How do monomorphic bacteria evolve? The *Mycobacterium tuberculosis* complex and the awkward population genetics of extreme clonality

Christoph Stritt, Sebastien Gagneux

Swiss Tropical and Public Health Institute, Allschwil, Switzerland; University of Basel, Basel, Switzerland

June 16, 2023

Abstract

Exchange of genetic material through sexual reproduction or horizontal gene transfer is ubiquitous in nature. Among the few outliers that rarely recombine and mainly evolve by de novo mutation are a group of deadly bacterial pathogens, including the causative agents of leprosy, plague, typhoid, and tuberculosis. The interplay of evolutionary processes is poorly understood in these organisms. Population genetic methods allowing to infer mutation, recombination, genetic drift, and natural selection make strong assumptions that are difficult to reconcile with clonal reproduction and fully linked genomes consisting mainly of coding regions. In this review, we highlight the challenges of extreme clonality by discussing population genetic inference with the Mycobacterium tuberculosis *complex, a group of closely related obligate bacterial pathogens of mammals. We show how uncertainties underlying quantitative models and verbal arguments affect previous conclusions about the way these organisms evolve. A question mark remains behind various quantities of applied and theoretical interest, including mutation rates, the interpretation of nonsynonymous polymorphisms, or the role of genetic bottlenecks. Looking ahead, we discuss how new tools for evolutionary simulations, going beyond the traditional Wright-Fisher framework, promise a more rigorous treatment of basic evolutionary processes in clonal bacteria.*

Introduction

Mutation, recombination, genetic drift, and natural selection are the basic evolutionary processes that drive the evolution of life. It is the aim and "great obsession" of population genetics to infer these processes from patterns of genetic variation observed in nature (Gillespie, 2004). Since the Modern Synthesis of evolutionary biology in the 1930s, a variety of mathematical models have been developed for this purpose, which today are in wide use in the analysis of genome sequencing data (Templeton, 2021).

A problem in the application of population genetic models to empirical data is that modeling assumptions can be a far cry from the biology and life history of real organisms. Archea and bacteria reproduce clonally through binary fission, frequently undergo horizontal gene transfer (HGT), and have genomes consisting mainly of coding regions. These characteristics are difficult to reconcile with models that are tailored to animals and plants (Woese and Goldenfeld, 2009) and commonly assume random mating, linkage equilibrium, and neutrality (Maynard Smith, 1995; Rocha, 2018). As a consequence, outside the laboratory, studies of bacterial population genetics have either remained descriptive, with much effort going into understanding the extent and effects of HGT (e.g. Denamur et al., 2021); or have resorted to models whose applicability remains an open question (discussed by Johri et al., 2022).

While the opportunistic, hardly predictable process of HGT has been highlighted as the most problematic breach of assumptions (Maynard Smith, 1995), a different, less frequently discussed challenge arises from the opposite extreme of the recombination spectrum: strictly clonal evolution, or the absence of any gene flow. HGT is not a general characteristic of bacteria (Hanage, 2016). Some bacteria are "monomorphic", that is, characterized by low levels of sequence diversity and an apparent absence of genetic exchange (Achtman, 2008). The causative agents of several devastating bacterial diseases of humans and animals belong to this group, including *Bacillus anthracis* (anthrax), *Salmonella enterica* serotype typhi (typhoid), *Yersinia pestis* (plague), *Mycobacterium leprae* (leprosy), and the members of the *Mycobacterium tuberculosis* complex (tuberculosis). Our understanding of the evolution of these bacteria is hampered not only by the low information content in their genomes, but also because there is little theoretical and conceptual work on population genetic inference under extreme clonality.

Here we highlight the obligate pathogens of the *Mycobacterium tuberculosis* complex (MTBC) as a model to study clonal evolution. The MTBC comprises a group of closely related obligate pathogens that cause tuberculosis (TB) in humans and a range of wild and domestic animals (Figure 1). Human TB mainly affects the global poor and has killed more than 1.6 million people in 2021 (World Health Organization, 2022). The evolution of antibiotic resistance is a main challenge and focus of research in TB. The genomes of thousands of MTBC strains from around the world have been sequenced, mainly to study epidemiological dynamics and drug resistance evolution, but also to infer the origin and biogeographic history of the species (Gagneux, 2018).

Members of the MTBC are among the more diverse of the predominantly clonal bacteria (Achtman, 2012), even though individual strains differ only by a maximum of ca. 2,400 SNPs across the 4.4 Mb genome (Figure 2a). At the molecular level, the MTBC is further characterized by a high GC content, a high proportion of nonsynomyous polymorphisms, and a low proportion of homoplastic mutations (Figure 2b-d). Different hypotheses have been put forward to explain these patterns and, more generally, what drives the evolution of the MTBC. Besides lack of HGT, prominent and conflicting propositions are that the dominant process in the evolution of the MTBC is genetic drift (Hershberg et al., 2008) or purifying selection (Namouchi et al., 2012; Pepperell et al., 2013).

In this review, we discuss these and other hypotheses about the basic processes driving the evolution of the MTBC. Given the unclear applicability of population genetics to highly clonal organisms, particular attention is paid to models, their assumptions, and the traits of the MTBC that might conflict with the latter. Evolutionary simulations are discussed as a way to achieve a more quantitative treatment of frequently invoked processes such as purifying selection or periodic bottlenecks.

Mutation

While in some bacteria new variants are more likely to be generated by HGT than by mutation (Vos and Didelot, 2009), under extreme clonality *de novo* mutations are the main source of genetic diversity and adaptation. The speed and direction in which a clonal prokaryote evolves is thus determined by the rate and spectrum of new mutations and by their effect on fitness. Numerous studies have investigated mutagenesis in the MTBC (reviewed by Mcgrath et al., 2014). As discussed below, in addition to methodological issues in estimating mutation rates, the life history of the bacteria, which can include extended periods of dormancy, poses a main challenge in understanding the rate at which variation originates *in vivo*.

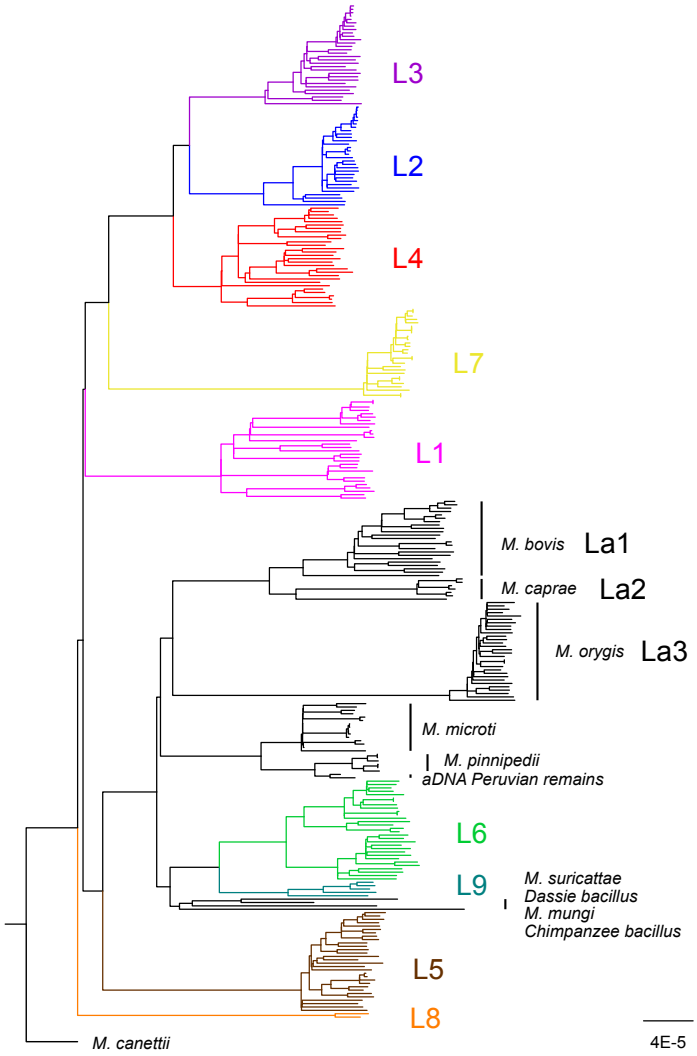

Figure 1: *Rooted maximum likelihood phylogeny of the MTBC estimated from genome-wide SNPs (tree adapted from Zwyer et al. (2021), for better readability large lineages were downsampled to max. 30 strains).* M. canettii *is the outgroup, human-adapted lineages (L1 to L9) are shown in colors, animal-adapted lineages in black. Species names represent the historically grown nomenclature, lineage names are a more recent classification based on genomic data. Lineages 1 to 4 and 7 are also referred to as* M. tuberculosis sensu stricto, *lineages 5 and 6 as* M. africanum. *Bootstrap supports for the lineages are above 0.95 and are not displayed in the figure.*

In the MTBC literature, as elsewhere, the mutation rate is sometimes confounded with the molecular clock rate. While the former refers to the rate at which mutations *originate* in the genome, the latter stands for an allegedly constant rate at which mutations accumulate through time (Ho et al., 2011). Both rates are subsumed in the more general concept of evolutionary rates. As discussed below, the power law that describes the slowing of evolutionary rates as one considers longer timescales is not as clear in the MTBC as in other bacteria: *in vitro* mutation rate

estimates can be similar to clock rate estimates from datasets including ancient DNA. How far methodological biases or evolutionary processes underlay this surprising finding remains to be understood.

Plasticity of mutation rates and generation times

Fluctuation assays suggest that point mutations in the MTBC appear at a rate of about 2.1×10^{-10} mutations per site per generation and at a similar rate during active disease in macaques if a generation time of 20 h is assumed (Ford et al., 2011, Figure 3). A later study, using the same fluctuation assay, found *in vitro* rates of 6.01×10^{-10} in a lineage 4 and 2.16×10^{-9} in a lineage 2 strain, suggesting somewhat faster and variable mutation rates within the MTBC (Ford et al., 2013). Comparatively fast rates were also proposed in two additional experimental evolution studies. After serial passaging of a MTBC strain through macrophage-like THP1 cells for 80 generations, Guerrini et al. (2016) inferred a rate of 5.7×10^{-9} per bp per generation. Copin et al. (2016), passaging bacteria in mice and assuming a generation time of 20 h, estimated a mutation rate of 3.8×10^{-9} in wild type mice and of 7.7×10^{-10} in T cell-deficient mice, suggesting that the presence of T cells leads to elevated mutation rates.

Overall, per-generation mutation rates estimated for the MTBC are well within the range of those in other bacteria, which typically are in the order 10^{-10} (reviewed by Katju and Bergthorsson, 2019). When trying to scale mutation rates to calendar time, however, complications due to the complex life history of these bacteria become apparent. The bacteria of the MTBC have long generation times ranging from 18 h in nutrient rich medium to potentially much longer time-spans *in vivo* (Colangeli et al., 2020). Scaled to clock time, mutation rates are thus indeed low in the MTBC compared to other bacteria, at least in the laboratory (Gibson et al., 2018).

In contrast to pathogens employing a "hit and run" strategy, bacteria of the MTBC can enter a state of reduced activity and persist for years in latent infections (Dutta and Karakousis, 2014). It is unclear whether latency and longer generation times imply a reduced mutation rate, as expected if mutation is driven by replication, or not, as expected if environmental stress drives mutation (Weller and Wu, 2015). Ford et al. (2011), in their experimental infection of macaques, found similar rates in latent and active disease (Figure 3), supporting stress-induced mutagenesis. A more complex, two-phased scenario was suggested by Colangeli et al. (2020), who investigated 24 paired TB cases with latently infected household contacts: mutation rates remained high up to two years, but then decreased with longer latency as the bacteria entered a quiescent state with longer generation times (Figure 3).

In summary, mutation rates estimated for the MTBC should be interpreted with some caution. Generation times are only known with confidence *in vitro*. At the same time, fluctuation assays reflect the mutation rate of a single gene (*rpoB*, the main drug resistance target of rifampicin) that might not be representative for the whole genome (Katju and Bergthorsson, 2019); and in the absence of stress, which *in vivo* might alter both the rate and the spectrum of new mutations (Fitzgerald and Rosenberg, 2019).

The time (in)dependence of evolutionary rates in the MTBC

Molecular dating has led to a re-evaluation of the origin and history of the MTBC, as for many other organisms. Earlier studies, assuming a synonymous mutation rate or a co-diversification of humans and the MTBC, located the most recent common ancestor of the existing lineages in Africa

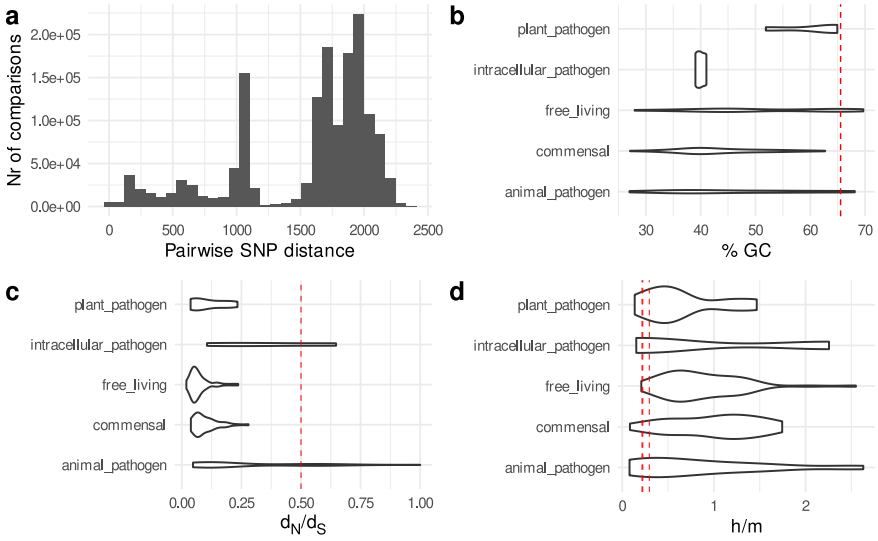

Figure 2: *Genetic diversity and molecular characteristics of the MTBC. a) Pairwise genetic differences between the strains shown in Figure 1, based on single nucleotide polymorphisms from Zwyer et al. (2021). b) to d) show molecular characteristics of the MTBC compared to 150 other bacterial species with diverse lifestyles (data from Bobay and Ochman, 2018). Red lines show the values for the bacteria of the MTBC (*M. tuberculosis sensu stricto, M. bovis, *and* M. africanum*). b) GC content, c)* d_N/d_S*, the genome-wide ratio of nonsynonymous to synonymous polymorphisms, d) the ratio of homoplastic to non-homoplastic mutations, a proxy for recombination.*

and suggested a scenario according to which humans and the MTBC have co-diversified across the globe (Comas et al., 2013; Kapur et al., 1994). Recent estimates, making use of tip dating, ancient DNA (aDNA) samples, and Bayesian phylogenetics, propose a more recent common ancestor in the Neolithic ca. 6,000 years ago (Bos et al., 2014; Kay et al., 2015; Sabin et al., 2020).

One caveat regarding these estimates is the poorly understood variability of evolutionary rates in the MTBC through time. For mitochondrial DNA, viruses, and bacteria, evolutionary rates usually appear faster when estimated from recent polymorphisms (Ho et al., 2011). For bacteria, Duchêne et al. (2016) found a clear negative association, described by an exponential decay curve, between clock rates and sampling time spans in 16 bacterial species, with an order of magnitude difference between a 10 year and a 100 year sampling period. The delayed effect of purifying selection is the most prominent explanation for this time dependence of evolutionary rates, although methodological biases might also contribute (Emerson and Hickerson, 2015; Ho et al., 2015). Time dependence can have a large effect on molecular dating: Membrebe et al. (2019) showed that accounting for purifying selection by using relaxed clock or epoch models can shift divergence times one order of magnitude back in time. Could this explain the surprisingly recent time to the most recent common ancestor (MRCA) estimated by the aDNA studies?

In the study of Duchêne et al. (2016), the MTBC does not follow the general pattern of time depence: almost identical rates were obtained from samples spanning 15 and 895 years. Similarly, Menardo et al. (2019) found only marginally lower rates when calibrating the clock with three samples of ancient DNA from Precolumbian human remains and an extensive MTBC dataset covering a sampling period of 30 years. An overview of evolutionary rates estimated for the MTBC

illustrates the large variability and uncertainty of rate estimates, but also suggest an overall trend of time dependence (Figure 3). As Menardo et al. (2019) showed in their extensive study of the molecular clock in the MTBC, clock rates vary substantially among lineages and clades of the MTBC and have large confidence intervals. Lineage 1, for instance, seems to have evolved faster than other lineages, and indeed faster than the L4 strain in the fluctuation assay of Ford et al. (2011). On the slow end of the spectrum is the long-term clock rate estimated by Sabin et al. (2020), for which all six aDNA samples available so far were included (1.4×10^{-8}, 95% HPD 9.46×10^{-9}, 1.96×10^{-8}).

The low diversity of the MTBC certainly contributes to the large variability and uncertainty in clock rate estimates. SNPs are not only few in the MTBC, but also to a large proportion singletons (Chiner-Oms et al., 2019; O'Neill et al., 2015) and thus not informative about tree topology. In a Bayesian setting, prior-posterior comparisons are therefore crucial to determine whether the data is informative when applying parameter-rich models such as relaxed clocks. This does not only apply to the clock but also to the tree model, which also biases clock rate estimates in data-limited scenarios (Menardo et al., 2021a; Möller et al., 2018). To our knowledge, prior-posterior comparisons have not been published in aDNA dating studies so far, and the limitations inherent to low-diversity MTBC genomes remain unclear.

Why are MTBC genomes so GC-rich?

In bacteria, newly arising mutations are biased towards adenines and thymines (Hershberg and Petrov, 2010; Hildebrand et al., 2010). If mutation bias and genetic drift alone would determine the nucleotide landscape (mutation-drift equilibrium), the expected GC content in the MTBC would be 41.5% (Hershberg and Petrov, 2010). MTBC genomes, however, consist to 65.6% of guanines and cytosines (Figure 2b; Cole et al., 1998), with values of 80% at synonymous and 60% at nonsynonymous sites. Such a discrepancy between observed and expected GC content is observed in many prokaryotes, whose genomes vary hugely in GC content (Figure 2b). It implies that an unknown process, unaccounted for in standard models of molecular evolution, affects the segregation of polymorphisms through time (Rocha and Feil, 2010).

Several large-scale comparative studies have attempted to find a general explanation for the discordance between expected and observed GC content in prokaryotes. One prominent hypothesis is that nucleotide composition reflects adaptation to environmental conditions, for example through selection for thermal stability of DNA (e.g. Reichenberger et al., 2015). An intriguing twist to this idea was recently added by Weissman et al. (2019), who described a correlation between GC content, environmental variables, and the presence of *Ku*, the key gene in the non-homologous end-joining (NHEJ) pathway for DNA break repair. The authors propose that high GC content could be beneficial in bacteria suffering stress-induced double strand breaks in periods of slow or no growth, when NHEJ is required for repair because only a single copy of the genome is present. This is an interesting scenario for the MTBC, where long periods of latency can occur (see above) and the *Ku* gene is present.

An alternative explanation for GC bias that does not imply a selective advantage is GC-biased gene conversion (gBGC). This process occurs during homologous recombination when mismatches in heteroduplex DNA are preferentially resolved into guanines and cytosines (reviewed by Duret and Galtier, 2009). The gBGC hypothesis predicts that GC content is higher in regions with high recombination rates, which is observed in mammalian genomes. In bacteria, the role of gBGC is contested. Whether comparative studies find associations between GC content and recombination

depends on the method used to infer recombination, and exceptions to general trends are common (Bobay and Ochman, 2017; Lassalle et al., 2015).

With its numerous genome sequences that can be placed in a robust phylogenetic framework, the MTBC provides an opportunity to study the evolution of base composition in detail and thus to complement broad comparative studies. A hypothesis to test is that the MTBC is evolving from the generally GC-rich state of mycobacteria (58 to 70%, *Mycobacterium* sp. genomes on NCBI) to a more AT-rich state characteristic of obligate pathogens (Rocha and Danchin, 2002, Figure 2b), including *Mycobacterium leprae* (58%).

Recombination

How "strict" is clonality in the MTBC? In the past, bacteria were classified as "clonal" or "monomorphic" based on a handful of housekeeping genes (Maynard Smith et al., 1993; Selander et al., 1987). With the full resolution of whole genome sequences, this classification needs to be reassessed. As discussed in the following, experimental and observational evidence agree that the MTBC is predominantly clonal, and that few to no new genes have found their way into the MTBC since the most recent common ancestor of the currently existing lineages. In contrast to interstrain recombination, intrachromosomal recombination is common and increasingly recognized as an important source of genetic variation.

Experimental evidence: genetic factors versus lack of opportunity

Most of the knowledge about the molecular mechanisms of HGT in mycobacteria stems from research with *Mycobacterium smegmatis*, a fast-growing, non-pathogenic mycobacterium more easily amenable to cultivation and genetic engineering than the bacteria of the MTBC. Mycobacteria lack the traditional components of HGT, possibly because transfer through the complex cell envelopes of these diderm bacteria requires other mechanisms (Madacki et al., 2021). Investigations of gene transfer in *M. smegmatis* have led to the description of a previously unknown form of bacterial conjugation: distributive conjugal transfer (DCT, reviewed by Gray and Derbyshire, 2018).

Of particular interest regarding the evolution of the MTBC is the observation of DCT in the closely related *Mycobacterium canettii*. *M. canettii* shares an average nucleotide identity of 97.5% with the MTBC, yet is strikingly more diverse: a handful of *M. canettii* strains from eastern Africa harbor more genetic diversity than the whole MTBC (Supply et al., 2013). Mating assays have shown that DCT occurs in *M. canettii*, while no DCT was observed between three MTBC strains (Boritsch et al., 2016). The same assays combining *M. canettii* and MTBC strains revealed that the latter can act as donors but not as receivers of DNA during DCT, as pieces of MTBC DNA were integrated into *M. canettii* genomes but not vice versa (Madacki et al., 2021). In *M. smegmatis*, polymorphisms in the *esxI* secretion locus underlay self identity and conjugal compatibility (Clark et al., 2022). In *M. canettii* and the MTBC, the molecular mechanisms underlying conjugal compatibility do not depend on *esxI* and remain to be elucidated (Madacki et al., 2021).

Lack of opportunity has been proposed to explain why intracellular pathogens such as the MTBC do not seem to recombine (Casadevall, 2008; Chiner-Oms et al., 2019). Against this scenario, it can be argued that there is more opportunity to recombine than the label "intracellular pathogen" might suggest. The bacteria of the MTBC are not confined to intracellular environments, but are also present in large extracellular populations after the induction of necrosis (Orme, 2014). Furthermore, mixed infections do occur (Moreno-Molina et al., 2021; Tarashi et al., 2017), such

that diverged strains might find themselves in close proximity. Rather than a mere side effect, as implied in the lack of opportunity hypothesis, absence of HGT could be an evolutionary strategy with a genetic basis. The predominance of clonality in a wide range of pathogenic organisms could indicate that clonality is adaptive by preventing the breakup of favorable allele combinations (Tibayrenc and Ayala, 2017). Further investigation into the genetic and environmental determinants of extreme clonality would be worthwhile, and the *M. canettii*-MTBC system provides a great opportunity to elucidate the poorly understood evolutionary transition to extreme clonality characteristic of many obligate pathogens.

Recombination between closely related strains: how strict is clonality?

Genome sequences from diverse MTBC strains are an important complement to experimental data, which leave open the question how far the observed outcome depends on the specific conditions and strains used in the laboratory. Various studies have investigated the extent of HGT in natural strains of the MTBC, motivated by the observation how HGT accelerates resistance evolution in other bacterial pathogens (Davies and Davis, 2010). Some have suggested that interstrain recombination does occur. Liu et al. (2006) found that mutation alone cannot explain the observed haplotype diversity, and identified a mosaic region in front of a *PPE* gene suggesting a recombination hotspot. They also point out the possibility that the pattern may have arisen through recombination between homologous sequences in the same genome. Namouchi et al. (2012) investigated 24 sequenced MTBC genomes and reported that "four different approaches showed evident signs of recombination in *M. tuberculosis*", with recombination typically involving small tracts of around 50 bp. On the other hand, the most extensive investigation to date, using different methods on genome-wide SNPs in 1,591 diverse strains, found "no measurable ongoing recombination among the MTBC strains" (Chiner-Oms et al., 2019).

Generalizing from these studies is difficult due to the diversity of datasets and methods used. It has been suggested that the signs of recombination described by Namouchi et al. are mainly artefacts as they are overrepresented in regions difficult to align or assemble, in particular repetitive and low-complexity regions in insertion sequences and the expanded *PE/PPE* gene families (Godfroid et al., 2018). Alternatively, signs of recombination can arise from gene conversion during intrachromosomal recombination, to which these repetitive sequences are prone (Liu et al., 2006). Gene conversion is the non-reciprocal transfer of DNA from one homologous sequence to another, which in the MTBC might account for recombination signatures in *ESX*, *PE*, *PPE*, *PE/PGRS* gene families (Karboul et al., 2008; Phelan et al., 2016; Uplekar et al., 2011).

Intrachromosomal recombination can also have more dramatic outcomes. More and more structural variants are described in MTBC genomes, ranging from insertion sequence (McEvoy et al., 2007) and gene copy number polymorphisms (Fishbein et al., 2015) to massive inversions (Merrikh and Merrikh, 2018) and tandem duplications (Wang et al., 2022). This is a vast topic deserving a dedicated review. It is brought up here to emphasize that recombination is an umbrella term for diverse processes of inter- and intrachromosomal exchange; and that clonality does therefore not imply absence of recombination, strictly speaking, but only of HGT. In the near future, long-read sequencing should allow more extensive studies of the repetitive "dark matter" in the MTBC genome and how it generates genetic variation intrachromosomally.

A basic limitation of methods to infer recombination is that they cannot distinguish *de novo* mutations from allelic recombination between closely related individuals, which might involve the exchange of a single nucleotide (Martin et al., 2011). Allelic recombination does not introduce new

genes, but it can affect the nucleotide landscape through recombination-associated processes like biased gene conversion (Duret and Galtier, 2009) or increased mutation rates around strand breaks (Fitzgerald and Rosenberg, 2019). While HGT between close relatives would be less restricted by opportunity, genetic incompatibilities might prevent gene transfer between close relatives, as in *M. smegmatis* (Clark et al., 2022).

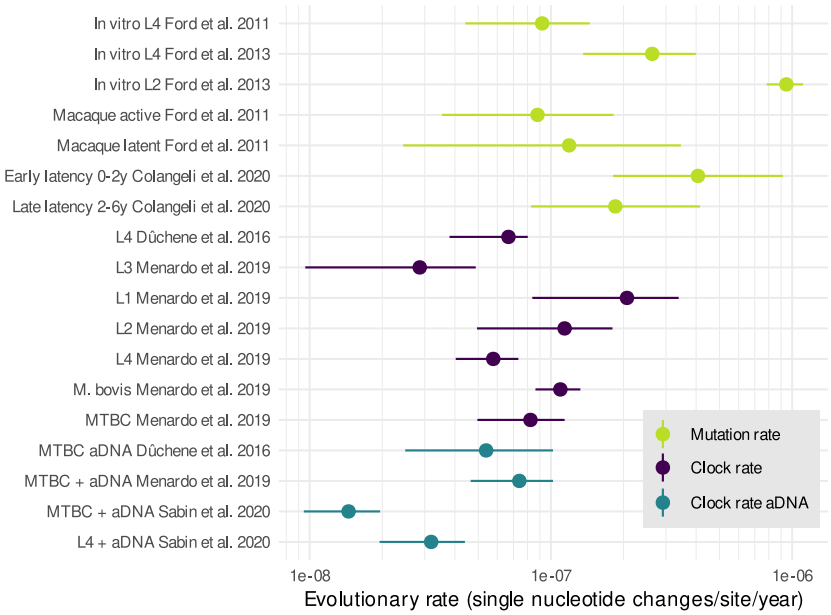

Figure 3: *Evolutionary rates in the MTBC. Only studies that report confidence intervals were considered. For the fluctuation assay estimates in Ford et al. (2011, 2013), a generation time $g = 20h$ was assumed to translate rates to calendar time. The rates of Colangeli et al. (2020) were translated back to calendar time by assuming $g = 18h$, as reported by the authors. From the molecular clock study of Menardo et al. (2019), BEAST estimates are reported for a $1/x$ clock rate prior and constant population size. For the BEAST analysis of Sabin et al. (2020), results for the birth-death skyline model with an uncorrelated lognormal clock are reported.*

Genetic drift and purifying selection

Once a mutation appears in a genome, its fate depends on the selective advantage or disadvantage it confers – and on chance. Genetic drift is the "chance factor" in evolution: it describes the undirected, stochastic change of allele frequencies due to sampling effects (Plutynski, 2007). Genetic drift sets limits to natural selection such that, by chance, deleterious alleles can increase and beneficial ones decrease in frequency (Kimura, 1983; Lynch, 2007). Increased genetic drift thus implies reduced purifying selection, and the same genomic evidence, discussed below, underlies claims as to the relative importance of the two processes. For this reason genetic drift and purifying selection are treated together, while positive selection is discussed in the next section.

Genetic drift is frequently invoked as an *ad hoc* explanation, but actually inferring and quantifying it is difficult. In the standard Wright-Fisher (WF) model with panmixia, discrete generations,

and no selection, drift occurs when the alleles to form the next generation are randomly sampled from the parental population (Fisher, 1930; Wright, 1931). In bacteria, population subdivision, linked selection, and demographic changes imply that sampling effects are stronger than under panmixia (Price and Arkin, 2015), and that effective population sizes (N_e) are orders of magnitude smaller than census sizes (Bobay and Ochman, 2018).

As discussed in this section, arguments about the strength of drift in the MTBC are largely based on indirect evidence in the form of low diversity and overabundant nonsynonymous polymorphisms. Estimates of N_e are sometimes obtained in Bayesian skyline analyses, but their underlying assumptions are problematic. Finally, we discuss transmission bottlenecks in the MTBC, a main mechanism of stochastic sampling whose mid- and long-term consequences go beyond simple reductions in genetic diversity and remain to be understood.

Do overabundant nonsynonymous polymorphisms indicate strong genetic drift?

In the MTBC, the drift-versus-selection discussion has mainly revolved around the large proportion of nonsynonymous polymorphisms observed in the species. The MTBC has a genome-wide ratio of nonsynonymous to synonymous polymorphisms (d_N/d_S) of around 0.5 when diverse strains from across the phylogeny are considered (Figure 2c). This is one third higher than in the closely related *M. canettii* (Supply et al., 2013) and more than six times higher than the median (0.076) of the 153 diverse species studied by Bobay and Ochman (2018).

Hershberg et al. (2008) have interpreted the high d_N/d_S in the MTBC as evidence for "extremely reduced purifying selection" – in other words strong genetic drift – which would allow the accumulation of deleterious nonsynonymous mutations. The authors refute the alternative explanation that nonsynonymous changes are due to positive selection by pointing out that d_N/d_S does not differ between housekeeping, surface-exposed, and virulence genes, as might be expected if host immunity would drive adaptive diversification. This interpretation of d_N/d_S fits well with the generalization that the intracellular niche of pathogens and symbionts implies smaller population sizes and stronger drift. Kuo et al. (2009) inferred strong drift in human pathogens including the MTBC and reported a strong inverse relationship between drift and genome size. A similar conclusion is reached by Balbi et al. (2009), who compared *E. coli* with the closely related pathogenic *Shigella* and found signs of increased drift in the latter, including an excess of nonsynonymous mutations and of transversions, which are proportionally more nonsynonymous and thus deleterious than transitions.

Different studies have challenged the view that purifying selection is "extremely reduced" in the MTBC. In the so far only attempt to quantify the strength of purifying selection across the genome, Pepperell et al. (2013) fitted a model including demographic expansion and a fraction of sites under selection to the site frequency spectrum obtained from a global sample of the MTBC. They infer purifying selection at nonsynonymous sites across 95% of the genome, with a selection coefficient s of -9.5×10^{-4}. This value is interpreted as "strong" compared to values in humans and *Drosophila*. The authors used simulations of completely linked genomes to evaluate their models, which assume linkage equilibrium between sites. They find that their best model performs poorly in some scenarios; specifically, strong selection can be misinferred when complete linkage is combined with weak purifying selection. Other model assumptions were not tested, for example the absence of population subdivision or that the population follows a simple demographic model of exponential growth.

Bringing in a temporal perspective on d_N/d_S, Namouchi et al. (2012) found 25% more non-

synonymous SNPs on terminal branches in their tree of 22 globally diverse strains. This suggests that deleterious nonsynonymous mutations are purged through selection over time, such that they become scarce in deeper parts of the phylogeny (Rocha et al., 2006). Trauner et al. (2017) present evidence that such purging might already occur within the host, as nonsynonymous within-host diversity is lower than expected under a model of random mutation. An implication of within-host purifying selection is that mutation rates estimated from *in vivo* experiments might be too low. In a simulation study Morales-Arce et al. (2020) suggest that genome-wide mutation rates in the MTBC might be two orders of magnitude faster, in the order 10^{-8}/bp/generation, if one accounts for progeny skew (Box 1) and the removal of mutations through purifying selection during within-host evolution.

Strong genetic drift leaves other signs than an excess of nonsynonymous mutations, including pseudogenization, proliferation of selfish genetic elements, or an increased proportion of transversions. With strong drift and clonal reproduction, such signatures can accumulate through Muller's ratchet, where lack of recombination and reduced efficacy of purifying selection lead to a build-up of deleterious mutations (Felsenstein, 1974; Muller, 1964). As pointed out by Namouchi et al. (2012), these signatures are hardly evident in the MTBC. There are 30 pseudogenes in the H37Rv reference genome (Cole et al., 1998), in line with the generally low number of pseudogenes in bacterial genomes (Lawrence et al., 2001). Also insertion sequences do not thrive in the MTBC: almost all IS activity is due to a single active element, IS6110, which is over-represented in intergenic regions, occurs at low frequencies, and thus seems to evolve under strong purifying selection (McEvoy et al., 2007). Finally, transitions occur well in excess of transversions (Payne et al., 2019). Taken together, there is scant evidence for genome erosion driven by Muller's ratchet in the MTBC.

Are synonymous sites under selection?

How could the high genome-wide d_N/d_S in the MTBC be explained if not by strong drift? An intriguing alternative scenario is purifying selection at synonymous sites (Namouchi et al., 2012). High d_N/d_S can reflect an overabundance of nonsynonymous mutations (numerator), but also a lower number of synonymous mutations (denominator) than in other species. Fitness effects of synonymous mutations can arise when different codons result in variation in RNA stability, protein folding, and translation efficiency and accuracy (reviewed by Hershberg and Petrov, 2008). Already weak selection on synonymous sites can inflate d_N/d_S, as shown in a recent study of codon usage in 13 bacterial genomes (Rahman et al., 2021).

In the MTBC, codon frequencies are associated with gene expression (Andersson and Sharp, 1996; Pan et al., 1998), but also with the hydrophobicity of proteins and sequence conservation (De Miranda et al., 2000). As suggested in the latter study, a combination of selective pressures may thus act on synonymous sites in the MTBC, including the more efficient and accurate translation of certain codons and constraints on protein folding. Wang and Chen (2013) assessed possible selection on synonymous sites by comparing synonymous (d_s) to intergenic (d_I) diversity across 13 MTBC genomes. Diversity varies strongly depending on the genomic position, suggesting variation in mutation rates or selective pressures across the genome. In the majority of windows, however, d_S is higher than d_I. Under the assumption that intergenic sites are free from selective pressures, Wang & Chen conclude that synonymous sites are more diverse than expected by chance and therefore evolve under diversifying, that is, positive selection.

Alternatively, and in line with the initial hypothesis of purifying selection at synonymous sites, higher synonymous than intergenic diversity is also expected when intergenic sites are

even more constrained than synonymous sites. Intergenic regions in bacteria are packed with regulatory motives and can hardly be assumed to evolve neutrally (Molina and Van Nimwegen, 2008; Rocha, 2018). Rather than comparing synonymous against assumed neutral sites, Thorpe et al. (2017) assessed the relative strength of purifying selection by comparing the proportion of singleton mutations among different site categories, reflecting that a higher proportion of singletons indicates stronger purifying selection. In five out of six species, site categories show a clear ranking, with the proportion of singletons increasing from synonymous, intergenic, non-synonymous, to non-sense mutations. In the MTBC, however, no differences between categories are apparent: there are similar proportions of singletons in all four categories. This surprising observation can at least partly be explained by the dataset used by the authors, which includes many near-identical MTBC strains sampled in a single country. Still, that even at short timescales non-sense mutations in the MTBC do not appear to be under stronger selection than synonymous mutations asks for clarification in future studies.

Bayesian skyline plots and the issue of storytelling

Neutral sites are in short supply in prokaryotes (Rocha, 2018). In contrast to eukaryotes, the streamlined genomes of archea and bacteria do not contain large swaths of decaying repeats and other DNA debris which can be assumed to be non-functional. This poses a particular challenge for the estimation effective population sizes and the quantification of genetic drift, which traditionally relies on the availability of sites not affected by natural selection (Charlesworth, 2009).

A popular approach to estimate effective population sizes and their change through time are Bayesian skylines (Ho and Shapiro, 2011). These models are frequently used in Bayesian phylogenetics, where N_e is treated as a nuisance parameter. Many studies, however, interpret N_e literally as historical change in population size and provide instructive examples of how strong assumptions are ignored for the sake of storytelling. Bayesian skyline models assume neutrality in order to translate coalescence times into population sizes. Several studies have shown that non-neutral processes confound demographic inference and should not simply be assumed away. Recombination (Hedge and Wilson, 2014), population structure (Heller et al., 2013), sampling design, gene conversion, and selection (Lapierre et al., 2016), as well as the skewness of reproductive success (Menardo et al., 2021a) all create spurious signs of population size changes. As observed by Lapierre et al., 2016, such methodological biases might explain why population size trajectories look suspiciously similar for a wide range of species.

Despite these caveats, Bayesian skyline plots continue to be used and interpreted liberally in the MTBC literature. Skyline plots were presented as evidence for a Neolithic expansion (Comas et al., 2013), expansions of specific lineages (Merker et al., 2022; Mulholland et al., 2019; O'Neill et al., 2019), or a recent co-expansion with humans in Tibet (Liu et al., 2021). That population size trajectories "make sense" in the historical narratives of these articles does not add to their credibility, but rather puts into question the way results are made sense of (Katz, 2013). Instead of literal interpretations of Bayesian skylines, an improved understanding is required of how far the demographic past can be reconstructed from the genomes of extremely clonal bacteria without taking into account confounding factors.

Box 1: Progeny skew in prokaryotes?

Recently, progeny skew was brought up as a neglected aspect of MTBC evolution with potentially significant effects on genetic diversity (Morales-Arce et al., 2020) and population genetic inference (Menardo et al., 2021a). Progeny skew refers to the unequal distribution of offspring among parental individuals in a population. Frequently mentioned examples are viruses, where a single parental sequence can give rise to numerous copies, or marine organisms reproducing through broadcast spawning. Wright-Fisher and coalescence models assume that variation in offspring number is small (Tellier and Lemaire, 2014), which leads to misinference when applied to such organisms (Sackman et al., 2019).

While progeny skew in viruses has a direct interpretation in the way these organisms reproduce, it is less straightforward to apply to prokaryotes. Archea and bacteria reproduce through binary fission, which can be thought of as each parent having two offspring and dying after division (Cury et al., 2021); or, in an age-structured population, as each parent having one offspring and surviving. Progeny skew can arise over multiple generations through rapid adaptation, superspreading events, or repeated bottlenecks, and it is thus a meaningful parameter in population-based models with a continuous timescale (Menardo et al., 2021a). In individual-based, discrete-generation models, it is preferable to simulate the processes giving rise to progeny skew explicitly.

How do bottlenecks affect genetic diversity?

In the MTBC, genetic drift is often associated with transmission bottlenecks or founder events, when few or even single strains initiate an infection or an outbreak (Pepperell et al., 2010; Smith et al., 2006). TB infections can be initiated by single to few cells (Ryndak and Laal, 2019); each transmission is thus a massive founder event where, from the millions of cells forming a within-host population, only a few cells are sampled to start a new population. Similar, small-scale colonization dynamics occur during within-host dissemination, as single to few cells "found" new granulomas in the highly structured habitat of the lung (Martin et al., 2017).

While genetic bottlenecks entail an immediate loss of genetic diversity, the mid- and long-term effects of periodic bottlenecks on genetic diversity and differentiation in clonal pathogens, where extreme bottlenecks alternate with clonal expansions, are less clear. Periodic bottlenecks have been investigated in the context of experimental evolution, where studies mainly focused on the effects of bottlenecks on the rate of adaptation (e.g. Windels et al., 2021). More general considerations can be found in the population genetics literature. One insight of potential relevance for the evolutionary dynamics of the MTBC is that, under predominant purifying selection, rates of evolution are accelerated when N_e is small because more deleterious mutations fix due to genetic drift (Lanfear et al., 2014). In the absence of homogenizing gene flow, founder events might thus be expected to increase genetic differentiation and overall diversity among lineages of the MTBC. Following this logic, the low global diversity of the MTBC (Figure 2a) is not evidence for strong bottlenecks. The puzzling observation rather is that there is not more diversity given the repeated bottlenecks during within- and between-host evolution and the absence of gene flow. As further discussed below, low diversity despite frequent bottlenecking could indicate purifying selection.

The purpose of these considerations is to show that genetic bottlenecks are more complex and interesting than they appear in the literature, where they often serve as *ad hoc* explanation for low

diversity. More work on periodic bottlenecks in bacterial pathogens is needed. This work could take into account some real-world complications such as the unclear number of cells actually transmitted, which is most likely larger than the minimum number required to start an infection (Namouchi et al., 2012). Furthermore, infection might not occur at a single time point, but extend through time as hosts are repeatedly exposed to bacteria-laden aerosol droplets (Ryndak and Laal, 2019). This situation resembles the source-sink dynamics of metapopulation models with repeated colonization events rather than a single bottleneck.

Positive selection

Most insights about how the MTBC has adapted to environmental challenges either regard pathoadaptation in the distant past before the MRCA, as revealed through comparative genomics (reviewed by Pepperell, 2022), or the recent evolution of antibiotic resistance (reviewed by Gygli et al., 2017). Much less is known about the genetics underlying adaptation to different mammalian host species, evident in host tropism (Brites et al., 2018; Zwyer et al., 2021), or about adaptation to different human populations, as suggested by sympatric patient-pathogen associations observed in cosmopolitan settings (Gagneux et al., 2006).

Identifying signatures of positive selection in linked genomes is challenging since most tests rely on the comparison of haplotypes within genomes (Shapiro et al., 2009). Two diversity-based signatures that are not haplotype-based have been used extensively to identify positive selection in MTBC genomes: homoplasy and excess of nonsynonymous polymorphisms. In the following, we discuss the properties and limitations of these measures and whether they can be used to elucidate the role of positive selection beyond the case of antibiotic resistance.

Homoplasies: how common is convergent adaptation?

Molecular homoplasy designates the independent appearance of identical mutations in different parts of a phylogeny through chance, recombination, or convergent selection (Stern, 2013). Chance homoplasy between genomes showing so little overall diversity is rare (Comas et al., 2009, Figure 2d), and its probability can be assessed through permutation tests (Farhat et al., 2013). Mutation hotspots can facilitate chance homoplasy (Galtier et al., 2006): in the MTBC, highly mutable tandem repeats frequently cause homoplasy (Outhred et al., 2020), while it is not known how rates of point mutations vary along the genome. Recombination has been argued against as a cause of homoplasies because homoplasies in the MTBC do not occur in clusters, as would be expected when recombination involves diverged DNA (Chiner-Oms et al., 2019). Non-clustering homoplasies, however, are also expected when recombinant genomes are similar (Bobay et al., 2015). Furthermore, intrachromosomal recombination can generate homoplasies, as suggested by their increased occurrence in homologous *PE/PPE* genes (Tantivitayakul et al., 2020).

Clear examples of convergent selection as a cause of homoplasy have been presented for genes involved in antimicrobial resistance (Comas et al., 2012; Farhat et al., 2013). Against a background of low diversity and rare homoplasy, some of these genes show exceptional patterns. In 1,161 strains sampled in Russia and South Africa, one specific mutation in the *katG* gene, which confers isoniazid resistance, has originated more than 70 times independently (Mortimer et al., 2017). This is an extreme pattern that arises because *katG* is a "tight target" of selection, that is, only single to few mutations can cause resistance without incurring high fitness costs. In other genes ("sloppy targets"), fewer homoplasies are observed but in more positions. The high

incidence of parallelism in resistance evolution, in combination with large datasets, allows the use of genome-wide association approaches to identify new drug resistance loci and to elucidate the genetic architecture of resistance phenotypes (e.g. Crook et al., 2022).

The basic limitation of homoplasies as a signature of selection is that they only reveal cases of convergent evolution. In the case of antibiotic resistance, convergence is ubiquitous. Thousands of parallel evolutionary experiments are conducted when people around the world are treated with the same antibiotics proposed by the WHO. For other selective pressures, things are less clear. Recently, two cases of convergent selection were shown in studies of experimental evolution with *M. canettii* and the MTBC. Selecting *M. canettii* strains for *in vivo* persistence in mice, Allen et al. (2021) identified two parallel mutations and demonstrated their effect on persistence through gene knock-out and complementation. Smith et al. (2022) selected for biofilm formation in experimentally evolved MTBC strains and identified two loci that mutated independently and are associated to biofilm-associated traits and fitness proxies. Both studies found that parallel mutations emerged in similar strains, suggesting that the genetic background constrains evolutionary trajectories. These studies also illustrate the rapidity with which mutations otherwise rare or absent can prevail in the presence of new selective pressures; and the significance of structural variation, as convergent evolution involved a large duplication (Smith et al., 2022) and a deletion of two genes (Allen et al., 2021).

Convergence might not only be favored by strong selective pressures, but also through demography and migration. Repeated introductions of sublineages into a region, as described for Tibet (Liu et al., 2021), are natural experiments where genetically highly similar strains are repeatedly confronted with a new environment. Liu et al. identified several genes that accumulate mutations independently after repeated introductions to the Tibetan Plateau, including *sseA*, a gene involved in the detoxification of reactive oxygen species, and three genes involved in DNA repair (*dnaE2*, *recB*, *mfd*). With the already large and still growing amount of data on MTBC outbreaks, such natural experiments of parallel evolution can provide valuable insights into the dynamics and genes involved in local adaptation.

Nonsynonymous polymorphisms: how frequent is positive selection?

The second widely used statistic to infer selection and its direction is the ratio of non-synonymous to synonymous polymorphisms d_N/d_S. Above, elevated genome-wide d_N/d_S was discussed as evidence for reduced purifying selection. The estimates presented there (Figure 2c) were obtained by averaging over pairs of sequences, yielding a coarse measure that does not take into consideration that selection might be restricted to few sites of a locus or certain branches in the phylogeny (Yang, Ziheng, 2014). To detect positive selection, a family of versatile maximum likelihood models have been developed that incorporate explicit models of codon evolution and allow to test for increased rates of nonsynonymous changes on particular branches or in particular codons of a gene (Yang and Bielawski, 2000). These methods are computationally intensive and not suitable for exploratory analyses on large phylogenies, while small MTBC datasets might not contain enough diversity to estimate parameters. They can be used, however, to obtain a more detailed picture of selective pressures in genes of interest and to formally test for selection using model comparisons (Yang, 1998).

A recent example of an exploratory selection scan followed by more rigorous statistical testing is the study of Menardo et al. (2021b). In a first step, they identified a hypervariable epitope at the *esxH* locus, which codes for a secreted effector interacting with the human immune system.

Codon models were then used to test for site- and branch-specific selection. Significant signatures were found in MTBC lineage 1 but not in other lineages and located to the N-terminal epitope of the gene. Further dissection of these signatures showed that they occur in strains collected in South and Southeast Asia, suggesting that this locus might be involved in adaptation to regional human host populations.

Two recent studies have proposed methods to estimate d_N/d_S for large datasets while avoiding site and branch averaging, respectively. Wilson et al. (2020) present a phylogeny-free (and thus fast) method to infer selection at the codon level. Applying their method to more than 10,000 MTBC genomes, they found a d_N/d_S significantly larger than 1 in 2,729 out of 3,979 genes. Chiner-Oms et al. (2022) investigated the temporal trajectories of p_N/p_S in a large phylogeny of 5,000 strains (p_N/p_S is based on simple counts while d_N/d_S includes correction through a substitution model, Yang, Ziheng, 2014, p. 47ff). Focusing on shifts in p_N/p_S along the tree, they found evidence for elevated nonsynonymous changes at some point in time in almost half the genes of the MTBC. While both studies generate long lists of candidate genes, they also lead to the inevitable follow-up question of selection scans: what to do with these candidates. Considering the difficulty of experimental validation in a human pathogen, further characterization of the candidates with phylogenetically explicit codon models (as implemented in PAML; Yang, 2007) could be useful.

Overall, homoplasies and d_N/d_S tell us little about the frequency and strength of positive selection in the MTBC. Recently, a method to infer selection coefficients from d_N/d_S under clonal reproduction was presented in the context of somatic evolution (Williams et al., 2020). The model developed in the study relaxes some assumptions of previous approaches (reviewed by Eyre-Walker and Keightley, 2007), in particular constant population sizes and evolution over long timescales. It would be worthwhile to explore whether this approach can be applied to bacterial within-host populations in order to better understand the contribution of positive selection in the MTBC.

Discussion

In this review, we have discussed the inference of basic evolutionary processes from patterns of genetic variation observed in the highly clonal bacteria of the MTBC. We took up a skeptical position, pointing out implicit or explicit assumptions underlying the inferential step from pattern to process, and why these assumptions are often problematic. In the following, we discuss a unifying scenario, the evolutionary optimum hypothesis, to connect the different threads laid bare above and to make a case for background selection as a key process in monomorphic bacterial pathogens. This speculative exercise is followed by a discussion of simulations as a key tool to transition to a more quantitative understanding of evolutionary dynamics under extreme clonality.

The bacteria of the MTBC are an outlier in the prokaryote world (Fig. 2) – and altogether outlandish when put aside the animal and plant models that have inspired evolutionary theory. Two patterns in particular demand explanation: the low levels of genetic diversity (a powerful deterrent for evolutionary biologists) and the high genome-wide d_N/d_S in the absence of other signs of genome erosion. Given the strong orientation of the MTBC field towards resistance evolution, only few studies have addressed these fundamental puzzles. Hershberg et al. (2008),Namouchi et al. (2012) and Pepperell et al. (2013) stand out and continue to be cited when genetic drift or purifying selection are invoked to explain genetic patterns in the MTBC. As shown in this review, however, these studies offer starting points rather than final answers. Much remains to be understood about how basic evolutionary processes contribute to evolution under extreme clonality.

The evolutionary optimum hypothesis and a case for background selection

An intriguing working hypothesis is that the bacteria of the MTBC have reached an evolutionary optimum and are well adapted to their hosts (Brites and Gagneux, 2015). This was initially proposed as a general scenario for monomorphic bacterial pathogens, and as a contrast to prevalent adaptive evolution in laboratory populations (Achtman, 2012). Once the key innovations had evolved that allowed these bacteria to infect humans, adaptation slowed or largely ceased. Using the adaptive landscape metaphor, we might envisage monomorphic bacterial pathogens as sitting on or close to a fitness peak. In the MTBC, host tropism (Fig. 1) implies at least some diversifying selection after the MRCA. Different lineages, or sublineages, might occupy different peaks in the adaptive landscape, reflecting the different immune environments of different mammalian species.

Crucially, fitness is a function of the environment: the same strain might find itself on a fitness peak when infecting a cow and at lower altitudes when in a Petri dish or a human treated with antibiotics. As evident in the contexts of resistance and experimental evolution, bacteria of the MTBC can climb the fitness landscape with surprising rapidity if challenged to do so. The commonplace that low mutation rates constrain evolution in the MTBC thus needs some qualification. The mutation rate is not some fixed species or lineage property, but a plastic trait that varies along the genome and is responsive to environmental changes (Fitzgerald and Rosenberg, 2019), for example the presence of T cells (Copin et al., 2016) and oxidative stress (Liu et al., 2020).

Through our focus on the empirical literature, one key aspect of clonal evolution has received little attention: linked selection. Under strict clonality, the fate of a mutation arising in any of the few thousand genes present in a typical bacterial genome is tied to all other sites in the genome. Selection acting on this mutation affects the fixation probability of linked variation and interferes with selection at other sites (Charlesworth, 2012; Neher, 2013). The dynamics and outcome of linked selection depend on a parameter that is usually unknown: the distribution of fitness effects (DFE) of new mutations (Eyre-Walker and Keightley, 2007). According to the evolutionary optimum hypothesis, beneficial mutations are rare and of small effect since populations already are well adapted. Evolutionary dynamics would thus be driven by the neutral and deleterious components of the DFE. Different outcomes are conceivable depending on how genetic drift interferes with purifying selection.

Strong drift in fully linked genomes is expected to lead to a build-up of deleterious mutations through Muller's ratchet (Felsenstein, 1974; Muller, 1964), pushing populations down the fitness slope and eventually to extinction. The restricted niche of bacterial endosymbionts has been considered to offer particularly favorable conditions for Muller's ratchet. In a classical study, increased d_N/d_S and transversion rates in endosymbionts compared to free-living relatives were interpreted as evidence for evolution under the ratchet driven by lack of recombination and small effective population size (Moran, 1996). Monomorphic bacterial pathogens have similarly restricted niches and share some genome characteristics with endosymbionts. *Mycobacterium leprae* is notable for its large number of pseudogenes (>1000), its reduced genome size (3.3 Mb), and its "low" GC content (58%) among the GC-rich mycobacteria (Cole et al., 2001). This peculiar genome composition has led to predictions that this pathogen will ultimately become extinct due to Muller's ratchet (Young and Robertson, 2001).

The generality of the ratchet in endosymbionts has been questioned: the old age of many symbionts seems hardly compatible with mutational meltdown, and both selection (Allen et al., 2009; Pettersson and Berg, 2007) and recombination (Naito and Pawlowska, 2016) might prevent such an outcome. Even clearer is the case against Muller's ratchet in *M. leprae*. Adding additional

M. leprae genomes to the picture, it becomes clear that pseudogenization and genome reduction largely preceded the MRCA of *M. leprae* (Monot et al., 2009). These are not ongoing processes reflecting strong drift in non-recombining genomes, but distant events during the transition to a pathogenic lifestyle. Regarding evolution under extreme clonality, the intriguing pattern are not the numerous pseudogenes, but that even functionally neutral pseudogenes show so little diversity.

A mechanism of linked selection that seems more compatible with low diversity in monomorphic bacterial pathogens is background selection (BS). BS refers to a scenario where purifying selection is effective (large N_e) and removes deleterious mutations and linked variants, leading to a reduction in linked neutral diversity (Charlesworth et al., 1993). Could BS explain the low diversity in pseudogenes of *M. leprae*, or the low synonymous diversity which might be responsible for the elevated d_N/d_S in the MTBC and other monomorphic bacterial pathogens? Little work has been conducted on BS in a prokaryote context. While some insights seem generalizable, such as its diversity-reducing effect, BS can have complex, non-intuitive outcomes (e.g. Cvijović et al., 2018; Kaiser and Charlesworth, 2009). To conclude this review, we illustrate and discuss how simulations can be used to better understand evolution under extreme clonality, including the poorly understood consequences of background selection.

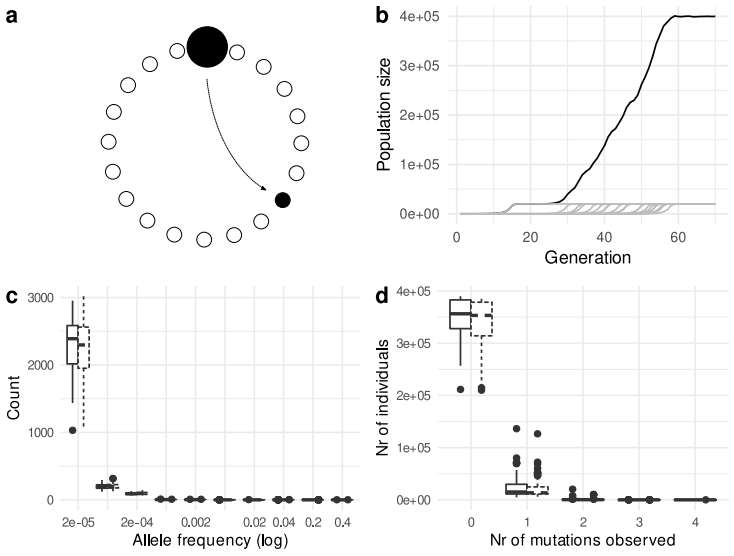

Figure 4: *A metapopulation model for within-host evolution, inspired by the study of Martin et al., 2017, who used DNA barcoding and infection mapping to infer the temporal and spatial dynamics of an MTBC infection in macaques. a) Infection begins with a single bacterium giving rise to an exponentially growing population through clonal reproduction. Once this population reaches carrying capacity $K = 20,000$, it can seed new populations which again grow exponentially. b) Exemplary growth dynamics of the model, the solid line showing total population size, dashed lines showing subpopulation sizes. c) Site frequency spectrum at generation 70. d) Number of individuals with 0 to 4 SNPs at generation 70. For further details and the simulation script are available on https://doi.org/10.5281/zenodo.8042695.*

Outlook: simulating a within-host metapopulation

With the large amount of sequencing data now available, covering evolutionary timescales from within-host evolution to global patterns of diversity, it would be a good moment to revisit some past hypotheses. We envisage focused studies that address specific hypotheses and pay more attention to methodological limitations. New tools for evolutionary simulations, in particular the versatile forward simulation tool SLiM (Haller and Messer, 2019), could provide a long-needed crutch to move forward.

Simulations are an invaluable tool in evolutionary genetics: they allow to test intuitions and methods, to compare alternative scenarios, and to fit models to data (Hoban et al., 2012; Johri et al., 2022). For bacterial population genetics, the use of simulations was so far rather limited. Most simulators are based on the coalescent – the backwards-in-time variant of the Wright-Fisher model. These are fast but usually limited to neutral scenarios of population size changes and migration. Recent advances in forward simulation, however, make it possible to simulate ever more realistic scenarios through improved computational efficiency and more flexible non-Wright-Fisher models (Cury et al., 2021, show some applications to bacteria).

To conclude this review, we present an exemplary simulation that captures some realistic aspects of the within-host population dynamics of a clonal pathogen (script and detailed description on https://doi.org/10.5281/zenodo.8042695). Such simulations could be used to better understand the patterns of genetic variation expected in an infected individual, and the bias introduced through punctual sampling of a structured population and culturing (Morales-Arce et al., 2021).

We envisage within-host dissemination dynamics as a metapopulation model with unidirectional migration from "full" to "empty" populations – as suggested by the study of Martin et al. (2017), who used DNA barcoding and infection mapping to infer the spatial and temporal dynamics of an MTBC infection in macaques. Infection begins with a single bacterium giving rise to an exponentially growing population through clonal reproduction and 19 "empty" populations. Once this population reaches carrying capacity $K = 20,000$, it can seed new populations (Figure 4a), which again grow and can seed new populations when K is reached (Figure 4b). Mutations are simulated at a rate $\mu = 5 \times 10^{-10}$/bp/gen in a genome of 4 Mb. Selection is either assumed to be absent ($s = 0$) or purifying ($s = -9.5e-4$ Pepperell et al., 2013). The simulation ends after 70 generations, which with a generation time of 24 h corresponds to a 10 week infection.

Independently of purifying selection, the dynamics of clonal growth and dissemination over 70 bacterial generations give rise to an extreme skew towards rare alleles (Figure 4c). A large proportion of the mutations are in fact singletons, that is, only present in a single individual. At generation 70, the vast majority of individuals have no mutation, except in few instances where a mutation arose early (Figure 4d). (Some simulations produced outlier values because not all populations were "filled" after 70 generations.)

The purpose of this simulation is to illustrate the simulation approach. Some assumptions might seem questionable (e.g. carrying capacity), but they are transparent and can easily be modified. Some further potential applications of evolutionary simulations are listed in the following. Simulations are not a panacea, but they allow to raise the debate to a more transparent, quantitative level than achieved by the so far largely verbal arguments. If nothing else, they could allow to better understand what kind of inference is at all possible, given the low levels of genetic diversity in monomorphic bacteria.

- Coupling within- and between-host evolution, periodic bottlenecking could be simulated to

study how diversity accumulates through time as a function of bottleneck size, purifying selection, or mutation rates. This would lead to a more nuanced understanding of transmission bottlenecks, which have more complex consequences than simple reduction of diversity.

- Synonymous and nonsynonymous mutations could be modeled, with variable distributions of fitness effects, to explore how d_N/d_S is affected by the interaction of genetic drift and purifying selection in fully linked genomes. Under what conditions, for example, would Muller's ratchet begin to click?

- Gene conversion between closely related strains could be simulated to test different methods to infer recombination. In general, methods should be tested on simulated data to understand their behavior and make an informed choice, instead of resorting to the typical bioinformatics approach of using multiple methods and reporting intersecting results, which leaves the door open to confirmation bias.

- Ultimately, approximate Bayesian computation could be used to fit models to data and to simultaneously infer demography and selection. It is difficult, however, to conceive what kind of data would be suitable for this. At the microevolutionary scale that is most straightforward to simulate, there is so little diversity that it is dubious that parameter-rich models could be fitted with any confidence.

Acknowledgments

We wish to thank two anonymous reviewers for their careful reading and constructive comments, and B. Jesse Shapiro for the editorial work. Our best thanks for their comments on earlier versions of the manuscripts to Daniela Brites, Etthel Windels, Michaela Zwyer, Selim Bouaouina, Fabrizio Menardo, Ana Morales-Arce, Galo Goig, and the members of the Gagneux group. This work was funded through grants from the European Research Council, grant number 883582, and the Swiss National Science Foundation, grant numbers 310030_188888 and CRSII5_177163.

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data and code availability

The data underlying the figures, the code for plotting the figures, and the simulation code (Figure 4) are available on Zenodo (https://doi.org/10.5281/zenodo.8042695). Figure 1 and figure 2a are based on data generated by Zwyer et al. (2021). Figures 2b-d is based on data from (Bobay and Ochman, 2018), additional file 1.

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