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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 *M. bovis* is not self-maintaining. Indeed, human-to-human transmission of *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 *M. bovis* and in the closely linked *Mycobacterium africanum* lineage 6 (L6) that likely account for this discrepancy. Genetic transfer of these mutations into the human TB agent, *Mycobacterium tuberculosis*, resulted in downregulation 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 *phoPR* mutations were partly compensated by a deletion, specific to the animal-adapted and *M. africanum* L6 lineages, that restores ESAT-6 secretion by a PhoPR-independent mechanism. Similarly, we also observed that insertion of an IS*6110* element upstream of the *phoPR* locus may completely revert the *phoPR-bovis*–associated fitness loss, which is the case for an exceptional *M. bovis* human outbreak strain from Spain. Our findings ultimately explain the long-term epidemiological data, suggesting that *M. bovis* and related *phoPR*-mutated strains pose a lower risk for progression to overt human TB, with major impact on the evolutionary history of TB.

evolution | phylogeny | adaptation | zoonosis

Tuberculosis (TB) is caused by bacilli from the genetically compact *Mycobacterium tuberculosis* complex (MTBC), which gathers eight defined phylogenetic lineages in addition to the more distantly related *Mycobacterium canettii* group (1–3): *M. tuberculosis sensu-stricto* from lineages L1–L4 and L7 form a large group of human-adapted strains responsible for the vast majority of global human TB cases, whereas *Mycobacterium africanum* lineages (L5, L6), which are restricted to humans from West Africa, are phylogenetically linked with the eighth lineage, comprising the various animal-adapted strains, with *Mycobacterium bovis* as the most downstream member in the phylogeny (Fig. 1*A*) (4, 5). Animal strains exhibit a wide host range that includes livestock animals in close contact with humans. Episodes of bovine TB in cattle herds have been reported in 128 of 155 countries during the period 2005–2008 (6). Although the bulk of these episodes is mainly found in developing countries (6), bovine TB remains a major problem even in some industrialized countries, best exemplified by the United Kingdom, which has experienced an important resurgence of bovine TB since the 1980s (7). Because *M. bovis* and other closely related animal-adapted strains are also capable of causing TB in humans, this situation raises concerns regarding the zoonotic risk. Indeed, human TB cases resulting from *M. bovis* are estimated to be around 2% worldwide (8), with higher incidence (18–30% of TB cases) in some developing countries, such as Tanzania (9). Despite this globally large number of cases, several lines of evidence suggest that *M. bovis* and phylogenetically related strains do not exhibit the same virulence and transmissibility for the human host as *M. tuberculosis sensu-stricto*. First, there are very few reports of human-to-human transmission of *M. bovis*. The infection occurs mostly through consumption of unpasteurized milk or close contact with infected animals (6, 10) suggesting that *M. bovis* exhibits a lower transmissibility among humans than *M. tuberculosis sensu-stricto*. Second, an important population study in Denmark showed that morbidity was much lower among patients infected with *M. bovis* than *M. tuberculosis* (11). Because human-to-human transmission is associated with overt disease, this conclusion is consistent with a lower transmissibility. Third, isolates from lineage L6) (also named West African 2 and corresponding to one group of *M. africanum* strains), which are very closely related phylogenetically to the strains of the animal lineage, exhibit features suggesting that they are also less virulent than *M. tuberculosis sensu-stricto*. *M. africanum* strains are restricted to West Africa and, despite

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, *Mycobacterium bovis*, together with other animal strains, forms a separate phylogenetic lineage apart from the human *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 *M. bovis* and related strains for the human host, likely obstructing the capacity of causing overt disease needed for efficient human-to-human transmission.

Author contributions: J.G.-A., W.M., A.P., C.M., R.B., and C.G. designed research; J.G.-A., W.M., A.P., C.A.-D., C.P., F.M., F.L., and C.G. performed research; J.G.-A., W.M., A.P., C.A.-D., C.P., F.L., M.D., C.M., R.B., and C.G. analyzed data; and J.G.-A., C.M., R.B., and C.G. wrote the paper.

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, www.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.

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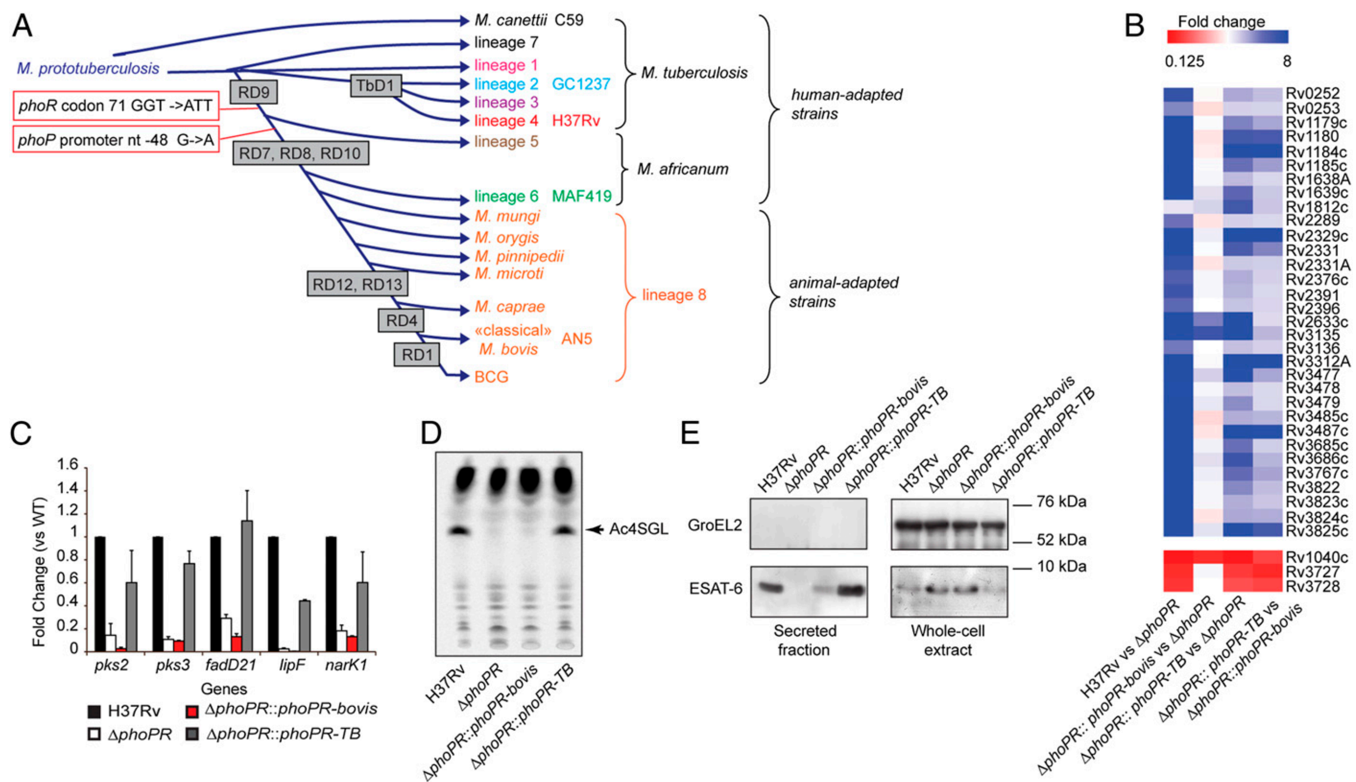

Fig. 1. The *phoPR* allele from animal-adapted and *M. africanum* L6 strains is deficient. (*A*) Schematic global phylogenetic tree of the MTBC (1, 2). The length of the branches does not correlate with phylogenetic distance. Names of the strains used in this study are indicated. (*B*) Genome-wide transcriptional profiles of WT *M. tuberculosis* H37Rv, Δ*phoPR* 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 Δ*phoPR* 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 [^{14}C] propionic acid-labeled cultures. The position of the major SL (the tetra-acylated sulfoglycolipids, Ac_4SGL) is highlighted (arrow). (*E*) Immunoblot of secreted and whole-cell fractions probed with ESAT-6– or GroEL2- (used as a lysis control) specific antibodies.

major population movements between this geographic area and the rest of the world, they have failed to disseminate worldwide. This observation has fueled speculations about a potential local zoonotic reservoir (12). Moreover, an epidemiological study showed that household contacts of TB patients progress less frequently to active disease when they are infected with *M. africanum* L6 than with *M. tuberculosis sensu-stricto* (13, 14), a situation similar to that observed for *M. bovis* (11). Consistently, *M. africanum* L6 strains exhibit a lower fitness than *M. tuberculosis sensu-stricto* in the mouse model of TB (15). Collectively, these observations infer that animal-adapted strains, along with related *M. africanum* L6 members, have acquired mutations that affect their fitness for humans. However, the nature of the genetic differences explaining these specific phenotypic traits has remained unknown. Identifying these alterations and their molecular consequences is of tremendous importance to understanding the critically relevant factors determining transmission efficiency and host-specific adaptation. This information is also highly relevant to evaluate the risk associated with the various MTBC strains.

In the present study, we combined comparative genomics, molecular genetics, and virulence tests to explore the impact of three SNPs evolutionarily conserved in animal-adapted and *M. africanum* L6 strains. These mutations affect a two-component regulation system known for its deep impact on the virulence of *M. tuberculosis*. Our findings establish that these SNPs impair the production and secretion of major protein- and lipid-virulence determinants and ultimately provide the likely genetic mechanism responsible for the lower prevalence and virulence of these lineages in humans.

Results and Discussion

SNPs Specific to the Animal and *M. africanum* 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. africanum* L6 lineages that were not seen in the *M. tuberculosis sensu-stricto* genomes (Fig. 1*A* 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 (Δ*phoPR*) 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. 1*A*). In both genetic backgrounds, most genes of the PhoP regulon (i.e., genes differentially expressed between the WT and corresponding Δ*phoPR* variant) exhibited clear expression differences between the *phoPR-bovis* and *phoPR-TB* complemented strains (Fig. 1*B* and Fig. S2*A*), a finding confirmed by quantitative RT-PCR (qRT-PCR) (Fig. 1*C* and Fig. S2*B*). 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)

and sulfolipid (SL) families (18, 19, 23–25). Consistently, the *M. tuberculosis*-specific lipids, PAT and SL, were barely detectable in the Δ*phoPR* and the *phoPR-bovis*–complemented mutants relative to WT or *phoPR-TB*–complemented strains (Fig. 1*D* and Fig. S2*C*). Similarly, export of ESAT-6 was strongly dependent on the presence of *phoPR-TB* allele in the *M. tuberculosis* WT and recombinant strains (Fig. 1*E* and Fig. S2*D*).

To define which mutation impairs the function of the *phoPR-bovis* allele, we first compared the production of PhoP in WT, Δ*phoPR*, 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 Δ*phoPR* 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 postinfection for the *M. tuberculosis* mutants expressing the *phoPR-bovis* allele relative to its *phoPR-TB* counterpart (Fig. 2*A*), a defect similar to that of the Δ*phoPR* 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 Δ*phoPR* 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. 2 *B* and *C*). Thus, the SNPs in the *phoPR-bovis* allele transferred to *M. tuberculosis* attenuate 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. 3*A* and Fig. S4*A*). 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. 3*B* 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. 3*C* 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. 4*A*). 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* Δ*phoPR* 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 Δ*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. canettii*

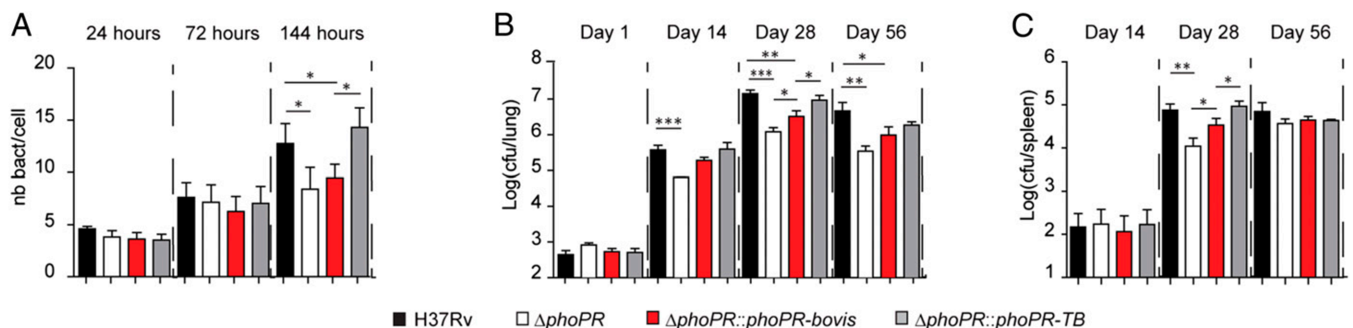

Fig. 2. The *phoPR-bovis* allele impacts the interaction of *M. tuberculosis* with cellular or animal hosts. (*A*) Quantification of intracellular bacteria at 24-, 72-, or 144-h postinfection. Human-monocyte-derived macrophages (hMDM) were infected (at a multiplicity of infection of 2) for 2 h with indicated 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, $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

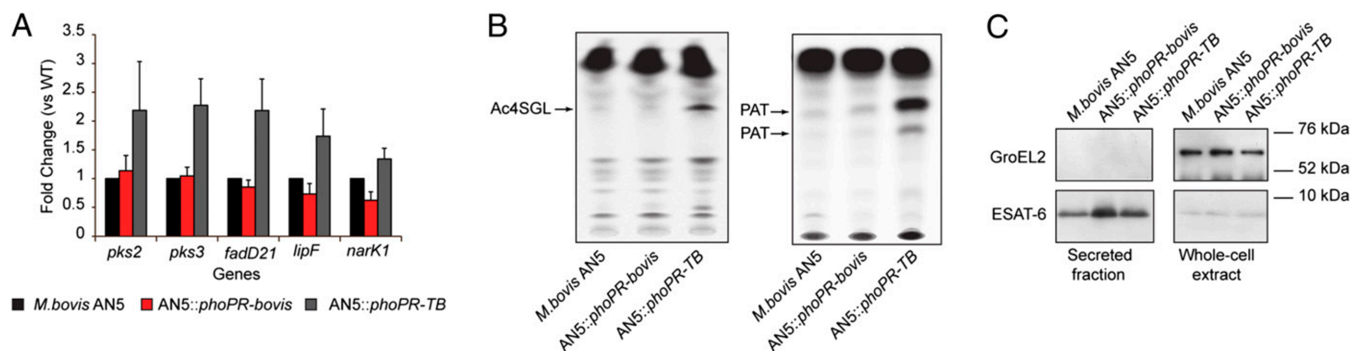

Fig. 3. The *phoPR-bovis* allele is deficient but ESAT-6 is secreted in animal-adapted strains. (*A*) qRT-PCR analysis of expression of main reporter genes of the PhoP regulon in WT and recombinant *M. bovis* AN5 grown liquid culture. (*B*) TLC analysis of lipids extracted from [^{14}C] propionic acid-labeled cultures. (*C*) Immunoblot of secreted and whole-cell fractions from WT or recombinant *M. bovis* strains probed with ESAT-6– or GroEL2-specific antibodies.

(Fig. 4*B*). The fact that *M. canettii*, which is an early-branching representative in the MTBC phylogenetic tree (3), 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* Δ*phoPR* 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. 4 *C* 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*.

IS*6110* 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, IS*6110*, inserted 75-bp upstream of the *phoPR* operon (36) (Fig. 5*A*). When we transferred this particular *phoPR* allele from *M. bovis* B (*phoPR-B*) into *M. tuberculosis* Δ*phoPR* and *M. bovis* Δ*phoPR*, we found increased *phoP* transcription and PhoP production in comparison

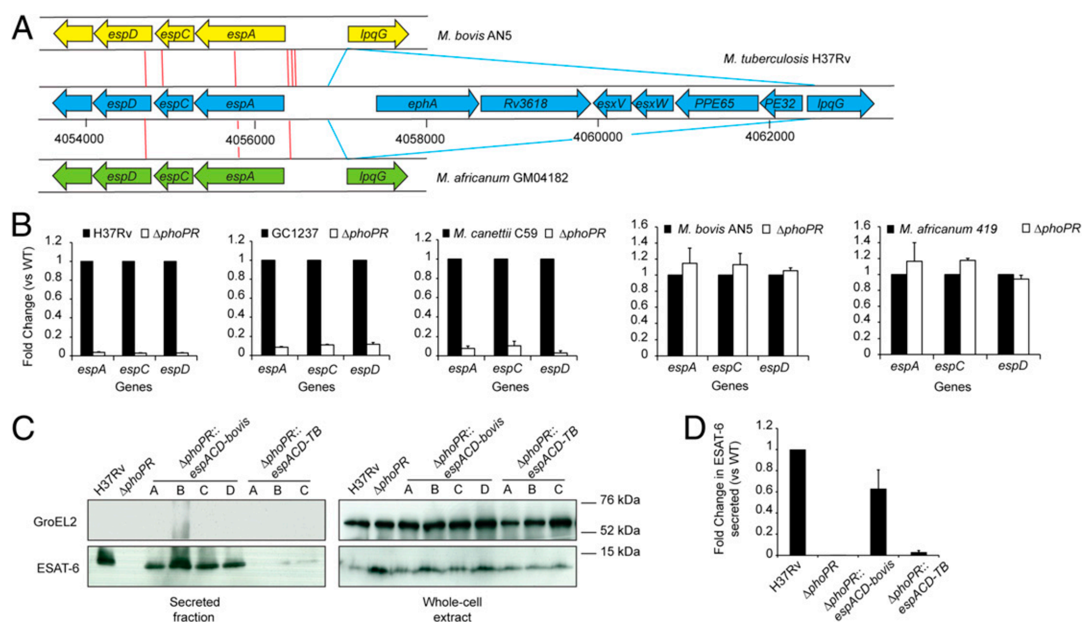

Fig. 4. RD8 deletion allows ESAT-6 secretion independently from PhoPR. (*A*) Schematic representation of the genetic structure at the *espACD* locus in the *M. tuberculosis sensu-stricto* and animal-adapted or *M. africanum* L6 strains. Red lines indicate individual SNPs identified between pairwise-compared genomes. Blue lines indicate the boundaries of the RD8 deletion. (*B*) qRT-PCR analysis of *espACD* genes expression in five strains of the MTBC harboring a Δ*phoPR* deletion. (*C*) Immunoblot of secreted ESAT-6 from H37Rv WT, Δ*phoPR* mutant, and Δ*phoPR* mutant carrying the *espACD* fragment from *M. bovis* (*espACD-bovis*) or the control similar fragment from H37Rv (*espACD-TB*). Several transformants were analyzed. The bacterial supernatants and cell pellets were probed for ESAT-6 or GroEL2. (*D*) Quantification of the ESAT-6 signals in secreted protein fractions from the indicated strains (relative to H37Rv).

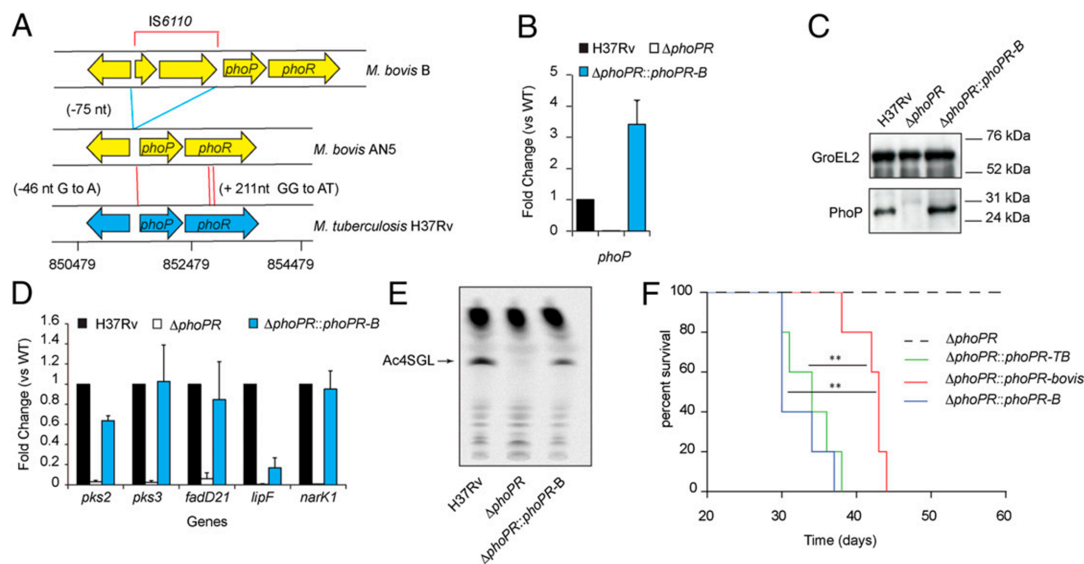

Fig. 5. Insertion of IS*6110* upstream *phoPR* in *M. bovis B* strain suppresses the *phoPR-bovis* deficiency. (*A*) Schematic representation of the genetic structure at the *phoPR* locus in the *M. bovis* B strain relative to "classic" *M. bovis* and *M. tuberculosis* strains. The positions of SNPs specific to the animal-adapted and *M. africanum* L6 and of the IS*6110* insertion site are indicated in brackets. Red lines indicate individual SNPs identified between pairwise-compared genomes. Blue lines indicate the boundaries of IS*6110*. (*B*) qRT-PCR analysis of *phoPR* expression in *M. tuberculosis* H37Rv strain, the *ΔphoPR* mutant, and the recombinant *M. tuberculosis* strain expressing the *phoPR-B* allele. (*C*) Western blot analysis of PhoP from WT and *phoPR-B* expressing strains. (*D*) qRT-PCR analysis of expression of PhoP regulon genes in the indicated strains. (*E*) TLC analysis of lipids extracted from [^{14}C] propionic acid-labeled cultures. (*F*) Survival of SCID mice infected with ~4×10^3 cfu of the indicated strains (five mice per group). Curves are significantly different between the *phoPR-bovis* expressing strain and the *phoPR-TB*– and *phoPR-B*–expressing strains ($P < 0.005$, according to the log-rank (Mantel– Cox) test).

with WT (Fig. 5 *B* and *C* and Fig. S9). Consistently, we detected increased transcription of PhoP regulon genes (Fig. 5*D*), production of SL and PAT (Fig. 5*E* and Fig. S9), and ESAT-6 secretion in the recombinant *M. tuberculosis* or *M. bovis* AN5 strains harboring the *phoPR-B* allele. Thus, the occurrence of IS*6110* 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 IS*6110*-contained promoter (36, 37).

Finally, we sought to determine the impact of the IS*6110* 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 Δ*phoPR* mutant (Fig. 5*F*).

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 IS*6110* 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 H37Ra 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 RD8 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

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compatible with previously proposed phylogenic 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. africanum*, or *M. canettii* 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 on the mycobacterial chromosome. Lipid analysis was performed on exponentially growing strains labeled for 24 h with [^{14}C] propionic acid or on stationary-phase bacteria cultured as pellicles for mass spectrometry. 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-wk-old cultures fractionated by centrifugation before protein extraction, separation by SDS/PAGE, membrane transfer, and hybridization with ESAT-6–, PhoP-, or GroEL2-specific antibodies. Animal studies were conducted on 7-wk-old mice infected intranasally and euthanized at various days postinfection for bacterial load evaluation in lung and spleens. Human macrophages infection assays were carried out in human monocyte-derived macrophages obtained from anonymous nontuberculous donors and infected at multiplicity of infection 2 for 2 h with GFP-expressing bacteria. Statistical analyses were performed using GraphPad Prism software.

A detailed materials and methods section is provided in the *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Geanncarlo Lugo-Villarino and Dr. Stephen V. Gordon for critical reading of the manuscript. We thank the members of the Genotoul platforms TRI and ANEXPLO (Institut de Pharmacologie et de Biologie Structurale) for epifluorescence microscopy imaging and for animal experiments, respectively; and Dr. Marie-Agnès Dillies and Dr. Jean-Yves Coppée from the Transcriptome Platform PF2 at the Institut Pasteur for advice. This work was supported in part by the Juan de la Cierva Programme JCI-2009-03799 from the Spanish Ministry of Science and Innovation (to J.G.-A.); the Centre National de la Recherche Scientifique; the Fondation pour la Recherche Médicale ("Equipe FRM" DEQ20090515399 and DEQ20130326471); the European Union 7th Framework Programme (Contracts NEWTBVAC 241745, MM4TB 260872, and FEDER/POCTEFA/REFBIO EFA237/11); the Spanish Ministry of Economy and Competitiveness (Contracts BIO2011-23555 and FIS12/1970); and the Région Midi-Pyrénées (CPER 2007-2013) for the scientific project and for the ASB3 animal facility.

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