Evolutionary history of tuberculosis shaped by conserved mutations in the PhoPR virulence regulator

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Edited by Peter M. Small, Institute for Systems Biology, Bill and Melinda Gates Foundation, Seattle, WA, and accepted by the Editorial Board June 20, 2014 (received for review April 11, 2014)

Although the bovine tuberculosis (TB) agent, Mycobacterium bovis, may infect humans and cause disease, long-term epidemiological data indicate that humans represent a spill-over host in which infection with \textit{M. bovis} is not self-maintaining. Indeed, human-to-human transmission of \textit{M. bovis} strains and other members of the animal lineage of the tubercle bacilli is very rare. Here, we report on three mutations affecting the two-component virulence regulation system PhoP/PhoR (PhoPR) in \textit{M. bovis} and in the closely linked \textit{Mycobacterium africanum} lineage 6 (L6) that likely account for this discrepancy. Genetic transfer of these mutations into the human TB agent, \textit{Mycobacterium tuberculosis}, resulted in down-regulation of the PhoP regulon, with loss of biologically active lipids, reduced secretion of the 6-kDa early antigenic target (ESAT-6), and lower virulence. Remarkably, the deleterious effects of the \textit{phoPR} mutations were partly compensated by a deletion, specific to the animal-adapted and \textit{M. africanum} L6 lineages, that restores ESAT-6 secretion by a PhoPR-independent mechanism. Similarly, we also observed that insertion of an IS6110 element upstream of the \textit{phoPR} locus may completely revert the \textit{phoPR}-bovis-associated fitness loss, which is the case for an exceptional \textit{M. bovis} human outbreak strain from Spain. Our findings ultimately explain the long-term epidemiological data, suggesting that \textit{M. bovis} and related \textit{phoPR}-mutated strains pose a lower risk for progression to overt human TB, with major impact on the evolutionary history of TB.

Significance

In 1901, when Robert Koch proposed that the bacilli causing human and bovine tuberculosis were not identical, this view caused much controversy. Now, 113 y later, we know that the bovine tuberculosis agent, \textit{Mycobacterium bovis}, together with other animal strains, forms a separate phylogenetic lineage apart from the human \textit{Mycobacterium tuberculosis} lineages, but the molecular reasons why bovine and animal strains only play minor roles in human tuberculosis epidemiology remain unknown. Herein, we show by genetic transfer and virulence experiments that specific mutations in a virulence regulator contribute to lower fitness and virulence of \textit{M. bovis} and related strains for the human host, likely obstructing the capacity of causing overt disease needed for efficient human-to-human transmission.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. P.M.S. is a guest editor invited by the Editorial Board.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, ncbi.nlm.nih.gov/geo (accession no. GSE54421: samples GSM1314812–GSM1314819).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1406693111/-/DCSupplemental.
**Results and Discussion**

**SNPs Specific to the Animal and M. africanaum Lineages Impair the Function of PhoPR.** Through genome comparison of 30 MTBC strains, we identified three SNPs affecting the *phoPR* genes of members of the animal-adapted and *M. africanaum* L6 lineages that were not seen in the *M. tuberculosis sensu-stricto* genomes (Fig. 1A and Fig. S1). The *phoPR* genes encode a two-component regulatory system that is known for its strong impact on virulence and immunogenicity of *M. tuberculosis* (16, 17) because of its key role in the regulation of lipid synthesis and secretion of the 6-kDa secreted antigenic target ESAT-6 (18–22). To explore whether these SNPs affect the expression of the PhoP regulon, we first compared the transcriptome of *M. tuberculosis* strains lacking the endogenous *phoPR* genes (*ΔphoPR* and their complemented derivatives expressing either the *M. bovis* or *M. tuberculosis* allele of *phoPR* (*phoPR-bovis* and *phoPR-TB*, respectively). These comparisons were performed in parallel in *M. tuberculosis* strains from two distinct genetic backgrounds: strain GC1237 from L2 (also named East Asia or Beijing cluster) and the reference strain H37Rv from L4 (also named Euro-American cluster) (Fig. 1B). To further explore the potential impact of these SNPs, we next compared the transcriptome of *M. tuberculosis sensu-stricto* strains harboring these *phoPR* mutations to closely related strains derived from *M. bovis* (Fig. 1C and Fig. S2A) or *M. africanum* (Fig. 1D and Fig. S2B). This observation is highly relevant for pathogenicity because PhoPR controls the synthesis and export of many *M. tuberculosis* virulence factors, such as LipF, ESAT-6, and lipids of the polyacyltrehalose (PAT)

**Fig. 1.** The *phoPR* allele from animal-adapted and *M. africanaum* L6 strains is deficient. (A) Schematic global phylogenetic tree of the MTBC (1, 2). The length of the branches does not correlate with phylogenetic distance. Names of the strains used in this study are indicated. (B) Genome-wide transcriptional profiles of WT *M. tuberculosis* H37Rv, *ΔphoPR* mutant, and *phoPR-bovis*– or *phoPR-TB*-complemented strains. Fold-change values from individual probes for each gene were averaged. Those genes showing a statistically significant average fold-change > 2 or < 0.5 in the WT or the *phoPR-TB*-complemented strains relative to the *ΔphoPR* mutant were selected as positively or negatively regulated by PhoP, respectively. (C) qRT-PCR analysis of expression of main reporter genes of the PhoP regulon. (D) TLC analysis of lipids extracted from [*13C*] propionic acid-labeled cultures. The position of the major SL (the tetra-acylated sulfoglycolipids, AcSGL) is highlighted (arrow). (E) Immunoblot of secreted and whole-cell fractions probed with ESAT-6– or GroEL2– (used as a lysis control) specific antibodies.
and sulfolipid (SL) families (18, 19, 23–25). Consistently, the M. tuberculosis-specific lipids, PAT and SL, were barely detectable in the ΔphoPR and the phoPR-bovis–complemented mutants relative to WT or phoPR-TB–complemented strains (Fig. 1D and Fig. S2C). Similarly, export of ESAT-6 was strongly dependent on the presence of phoPR-TB allele in the M. tuberculosis WT and recombinant strains (Fig. 1E and Fig. S2D).

To define which mutation impairs the function of the phoPR-bovis allele, we first compared the production of PhoP in WT, ΔphoPR, or complemented strains carrying the phoPR-bovis or phoPR-TB allele (Fig. S3). A similar amount of the PhoP protein was detected in the WT and the complemented strains, suggesting that the SNP located in the promoter region of the phoPR-bovis allele does not impair the expression of the phoPR operon. We next produced two phoPR allele chimeras combining phoP-bovis, carrying the promoter SNP, with phoR-TB (phoP-bovis+phoR-TB) or phoR-bovis, harboring the missense mutation, with phoP-TB (phoP-TB+phoR-bovis). These chimeras were used to complement the ΔphoPR mutant. Both the qRT-PCR and lipid analyses demonstrated that the phoR-bovis allele is defective, whereas the promoter mutation seems to have no impact on its own (Fig. S3).

SNPs in the phoPR-bovis Allele Impact Virulence in M. tuberculosis. Next, we assessed whether the modulation of the PhoP regulon, caused by the SNPs in the phoPR-bovis allele, affects host–pathogen interaction. M. tuberculosis is an intracellular pathogen that thrives inside macrophages and other phagocytes in infected individuals. Therefore, we first infected human primary macrophages with the different recombinant strains and monitored the intracellular bacterial load at various time points postinfection. This experiment revealed a lower bacterial load at 6 d post-infection for the M. tuberculosis mutants expressing the phoPR-bovis allele relative to its phoPR-TB counterpart (Fig. 2A), a defect similar to that of the ΔphoPR mutant. We also conducted low-dose intranasal infection of immunocompetent BALB/c mice (200 cfu per mouse) using the M. tuberculosis H37Rv strains carrying the phoPR-TB or phoPR-bovis allele, as well as WT and ΔphoPR strains as controls. We found that complementation with the phoPR-bovis allele only partially restored the capacity of the recombinant H37Rv strain to multiply in infected animals, in contrast to the strain with the phoPR-TB allele (Fig. 2B and C). Thus, the SNPs in the phoPR-bovis allele transferred to M. tuberculosis attenuates its virulence both in human cells and in the mouse infection model.

The PhoPR System Is Defective in Animal-Adapted and M. africanum L6 Strains But Compensatory Evolution Has Restored ESAT-6 Secretion. Given the strong phenotype of the phoPR-bovis allele in M. tuberculosis sensu-stricto, we sought to determine whether the phoPR-bovis allele is indeed responsible for an impaired PhoPR regulation in the M. bovis and M. africanum L6 genetic backgrounds. To this end, representative strains of these lineages were transformed with the phoPR-TB allele and assessed for the expression levels of genes from the PhoP regulon by qRT-PCR. Expression of indicator genes pks3, pks2, lipF, fadD21, and narK1 was much stronger in M. bovis and M. africanum recombinants expressing phoPR-TB compared with strains expressing phoPR-bovis only (Fig. 3A and Fig. S4A). Consistently, lipids (i.e., SL and PAT) described as specific for M. tuberculosis sensu-stricto (26) were now detectable in the recombinant M. bovis and M. africanum strains carrying the phoPR-TB allele, as demonstrated by thin-layer chromatography (TLC) and mass spectrometry of the purified compounds (Fig. 3B and Figs. S4 and S5). These findings indicate the direct link between the phoPR mutations and the lipid profile of various tubercle bacilli. In contrast, ESAT-6 was secreted at comparable levels in WT and recombinant strains (Fig. 3C and Fig. S6), suggesting that the animal-adapted and M. africanum L6 lineages have evolved to retain ESAT-6 secretion despite the mutations in phoPR.

Secretion of ESAT-6 requires the espACD operon (27, 28), which is part of the PhoP regulon (18, 20–22). In addition to PhoPR, several regulators, such as the nucleoid-associated proteins Lsr2 (29) and EspR (30–32) and the two-component system MprAB (33), were found to modulate the transcription of the espACD operon in response to environmental signals and stresses. Interestingly, both animal-adapted and M. africanum L6 strains harbor a specific deletion, named region of difference 8 (RD8) (34), and several SNPs just upstream of espACD relative to M. tuberculosis sensu-stricto strains (Fig. 4A). The RD8 deletion removes binding sites for EspR and MprAB, and therefore may impact the expression of espACD. These observations led us to assess espACD gene expression in a collection of animal-adapted or M. africanum L6 strains and compare it to M. tuberculosis sensu-stricto WT or recombinant strains. We found unexpected high levels of espACD expression in all animal-adapted or M. africanum L6 strains, in contrast to the situation in M. tuberculosis ΔphoPR or recombinant strains expressing phoPR-bovis (Fig. S7), suggesting that espACD expression escaped the PhoPR control in these strains. This exceptional result was confirmed in phoPR mutants of representatives of the animal-adapted and M. africanum L6 strains showing no reduction of the transcript level relative to WT, in contrast to M. tuberculosis or M. canetti.

**Fig. 2.** The phoPR-bovis allele impacts the interaction of M. tuberculosis with cellular or animal hosts. (A) Quantification of intracellular bacteria at 24-, 72-, or 144-h postinfection. Human-macrophage-derived macrophages (HMDM) were infected (at a multiplicity of infection of 2) for 2 h with indicated strains. Data are mean ± SD of three independent experiments performed in duplicate. (B and C) Seven-week-old BALB/c mice were infected intranasally with ~200 cfu of the indicated strains. At the indicated time points, lungs (B) or spleens (C) were homogenized and plated for colony-forming unit determination. Data are means ± SD of four mice at days 14 and 56 and eight mice at days 1 and 28, from two independent experiments. The difference between experimental groups was evaluated by the two-tailed Student t test: P values, *P < 0.05, **P < 0.01, ***P < 0.005.
The fact that *M. canettii*, which is an early-branching representative in the MTBC phylogenetic tree, exhibits a similar PhoPR control on *espACD* expression as *M. tuberculosis* strongly supports the hypothesis that animal-adapted and *M. africanum* L6 strains specifically acquired this property during evolution. In line with these observations, transfer of the *espACD* allele from *M. bovis* (*espACD-bovis*) into *M. tuberculosis ΔphoPR* increases *espACD* expression and restores ESAT-6 secretion, whereas transfer of a second copy of the corresponding region from *M. tuberculosis* (*espACD-TB*) has only marginal impact (Fig. 4C and D and Fig. S8). Overall, our results demonstrate that mutations of the PhoPR system impair the production of molecules, such as SL, PAT, and LipF, important for pathogenicity in the animal-adapted and *M. africanum* L6 lineages. Nevertheless, these strains acquired compensatory mutations that short-cut the regulation loop controlling *espACD* expression in *M. tuberculosis*.

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**IS6110 Insertion Upstream phoPR in the Hypervirulent *M. bovis* B Compensates the phoPR-bovis Deficiency.** Despite the compensatory genetic events affecting ESAT-6 secretion, epidemiological data support the hypothesis that animal-adapted and *M. africanum* L6 strains have not recovered the same virulence for humans as *M. tuberculosis sensu-stricto* strains (likely because of the above-mentioned expression differences in other members of the PhoPR regulon). Thus, documented outbreaks of human TB caused by strains from the animal-adapted lineage are extremely rare. Of note, a unique multidrug-resistant *M. bovis* isolate, *M. bovis* B, was responsible for 36 TB cases in Spain (35), where this specific strain successfully spread via human-to-human transmission. Interestingly, this *M. bovis* B isolate harbors a mobile element, IS6110, inserted 75-bp upstream of the *phoPR* operon (36) (Fig. 5A). When we transferred this particular *phoPR* allele from *M. bovis* B (*phoPR-B*) into *M. tuberculosis ΔphoPR* and *M. bovis ΔphoPR*, we found increased *phoP* transcription and PhoP production in comparison...
with WT (Fig. 5 B and C and Fig. S9). Consistently, we detected increased transcription of PhoP regulon genes (Fig. 5D), production of SL and PAT (Fig. 5E and Fig. S9), and ESAT-6 secretion in the recombinant M. tuberculosis or M. bovis ANS strains harboring the phoPR-B allele. Thus, the occurrence of IS6110 upstream phoPR globally compensates the deleterious effect of the SNPs found in the phoPR-bovis allele, and this suppressive effect is likely a result of increased expression of phoPR driven by a strong IS6110-contained promoter (36, 37).

Finally, we sought to determine the impact of the IS6110 insertion on virulence. Because we observed in our initial experiments (Fig. 2 B and C) that the attenuation phenotype associated with the phoPR-bovis allele was clearly seen in the acute phase of infection in mice, we used for these experiments severe combined immunodeficient (SCID) mice, which reproduce this initial phase (38). Consistent with the phenotype in vitro, the phoPR-B allele provides enhanced in vivo virulence in mice. Indeed, infection of SCID mice showed the median survival times were similar for M. tuberculosis strains expressing phoPR-TB (34 d) or phoPR-B (30 d) and significantly higher for the recombinant strain expressing phoPR-bovis (43 d) or the ΔphoPR mutant (Fig. 5F).

Taking these data together, it seems very likely that the highly virulent, human-to-human transmission-associated phenotype of the M. bovis B strain is caused by the specific IS6110 insertion, which suppresses the various phoPR-bovis–associated deficiencies.

Conclusions
In this study we demonstrated that the PhoPR regulation system is deficient in animal-adapted and M. africanum strains primarily because of a mutation within the phoR gene. The key role of PhoPR for the M. tuberculosis pathogenicity was previously established: a spontaneous punctual mutation altering the DNA-binding capacity of PhoP in the H37Rv strain or insertional inactivation of the phoP gene in two M. tuberculosis strains significantly impaired the multiplication in mice or macrophages (17, 18, 20, 21). Here, we found that specific mutations present within the phoPR-bovis allele, when transferred into M. tuberculosis, also strongly impact the virulence of the recombinant strain, raising important questions about the consequences of these mutations for the evolutionary history of the concerned lineages of tubercle bacilli.

Overall, our results provide a molecular explanation for the intriguing epidemiological data showing that M. bovis and other animal-adapted strains are only rarely found in human TB outbreaks, despite their close genetic relationship with M. tuberculosis sensu-stricto and their ability to infect humans through contaminated milk. Our findings may also be extended to the closely related M. africanum L6 strains, which exhibit a similar impaired fitness relative to M. tuberculosis sensu-stricto (13). Our results suggest an evolutionary scenario (Fig. S10) in which mutations at the phoPR locus occurred in the common ancestor of the animal-adapted and M. africanum strains. These mutations impacted the synthesis and secretion of lipid- and protein-pathogenicity factors, with the likely consequence of dramatically reduced fitness for the human host. The strains, which successfully got through this low-virulence bottleneck, acquired compensatory mutations, such as the R8 deletion, that restored the secretion of ESAT-6 via EspACD independently of phoPR but still failed to provide the same virulence for humans as the ancestral phoPR-TB allele. The animal-adapted strains may have acquired later additional mutations, allowing them to persist in various animal species that might exhibit a different susceptibility than humans, such as Mycobacterium microti in voles and cats (39), the Dassie bacillus in dassies (40), Mycobacterium mungi in the banded mongooses (41), Mycobacterium pinnipedii in seals (42), or M. bovis in cattle, deer, and badgers (6). Of note, specific adaptation of M. tuberculosis sensu-stricto to the human host seems to be conversely associated with lower virulence than M. bovis strains in certain animal species, such as rabbits, goats, or cattle (43–45).

Finally, in some exceptional animal-adapted isolates, such as the M. bovis B strain, additional fitness for the human host was gained through insertion of a mobile element upstream phoPR, leading to overexpression of this operon, favoring aerosol transmission of bacilli via patients who have developed active pulmonary TB. This succession of genetic events is fully
compatible with previously proposed phylogenetic schemes (1, 2, 46) and underlines how a few point mutations in important genes, combined with selected compensatory mutations, can have a long-lasting and powerful impact on the evolution and adaptation of a pathogen to specific hosts.

Materials and Methods

Mutations in the various *M. tuberculosis* sensu stricto, *M. bovis*, *M. africanaum*, or *M. caratti* strains were performed by allelic exchange. The resulting mutants were characterized by PCR using specific primers. The complemented strains were produced by inserting a single copy of the indicated genes in the mycobacterial chromosome. Lipid analysis was performed on exponentially growing strains labeled for 24 h with [14C] propionic acid or on stationary-phase cultures using a plate scintillation counter. RNA levels were determined by quantitative real-time PCR using SYBR green and specific primers. Transcriptome analyses were performed using Agilent manufactured customized microarrays with RNA extracted from exponentially growing bacteria, labeled with Cy5 or Cy3, and hybridized competitively. Western-blot of proteins secreted or associated with bacterial cells were performed on 3- to 9-μm thick membranes obtained by centrifugation before protein extraction, separation by SDS-PAGE, and membrane trankration. ESAT-6 secretion, a virulence gene cluster of *M. africanum* complex: experimental infection shows its role in a bronchoscopic rabbit model of cavitary tuberculosis in South Africa. The Infect Microbio 51(4):577–597.


