Shared Pathogenomic Patterns Characterize a New Phylotype, Revealing Transition toward Host-Adaptation Long before Speciation of *Mycobacterium tuberculosis*

Guillaume Sapriel\(^1,2,*\) and Roland Brosch \(^3\)

\(^1\)UFR des Sciences de La Santé, Université de Versailles St. Quentin, Montigny le Bretonneux, France
\(^2\)Atelier de Bioinformatique, ISYEB, UMR 7205, Paris, France
\(^3\)Unit for Integrated Mycobacterial Pathogenomics, Institut Pasteur, CNRS UMR 3525, Paris, France

*Corresponding author: E-mail: guillaume.sapriel@uvsq.fr.
Accepted: July 9, 2019
Data deposition: No new sequence data.

Abstract

Tuberculosis remains one of the deadliest infectious diseases of humanity. To better understand the evolutionary history of host-adaptation of tubercle bacilli (MTB), we sought for mycobacterial species that were more closely related to MTB than the previously used comparator species *Mycobacterium marinum* and *Mycobacterium kansasii*. Our phylogenomic approach revealed some recently sequenced opportunistic mycobacterial pathogens, *Mycobacterium decipiens*, *Mycobacterium lacus*, *Mycobacterium riyadhense*, and *Mycobacterium shinjukuense*, to constitute a common clade with MTB, hereafter called MTB-associated phylotype (MTBAP), from which MTB have emerged. Multivariate and clustering analyses of genomic functional content revealed that the MTBAP lineage forms a clearly distinct cluster of species that share common genomic characteristics, such as loss of core genes, shift in d\(\text{N}\)/d\(\text{S}\) ratios, and massive expansion of toxin–antitoxin systems. Consistently, analysis of predicted horizontal gene transfer regions suggests that putative functions acquired by MTBAP members were markedly associated with changes in microbial ecology, for example adaption to intracellular stress resistance. Our study thus considerably deepens our view on MTB evolutionary history, unveiling a decisive shift that promoted conversion to host-adaptation among ancestral founders of the MTBAP lineage long before *Mycobacterium tuberculosis* has adapted to the human host.

Key words: tuberculosis, mycobacteria, host adaption, pathogenomic, pathogen evolution, evolutionary transition.

Introduction

Obligate pathogens are specifically adapted to host cells, which represent their ecological niche. Most of these host-adapted organisms are descendants of free-living extracellular ancestors. Their emergence is associated with drastic adaptive changes to cope with new constraints, such as nutrient availability or host defenses (Toft and Andersson 2010). For many bacterial pathogens, comparative genomics identified genomic changes that correlate with this transition, including genome reduction, virulence factor acquisition, and loss of genetic diversity (Pallen and Wren 2007).

Presently, tuberculosis remains the deadliest human infectious disease (Dye and Williams 2010). However, up to now, the evolutionary steps leading to the tubercle bacilli (MTB), responsible for this disease, remain uncertain. MTB belong to the slow growing mycobacteria (SGM), a subgroup of the mycobacterial genus that contains most of the pathogenic mycobacterial species. MTB mainly consist of strains from the *Mycobacterium tuberculosis* complex (MTBC), a clonal group of closely related bacteria that cause TB in selected mammalian species, and a clade of *Mycobacterium canettii* strains, representing recombinogenic, rare tubercle bacilli, with unusual smooth colony morphology (Fabre et al. 2004; Boritsch et al. 2016a; Madacki et al. 2018). *Mycobacterium tuberculosis* and *M. canettii* share more than 98% nucleotide identity (Supply et al. 2013), and thus can theoretically be classified into a single species (Riojas et al. 2018), whereby often the traditional, host-related names are continued to be
used. Comparative genomic approaches, using Mycobacterium marinum or Mycobacterium kansasii as outgroups suggested that massive genomic changes led to MTB differentiation (Stinear et al. 2008; Wang et al. 2015), including marked genomic reduction, horizontal gene transfer (HGT), and toxin–antitoxin massive expansion. These studies revealed a wide evolutionary gap separating the genomic structures of MTB from those of related environmental mycobacteria, suggesting the existence of unknown intermediate evolutionary steps that may have paved the way to MTB host-adaptation (Veyrier et al. 2011; Wang and Behr 2014). Taking advantage of recently released mycobacterial genus sequence data (Tortoli et al. 2017), we investigated a distinct phylogenetic cluster of species associated with MTB, hereafter called MTB-associated phylotype (MTBAP). A dedicated comparative genomic analysis approach revealed that MTBAP members share most of the MTB attributes associated with host adaptation, suggesting ancestral transitional forms leading to host-adaptation conversion.

Our results allowed us to propose a novel phylogenetic scheme of MTB deep evolutionary roots, which is also linked to apparent changes in the ecology of MTB. They unveil a major ancestral evolutionary leap that shaped most genomic characteristics related to host-adaptation, inaugurating an extended co-evolution period, long before MTB speciation.

Materials and Methods
Research Strategy Overview
To evaluate the deeper phylogenetic and pathogenomic relationships between MTB and selected, recently genome-sequenced mycobacterial species, we used following approaches: At first, Average Nucleotide Identity (ANI) analyses were performed on whole genome sequences from selected mycobacterial species, followed by the generation of a phylogenetic tree based on maximum likelihood similarities of widely distributed essential bacterial gene products, preselected by the bcgTree software. In addition, synonymous mutation rates were calculated. In a second step, species-specific evolutionary dynamics were investigated by analyzing gene gains and gene losses as well as determination of gene flux by HGT and identification of putative genomic islands. Based on the different results, an evolutionary scenario was proposed that integrates the putative contributions of a group of closely related mycobacterial species on the shaping of host-associated traits of MTB.

ANI and Phylogeny Analyses
We performed mutual ANI calculations on genome data from 10 defined SGM species, consisting of strains M. tuberculosis H37Rv, M. canettii STB-K, Mycobacterium decipiens TBL1200985, Mycobacterium shinjukuense CCUG 53584, Mycobacterium lacus DSM 44577, Mycobacterium niyadense DSM 45176, M. kansasii ATCC 12478, M. marinum E11, Mycobacterium szulgai DSM 44166, and Mycobacterium gordoniae DSM 44160 (table 1), which were selected based on reported findings from genus-wide mycobacterial sequence analyses (Fedrizzi et al. 2017; Tortoli et al. 2017). Pairwise genomic distances between genomes were calculated according to BLAST-based ANI scores (Goris et al. 2007), determined by using JSpeciesWS (Richter et al. 2016).

For phylogenetic analyses, a larger, representative study data set was established, to obtain robust bootstrap values. Twenty-eight mycobacterial species were selected for the analysis (supplementary table 1, Supplementary Material online), including 25 SGM species, Mycobacterium terrae, belonging to the intermediate M. terrae complex group, as well as Mycobacterium smegmatis and Mycobacterium abscessus, representing rapidly growing mycobacteria. The latter three species were used as outgroup. Putative coding sequences and associated protein sequences were identified, and annotated from contigs and whole genome data, using the Microbial Genome Annotation and Analysis platform “MicroScope” (Vallenet et al. 2017).

For the calculations, 107 essential bacterial genes, which were defined by the bcgTree (bacterial core genome Tree) software (Ankenbrand and Keller 2016) as being present as single copy genes in the majority of bacterial genomes, were retrieved for each of the 28 species. Sequences were concatenated, aligned using clustal Omega, and well-conserved regions were selected using Gblocks. The phangorn R package (Schliep 2011) was then used to generate a phylogenetic tree based on maximum likelihood (ML) estimates (via the pml algorithm), using a JTT_DCMut+G substitution model.

CoreVariable Genome and dN/dS Analyses
Gene families were determined using the MICFAM (Microscope gene families) tool, computed with the SiLiX software (Miele et al. 2011) by using a 50% amino acid identity threshold and 80% alignment coverage. Families with exactly one gene per species were selected for core-genome orthologs. For dN/dS ratio calculations, core-genome amino-acid sequences were retrieved, and aligned using clustalW2 (Larkin et al. 2007). Nucleic sequences were then aligned according to the deduced codons from amino-acid alignment using the pal2nal software (Suyama et al. 2006), and gaps were removed. dN/dS calculation was then performed using the PAML program (Yang 1997). To remove saturation effects, genes having dS < 0.01, dS > 2, and dN > 2 were removed from the analysis. For each species, dN/dS distribution values relative to the M. gordoniae outgroup were generated and visualized using R boxplot functions (R-Core-Team 2014), and a Wilcoxon–Mann–Whitney test was used to identify median values significantly lower than M. tuberculosis median values. *P value < 0.05. **P value < 0.001.
Table 1  

Pairwise Genomic Distances of MTB and Closely Related SGM Species

| Species | Mtub | Mcan | Mdec | Mshi | Mlac | Mry | Mkan | Mmar | Mszu | Mgor |
|---------|------|------|------|------|------|-----|------|------|------|------|
| Mtub    | —    | 97.79 | 98.15 | 82.22 | 81.46 | 80.51 | 79.63 | 78.57 | 79.28 | 77.70 |
| Mcan    | 97.71 | —    | 85.18 | 81.99 | 80.42 | 79.54 | 78.57 | 79.28 | 77.70 | 76.76 |
| Mdec    | 84.34 | 84.39 | —    | 80.91 | 80.42 | 80.49 | 80.17 | 80.49 | 80.46 | 80.46 |
| Mshi    | 82.21 | 82.08 | 81.48 | —    | 81.43 | 80.17 | 81.30 | 81.43 | 80.46 | 80.46 |
| Mlac    | 81.15 | 81.25 | 81.63 | 81.48 | —    | 79.13 | 80.17 | 79.13 | 80.07 | 79.70 |
| Mriy    | 79.54 | 79.59 | 80.17 | 79.98 | 79.97 | —    | 79.70 | 79.90 | 79.07 | 77.24 |
| Mkan    | 78.62 | 78.63 | 78.53 | 78.53 | 78.53 | 78.53 | —    | 77.24 | 77.24 | 77.24 |
| Mmar    | 77.31 | 77.35 | 78.31 | 78.31 | 78.31 | 78.31 | 78.31 | —    | 77.24 | 77.24 |
| Mszu    | 77.82 | 77.84 | 78.53 | 78.53 | 78.53 | 78.53 | 78.53 | 78.53 | —    | 77.24 |
| Mgor    | 76.20 | 76.23 | 76.76 | 77.29 | 77.22 | 76.96 | 76.96 | 76.96 | 76.96 | —    |

Notes.—ANI values were calculated from genome to genome BLAST-based comparison. Within brackets: aligned genome percentage. Bold: higher ANI values with MTBC, as compared with previously studied reference outgroups M. kansasii and M. marinum.

Mtub, M. tuberculosis H37Rv; Mcan, M. canettii STBK; Mdec, M. decipiens; Mlac, M. jacus; Mriy, M. riadhense; Mkan, M. kansasii; Mmar, M. marinum E11; Mszu, M. szulgai; Mgor, M. gordonae.
Together with MTB, they represent a novel phylogenetic group that is descending from a common ancestor and will be referred to hereafter as the MTBAP lineage. Using core-genome data from MTBAP species and four closely related outgroup-species, namely *M. kansasii*, *M. marinum*, *M. szulgai*, and *M. gordonae*, referred hereafter as MGS–MKM outgroup, synonymous mutations were extracted to get an estimate of divergence time between *M. marinum* (fig. 1A).
these species and MTB (fig. 18). From these data, the most recent common ancestor (MRCA) of MTB and other species from the MTBAP lineage was found to be more recent than the MRCA of MTB and the MGS–MKM outgroup species. These data also suggest that MTBAP species are phylogenetically related descendants originating from a common progenitor, with a divergence time estimated to be 15–30 times longer than the one considered for *M. tuberculosis* and *M. canettii* within MTB.

**Shared Genomic Reduction Profiles**

Because MTB are well-known to have undergone genome size reduction (<4.5 Mb) compared with *M. kansasii* (6.4 Mb) and *M. marinum* (6.6 Mb) outgroups, we assessed if other species from the MTBAP lineage displayed similar genomic reduction patterns. A preliminary study using EGGNOG gene functional classification from MicroScope (Vallenet et al. 2017) suggests that besides MTB, *M. decipiens*, *M. shinjukuense*, and *M. lacus* harbor significantly less genes in many functional categories (excluding basic, essential cellular functions), compared with other mycobacterial species (table 2), whereas *M. ryadhense* exhibits a less significant, intermediate pattern that is also associated with a relatively large genome size of 6.3 Mb (supplementary table 1, Supplementary Material online). To evaluate this trend in more detail, we used core-genome data to identify presence or absence of MICFAM gene families in each genome of the MTBAP and MGS–MKM lineages, which were then categorized by large-scale hierarchical clustering. Distance-tree analysis of species based on MICFAM gene family content shows that the MTBAP lineage represents a specific cluster, clearly distinct from the MGS–MKM outgroup, a finding that is further emphasized by the large number of gene families present in the MGS–MKM outgroup that are under-represented in all species of the MTBAP lineage (fig. 2A). A model of gene gain and gene loss (fig. 2B) confirms that loss of certain genes and gene families occurred within the MTBAP lineage. Most of the gene loss occurred within the branches leading to each species. In addition, some gene loss signatures are also shared between different lineages, which suggest that some of these genetic events might have been initiated at deep branching nodes at the base of the MTBAP lineage.

dNdS Ratios Suggest Common Genome Evolution Dynamics within the MTBAP

To characterize the genome evolution dynamics of the different species, non-synonymous/synonymous mutation rates (dNdS) were compared for the core genomes of the MTBAP and MGS–MKM group. As shown in figure 2C, by using the commonly found environmental *M. gordonae* as comparator reference species, we observed dNdS ratios that were significantly higher for *M. tuberculosis* than for *M. szulgai*, *M. marinum*, and *M. kansasii*, the latter considered as environmental mycobacteria. Of note, high dNdS ratios were also obtained for *M. canettii*, *M. decipiens*, and *M. shinjukuense*, whereas *M. lacus* showed intermediate values.

**Functional Profile Analysis Reveals Similar Protein Family Clustering**

To explore if members of the MTBAP lineage were characterized by a specific protein content in terms of selected functional features, we retrieved the repertoire of PFAM protein family domains (Finn et al. 2006) associated specifically with each of the genomes of the MTBAP and MGS–MKM lineages. Multivariate analysis of these data, performed by PCA, showed that in agreement with aforementioned MICFAM clustering analyses, the MTBAP lineage constitutes a specific cluster (fig. 3A). The first projection axe (PC1) explains 30% of the variance, and allows to clearly distinguish the MTBAP lineage from the species of the MGS–MKM outgroup (fig. 3A). A striking consistency between data derived from MICFAM and PFAM domain analyses emphasize that genome contents of the MTBAP lineage members are substantially different from those of the MGS–MKM outgroup. In addition, BCA results showed that many PFAM domains were under-represented in the MTBAP lineage (negative BCA scores), apparently due to genomic reduction (fig. 3B). However, we also noticed some positive BCA scores, indicating a particular over-representation of selected PFAM domains in MTBAP members, arguing that this lineage is not only defined by gene loss, but also by acquisition of specific functional features. Careful study of these PFAM domains over-represented in the MTBAP lineage revealed that many of them corresponded to toxin–antitoxin domains (red and green dots, respectively, fig. 3B). Among these toxin–antitoxin domains, VapBC and MazEF type II systems appeared to be the most abundant in the MTBAP lineage (highest scores in y axis of fig. 3B). Specific counts of the genes coding for VapC or MazF toxins (containing respectively PIN and PemK domains) confirmed that these families were significantly over-represented in *M. shinjukuense*, *M. lacus*, and *M. ryadhense* together with MTB members *M. canettii* and *M. tuberculosis* (fig. 4A). It is clear from this analysis that certain mycobacterial species may harbor a greater repertoire of toxin–antitoxin systems, which were previously only reported for MTB (Ramage et al. 2009; Wang et al. 2015). Our results show now that with some exceptions found for *M. decipiens*, the MTBPA lineage members share an unexpectedly high number of VapBC and MazEF type II toxin–antitoxin systems. The high diversification of these toxin–antitoxin systems may be explained either by late genomic events that occurred independently in each of the species of the lineage, or by early genomic events that had occurred in a common ancestor of the lineage, followed by subsequent diversification. For the purpose of determining which of the two hypotheses was more likely, BLAST and BBH-based synteny analyses of VapC and MazF sequences...
of *M. tuberculosis* were undertaken in a variety of species. This approach revealed that among the 55 studied toxins, more than a third (35%) may be considered as orthologous proteins (BBH + synteny) that are shared among selected species of the MTBAP lineage, without showing orthologs of any of the other SGM species used in our study (supplementary table 1, Supplementary Material online), suggesting that many of these genes, previously considered as M. tuberculosis-specific genomic traits, were acquired by HGT by a common ancestor in the MTBAP lineage before MTB speciation. We then asked if among these genes, some might play a role in pathogenicity. A literature review performed on these 169 genes, allowed us to identify 35 of them that were reported to be involved in survival or growth of MTB during infection of mononuclear phagocytic cells or in animal models (table 3), representing 21% of the initial gene pool, and 33% of the above-identified genes. Among these genes, some were encoding regulation systems, comprising transcriptional regulation factors such as MosR, VirS, or Rv1359 adenyl cyclase, as well as several toxin–antiotxin systems (VapC44). Strikingly, more than a third of these likely virulence-associated genes encode proteins involved in lipid metabolism and/or lipid transport (mosR, mce2B, Rv0895, Rv1288, Rv2954c, Rv2955c, virS, Rv3087, Rv3376, Rv3377c, lipF, fadD23). Many of them are predicted to be involved in biochemical processes linked to the mycomembrane of MTB, being implicated in mycolic acid composition (virS, Rv3087) (Singh et al. 2005), phenolic glycolipid glycosylation (Rv2954c, Rv2955c) (Simeone et al. 2013), and sulfolipid synthesis and accumulation (mce2B, fadD23) (Lynett and Stokes 2007; Marjanovic et al. 2011). Many genes were acquired during the evolution of the MTBAP lineage before MTB speciation. These genes were present in 32 of the 46 identified *M. tuberculosis* RGP (supplementary table 2, Supplementary Material online), arguing for an early branching of *M. tuberculosis* lineages). Such genes were determined by BBH-analysis to find synteny (fig. 4B), arguing for an early acquisition of a substantial portion of toxin–antiotxin systems in species of the MTBAP lineage and subsequent diversification.

### HGT and Acquisition of Rare Virulence and Cellular Stress Factors

As toxin–antiotxin systems are often found within genomic islands acquired by HGT (Ramage et al. 2009) and may play important roles in shaping MTB gene content (Becq et al. 2007; Supply et al. 2013; Levillain et al. 2017), we performed a genome-wide screen to search for *M. tuberculosis* genes that likely were acquired in a common progenitor during the evolution of the MTBAP lineage before MTB speciation (i.e., after divergence from the MGS–MKM outgroup, but before branching of *M. canetti* and *M. tuberculosis* lineages). Such genes were determined by BBH-analysis to find *M. tuberculosis*/M. canetti genes with putative orthologs in other species from the MTBAP lineage that had no ortholog in the genomes of any of the other SGM species used in our study (supplementary table 1, Supplementary Material online). Our screen identified 169 genes of *M. tuberculosis* that were putatively

---

**Table 2**

EGGNOG Functional Category Counts of Annotated Genes in Genomes of MTB and Closely Related Mycobacterial Species

| Function                                    | Mtub | Mcan | Mdec | Mshi | Mlac | Mriy | Mkan | Mmar | Mszu | Mgor |
|---------------------------------------------|------|------|------|------|------|------|------|------|------|------|
| Energy production and conversion           | 212a | 214a | 293a | 230a | 282a | 322b | 347  | 322a | 366  | 379  |
| Cell cycle control, cell division, chromosome partitioning | 45   | 45   | 35   | 44   | 41   | 49   | 38   | 32   | 42   | 57   |
| Amino acid transport and metabolism        | 197a | 196a | 243  | 187a | 210a | 224b | 236  | 265  | 242  | 242  |
| Nucleotide transport and metabolism        | 74b  | 77   | 77   | 72   | 80   | 82   | 80   | 79   | 74   | 75   |
| Carbohydrate transport and metabolism      | 147b | 141a | 175b | 166a | 170a | 197  | 202  | 193  | 232  | 240  |
| Coenzyme transport and metabolism          | 136a | 140a | 148a | 115a | 147a | 173  | 158  | 162  | 171  | 157  |
| Lipid transport and metabolism             | 213a | 210a | 273b | 216a | 265a | 289b | 311  | 318  | 313  | 386  |
| Translation, ribosomal structure, and biogenesis | 149  | 147  | 150  | 139b | 140b | 150  | 141  | 154  | 146  | 151  |
| Transcription                              | 227a | 235a | 281b | 221a | 273b | 356b | 306  | 325  | 356  | 429  |
| Replication, recombination, and repair      | 233  | 257  | 176  | 176  | 188  | 262  | 248  | 192  | 152  | 328  |
| Cell wall/membrane/envelope biogenesis     | 124a | 124a | 152  | 127a | 123a | 152  | 168  | 149  | 152  | 175  |
| Cell motility                               | 35a  | 37a  | 36a  | 40b  | 43b  | 45   | 47   | 42   | 52   | 50   |
| Post-translational modification, protein turnover, chaperones | 103a | 108a | 106a | 115b | 115b | 126b | 142  | 136  | 132  | 167  |
| Inorganic ion transport and metabolism      | 138a | 139a | 164a | 139a | 151a | 170a | 197  | 202  | 204  | 235  |
| Secondary metabolites biosynthesis, transport, and catabolism | 155a | 150a | 292  | 145a | 217a | 273b | 263  | 303  | 351  | 342  |
| Signal transduction mechanisms             | 110b | 105a | 131b | 109a | 111b | 147b | 182  | 146  | 186  | 240  |
| Intracellular trafficking, secretion, and vesicular transport | 22   | 21   | 22   | 22   | 24   | 28   | 22   | 20   | 23   | 28   |
| Defense mechanisms                          | 59   | 59   | 62   | 61   | 64   | 67   | 77   | 59   | 62   | 87   |

*Note:* Number of genes in each EGGNOG category.

1. Values below two standard deviation quantities as compared with the MGS–MKM outgroup.
2. Values below one standard deviation quantity, as compared with the MGS–MKM outgroup.

Mtub, *M. tuberculosis* H37Rv; Mcan, *M. canetti* STB-K; Mdec, *M. decipiens*; Mlac, *M. lacus*; Mriy, *M. riyadhense*; Mkan, *M. kansasii*; Mmar, *M. marinum* E11; Mszu, *M. szulgai*; Mgor, *M. gordoniae*.
Fig. 2.—Genomic evolution in MTBAP lineage. (A) Color coded table representing a heatmap for which the rows and columns were sorted by hierarchical clustering approaches. The row tree shows the clustering of MICFAM protein families based on species profile similarity calculated by the Ward agglomerative hierarchical clustering algorithm. The column tree represents the clustering of species based on MICFAM profile. Red: under-represented gene families. Green: over-represented gene families. Color-key for Row Z-scores is shown together with a histogram indicating the number of MICFAM protein families associated with each of the Z-scores. (B) Estimated gene gain and loss in the MTBAP lineage and the MGS–MKM outgroup. Variable genome parts of the MTBAP lineage and the MGS–MKM outgroup were computed using the MICFAM tool with a 50% amino-acid identity threshold and 80% alignment coverage. A table representing presence or absence of gene families for each species was then analyzed by Gain Loss Mapping Engine. Red: branches showing more gene loss than gene gain. (C) dNdS distribution of core-genome orthologs as compared with the M. gordonae outgroup. Bold bars indicate the median dNdS values for each species. Notch estimates correspond to 95% confidence intervals for median values. Box edges represent 25th and 75th percentiles. Whiskers represent estimated extreme values. Mcan, M. canetti STB-K; Mdec, M. decipiens; Mshi, M. shinjukuense; Mlac, M. lacus; Mry, M. riyadhense; Mkan, M. kansasii; Mmar, M. marinum E11; Mszu, M. szulgai; Mgor, M. gordonae.
reported as induced under phagosome-associated stress, such as acidic pH (virS, lipF), and nutrient starvation (Rv0064, mosR, Rv1359, frdA, Rv2275, Rv3087, vapC44) as well as under specific conditions within the phagosome, the granuloma or the lungs (Rv0064, vapC24, frdA, vapC19, vapB19, vapB20, virS, PE_PGRS50) (Betts et al. 2002; Rachman et al. 2006; Watanabe et al. 2011; Mendum et al. 2015; Hudock et al. 2017). Moreover, genes encoding diterpene synthase
(Rv3377c), implicated in production of diterpene nucleoside 1-TbAd involved in phagosome maturation arrest, or cyclopeptide synthase (Rv2275), producing mycocyclosin involved in intra-macrophage survival, are shared by selected species of the MTBAP lineage. Finally, we found that some of these potential virulence factors were encoded on obvious genomic islands (fig. 5), for example the fumarate reductase GI (Rv1552-1555), Myma GI (Rv3082c-3087), or sulfolipid synthesis GI (Rv3820c-3826). As summarized in figure 6, our results suggest that many genetic and genomic factors, formerly thought to correspond to MTB-specific virulence factors, are also present in certain other MTBAP species, and were probably acquired long time before MTB speciation, supporting an evolutionary scenario in which tuberculosis-causing mycobacteria emerged from an ancient family of mycobacteria that had “learned” to cope with intracellular stress conditions, likely through long-lasting interaction with yet unknown eukaryotic host organisms.

Discussion

In the more than 20 years since the publication of the first complete mycobacterial genome sequence, featuring the reference strain *M. tuberculosis* H37Rv (Cole et al. 1998), the phenomenal increase in available sequence data has allowed an important refinement of the phylogenetic structure of the clonal population of *M. tuberculosis* and the MTBC to be achieved (Brosch et al. 2002; Mostowy et al. 2002;
This information was further enriched by genome data from *M. canettii* strains, which share at least 98% sequence identity with *M. tuberculosis* strains, but differ from them by a highly recombinogenic population structure (Supply et al. 2013; Blouin et al. 2014; Boritsch et al. 2016b) and a yet unknown reservoir or environmental source. Apart from *M. canettii*, the closest known outgroups used until now for identifying *M. tuberculosis*-genome characteristics, and inferring its evolutionary history, were *M. marinum* (Stinear et al. 2008) and *M. kansasii* (Wang et al. 2015), which are both environmental mycobacteria characterized by substantially larger genomes (6.6 and 6.4 Mb, respectively) than *M. tuberculosis* (4.4 Mb) or *M. canettii* (4.5 Mb). This situation means that there was...
show optimal growth temperatures higher than 25 °C. Our results show that the nontuberculous members of the MTBAP lineage constitute a specific group of mycobacterial species sharing several genomic characteristics associated with host-adaptation, strongly supporting the hypothesis that ancestral founders of this lineage might represent pivotal evolutionary intermediates between the non- or low-virulent environmental mycobacteria and the highly virulent professional pathogens such as M. tuberculosis.

The study unveiled that specific genomic characteristics of MTB genomes, such as massive acquisition of toxin–antitoxin systems, were shared with most of the members of the MTBAP lineage. Indeed, our results suggest that the acquisition of a substantial part of the repertoire of M. tuberculosis VapBC and MazEF type II toxin–antitoxin systems was acquired in the MTBAP lineage before MTB speciation. In this perspective, M. decipiens represents as exception to the trend as this recently defined species does not seem to harbor the plethora of toxin–antitoxin encoding genes present in other members. To answer the question how this could happen, two working hypotheses may be proposed. The first hypothesis evokes that massive toxin–antitoxin acquisition occurred twice in the MTBAP lineage, due to convergent evolution dynamics whereas the second possible hypothesis suggests that massive toxin–antitoxin acquisition was initiated early in the MTBAP lineage ancestor, and that M. decipiens underwent a subsequent loss of most of these systems due to yet unknown reasons. Gene orthology and synteny analyses of toxin–antitoxin encoding genes suggest an ancestral acquisition for a significant part of these genes, and partial loss by certain members of the MTBAP lineage. This scenario means that a pivotal evolutionary breakpoint was initiated a long time before MTB speciation, giving rise to a distinct group of mycobacteria that likely evolved into opportunistic pathogens and acquired many of the characteristic features that today define the professional pathogen M. tuberculosis. Our attempts to estimate the putative emergence time of the last common ancestor of the MTBAP lineage using synonymous substitution comparisons with M. tuberculosis H37Rv, are consistent with previous calculations performed on a small gene set for M. canettii (Gutierrez et al. 2005). Synonymous mutations can be considered as neutral markers (Zuckerkandl and Pauling 1965; Kimura 1979) that linearly accumulate over time (Kishimoto et al. 2015). If we infer a constant mutation rate, our results suggest that the divergence between the MRCA of reference strain M. tuberculosis H37Rv and M. riyadhense occurred about 30 times earlier than the divergence between M. tuberculosis H37Rv and M. canettii STB-K, which is the most distantly related M. canettii strain relative to M. tuberculosis, known today (Supply et al. 2013; Blouin et al. 2014; Boritsch et al. 2014). Moreover, the MRCA of M. tuberculosis and either M. riyadhense, M. lacus, or M. shinjukuense is very close, suggesting an adaptive radiation process that might have occurred during, or soon after, the genomic
Table 3
Mycobacterium tuberculosis Virulence Genes Probably Acquired before MTB Speciation, and within MTBAP Lineage Evolution

| Label | Gene | Product | Function | Phenotype | Intracellular Survival | Animal Infection Model | Mcan | Mdec | Mshi | Mlac | Mriy | SGM |
|-------|------|---------|----------|-----------|------------------------|------------------------|------|------|------|------|------|------|
| Rv0064 | — | Membrane protein | — | — | DC (Mendum et al. 2015) | — | ++ | ++ | — | — | — |
| Rv0071 | — | — | — | Deleted in L2/Beijing lineage M. tuberculosis strains (RD105) | M (Stewart et al. 2005) | — | — | + | — | — | — |
| Rv0240 | vapC24 | Toxin-antitoxin | Regulation | Induced under lysosomal stress conditions (Lin et al. 2016) | — | Macaque (Dutta et al. 2010) | — | — | — | + | — | — |
| Rv0348 | mosR | Transcriptional regulator | Regulation | Hypoxia responsive regulator (Abomolak et al. 2009) | — | Mouse (Abomolak et al. 2009) | — | — | + | — | — | — |
| Rv0590 | mce2B | Transporter | Transport (lipids) | Belongs to mce2 operon, involved in sulfolipid accumulation (Marjanovic et al. 2011) | — | Mouse (Marjanovic et al. 2010) | ++ | ++ | — | — | — | — |
| Rv0890c | — | Transcriptional regulator | Regulation | — | DC (Mendum et al. 2015) | — | ++ | ++ | — | — | — |
| Rv0893c | — | SAM methyltransferase | Secondary metabolism | — | M (Zhang et al. 2013) | — | ++ | ++ | — | — | — |
| Rv0895 | DAG-O-acyltransferase | Secondary metabolism (lipids) | — | M (Rengarajan et al. 2005) and DC (Mendum et al. 2015) | — | ++ | ++ | — | — | + |
| Rv0977 | PE_PGRS16 | Secreted and surface proteins | PE_PPE | Secreted and surface proteins | DC (Mendum et al. 2015) | — | ++ | — | ++ | — | ++ |
| Rv1288 | — | Putative mycolyltransferase | Secondary metabolism (lipids) | — | DC (Mendum et al. 2015) | — | ++ | ++ | — | — | + |
| Rv1359 | — | Transcriptional regulator | Regulation | — | DC (Mendum et al. 2015) | — | ++ | — | — | — | — |
| Rv1442 | bisC | Biotin sulfoxide reductase | Electron transfer activity | Oxidative stress resistance (putative) | DC (Mendum et al. 2015) | — | ++ | ++ | (p) | + | ++ (p) |
| Rv1552 | frdA | Fumarate reductase subunit | Electron transfer activity | Hypoxia adaptation (Watanabe et al. 2011), Phagosome acidification arrest (Stewart et al. 2005) | DC (Mendum et al. 2015) | — | ++ | ++ | ++ | — | + |
| Rv1793c | sulP | Transporter | Transport | Sulfate uptake (Zolotarev et al. 2008) | DC (Mendum et al. 2015) | — | ++ | — | ++ | + | — |
| Rv1981c | nrdF1 | DNA-methylase | Regulation | — | DC (Mendum et al. 2015) | — | ++ | ++ | — | — | + |

(continued)
Table 3

| Label | Gene | Product | Function | Phenotype | Intracellular Survival | Animal Infection Model | Mcan | Mdec | Mshi | Mlac | Mry | SGM |
|-------|------|---------|----------|-----------|------------------------|------------------------|------|------|------|------|-----|-----|
| Rv2275 | — | Cyclopeptide synthase | Secondary metabolism (mycocyclosin) | Deleted in *M. bovis* BCG strains (RD2) | — | — | Mouse (Kozak et al. 2011) | — | ++ | — | — | — | — |
| Rv2328 | PE23 | PE_PPE | Secreted and surface proteins | — | — | M (Stewart et al. 2005) | — | ++ | — | — | — | — |
| Rv2547 | vapC19 | Toxin-antitoxin | Regulation | Induced within granulomas (Hudock et al. 2017) | M (Stewart et al. 2005) | — | — | ++ | — | — | — |
| Rv2548 | vapB19 | Toxin-antitoxin | Regulation | Induced within granulomas (Hudock et al. 2017) | M (Stewart et al. 2005) | — | — | ++ | — | — | — |
| Rv2549c | vapC20 | Toxin-antitoxin | Regulation | — | M (Stewart et al. 2005) | — | — | ++ | — | — | — |
| Rv2550c | vapB20 | Toxin-antitoxin | Regulation | — | M (Stewart et al. 2005) | — | — | ++ | — | — | — |
| Rv2735c | — | — | — | — | DC (Mendum et al. 2015) | — | — | — | ++ | — | — |
| Rv2954c | — | Fucosyltransferase | Secondary metabolism (phenolglycolipid) | — | M (Rosas-Magallanes et al. 2007) | — | ++ | — | — | — | — |
| Rv2955c | — | Fucosyltransferase | Secondary metabolism (phenolglycolipid) | — | M (Rosas-Magallanes et al. 2007) | — | ++ | — | — | — | — |
| Rv3082c | virS | — | Transcriptional regulator | — | M (Singh et al. 2005) | — | ++ | — | — | — | — |
| Rv3087 | — | DAG-O-acyltransferase | Secondary metabolism (lipids) | — | M (Singh et al. 2005) | — | ++ | — | — | — | — |
| Rv3179 | — | — | — | — | DC (Mendum et al. 2015) | — | ++ | — | — | + (p) | — |
| Rv3320c | vapC44 | Toxin-antitoxin | Regulation | — | DC (Mendum et al. 2015) | — | ++ | — | — | — | — |
| Rv3343c | PPE54 | PE_PPE | Secreted and surface proteins | Oxidative stress resistance (Mestre et al. 2013) | DC (Mendum et al. 2015) | — | ++ | — | — | — | — |
| Rv3345c | PE_PGR550 | PE_PPE | Secreted and surface proteins | — | DC (Mendum et al. 2015) | — | — | — | — | — | — |
| Gene   | Function               | Secondary metabolism                     | Phenomenon                                      | Source                          | Score | Scores                  |
|--------|------------------------|------------------------------------------|------------------------------------------------|---------------------------------|-------|------------------------|
| Rv3376 | Phosphatase            | Phagolysosome maturation arrest (Pethe et al. 2004) | M (Renganarajan et al. 2005)                   | —                              | ++    | +                      | –       | +                    | –       | –                      |
| Rv3377c| Diterpene synthase     | Phagolysosome maturation arrest (Pethe et al. 2004) | DC (Mendum et al. 2015)                        | —                              | ++    | ++                     | –       | ++                   | –       | –                      |
| Rv3476c| kgtP                   | Transport                                | DC (Mendum et al. 2015)                        | —                              | ++    | ++                     | –       | –                    | –       | –                      |
| Rv3487c| lipF                   | Lipase-esterase                          | Upregulated under acidic pH conditions (Richter and Saviola 2009) | Mouse (Camacho et al. 1999)    | ++    | ++                     | –       | –                    | –       | –                      |
| Rv3826 | fadD23                 | Acyl-CoA synthetase                      | Lower binding affinity for macrophages (Lynett and Stokes 2007) | DC (Mendum et al. 2015)        | ++    | +                      | –       | –                    | –       | –                      |

**Notes:** List of experimentally confirmed *M. tuberculosis* virulence genes that have at least one ortholog among other species of the MTBAP lineage (*M. canettii* is not included in the analysis) and no ortholog in any SGM depicted in the representative SGM database.

+ Presence of a putative ortholog by BBH-analysis.
+ + Presence of a putative ortholog by BBH-analysis and synteny confirmation.
- Absence of putative ortholog. (p)Putative pseudogene.

Mtub, *M. tuberculosis* H37Rv; Mcan, *M. canettii* ST8K; Mdec, *M. decipiens*; Mlac, *M. lacus*; Mriy, *M. riyadhense*.
evolutionary breakpoint that we can observe in the data sets. The estimations on the molecular clock rates for *M. tuberculosis* and the MTBC are still debated, as is it is difficult to calculate such rates for clonal organisms given certain evolutionary bottlenecks that may induce strong biases in long-term fixed mutation data (Comas and Gagneux 2009). Recent estimations based on genome analyses of ancient mycobacterial DNA from 1,000-year-old mummies placed the divergence of the clonal MTBC at about 6,000 years before present (Bos et al. 2014), which is one of the shortest evolutionary estimates for the MTBC, as by the use of other models the divergence time of the MTBC has been postulated to be up to 70,000 years (Comas et al. 2013). Using the shortest estimates, this would still suggest that the evolutionary breakpoint at the root of the MTBAP lineage occurred at least several hundred thousand years before *M. tuberculosis* specialization to the human host. However, previous studies on *M. canettii* genomic diversity reported phylogenetic profiles suggesting that *M. canettii* diversification might be 5–25 time more ancient than the rise of the clonal MTBC (Supply et al. 2013). Hence, under constant mutation rates, a rough estimate of the emergence of the MTBAP lineage would be 150–750 times earlier than the rise of the clonal MTBC. Although precise time estimates about the emergence of the MTBC are still under debate and will require further investigations on ancient DNAs (Donoghue 2016), our study reveals that major transitions in the evolutionary history of tuberculosis-causing mycobacteria occurred much earlier than previously thought, and were marked by early acquisition of host-adaptation characteristics shared with other nontuberculous mycobacteria of the MTBAP lineage. These reflections suggest a very long initiating period of co-evolution, with yet unknown hosts, that pre-dated MTB specialization and *M. tuberculosis* specialization as a human pathogen. Genomic reduction and expansion of toxin–antitoxin systems has been suggested to be correlated with gain in virulence (Dawson et al. 2018). Thus, the evolutionary breakpoint that gave rise to the MTBAP lineage might correspond to an, at least partial, transition point toward host-adapted lifestyle. Other features observed in our study, such as the loss of core genes observed in MTB, *M. decipiens*, *M. shinjukuense*, *M. latus*, and to a lesser extent in *M. riyadhense*, and the specific MICFAM profiles support this hypothesis. The shift in core-genome dWdS distribution pattern that we observe for MTB, *M. decipiens*, *M. shinjukuense*, and to a lesser extent for *M. latus*, is a characteristic trait usually interpreted as a genomic marker associated with reduced population size and/or a genetic bottleneck that is consistent with specialized pathogen population dynamics. In agreement with previous reports, this pattern is considered as highly correlated with host-adaptation (Kuwahara et al. 2008; Kuo and Ochman 2009; Kjeldsen et al. 2012). Conversely, as observed for *M. szulgai*, *M. marinum*, and *M. kansasi*, this parameter is very stable within free-living, related bacteria (Kuo and Ochman 2009; Novichkov et al. 2009). Taken together, these trends are consistent with an early transition from free-living environmental to a specialized life-style within reduced ecological niches. Thereby, our results further support the hypothesis that the emergence of the MTBAP lineage included important steps toward a host-adapted lifestyle. This framework is consistent with a “quantum” evolutionary dynamic initiated by extensive genome-scale shifts, rather than by gradual evolution (Simpson 1944; Gould 1980). In this scenario, *M. riyadhense*, which is most distantly related to MTB within the MTBAP lineage, shows an intermediate pattern (with large toxin–antitoxin expansion, but only limited core-gene loss, a still-large genome size, and a dWdS ratio similar to environmental species), suggesting a possible transitional form (“missing link”) at the root of the MTBAP lineage, that acquired some host-adaptation traits, but also conserved several traits associated with classical environmental life-style. These and all other results shed light on the emergence of MTB, and unveil pivotal transition that early shaped its major genomic characteristics, allowing us to propose a large-scale evolutionary model (fig. 6). Interestingly, this evolutionary scenario shows some similarities to a recently established extended model of the leprosy bacilli (Stinear and Brosch 2016). For many years it was thought that the etiological agent of human leprosy, *Mycobacterium leprae*, was an isolated, clonal species that had undergone massive genome decay (Cole et al. 2001) and was the sole agent of all forms of leprosy. However, more recent genome-based studies revealed that related leprosy bacilli, named *Mycobacterium lepromatosis*, shared significant genomic characteristics with *M. leprae* (Singh et al. 2015) and were responsible for particular leprosy-like pathologies in humans and in squirrels (Avanzi et al. 2016). Extensive genome analyses also established that these leprosy bacilli were closely related to the emerging pathogen *Mycobacterium haemophilum* (Tufariello et al. 2015), as well as to the most recently defined *Mycobacterium iberis*, an emerging skin pathogen in dairy animals (Benjak et al. 2018). It is clear from these observations that with the ongoing massive increase in the availability of bacterial genome sequences, important new insights into the evolution, phylogeny and population structure of key human pathogens may be obtained that can substantially revise our view on these agents and the diseases they cause.

Genome comparisons can reveal the molecular tools bacteria employ to establish a pathogenic lifecycle in selected hosts, that is to survive and proliferate within intracellular and/or extracellular host environments and induce lesions and disease. For *M. tuberculosis* these features represent key requirements for its efficient transmission to new hosts. Some major virulence factors of *M. tuberculosis* are very common functions shared by many mycobacteria, such as ESX systems (Dumas et al. 2016; Newton-Foot et al. 2016), which were refined and adapted by SGM pathogens for survival in the host...
Closest Relatives of Mycobacterium tuberculosis revealed

(Groschel et al. 2016), whereby the independent acquisition of an apparent genomic island harboring ESX-associated genes espACD has likely played an important role (Ates and Brosh 2017; Orgeur and Brosh 2018). Other examples are the many genes involved in lipid metabolism, a most important feature for M. tuberculosis, given its lipid-rich cell envelope and its capacity to use host lipids as nutrients (Madacki et al. 2018). However, although such functions are necessary for virulence of M. tuberculosis, they cannot solely explain the specific transition to host-adaptation and pathogenicity, because many of them are also found in environmental mycobacteria. Previous studies on genomic comparisons of M. kansasii or M. marinum and MTB, detected major HGT events which were thought to have contributed to MTB emergence as host-associated mycobacteria (Rosas-Magallanes et al. 2006; Becq et al. 2007; Stinear et al. 2008; Wang and Behr 2014; Wang et al. 2015). However, in these former studies a wide evolutionary gap remained between MTB and the environmental mycobacteria that served as a comparison point, leaving decisive steps of MTB differentiation unexplored. Here, our RGP analysis showed that most of these genomic domains, previously thought to have been acquired by HGT during MTB differentiation from M. kansasii and/or M. marinum common ancestors, were also present in other species of the MTBAP lineage and thus apparently occurred already before MTB speciation. This observation was particularly striking for the toxin-antitoxin systems, which are reported to be involved in dormancy and resistance against environmental stresses induced by antibiotics or host immune defenses (Coussens and Daines 2016), as well as in pathogenicity (Lobato-Marquez et al. 2016). Indeed, our results suggest that some of these systems, which were apparently already acquired by early members of the MTBAP lineage, are involved in virulence, because M. tuberculosis vapC24 and vapC44 transposon insertion mutants are attenuated in selected animal infection models (Dutta et al. 2010; Zhang et al. 2013), whereas vapBC19 and vapBC20 were reported to be induced in granulomas (Hudock et al. 2017). Our study highlights the likely early acquisition of other major regulatory functions such as transcriptional regulators linked with pathogenicity (MosR, VirS, Rv1359 adenylate cyclase). All of these regulators are associated with mycobacterial lipids. A deletion mutant for the gene virS, for example, is affected in mycolic acid composition, spleen persistence in guinea pigs, and resistance to acidic stress (Singh et al. 2005), whereas a strain with a mosR deletion is deficient in phthiocerol dimycocerosate (PDIM) synthesis (Abomoelak et al. 2009). Moreover, the Rv1359 adenylate cyclase is probably involved in PDIM regulation (Camacho et al. 1999). PDIMs are among the first virulence factors that were identified for M. tuberculosis by using transposon insertion mutants and mouse infection models (Camacho et al. 1999; Cox et al. 1999). PDIMs were also shown to be involved in resistance to oxidative burst produced by macrophages, and modulation of immunity (Rousseau et al. 2004). Tight regulation of PDIM was shown to impact phagosomal damage and autophagy in host cells (Quigley et al. 2017), a finding that is also linked to the combined action with the ESX secretion system (Augenstreich et al. 2017). Our study shows that many potential virulence factors that were likely acquired during evolution of the MTBAP lineage before speciation of MTB exhibit many lipid-associated functions, and are involved in M. tuberculosis synthesis of specific mycolipids (such as sulfolipid synthesis, glycolipid glycosylation, and mycolic acid composition) (table 3). As outlined in the “Results” section, acquisition of concern-gene or gene clusters by HGT seems to have paved the way of selected MTBAP members to adopt a pathogenic lifestyle. The obtained insights allow prediction of the lipid content of the different MTBAP members, a feature that can thus be experimentally compared in future wet-lab-based lipid analyses and cell culture infection experiments. Based on our results from genome-based comparisons and analyses that place several species of nontuberculous mycobacteria into the immediate phylogenetic proximity of M. tuberculosis, a range of experiments can be designed to explore the specific roles played by the various molecular systems potentially involved in dormancy, survival under anaerobic conditions, and persistence of M. tuberculosis. Selected members of the MTBAP lineage could thereby serve as promising model organisms to find new, vulnerable features of M. tuberculosis, which can then be targeted by innovative new intervention strategies.

Supplementary Material

Supplementary data are available at Genome Biology and Evolution online.

Acknowledgments

This work was supported in part by the French National Research Council ANR (ANR-10-LABX-62-IBIED, ANR-16-CE35-0009, ANR-16-CE15-0003), the Fondation pour la Recherche Médicale (DEQ20130326471) and the European Union’s Horizon 2020 Research and Innovation Program (643381). We thank Wafa Frigui and Jan Madacki for help and fruitful discussions. We are also grateful to the whole team of the Atelier de Bioinformatique (Museum d’Histoire Naturelle, ISYEB) for support and advice, and to Ariane Chaudot for participating in preliminary datamining approaches during a student internship.
Literature Cited

Ab mommy, L, et al. 2009. mosR, a novel transcriptional regulator of hypoxia and virulence in Mycobacterium tuberculosis. J Bacteriol. 191(19):5941–5952.

Albretsen J, et al. 2013. Proteomic profiling of Mycobacterium tuberculosis identifies nutrient-stationary-responsive toxin–antitoxin systems. Mol Cell Proteomics 12(5):1180–1191.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215(3):403–410.

Ankenbrand M, Keller A. 2016. bgtree: automated phylogenetic tree building from bacterial core genomes. Genome 59(10):783–791.

Ates LS, Brosch R. 2017. Discovery of the type VII ESX-1 secretion needle? Mol Microbiol. 103(1):7–12.

Augenstreich J, et al. 2017. ESX-1 and phthiocerol dimycocerosates of Mycobacterium tuberculosis act in concert to cause phagosomal rupture and host cell apoptosis. Cell Microbiol. 19(7):e12726.

Avanzi C, et al. 2016. Red squirrels in the British Isles are infected with leprosy bacilli. Science 354(6313):744–747.

Beec J, et al. 2007. Contribution of horizontally acquired genomic islands to the evolution of the tubercle bacilli. Mol Biol Evol. 24(8):1861–1871.

Benjak A, et al. 2018. Highly reduced genome of the new species Mycobacterium uberis, the causative agent of nodular thelitis and tuberculoid scrotitis in livestock and a close relative of the leprosy bacilli. mSphere 3(5):e00404–18. doi: 3/5/e00405-00418

Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. 2002. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol Microbiol. 43(3):717–731.

Blouin Y, et al. 2014. Progenitor “Mycobacterium canetti” clone responsible for lymph node tuberculosis epidemic. Emerg Infect Dis. 20(1):21–28.

Boritsch EC, et al. 2014. A glimpse into the past and predictions for the future: the molecular evolution of the tuberculosis agent. Mol Microbiol. 93(5):835–852.

Boritsch EC, et al. 2016a. pks5-recombination-mediated surface remodeling in Mycobacterium tuberculosis emergence. Nat Microbiol. 1(2):15019.

Boritsch EC, et al. 2016b. Key experimental evidence of chromosomal DNA transfer among selected tuberculosis-causing mycobacteria. Proc Natl Acad Sci U S A. 113(25):9876–9881.

Bos KI, et al. 2014. Pre-Columbian mycobacterial genomes reveal seals as a source of New World human tuberculosis. Nature 514(7523):494–497.

Brosch R, et al. 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl Acad Sci U S A. 99(6):3684–3689.

Brown-Elliott BA, et al. 2018. Mycobacterium decipiens sp. nov., a new species closely related to the Mycobacterium tuberculosis complex. Int J Syst Evol Microbiol. 68(11):3557–3562.

Camacho LR, Ensergueix D, Perez E, Gicquel B, Guilhot C. 1999. Identification of a virulence gene cluster of Mycobacterium tuberculosis by signature-tagged transposon mutagenesis. Mol Microbiol. 34(2):257–267.

Cole ST, et al. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 393(6685):537–544.

Cole ST, et al. 2001. Massive gene decay in the leprosy bacillus. Nature 409(6823):1007–1011.

Comas I, et al. 2013. Out-of-Africa migration and Neolithic coexpansion of Mycobacterium tuberculosis with modern humans. Nat Genet. 45(10):1176–1182.

Comas I, Gagneux S. 2009. The past and future of tuberculosis research. PLoS Pathog. 5(10):e1000600.
Kozak RA, Alexander DC, Liao R, Sherman DR, Behr MA. 2011. Region of difference 2 contributes to virulence of Mycobacterium tuberculosis. Infect Immun. 79(1):59–66.

Kuo CH, Ochman H. 2009. Inferring clocks when lacking rocks: the variable rates of molecular evolution in bacteria. Biol Direct 4(1):35–10.

Kuwahara H, et al. 2008. Reductive genome evolution in chemoautotrophic intracellular symbionts of deep-sea Calyptogena clams. Extremophiles 12(3):365–374.

Larkin MA, et al. 2007. ClustalW and ClustalX version 2.0. Bioinformatics 23(21):2947–2948.

Le Dantec C, et al. 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl Environ Microbiol. 68(11):5318–5325.

Leoni E, Legnani P, Mucci MT, Pirani R. 1999. Prevalence of mycobacteria in a swimming pool environment. J Appl Microbiol. 87(5):683–688.

Levillain F, et al. 2017. Horizontal acquisition of a hypoxia-responsive mono-lysin cofactor biosynthesis pathway contributed to Mycobacterium tuberculosis pathoadaptation. PLoS Pathog. 13(11):e1006752.

Lin W, et al. 2016. Transcriptional profiling of Mycobacterium tuberculosis exposed to in vitro lysosomal stress. Infect Immun. 84(9):2505–2523.

Lobato-Marquez D, Diaz-Orejas R, Garcia-Del Portillo F. 2016. Toxicity and bactericidal virulence. FEMS Microbiol Rev. 40:592–609.

Lynett J, Stokes RW. 2007. Selection of transposon mutants of Mycobacterium tuberculosis with increased macrophage infectivity identifies fadD23 to be involved in sulfolipid production and association with macrophages. Microbiology 153(Pt 9):3133–3140.

Madacki J, Mas Fiol G, Boshch R. 2018. Update on the virulence factors of the obligate pathogen Mycobacterium tuberculosis and related tuberculosis-causing mycobacteria. Infect Genet Evol. 10:30955–30959.

Marjanovic O, lavarone AT, Riley LW. 2011. Sulfolipid accumulation in Mycobacterium tuberculosis disrupted in the mce2 operon. J Microbiol. 49(3):441–447.

Marjanovic O, Miyata T, Goodridge A, Kendall LV, Riley LW. 2010. Mce2 operon mutant strain of Mycobacterium tuberculosis is attenuated in C57BL/6 mice. Tuberculosis (Edinburgh) 90(1):50–56.

Mendum TA, Wu H, Kierzek AM, Stewart GR. 2015. Lipid metabolism and Type VII secretion systems dominate the genome scale virulence profile of Mycobacterium tuberculosis in human dendritic cells. BMC Genomics 16(1):372.

Mestre O, et al. 2013. High throughput phenotypic selection of Mycobacterium tuberculosis mutants with impaired resistance to reactive oxygen species identifies genes important for intracellular growth. PLoS One 8(1):e53486.

Miele V, Penel S, Duret L. 2011. Ultra-fast sequence clustering from similarity networks with SILIX. BMC Bioinform. 12:116.

Mostowy S, Cousins D, Brinkman J, Aranan A, Behr MA. 2002. Genomic deletions suggest a phylogeny for the obligate pathogen Mycobacterium tuberculosis-causing mycobacteria. Infect Genet Evol. 2:100-110.

Mutchler JM, Watson J, Parish C. 2011. Transcriptional profiling of Mycobacterium tuberculosis exposed to in vitro lysosomal stress. Infect Immun. 84(9):2505–2523.

Newton-Foot M, Warren RM, Sampson SL, van Helden PD, Gey van Pittius NC. 2016. The plasmid-mediated evolution of the mycobacterial ESX (Type VII) secretion systems. BMC Evol Biol. 16:62.

Novichkov PS, Wolf YI, Dubchak I, Koonin EV. 2009. Trends in prokaryotic evolution revealed by comparison of closely related bacterial and archaean genomes. J Bacteriol. 191(1):65–73.

Orgeur M, Brosch R. 2018. Evolution of virulence in the Mycobacterium tuberculosis complex. Curr Opin Microbiol. 41:68–75.

Pallen MJ, Wren BW. 2007. Bacterial pathogenomics. Nature 449(7164):835.

Peth K, et al. 2004. Isolation of Mycobacterium tuberculosis mutants defective in the arsiter of phagosomal maturation. Proc Natl Acad Sci U S A. 101(37):13642–13647. Epub 2004 Aug 13631.

Quigley J, et al. 2017. The cell wall lipid PDIM contributes to phagosomal escape and host cell exit of Mycobacterium tuberculosis. MBio 8(2):e00148-00117.

Rachman H, et al. 2006. Unique transcriptome signature of Mycobacterium tuberculosis in pulmonary tuberculosis. Infect Immun. 74(2):1233–1242.

Ramage HR, Connolly LE, Cox JS. 2009. Comprehensive functional analysis of Mycobacterium tuberculosis toxin–antitoxin systems: implications for pathogenesis, stress responses, and evolution. PLoS Genet. 5(12):e1000767.

R-Core-Team. 2014. R: A language and environment for statistical computing. [Internet]. Vienna (Austria): R Foundation for Statistical Computing.

Rehangejan J, Bloom BR, Rubin EJ. 2005. Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. Proc Natl Acad Sci U S A. 102(23):8327–8332.

Richter L, Saviola B. 2009. The lipf promoter of Mycobacterium tuberculosis is upregulated specifically by acidic pH but not by other stress conditions. Microbiol Res. 164(2):228–232.

Richter M, Rossello-Mora R, Oliver Glockner F, Peplies J. 2016. *SpeciesWS*: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 32(6):929–931.

Rios JA, McGough KJ, Rider-Rios CJ, Rastogi N, Hazbon MH. 2016. Phylogenetic analysis of the species of the Mycobacterium tuberculosis complex demonstrates that Mycobacterium africanum, Mycobacterium bovis, Mycobacterium caprae, Mycobacterium microtuberculosis and Mycobacterium pinnipedi are later heterotypic synonyms of Mycobacterium tuberculosis. Int J Syst Evol Microbiol. 68:324–332.

Rogas-Magallanes V, et al. 2006. Horizontal transfer of a virulence operon to the ancestor of Mycobacterium tuberculosis. Mol Biol Evol. 23(6):1129–1135.

Rogas-Magallanes V, et al. 2007. Signature-tagged transposon mutagenesis identifies novel Mycobacterium tuberculosis genes involved in the parasitism of human macrophages. Infect Immun. 75(1):504–507.

Rousseau C, et al. 2004. Production of phthiocerol dimycocerosates protects Mycobacterium tuberculosis from the cidal activity of reactive nitrogen intermediates produced by macrophages and modulates the early immune response to infection. Cell Microbiol. 6(3):277–287.

Saad MM, et al. 2015. Mycobacterium ngathidense infections. Saudi Med J. 36(5):620–625.

Sasseur CM, Rubin EJ. 2003. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A. 100(22):12989–12994.

Schliep KP. 2011. phangorn: phylogenetic analysis in R. Bioinformatics 27(4):592–593.

Simone R, et al. 2013. Functional characterisation of three α-methyltransferases involved in the biosynthesis of phenolglycolipids in Mycobacterium tuberculosis. PLoS One 8(3):e58954.

Simpson GG. 1944. Tempo and mode in evolution. New York (NY): Columbia University Press.

Singh P, et al. 2015. Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium leprae. Proc Natl Acad Sci U S A. 112(14):4459–4464.

Singh R, et al. 2003. Disruption of mptpB impairs the ability of Mycobacterium tuberculosis to survive in guinea pigs. Mol Microbiol. 50(3):751–762.

Singh R, Singh A, Tyagi AK. 2005. Deciphering the genes involved in pathogenesis of Mycobacterium tuberculosis. Tuberculosis (Edinburgh) 85(5–6):325–335.

Stewart GR, Patel J, Robertson BD, Rae A, Young DB. 2005. Mycobacterial mutants with defective control of phagosomal acidification. PLoS Pathog. 1(3):269–278.

Stinear TP, Brosch R. 2016. Leprosy in red squirrels. Science 354(6313):702–703.
Stinear TP, et al. 2008. Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. Genome Res. 18(5):729–741.

Supply P, et al. 2013. Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*. Nat Genet. 45(2):172–179.

Suyama M, Torrents D, Bork P. 2006. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. Nucleic Acids Res. 34(Web Server issue):W609–12.

Takeda K, et al. 2016. Six cases of pulmonary *Mycobacterium shinjukuense* infection at a single hospital. Intern Med. 55(7):787–791.

Thioulouse J, Chessel D, Dolédec S, Olivier JM. 1997. ADE-4: a multivariate analysis and graphical display software. Stat Comput. 7(1):75–83.

Toft C, Anderson SGE. 2010. Evolutionary microbial genomics: insights into bacterial host adaptation. Nat Rev Genet. 11:465.

Tortoli E, et al. 2017. The new phylogeny of the genus *Mycobacterium*: the old and the news. Infect Genet Evol. 56:19–25.

Tufariello JM, et al. 2015. The complete genome sequence of the emerging pathogen *Mycobacterium haemophilum* explains its unique culture requirements. MBio 6(6):e01313-01315.

Turenne C, et al. 2002. *Mycobacterium lacus* sp. nov., a novel slowly growing, non-chromogenic clinical isolate. Int J Syst Evol Microbiol. 52(Pt 6):2135–2140.

Vaerewijck MJ, Huys G, Palomino JC, Swings J, Portaels F. 2005. *Mycobacteria* in drinking water distribution systems: ecology and significance for human health. FEMS Microbiol Rev. 29(5):911–934.

Wang J, Behr MA. 2014. Building a better bacillus: the emergence of *Mycobacterium tuberculosis*. Front Microbiol. 5:139.

Wang J, et al. 2015. Insights on the emergence of *Mycobacterium tuberculosis* from the analysis of *Mycobacterium kansasii*. Genome Biol Evol. 7(3):856–870.

Watanabe S, et al. 2011. Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. PLoS Pathog. 7(10):e1002287.

Yang Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci. 13:555–556.

Zhang YJ, et al. 2013. Tryptophan biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. Cell 155(6):1296–1308.

Zolotarev AS, et al. 2008. Increased sulfate uptake by *E. coli* overexpressing the SLC26-related SuP protein Rv1739c from *Mycobacterium tuberculosis*. Comp Biochem Physiol Part A: Mol Integr Physiol. 149(3):255–266.

Zuckerkandl E, Pauling L. 1965. Molecules as documents of evolutionary history. J Theor Biol. 8(2):357–366.

Associate editor: Eric Bapteste