identified as being upregulated (Voskuil et al., 2004), 10 genes were significantly activated in H37Ra after 2 weeks of culture as compared to gene expression in H37Rv (Figure 3C and Table S2;  $p$  value  $< 0.001$ ).

Conditions of the 1-year time point likely resemble starvation. Seven genes that were upregulated in this time point were also induced in cultures kept in phosphate-buffered saline (Betts et al., 2002). Three of these are coded in the genomic region between *Rv2660c* and *Rv2664* and belong to operons that are highly induced during starvation. However, other starvation-associated genes (e.g., those involved in energy metabolism) were not differentially transcribed during growth in nutrient-poor media (Betts et al., 2002).

### Complementation of H37Ra with *phoP* from H37Rv

Physiological features of *phoP* KO mutants differ from wild-type bacteria in that they develop smaller colonies on agar-based media and show different cording properties, a feature associated with virulence (Glickman et al., 2000; Perez et al., 2001; Walters

et al., 2006). The *phoP* mutant's colony morphology differed distinctly from the wild-type strain when cultured on 7H10 Middlebrook agar plates. We observed similar morphological differences between H37Rv and H37Ra. Colonies were smaller in H37Ra than in H37Rv, and wrinkling on the colony surface was reduced in H37Ra as compared to H37Rv. As depicted in Figure 4A, these differences were reduced when H37Ra was complemented with pSO5-expressing wild-type PhoP (H37Ra-phoP-Rv). Previously, Perez et al. (2001) found no significant growth differences between wild-type and *phoP* KO strains when cultured in liquid medium. However, H37Ra took slightly longer to reach the logarithmic and stationary phase. The *phoP*-complemented H37Ra strain revealed a growth pattern similar to H37Rv (Figure S4).

Next, we were interested if the complementation of H37Ra with the original *phoP* gene from H37Rv increased bacterial survival in immune cells, which is strongly diminished in H37Ra (McDonough et al., 1993). To this end, we infected murine bone marrow-derived macrophages with equal multiplicities of infection of H37Ra, H37Rv, and H37Ra-phoP-Rv (Figure 4B). This resulted in similar bacterial loads on day 0 for the three strains (Figure S5). At later time points, the bacterial burden of macrophages infected with H37Ra was reduced to about 10% of the colony-forming unit (CFU) count of macrophages infected with H37Rv. Cells challenged with H37Ra-phoP-Rv reached a CFU count, which was between 24% and 35% of that of H37Rv depending on the time point investigated. Overall CFU of H37Ra-phoP-Rv were about three times higher than for the noncomplemented H37Ra, showing that the intracellular bacterial persistence, and thus its virulence, was increased by the complementation.

## DISCUSSION

We identified three point mutations in coding regions that are likely to cause the attenuation of H37Ra. The additional confirmation of sequencing errors in the original reference sequence for H37Rv underscores the accuracy of the high-throughput analysis. However, it is important to realize that not all confirmed differences necessarily represent sequencing errors, as it is difficult to estimate the number of generations between individual sequencing attempts. Only a large-scale study of H37Rv strains from different sources would shed light on this question as it has been performed recently for *M. bovis* BCG (Brosch et al., 2007).

Only 19 of 22 loci differed in H37Rv as compared to CDC1551 and H37Ra. The far more frequent use of H37Rv over H37Ra could have led to the accumulation of gene alterations specific for laboratory conditions. The small number of bona fide polymorphisms between H37Ra and H37Rv can be explained satisfactorily by the long generation times of mycobacteria as compared to other bacteria and is consistent with the relatively few differences between H37Rv, the recent clinical isolate CDC1551 (Fleischmann et al., 2002), and *M. bovis* (Garnier et al., 2003). We consider the mutation in PhoP most critical for the phenotypic differences. Four pieces of evidence support this. (1) Of the three mutated genes that are different between all virulent strains and H37Ra, PhoP is the only protein known to be involved in virulence. (2) The mutation occurs in a region that interferes with the biological function of this transcription factor as determined by gel shift assays. (3) We revealed a significant overlap between genes with altered expression levels between H37Ra and the *phoP* KO mutant of H37Rv. (4) Complementation of H37Ra with the *phoP* gene of H37Rv reverted the colony shape phenotype and was more virulent than the original H37Ra in a mouse macrophage infection model.

However, we do not formally rule out the possibility that additional mutations observed here or mutations in gaps or regions of low sequencing quality, which were not pursued, contributed to phenotypic differences between H37Rv and H37Ra. Several gap regions span PE and PPE genes, which have been implicated in virulence although mechanistic insight is lacking (Gordon et al., 1999).

The crucial role of PhoP in *M. tuberculosis* virulence has been well characterized in models of mycobacterial infections (Martin et al., 2006; Perez et al., 2001; Walters et al., 2006). Our data revealed a highly significant overlap of genes repressed in H37Ra and the *phoP* KO mutant of H37Rv. Interestingly, this correlation was not observed for genes that are more actively transcribed in the *phoP* KO strain and H37Ra. The most likely reason for these differences is that PhoP is completely absent in the experiments by Walters et al. (2006), whereas the H37Ra strain still comprises mutated PhoP. Since the mutation is located in the DNA-binding domain, one would expect that activating and repressing functions of a transcription factor are compromised to the same degree, which is apparently not the case. One hypothesis suggests that PhoP interacts with hitherto unknown additional proteins that act together as transcriptional repressors. A detailed characterization of the PhoP protein and its interaction partners will be necessary to elucidate this issue. We have not determined whether PhoP is a direct regulator of the differentially expressed genes, and therefore, secondary effects should be considered as well: wild-type PhoP could induce the expression of transcriptional repressors; accordingly, in the *phoP* KO mutant the relief of this repression would cause indirect gene activation. Chromatin immunoprecipitation experiments could help to identify direct target genes of PhoP.

We also observed a significant similarity between gene expression patterns of H37Ra and genes upregulated under dormancy-like conditions at the 2-week time point. According to the work by Wayne and Hayes (1996) and Voskuil et al. (2003, 2004), mycobacteria have reached a state of nonreplicating persistence in this stationary culture phase, which is characterized by the upregulation of the dormancy regulon. Since this was accompanied by a transcriptional induction of *dosR*, the essential transcription factor mediating dormancy in H37Ra, it is tempting

to speculate that the attenuation of this strain is caused by hyperdormancy.

Previously, a particular correlation of H37Ra with dormancy has not been observed, since gene expression patterns have only been investigated in exponentially growing bacteria. Recently, the constitutive upregulation of genes belonging to the dormancy regulon has been found in the Beijing lineage of *M. tuberculosis* during the exponential growth phase (Reed et al., 2007). It was speculated that this induction confers a growth advantage to Beijing strains since they could be preadapted to the conditions inside macrophages during infection and that this might underlie the increased virulence of this mycobacterial subfamily. The interplay between dormancy and virulence of mycobacteria is not yet resolved. Other avirulent strains such as *M. smegmatis* exhibit dormancy-like phenotypes, and many virulence factors do not belong to the dormancy regulon (Mayuri et al., 2002; Smith, 2003). In addition, the targeted deletion of key dormancy genes, including *dosR* and *Rv2031c*, results in hypervirulent phenotypes (Hu et al., 2006). If the higher expression of dormancy genes is the key to virulence as postulated, the deletion of these genes should have generated hypovirulent mycobacteria. Therefore, it remains unresolved why the constitutive overexpression of *dosR* in the Beijing lineage should be associated with its hypervirulence. As a caveat it should be mentioned that deleting the *dosR* gene in a guinea pig model (Malhotra et al., 2004) led to attenuation. Further studies will be necessary to clarify the meaning of dormancy for virulence and persistence of mycobacteria.

The 1-year sample of our time course study exhibited a different pattern of gene expression: almost all genes of the cluster between *Rv2660c* and *Rv2672*, probably comprising four different operons, were strongly induced in H37Ra. The *Rv2660c* operon was also highly induced under starvation conditions in H37Rv (Betts et al., 2002). Thus—as under dormancy-like conditions—it needs to be determined whether the stronger expression of some genes involved in starvation are related to the loss of virulence in H37Ra.

The complementation of H37Ra with *phoP* from H37Rv reverted the colony morphology and increased bacterial persistence within macrophages. Nevertheless, the complemented bacteria were still less virulent than H37Rv. It is conceivable that other mutations in the genome of H37Ra might affect the bacterial persistence in macrophages. Alternatively, the reduced virulence of the complemented strain could be caused by extra-chromosomal complementation of *phoP*, which does not mirror all aspects of its naturally occurring gene regulation, because PhoP is autoregulatory (Gupta et al., 2006).

Our combined approach demonstrates the feasibility of identifying minute but distinct differences between isogenic strains with respect to their genomes and transcriptomes. Perhaps more importantly, our experiments revealed paramount consequences of single point mutations on the survival stratagem of *M. tuberculosis* from which we conclude that the mutation in the *phoP* gene is of particular interest and at least partially responsible for the decreased virulence of H37Ra.

#### EXPERIMENTAL PROCEDURES

A detailed description of all procedures and protocols is available as [Supplemental Data](#).

**Bacterial Strains**

*M. tuberculosis* H37Ra ATCC 25177 was used as test strain. The *M. tuberculosis* H37Rv reference strain was kindly provided by Stewart Cole (Institut Pasteur, Paris, France), which is the same strain used for original sequencing (Cole et al., 1998). The generation of the complemented strain is described in the Supplemental Data.

**Genomic DNA Preparation and Pyrosequencing**

Bacterial cultures were harvested at mid-log phase. For each 50 ml culture pellet, 5 ml phenol and 5 ml chloroform-methanol (3:1) were applied and mixed by pipetting one after another. DNA was extracted with 4 ml RLT buffer (QIAGEN, Hilden), precipitated, and washed. The sequencing system has been described in detail for the resequencing of *Mycoplasma genitalium* (Margulies et al., 2005).

**RNA Preparation, Transcriptomic, and qRT-PCR Analysis**

Total RNA from mycobacterial cells was prepared as previously described (Dietrich, 2001) for all samples except the 1-year time point. For the 1-year time point we applied an optimized amplification protocol (Rachman et al., 2006). We validated array results using qRT-PCR (Figure S3) and could qualitatively confirm microarray results in approximately 90% of tested cases (40 out of 44 qRT-PCRs with single peaks in the dissociation curves).

Fluorescence labeling of cDNA was performed using a LabelStar Array kit (QIAGEN, Hilden). Microarrays were hybridized and scanned using an Agilent Version B scanner (Agilent Technologies, Palo Alto, CA). After combining results of two biological replicates, each analyzed in color-swapped triplicates, genes were assigned to be differentially regulated if they exhibited at least a two-fold change and passed the Student's *t* test of the Rosetta Resolver Software.

**Infection of Mouse Bone Marrow Macrophages**

Bone marrow-derived macrophages were infected with bacterial suspensions of H37Rv, H37Ra, or H37Ra-*phoP*-Rv at multiplicities of infection (MOI) of 5:1. After 4 hr incubation, infection was terminated by washing, and fresh culture medium was added. At days 0, 3, 6, and 9 the number of intracellular CFU was evaluated.

**Gel Shift Assay**

IRDye 800-labeled DNA oligonucleotides containing the PhoP-binding site were incubated with PhoP proteins from H37Ra and H37Rv. The proteins were overexpressed in *E. coli* (BL21) as His-tagged fusion proteins and purified using Ni-NTA beads.

**Supplemental Data**

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and three supplemental tables and can be found with this article online at <http://www.cellhostandmicrobe.com/cgi/content/full/3/2/97/DC1/>.

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#### Accession Numbers

Microarray data have been deposited at the GEO database under the preliminary ID GSE7539.