**Mycobacterium tuberculosis** H37Rv has a single nucleotide polymorphism in PhoR which affects cell wall hydrophobicity and gene expression

L. J. Schreuder,1 P. Carroll,1 J. Muwanguzi-Karugaba,1 Rachel Kokoczka,2 Amanda C. Brown1,3 and T. Parish1,2

Correspondence
T. Parish
tanya.parish@idri.org

1Queen Mary University of London, Barts and The London School of Medicine and Dentistry, London, UK
2TB Discovery Research, Infectious Disease Research Institute, Seattle, WA, USA

**INTRODUCTION**

Tuberculosis (TB) is responsible for the death of more than 1 million people annually (WHO, 2013), and although the number of global TB cases is decreasing, the emergence of multi-drug resistant and extensively drug resistant strains of the causative agent *Mycobacterium tuberculosis* is of severe concern (WHO, 2013).

The complete genome sequence of the *M. tuberculosis* laboratory strain H37Rv was published in 1998 and has provided important insights into its biology (Cole et al., 1998). Although this strain is widely used for experimental studies, there remains genetic diversity among laboratory isolates of H37Rv, which include single nucleotide polymorphisms (SNPs) with important functional consequences. One example is a frameshift mutation in the mycocerosic acid synthase (*mas*) gene, which causes deficiency in the biosynthesis of phthiocerol dimycocerosate (PDIM) (Ioerger et al., 2010), a key cell wall component and major virulence factor of *M. tuberculosis* involved in the prevention of phagosomal acidification (Astarie-Dequeker et al., 2009). A number of other *M. tuberculosis* strains are currently in use in laboratories worldwide. One such strain is CDC1551, a relatively recent clinical isolate responsible for a cluster of tuberculosis cases in the 1990s (Valway et al., 1998). CDC1551 is known to be transmissible and virulent in humans (Valway et al., 1998), and comparable in virulence to H37Rv in animal models (Bishai et al., 1999), but has greater immune-reactivity through a vigorous induction of TNF-α, IL-6, IL-10 and IL-12 (Manca et al., 1999). An early proteome comparison study of H37Rv and CDC1551 found that the two strains are highly similar, despite the lengthy *in vitro* passaging of H37Rv (Betts et al., 2000).
Bacterial two-component regulatory systems (2CRs) are key regulatory systems used to respond to environmental changes (Stock et al., 2000), altering gene expression in response to external stimuli to generate adaptive responses (Ashby, 2004; Hoch, 2000). Prokaryotic 2CRs are highly conserved, and in their simplest forms consist of a sensor histidine kinase (HK) and an effector response regulator (RR). In response to specific signals the HK autophosphorylates and then transfers this phosphate to the RR. In most cases the activation of the RR leads to modulation of gene expression through DNA binding, enabling bacterial adaptation to the initial environmental stimulus (Ryndak et al., 2008). *M. tuberculosis* has 12 complete 2CRs, a small number compared to other bacterial species with similar genome sizes (Parish, 2013).

PhoPR is one of the few mycobacterial 2CRs that are relatively well defined, with a key regulatory role in controlling cell wall composition and virulence (Gonzalo-Asensio et al., 2008a; Goyal et al., 2011; Gupta et al., 2006; Walters et al., 2006). PhoP, the RR, positively regulates several major processes, including aerobic and anaerobic respiration, lipid metabolism, the immediate and enduring hypoxic responses, stress responses and persistence (Cimino et al., 2012; Das et al., 2013; Gonzalo-Asensio et al., 2008b). It is estimated that PhoPR controls the expression of about 2% of the *M. tuberculosis* genome (Cimino et al., 2012).

Inactivation of *phoP* results in a reduced ability to multiply inside macrophages and alterations in the proportion of acyl forms of mannolsylated lipoarabinomannan (manLAM) (Ludwigczak et al., 2002), an essential cell wall component (Brennan, 2003; Goude & Parish, 2008a). ManLAM is known to play a key role in pathogenicity and immune-modulation, suggesting that PhoP is a key regulator of virulence (Brennan, 2003; Ludwigczak et al., 2002; Pérez et al., 2001). The attenuation caused by deletion of the PhoPR is so severe, that deletion strains are under consideration in current vaccine strain trials (Leung et al., 2008). An SNP in the DNA binding domain of PhoP is found in the attenuated H37Ra strain of *M. tuberculosis* (Chesne-Seck et al., 2008; Lee et al., 2008; Wang et al., 2007). This SNP (S219L) results in a loss of DNA binding to its own promoter, and has pleiotropic effects including lack of secretion of the ESAT-6 antigenic protein (Frigui et al., 2008; Gao et al., 2011) and loss of virulence (Lee et al., 2008).

In comparison, relatively little is known about PhoR, the HK sensor of this complex. PhoP and PhoR are co-transcribed and the operon is positively auto-regulated with PhoP binding to direct repeats in its own promoter (Gonzalo-Asensio et al., 2008a; Gupta et al., 2006). The structure of PhoR has been modelled using information from the *Escherichia coli* homologue (Ryndak et al., 2008). PhoR is predicted to be an integral membrane protein with an external domain involved in sensing external signals. The specific signals to which PhoR responds are unknown, although PhoPR signalling was recently linked to pH sensing, since it controls the expression of the *AprABC* (Acid and Phagosome Regulated) locus in *M. tuberculosis* (Abramovitch et al., 2011; Tan et al., 2013). In addition, recent work revealed that an SNP found in PhoR in *Mycobacterium bovis* isolates results in pleiotropic effects on lipid production, secretion and virulence (Gonzalo-Asensio et al., 2014).

We were interested in the functional and phenotypic consequences of SNPs in the PhoPR system and we identified an SNP in PhoR. We hypothesized that this would have downstream effects on the PhoPR regulon and might lead to changes in cell wall composition and gene expression in response to acidic conditions.

**METHODS**

**Bacterial strains and culture.** *M. tuberculosis* H37Rv (ATCC 25618) and CDC1551 were grown in Middlebrook 7H9 medium plus 10% AD (5%, w/v, BSA; 2%, w/v, glucose) and 0.05%, w/v, Tween 80, or on Middlebrook 7H10 agar plus 10% (v/v) OADC (oleic acid, albumin, glucose, catalase) supplement (Becton Dickinson). Cultures were grown without agitation at 37°C/5% CO₂ and 2% humidity for 10 days at pH 6.8. cDNA was made from 1.5 μg of total RNA using the Roche Transcriptor.
cDNA kit and random primers. Primer/probe sets were as follows: *sigA* primers, 5'-CCGATGACGGACGGAGATC-3', 5'-GCCCTCGCCAC-TGCTTTCA-3' and probe 5'-CTCCGGTGATTTC-3'; *phoR* primers, 5'-AGATTTCTGACCCACACCTG-3', 5'-ACGAGGACTCGACCGAGT-3' and probe 5'-TGGTGATAG-3'; *phoR* primers, 5'-CTGCCTCGGC-ATTGAGACG-3', 5'-CAAGAGCAGCAGAATGCTG-3' and probe 5'-GGTGTCGCTG-3'; *lipF* primers, 5'-ATGCCGGAAAGTGGTAATAG-3' and probe 5'-TCGGCCGGC-3'; *mce1* primers, 5'-TCGCCCTATATGACTGGTGA-3', 5'-CATTGGGTTGATCGTGTATCC-3' and probe 5'-CTGCGGGCC-3'. Quantitative reverse transcriptase-polymerase chain reactions (RT-PCRs) were prepared containing Roche LightCycler 480 Taqman Master mix, 2.4 μl of cDNA reaction diluted 1:2, 0.9 μM of each primer and 0.25 μM probe. A no DNA control was included for each run. Cycle conditions were initial denaturation at 95 °C for 10 min, 45 cycles of denaturation (95 °C for 10 s), amplification (56 °C for 1 min) and extension (72 °C for 1 s). A standard curve was generated using genomic DNA and used to calculate copy number. *PhoP*, *phoR*, *lipF*, and *mce1* expression were normalized to *sigA* for each condition. For three biological replicates were assayed in duplicate.

**Fluorescent reporter strains.** Fluorescent reporter strains of *M. tuberculosis* were constructed using codon-optimized mCherry or Turbo-635 in pSMT3 (Carroll et al., 2010). Briefly, actively growing mycobacteria were constructed using codon-optimized mCherry or Turbo-635 in pSMT3 (Carroll et al., 2010). Fluorescent reporter strains. Fluorescent reporter strains of *M. tuberculosis* were constructed using codon-optimized mCherry or Turbo-635 in pSMT3 (Carroll et al., 2010). For the acid-inducible reporters, primers were designed to amplify the upstream region of *aprA* (Abramovitch et al., 2011) using primer pair pHIM2-F (CCC TCT AGA GGC CCG TCT GCT GAT CAA G) and pHIM2-R (CCC GGA TCC CTC TGT CCC CCT TCC GAG CCA G) from *M. tuberculosis* genomic DNA. The product was cloned into pCherry1 or pChargen (Carroll et al., 2010) as a BamHI/XbaI fragment, replacing PhoP from Mtb H37Rv has an SNP with functional consequences

**RESULTS**

**PhoR from H37Rv and CDC1551 differ by a single SNP**

We were interested in understanding the consequences of SNPs in the function of the PhoPR 2CR. We compared the sequence of the complete *phoR* locus from the three of the commonly used strains of *M. tuberculosis* – H37Rv, H37Ra and CDC1551. We found two polymorphisms; an SNP in *phoP* in H37Ra previously reported (Chesne-Seck et al., 2008; Lee et al., 2008) and an SNP in *phoR* with P152 in the H37Rv and H37Ra strains and L152 in the CDC1551 strain (Fig. 1). No other differences were noted in the operon. The SNP was found in all strains of H37Rv previously sequenced (Loeger et al., 2010). SNPs in RR can have severe consequences for protein function and in particular a proline could result in structural changes since it introduces structural constraints. We were interested to determine if this SNP had any functional consequences for processes controlled by the PhoPR system. The *apr locus* is not acid-inducible in the H37Rv strain

PhoPR controls the expression of a significant proportion of the *M. tuberculosis* genome, including the *aprABC* operon. In order to monitor gene expression controlled by PhoPR, we constructed a reporter construct carrying mCherry under the control of the *P* _aprA_ operon. We constructed a reporter construct carrying mCherry under the control of the *P* _aprA_ operon. We were interested to determine if this SNP had any functional consequences for processes controlled by the PhoPR system.

**Lack of *AprABC* induction is not dependent on *PhoRL152* in the H37Rv background**

We hypothesized that the lack of repression of *aprABC* in H37Rv was due to PhoR_L152 activity being suboptimal. In order to address this directly, we engineered H37Rv to carry the PhoR_L152 allele; we constructed an isogenic mutant of H37Rv carrying PhoR_L152 by homologous recombination. The *P* _aprA*-mCherry construct was transformed into the strain, and the promoter activity measured in neutral and acidified medium (Fig. 3a). Surprisingly, a complete lack of induction of expression was seen in both neutral and acidic pH over the 7 day time-course, with *P* _aprA_ activity being constitutive. The growth-phase dependence seen in both other strains was lost, with expression remaining at the same level over 7 days. This was different from both H37Rv and CDC1551, and suggested that although PhoR plays a role in the acid-inducibility, other factors are responsible for the lack of induction in H37Rv.
We excluded the possibility that other SNPs in the PhoPR system were responsible by sequencing the entire locus and promoter region from H37Rv and CDC1551, but no SNPs (other than the two identified) were seen.

We generated reporter constructs with P_{aprA} linked to either GFP or Turbo-635 to exclude any artefacts from the reporter protein expression. In both cases we saw high level constitutive expression in both H37Rv and CDC1551.

**Fig. 1.** Alignment of PhoR from *M. tuberculosis* strains H37Rv, CDC1551 and H37Ra. PhoP and PhoR from H37Rv, CDC1551 and H37Ra were aligned. The PhoP S219L and the PhoR P152L polymorphisms are marked with an asterisk. The non-coding region (44 bp) is indicated by dashes.

**Fig. 2.** AprA promoter activity in response to pH in *M. tuberculosis* H37Rv and CDC1551. mCherry expression driven by P_{aprA} was measured in *M. tuberculosis* grown in medium at neutral pH 6.8 (filled diamonds) or at acidic pH 5.5 (open squares) for 7 days. (a) CDC1551. (b) H37Rv. Fluorescence was quantified at 587/610 nm. Data are the mean and standard deviation from three independent transformants. Significant differences between culture conditions are indicated using Student’s t-test (*P<0.05; **P<0.01).
H37Rv::PhoR<sub>L152</sub> (Fig. 3b), confirming that this was not dependent on mCherry.

In order to exclude effects from differences in gene expression, we measured the mRNA levels of phoPR in all three strains in both neutral and low pH (Fig. 4). Expression of PhoR and PhoP was very similar between the three strains. There was a statistically significant decrease in PhoR in the isogenic mutant strain, but this was a minor change (0.25 to 0.20) and is not likely to be physiologically relevant. There was a trend towards higher expression of PhoP in the strains carrying the PhoR<sub>L152</sub> allele at low pH (but not H37Rv with its native PhoR<sub>P152</sub> allele), but this was not statistically significant due to larger variation between the biological replicates, and the fold-change was low (~2-fold). Thus we concluded that changes in expression levels do not play a major role in the differences between the three strains.

Expression of LipF is acid-responsive in all strains

In order to determine if differences in acid-responsive gene expression were the same for other acid-responsive genes, we looked at expression of lipF in the three strains, since it is also known to be regulated by PhoPR in an acid-dependent fashion. In all three strains, expression of lipF mRNA was increased at pH 5.5, confirming that acid-responsiveness was maintained with both alleles (Fig. 4). There was a slight variation in the level of expression, with expression being higher in the PhoR<sub>P152</sub> strain (H37Rv) than for the PhoR<sub>L152</sub> strains (CDC1551 and H37Rv:PhoR<sub>L152</sub>). This reinforces our suggestion that the PhoR<sub>P152</sub> allele has reduced effectiveness as a repressor, leading to a higher basal level, although it is still a functional regulator. We also looked at mce1 expression as a representative member of the PhoPR regulon (Fig. 4); in this case we saw acid-responsive expression only in the H37Rv background, regardless of the PhoR allele, again suggesting that there are alternative regulatory elements involved in its control and that differences in the pattern of expression between H37Rv and CDC1551 are not wholly PhoR-dependent.

H37Rv and CDC1551 have different outer cell wall layers

PhoPR controls the expression of a large number of genes, and so has control over major processes including cell wall composition (Walters et al., 2006). We hypothesized that the PhoR mutation could lead to differences in the cell wall and its major components. We first confirmed that there was no difference in growth rate between the strains, which might confound any phenotypic assays (Fig. 5a). We used

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**Fig. 3.** The AprA promoter is constitutively expressed in M. tuberculosis H37Rv::PhoR<sub>L152</sub>. (a) P<sub>aprA</sub> activity was measured using mCherry as a reporter in M. tuberculosis H37Rv::PhoR<sub>L152</sub> grown in medium at neutral pH 6.8 (filled diamonds) or at acidic pH 5.5 (open squares) for 7 days. Fluorescence was quantified at 587/610 nm. Data are the mean and standard deviation from three independent transformants. (b) Expression of GFP, mCherry and Turbo-635 from P<sub>aprA</sub> in M. tuberculosis H37Rv and H37Rv::PhoR<sub>L152</sub>. Recombinant strains were culture on solid medium at pH 6.8.

**Fig. 4.** Expression of PhoPR and representative regulon members. Gene expression at the mRNA level was measured in strains grown in medium at pH 6.8 or pH 5.5 for 5 days using qPCR on cDNA. Data are mean plus standard deviation from three independent cultures assayed in duplicate (n=6). H, H37Rv; C, CDC1551; P, H37Rv::PhoR<sub>L152</sub>.
cell hydrophobicity as a gross measure of cell wall composition (Fig. 5b). There was a significant difference between H37Rv and CDC1551 suggesting that these strains do have different cell wall structures, in particular with respect to the outermost layer. We determined whether these differences could be attributed to PhoR using the isogenic mutant H37Rv : : PhoR_{L152}; this strain showed an intermediate phenotype, with hydrophobicity between the two wild-type strains. This suggests that the SNP in PhoR is at least partly responsible for the altered cell wall. We also monitored the uptake of malachite green, as an indicator of permeability (Fig. 5c), but no differences between the strains were noted, suggesting that the gross architecture and transport pathways are unaffected by the PhoR polymorphism. Since PhoPR controls acid-responsive genes, we also looked at the ability of the strains to survive extreme pH (Fig. 5d); of interest the H37Rv strain seemed to show a slightly higher ability to survive. The introduction of PhoRL152 reduced its ability to survive to the level of CDC1551. We speculate this may be due to changes in acid-regulated gene expression, for example if acid-responsive genes are already turned on, this may enhance survival at extreme pH where there is limited time for adaptive responses.

Neither PhoPR allele shows genetic dominance

The polymorphism in PhoR has phenotypic consequences, which could be as a result of loss of function. We wanted to establish whether one M. tuberculosis phoR allele was dominant over the other. We constructed strains in which a second copy of phoPR was integrated into the chromosome (using a phage-based integrating plasmid). In order to avoid disrupting the relative amounts of PhoP and PhoR, we incorporated the complete phoPR operon. PhoP-phoR_{L152} or phoP-phoR_{L152} operons were introduced into the three strains (H37Rv, H37Rv : : PhoR_{L152}, and CDC1551).

We first determined whether this had any effect on $P_{apRA}$ activity in neutral pH (Fig. 6). Expression of PhoR_{L152} in the H37Rv background significantly reduced the basal level of expression from $P_{apRA}$, consistent with the idea that PhoRL152 is less active than PhoR_{L152}. In addition, expression of
PhoR<sub>P152</sub> in CDC1551 resulted in an increased basal level under neutral conditions, which could result from competition/interference for the DNA binding site. Contrary to expectation, introduction of PhoR<sub>P152</sub> into the H37Rv::PhoR<sub>L152</sub> strain resulted in a decrease in the basal level under neutral pH (Fig. 6a). The basal level was 1.5 to 2-fold lower in this strain than in H37Rv or CDC1551, again suggesting that factors other than PhoPR are involved in regulation of AprABC expression.

We also looked at acid-inducibility of P<sub>aprA</sub> in these strains; reporter activity was measured after 3 days in neutral or acidic medium (Fig. 6b). Introduction of PhoR<sub>L152</sub> into H37Rv was unable to confer acid-inducibility, whereas introduction of PhoR<sub>P152</sub> into CDC1551 led to growth-phase regulated expression under neutral pH and a partial loss of acid-inducibility. In the H37Rv::PhoR<sub>L152</sub> strain there was little induction of expression regardless of the extra allele.

In contrast, the results for cell wall hydrophobicity were much clearer, with the PhoR<sub>P152</sub> showing a dominant effect (Fig. 7). Introduction of the PhoR<sub>P152</sub> allele into the CDC1551 strain resulted in an increase in hydrophobicity, to the level of the H37Rv strain, whereas introduction of the PhoR<sub>L152</sub> allele had no effect on either CDC1551 or H37Rv::PhoR<sub>L152</sub> strain (Fig. 7). These data suggest that the difference in cell wall hydrophobicity between the strains is largely due to PhoR.

**DISCUSSION**

A number of virulent <i>M. tuberculosis</i> strains, originally isolated from clinical cases of TB, are widely used in laboratories around the world. Reports of genetic variation between strains are known, with many cases of unexplored scientific variables. The virulent strains of <i>M. tuberculosis</i> H37Rv and CDC1551, and the attenuated strain H37Ra have been the focus of comparative studies (Fleischmann et al., 2002; Zheng et al., 2008). The S219L PhoP SNP in H37Ra contributes to its attenuation through impaired PhoP DNA binding and transcriptional activation (Chesne-Seck et al., 2008; Zheng et al., 2008). Here we report on additional genetic and phenotypic differences...
between H37Rv and CDC1551, and discuss the significance of a PhoR polymorphism.

As the polymorphism in PhoR has phenotypic consequences we attempted to establish whether one M. tuberculosis phoR allele could compensate for this potential loss of function. However, the double phoPR allele strains behaved unexpectedly in that three strains with the same allele combination all had different levels of expression from the apr promoter. This could suggest that other regulatory systems are involved. Alternatively, it could be due to the difference in expression levels of the integrated phoPR operon, as although it was integrated as a single copy with its native promoter, the expression levels from the L5 att locus were not always the same as for the normal chromosomal location.

Interestingly, in our study neither phoR allele seemed dominant. In contrast, the M. tuberculosis PhoR allele was dominant over the M. bovis allele, presumably since the latter sensor appears to be defective (Gonzalo-Asensio et al., 2014). In the case of the two M. tuberculosis alleles, it may be that both proteins are functional, but possibly to different extents.

It is interesting that the cell hydrophobicity phenotype appeared to be largely PhoPR-dependent, whereas the acid-induced expression of AprABC was only partly controlled by PhoPR. Presumably other genetic differences between CDC1551 and H37Rv also contribute to these changes, although there are no other polymorphisms in the PhoPR 2CR. PhoR is a sensor which is likely to be located in the cytoplasmic membrane; the stimulus for this protein is not known, but presumably it needs to first be transmitted through the cell wall. Differences in the cell wall between the strains could therefore mean that the same stimulus is transduced with different effectiveness. Our strain of H37Rv (London Pride) has low amounts of PDIM in the cell wall; M. marinum strains deficient in PDIM and/or peptidoglycans are avirulent and hypersensitive to antibiotics and have increased cell wall permeability (Yu et al., 2012), and so our strain is likely to have different/increased permeability (Loerger et al., 2010). We could envisage a model to account for the differences in which a combination of signal transduction through the cell wall and differences in HK activity produces the three different phenotypes seen. For example, if there is increased signal transduction in the H37Rv strain coupled with a fully active PhoPR system derived from CDC1551, then this could result in the complete derepression of the system seen in the H37Rv: :PhoR152 strain under neutral conditions as seen. In support of this idea, the SNP in PhoR is located in the intracellular PAS domain, the region responsible for sensing the environment (Taylor & Zhulin, 1999).

In conclusion, we have demonstrated that an SNP between CDC1551 and H37Rv has functional consequences for both the cell wall and gene expression and we have demonstrated that regulation of AprABC expression is more complex than previously thought.

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REFERENCES


Frigui, W., Bottai, D., Majlessi, L., Monot, M., Josselin, E., Brodin, P., Garnier, T., Gicquel, B., Martin, C. & other authors (2008). Control of...


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