Published in final edited form as:

Microbiol Spectr. 2017 March ; 5(2): . doi:10.1128/microbiolspec.TB2-0027-2016.

DNA Replication in *Mycobacterium tuberculosis*

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Abstract

Faithful replication and maintenance of the genome are essential to the ability of any organism to survive and propagate. For an obligate pathogen such as *Mycobacterium tuberculosis* that has to complete successive cycles of transmission, infection, and disease in order to retain a foothold in the human population, this requires that genome replication and maintenance must be accomplished under the metabolic, immune, and antibiotic stresses encountered during passage through variable host environments. Comparative genomic analyses have established that chromosomal mutations enable *M. tuberculosis* to adapt to these stresses: the emergence of drug-resistant isolates provides direct evidence of this capacity, so too the well-documented genetic diversity among *M. tuberculosis* lineages across geographic loci, as well as the microvariation within individual patients that is increasingly observed as whole-genome sequencing methodologies are applied to clinical samples and tuberculosis (TB) disease models. However, the precise mutagenic mechanisms responsible for *M. tuberculosis* evolution and adaptation are poorly understood. Here, we summarize current knowledge of the machinery responsible for DNA replication in *M. tuberculosis*, and discuss the potential contribution of the expanded complement of mycobacterial DNA polymerases to mutagenesis. We also consider briefly the possible role of DNA replication—in particular, its regulation and coordination with cell division—in the ability of *M. tuberculosis* to withstand antibacterial stresses, including host immune effectors and antibiotics, through the generation at the population level of a tolerant state, or through the formation of a subpopulation of persister bacilli—both of which might be relevant to the emergence and fixation of genetic drug resistance.

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INTRODUCTION

The transfer of genetic material through successive generations is essential to the survival and evolution of all living organisms, including bacteria. As causative agent of tuberculosis (TB), *Mycobacterium tuberculosis* must complete successive cycles of transmission, infection, and disease in order to maintain a viable presence in the human population. And, like other pathogens (1), *M. tuberculosis* is faced with the extra problem of regulating DNA replication, chromosomal segregation, and cell division while residing in diverse anatomical and cellular loci within its human host—including extra- and intracellular compartments (2, 3). Therefore, in addition to the metabolic challenges faced during infection of dynamic and often hostile environments (4, 5), *M. tuberculosis* is likely to encounter multiple stresses that are directly or indirectly genotoxic (6–8). In patients with active TB disease, these stresses might arise from host-derived antimicrobial immune effectors, generation of toxic by-products from host and/or mycobacterial metabolism, changes in intracellular redox potential as a function of shifts in metabolic activity, pH, or oxygen availability, or even exposure to anti-TB drugs. However, given that the number of active TB cases (although devastatingly high in absolute terms) is small relative to the total number of estimated infections (9), an additional feature of *M. tuberculosis* is the ability of infecting bacilli to persist for decades in a poorly understood subclinical state (10, 11), in some cases reactivating decades later to cause postprimary TB (12, 13). Under these conditions, DNA replication and repair pathways are predicted to be essential for preserving the genetic content and viability of bacilli located in lesions characterized by different states of immune activation at various stages throughout the disease cycle (14).

Of course, the evolutionary imperative to survive while adapting to the stresses and fluctuating environments encountered during long-term infection requires that there must be an intrinsic capacity for error even in the processes that function to maintain genomic integrity (15). This reinforces the importance of understanding DNA metabolism in *M. tuberculosis* as a key component of both mycobacterial virulence and mycobacterial evolution (including the development of drug resistance) and, in turn, identifies the mycobacterial DNA metabolic machinery as a potential source of new targets for novel anti-TB chemotherapies designed to inhibit growth while limiting emergence of drug resistance. Unlike many other bacterial pathogens, drug resistance in *M. tuberculosis* arises exclusively from mutations in chromosomal genes that are associated with drug action: there is no evidence of horizontal gene transfer in the modern evolution of *M. tuberculosis* strains (16–18). So, chromosomal mutagenesis drives the microevolution of this obligate pathogen within its human host, in which case the interplay between high-fidelity DNA replication and repair, on the one hand, and low-fidelity damage tolerance pathways, on the other, might be critical to the ability of *M. tuberculosis* to maintain viability under otherwise lethal antibiotic exposure, and to adapt under changing selection pressures (19).

In this review, we summarize recent progress in our understanding of the machinery responsible for DNA replication in *M. tuberculosis*, with a specific focus on the different mycobacterial DNA polymerases and their potential roles as specialists in different aspects of DNA replication and repair. In addition, we highlight key results suggesting the utility of

targeting DNA replication for anti-TB drug development. Finally, given that the regulation of DNA replication and its coordination with cell division are critical to cell cycle progression, we consider the impact of transient interruptions of DNA replication on mycobacterial drug susceptibility as well as the emergence and fixation of genetic mutations in a pathogen increasingly associated with multidrug resistance. As applies to all specialist review articles, the treatment in this case of DNA replication in *M. tuberculosis* and its potential role in pathogenesis is neither exhaustive nor definitive: the reader is encouraged to consult the large number of related articles on this subject, some of which are cited here as well (8, 20–24).

BACTERIAL DNA REPLICATION

Bacterial DNA replication is performed by a large, multiprotein replisome that synthesizes leading and lagging strands in a highly coordinated fashion. Together, the replisome proteins catalyze a large number of events such as DNA unwinding, RNA primer synthesis, clamp loading, and DNA synthesis (Fig. 1). From comparative genomic analyses, it is evident that most of the replisome components are conserved across bacteria (25). This observation remains valid for a small panel of mycobacteria including *Mycobacterium leprae* (Tables 1 and 2), an obligate pathogen whose genome displays evidence of extensive decay (26), as well as the nonpathogenic environmental mycobacterium *Mycobacterium smegmatis*, which has served as tractable model in the majority of live-cell investigations of mycobacterial DNA replication and cell division (27–33).

As noted elsewhere (22,34,35), replisome function has been most thoroughly studied in *Escherichia coli* (36) and *Bacillus subtilis* (37), from which models of the bacterial replisome have been constructed (Fig. 1). The replisome can be divided into three catalytic centers (Fig. 2): the helicase-primase complex (25), the core complex, and the clamp loader complex (35,38). Together with the β clamp, the core complex and clamp-loader complex form the DNA polymerase III holoenzyme (PolIII HE) (39,40). The helicase-primase complex contains a DnaB helicase that unwinds the two DNA strands, and a DnaG primase that synthesizes the short RNA primers on the lagging strand that form the initiation site for the replicative DNA polymerase, PolIII α . Two core complexes—comprising PolIII α , the exonuclease ϵ , and the small subunit θ —synthesize the new DNA strand on both the leading and lagging strands. To ensure processivity, the core complex binds to the β clamp, a toroidal protein that encircles the DNA. Together, the core-clamp complex synthesizes DNA fragments with lengths of up 100,000 base pairs (41). The $\tau_3\delta_1\delta'_1\chi_1\psi_1$ clamp-loader complex loads β clamps onto newly synthesized RNA primers. The τ subunits furthermore bind to the PolIII α subunits, thus coupling leading- and lagging-strand synthesis. Finally, the χ/ψ subunits guide SSB molecules onto the single-stranded DNA lagging strand.

The genes predicted to be involved in DNA replication in *M. tuberculosis* are shown in Tables 1 and 2. The mycobacterial replisome lacks obvious homologs of several components that perform key functions in the model organism; for example, there are no obvious homologs of the initiation proteins, DnaC, DnaT, PriB, and PriC; the *holE*-encoded θ subunit; or the *holC*- and *holD*-encoded χ and ψ clamp-loader subunits (22). However, comparative genomics has established that this reduced gene complement is typical of many

bacteria. In fact, the *M. tuberculosis* genome was included among the set that contributed to the definition of a basic bacterial replication module comprising the replication initiator protein, DnaA, the DnaB helicase, DnaG primase, PolIII α , the β_2 -sliding clamp, ϵ proofreading subunit, τ , δ , and δ' , SSB, DNA ligase, and PolI (25, 35). Key recent studies have provided additional insights into the composition of the mycobacterial replisome and its functional organization (42,43) (detailed below). Nevertheless, it remains relatively poorly characterized, and the working model of the full *M. tuberculosis* replisome is therefore inferred largely through comparison with model organisms (39, 40), and from the limited number of genetic and biochemical studies that have focused on specific mycobacterial replication proteins (Tables 1 and 2). As a result, there are a number of important gaps that need to be addressed. For example, the stoichiometry and architecture of the mycobacterial replisome are unknown; what is the complement of replisome components present at the replication fork during active replication? And how does this alter under conditions of slow growth, metabolic quiescence, or stress? What are the factors that determine the rates of chromosomal replication and cell division in *M. tuberculosis* during host infection? In addition, the potential for other cellular factors to affect (or modify) both function and composition of the replisome—such as the relative levels of deoxyribonucleotide triphosphates (dNTPs) and ribonucleotide triphosphates (rNTPs), the building blocks of DNA and RNA, respectively, or the presence of specialist DNA polymerases and other repair enzymes—remains unresolved. Some of these questions are explored further below.

The Mycobacterial Replication Machinery

Despite significant genetic differences across bacterial phyla, there appears to be strong functional conservation in the mechanics of chromosomal replication (25, 34). For simplicity, it is useful to reference the *E. coli* model when considering the overall process of DNA replication in *M. tuberculosis*, although a number of recent observations have suggested that the mycobacterial system is likely to differ in some key respects.

The DnaA-ATP interaction is critical for replication initiation, since it results in the opening of the DNA duplex to allow loading of DnaB, and the *dnaA* promoter remains active during replication to ensure progression through the cell cycle (44). *M. tuberculosis oriC* is located in the 527-bp intergenic region between *dnaA* and *dnaN*, and contains multiple predicted and confirmed DnaA-binding sites. Interestingly, this region also serves as a common locus for the insertion of IS6110 transposable elements. To date, however, there is no evidence to suggest the insertions have any effect on the replication process, including the timing of replication initiation. Instead, these sites have been exploited as useful markers for restriction fragment length polymorphism (RFLP) fingerprinting of clinical *M. tuberculosis* isolates (45). In *E. coli*, DnaA recruits the hexameric DnaB replicative helicase to the origin to initiate strand separation. Recent work has confirmed the physical interaction of *M. tuberculosis* DnaA and DnaB, and has further implicated DnaB in controlling DnaA complex formation and the interaction with *oriC* (46). In contrast, *M. tuberculosis* does not possess a homolog of the DnaC helicase loader, which is required for loading DnaB helicase onto the DNA in *E. coli* (Table 1). This suggests that the DnaC function must be performed by another protein, or that DnaA alone might be sufficient for DnaB loading, as has been

suggested for other organisms that lack a DnaC helicase loader (47). Similarly, and as noted above, *M. tuberculosis* does not possess either θ , χ , or ψ subunits; moreover, the mycobacterial *dnaX* gene does not contain the alternative STOP codon that, in *E. coli*, creates the γ protein through ribosomal slippage (48). All four proteins are, however, not generally conserved in bacterial replisomes and, in some cases, may even be unique to the *E. coli* replisome. The θ subunit is nonessential and found only in a small group of *Enterobacteriaceae* (49, 50). The clamp-loader subunits, χ and ψ , connect to SSB on the lagging DNA strand (51, 52), and their deletion in *E. coli* leads to a reduction in viability (53). Yet, in several organisms, χ and ψ appear to be absent (54). In *E. coli*, the γ protein is a shorter version of the τ protein, containing only the first ~430 residues that oligomerize into the pentameric clamp-loader complex, but lacking the C-terminal ~210 residues that bind the DNA polymerase. Deletion of γ has no effect on *E. coli* viability (55), and the subunit is absent in several bacteria (56–58), supporting the notion that it is nonessential for replication.

Leading-strand synthesis is highly processive and involves the continuous extension of DNA; this is in contrast to lagging-strand synthesis, which requires discontinuous replication via the extension and ligation of Okazaki fragments (Fig. 1). DnaG primase fulfills the essential function of producing the short RNA primers for extension by PolIII, and has been identified as a potential target for novel TB drugs (59).

THE MYCOBACTERIAL C-FAMILY DNA POLYMERASES

As detailed above, the core complex in the well-studied bacterium, *E. coli*, contains three subunits: the PolIII α subunit that functions as active DNA polymerase, the *dnaQ*-encoded ϵ subunit possessing 3′-5′ exonuclease proofreading activity, and the *holE*-encoded θ subunit, that stabilizes the ϵ subunit. PolIII α subunits are C-family DNA polymerases (60), restricted exclusively to the bacterial kingdom and a few bacteriophages (61) and classified into two major groups: PolC-type (62) and DnaE-type (63,64), the last of which is further subdivided into the DnaE1, DnaE2, and DnaE3 groups. PolC is present in low-GC Gram-positive bacteria such as *B. subtilis*, whereas DnaE1 serves as PolIII α in the widely studied Gram-negative model organism, *E. coli*, and also in *M. tuberculosis*.

C-family DNA polymerases possess a set of four highly conserved and ordered domains within a single polypeptide that folds broadly into the shape of a cupped right hand (Fig. 3). These are the Polymerase and Histidinol Phosphatase (PHP) domain, which is limited to the C-family polymerases as well as some PolX members and, in some organisms, possesses proofreading activity; the Palm domain, which contains the catalytic residues required for template elongation; the Thumb domain, which binds the DNA backbone and inserts a loop into the major groove; and the Fingers domain, which, together with the palm domain, forms a preinsertion nucleotide-binding pocket that positions the incoming dNTP for transfer to the catalytic binding site (35). The arrangement of additional domains, such as the oligonucleotide/oligosaccharide binding (OB) domain that binds single-stranded DNA (35, 65, 66), distinguishes DnaE and PolC subfamilies: in the DnaE family, the OB domain is located C-terminal from the polymerase, whereas in the PolC family it is located at the N-terminal end (Fig. 3B). DnaE2 polymerases differ from both DnaE1 and DnaE3 types in that

no DnaE2 appears to possess the C-terminal domain, which includes both OB-fold and τ -binding domains. In contrast, the DnaE3 polymerases are characterized by a domain organization that is similar to the DnaE1 group, albeit with a smaller (degraded), disordered PHP domain (61). DnaE2 also lacks the C-terminal τ -binding domain that is critical for connecting DNA to the rest of the replisome during DNA replication, and is instead characterized by a C-terminal pentapeptide motif, SRDF[H/R], which is conserved among most DnaE2 homologs (61). It has been hypothesized that this motif is required for mediating protein-protein interactions, including during function of the mycobacterial mutasome (67); however, this remains to be demonstrated.

The majority of bacterial genomes sequenced to date contain two, three, or even four putative C-family polymerases (61). Most encode a single replicative polymerase of the DnaE1 type, which functions as the sole high-fidelity replicative C-family DNA polymerase in the cell (61, 68); this is the case for *M. tuberculosis*, which encodes a single DnaE1 subunit, Rv1547 (Tables 1 and 2). Based on distributions across bacterial genomes, DnaE1 appears to be the only C-family polymerase that is able to exist alone or in combination with DnaE2 and PolC (68); in contrast, the other C-family members (PolC, DnaE2 or DnaE3) always occur in combination with representatives from at least one of the other groups. Only DnaE2 subunits—which are common among aerobic bacteria with large, GC-rich genomes—do not conform to phylogenetic boundaries, and can coexist with DnaE1 or PolC (61). Consistent with this observation, in addition to the DnaE1 replicative subunit, *M. tuberculosis* possesses a second, DnaE2-type polymerase, which has been implicated in DNA damage-induced mutagenesis as part of the mycobacterial DNA damage (or SOS) response (61, 69).

DnaE1 PHP Domain Proofreading Activity in Maintaining Replication Fidelity

The *in vitro* mutation rate in *M. tuberculosis* has been calculated at $\sim 2.9 \times 10^{-10}$ per base pair per round of replication (70), a figure comparable to that determined for *E. coli* (71). However, whereas *E. coli* has a well-characterized pathway for postreplicative mismatch repair (MMR), like all actinomycetes, *M. tuberculosis* does not possess an identifiable MMR system (21). Loss of MMR function in *E. coli* results in a mutator phenotype, with MMR-deficient cells exhibiting mutation rates more than 100-fold higher than wild-type, MMR-proficient cells (71). This is not surprising: the overall error rate of 10^{-10} is a function of the intrinsic replication fidelity of the replicative polymerase (which contributes an error rate of $\sim 10^{-5}$), the 3′–5′ exonuclease activity of the replicative polymerase itself or its interacting proofreading subunit, DnaQ (contributing an error rate $\sim 10^{-2}$), and MMR (for which the escape rate is estimated at $\sim 10^{-3}$); therefore, the loss of this critical fidelity mechanism can be expected to impact mutation rates (72). What is surprising, however, is that *M. tuberculosis* is not a mutator despite the natural absence of MMR (20), which suggests that intrinsic polymerase fidelity and/or proofreading are able to preserve the mycobacterial error rate. It is possible that an alternative, nonorthologous system catalyzes MMR in mycobacteria (20,21), or that a recently identified archaeal mismatch-specific endonuclease (73) is functional in *M. tuberculosis*, but this remains to be tested.

A key recent study by Rock and colleagues has highlighted the hazards of investigating all bacterial systems in the context of the well-established *E. coli* model in which proofreading is predominantly the function of the exonuclease subunit. Using a combination of biochemical, microbiological, and bioinformatics approaches, the authors located intrinsic exonuclease function in the PHP domain of the essential PolIII α subunit, DnaE1 (43). Critically, this study established that, although *M. tuberculosis* encodes two putative homologs of the *E. coli* ϵ subunit (74), Rv3711c (*dnaQ*) and Rv2191 (comprising a putative N-terminal 3'–5' exonuclease domain fused to a C-terminal UvrC-like endonuclease domain) (Table 2), neither appears essential to the maintenance of replication fidelity during normal growth *in vitro*: deletion of *Rv3711c* had no impact on the *M. tuberculosis* mutation rate in fluctuation assays, an observation reinforced in the faster-growing *M. smegmatis* in which knockouts lacking either one or both DnaQ homologs retained wild-type mutation rates. In contrast, *M. smegmatis* mutants carrying targeted substitutions of amino acids essential for PHP exonuclease function (owing to their roles in metal cofactor coordination) exhibited severe growth defects that were coupled with massively elevated mutation rates in excess of 2,300-fold greater than the wild-type parental strain. Moreover, whole-genome sequence (WGS) analyses of the PHP domain mutants revealed an accumulation of insertion and deletion events, confirming the requirement for a functional PHP domain in maintaining genomic integrity.

In *E. coli*, the replicase maintains a weak interaction between the ϵ proofreading subunit and the β clamp in the polymerization mode of DNA synthesis, which is disrupted to enable the transition to alternative conformational states which allow transient access of other polymerases and clamp-binding proteins, for example, during proofreading or translesion synthesis (75). In their study of mycobacterial replication fidelity, Rock and colleagues raised important questions about the applicability of this *E. coli* paradigm to *M. tuberculosis*: in particular, they observed that, while *M. tuberculosis* DnaQ possessed exonuclease activity in biochemical assays *in vitro*, the putative proofreading subunit did not interact stably with the *dnaE1*-encoded α subunit (43). This result was confirmed subsequently in another key study by Lijun Bi and colleagues (42), who reconstituted a functional *M. tuberculosis* DNA PolIII HE comprising recombinant α (DnaE1), ϵ (DnaQ), β (DnaN), τ (DnaZX), δ (HolA), δ' (HolB), and SSB (Ssb) subunits *in vitro*. From a series of biochemical assays, the authors concluded that the core mycobacterial replicase consists of $\alpha\beta_2\epsilon$, with β_2 functioning as bridge protein to compensate for the lack of interaction between the α and ϵ proteins. Consistent with the *E. coli* model, however, *M. tuberculosis* DNA PolIII replicase was shown to transition between polymerization and proofreading modes, with these results implicating the β_2 clamp (which maintains the $\alpha\beta_2\epsilon$ replicase in polymerization mode) and dNTP availability in mediating this switch.

Although largely in agreement, these studies did highlight a potential disconnect between the phenotypes observed in whole-cell (microbiological) assays (43) and the enzymatic properties characterized *in vitro* (42): that is, no increase in mutation rate was detected in any of the *dnaQ*-deficient deletion mutants (43) despite the apparently integral role of DnaQ in the mycobacterial replicase that was inferred from biochemical assays. This suggests that there may be additional factors that determine the relative contributions of PHP and DnaQ to proofreading in live *M. tuberculosis* cells, including during host infection. While this

remains to be resolved, it is worth noting that comparative genomics of clinical strains has identified *dnaQ* as highly polymorphic (76) and possibly linked to the emergence of *M. tuberculosis* drug resistance (77). Both observations suggest that *dnaQ* is under strong selective pressure; however, further work is required to verify this inference.

Specialist Function: DnaE1 Versus DnaE2

M. tuberculosis is unusual in that it contains both SOS-dependent (78) and SOS-independent (79, 80) DNA damage responses, with some repair components induced by both mechanisms (81). Of the complement of DNA polymerases encoded in the *M. tuberculosis* genome, only *dnaE1* and *dnaE2* are upregulated in the mycobacterial DNA damage response (67, 69) (discussed further below). Given the essentiality of the DnaE1 subunit for chromosomal replication (and hence bacillary survival), it is challenging to determine the specific reason for increased *dnaE1* expression under damage conditions. In contrast, the nonessential nature of *dnaE2* has enabled the elucidation of a DnaE2-dependent mutagenic pathway that might be considered the functional analog of the PolV mutasome in *E. coli* (67).

Loss of DnaE2 activity rendered *M. tuberculosis* hypersensitive to DNA damage and eliminated induced mutagenesis *in vitro* (67,69). Moreover, a *dnaE2* deletion mutant was associated with attenuated virulence and reduced frequency of drug resistance in a mouse model (69). Coupled with the induction of *dnaE2* during stationary infection, these observations implicated a DnaE2-mediated mutagenic mechanism in both pathogenesis and the evolution of drug resistance during therapy. Subsequent studies demonstrated that DnaE2 operates in a novel mutagenic pathway comprising two additional accessory proteins, ImuA' and ImuB. ImuB is one of three putative Y-family polymerase homologs in the *M. tuberculosis* genome, but it appears from sequence analysis to be catalytically inactive (67). Although this prediction requires formal demonstration, the current model for the mycobacterial "mutasome" holds that DnaE2 functions as the translesion synthesis (TLS) polymerase with ImuB acting as hub protein that interacts with both ImuA' and DnaE2 via the C-terminal domain, and with the β_2 clamp via a clamp-binding motif (67). The basis for the functional specialization of C-family replicative and TLS polymerases in *M. tuberculosis* and other Gram-positive bacteria remains unclear (82, 83). Unlike Y-family polymerases whose structures are adapted to specialist lesion bypass (84), sequence analysis reveals few clues about DnaE2 function. DnaE1 and DnaE2 are similar in terms of amino acid identity, yet perform different functions (69). Moreover, although DnaE2 appears to be a central player in the DNA damage response in *M. tuberculosis*, its role as an error-prone TLS polymerase is not generally conserved across bacterial phyla. For example, *Pseudo-monas putida* DnaE2 has been shown to have anti-mutator properties (85), while the homologous protein in *Pseudomonas aeruginosa* appears dispensable for damage tolerance (86) but not for induced mutagenesis (87). In addition, in *Streptomyces coelicolor*, DnaE2 is SOS inducible, but dispensable for DNA replication, linear chromosome end patching, ultraviolet resistance, and mutagenesis (88). This suggests that, in addition to intrinsic structural determinants such as active-site architecture, differential interactions with other DNA metabolic proteins might modulate polymerase function and fidelity.

Most major DNA PolIII α structural features are readily identifiable in both DnaE1 and DnaE2, except for the C-terminal τ -interacting domain that is absent in DnaE2 (Fig. 3B) (61, 67). The α - τ interaction enables dimerization of leading- and lagging-strand polymerases in *E. coli* (35), which suggests that the absence of this region might account (at least partially) for the inability of DnaE2 and other nonessential polymerases to substitute essential replicative function (69, 82, 83). DnaE2 also lacks a consensus β_2 -clamp-binding motif, QL[S/D] LF, which suggests that it must bind another β_2 -clamp-binding protein(s) for access to the DNA (89) and, in addition, does not possess the complete set of metal coordinating residues required for PHP domain proofreading function (90, 91). As noted above, the interaction between PolIII α and the *dnaQ*-encoded ϵ subunit is essential for fidelity in *E. coli*; moreover, disruptions to proofreading activity enable PolIII-mediated TLS in the absence of specialist DNA repair pols IV and V (92, 93), while in organisms such as *Streptococcus pyogenes*, the essential DnaE subunit that catalyzes error-prone TLS does not bind DnaQ (83). In combination, these observations raise the possibility that differential interactions with DnaQ (Rv3711c) or Rv2191 might impact DnaE2 function in *M. tuberculosis*, but this remains to be determined.

OTHER DNA POLYMERASES AT THE REPLICATION FORK?

In addition to the C-family DNA polymerases, DnaE1 (42, 43) and DnaE2 (67, 69), the *M. tuberculosis* genome encodes six other DNA polymerases (Tables 1 and 2): the A-family polymerase, PolI (*polA*; Rv1629) (94, 95); three polymerases of the archaeo-eukaryotic primase (AEP) superfamily, LigD-POL (Rv0938) (96), PolD1 (Rv3730c), and PolD2 (Rv0269c) (97, 98); and two Y-family polymerases, DinB1 (Rv1537) and DinB2 (Rv3056) (99–101). Homologs of *E. coli* PolIII (*polA*) or PolV (*umuD*, *umuC*) are not found in *M. tuberculosis*. As discussed previously (22), dynamic polymerase exchange is critical to chromosomal replication and repair in bacteria (72). For example, both PolI (*polA*) and PolII (*polB*) contribute directly to the fidelity of normal chromosomal replication in *E. coli*, (35,72), as well as functioning with the Y-family polymerases, PolIV (*dinB*) and PolV (*umuD*, *umuC*), as specialist TLS polymerases in the DNA damage response (72). Owing to their roles in response to stress, many specialist polymerases—especially Y-family TLS polymerases—have been implicated in induced mutagenesis (102). In turn, this suggests that the different DNA polymerases in *M. tuberculosis* are likely also to encode specialist functions, and so highlights the importance of elucidating the conditions under which each (or a combination thereof) is preferentially active.

M. tuberculosis does not encode a PolIII enzyme, but possesses two PolIV homologs, DinB1 and DinB2 (99), which are Y-family TLS polymerases. The *E. coli* genome contains three TLS polymerases, all of which are upregulated in the DNA damage or SOS response: the B-family polymerase PolIII, and the Y-family polymerases PolIV and PolV that are encoded by *dinB* and *umuDC*, respectively (103). Since *M. tuberculosis* does not encode a B-family DNA polymerase, it was assumed that all specialist bypass function in *M. tuberculosis* depended on the two PolIV homologs, originally annotated as DinP (DinB2) and DinX (DinB1) (21). However, numerous studies have confirmed that, in contrast to most bacterial systems, neither *dinB1*- nor *dinB2*-encoded PolIV homolog is upregulated in the mycobacterial damage response (67, 69, 81,104); instead, both genes are expressed

constitutively during logarithmic growth and stationary phase (99). Notably, *M. tuberculosis* deletion mutants lacking one or both of *dinB1* and *dinB2* were not hypersensitive to multiple DNA-damaging agents *in vitro*, and showed no phenotype in a mouse model of TB; furthermore, overexpression of either *M. tuberculosis dinB1* or *dinB2* gene in *M. smegmatis* did not increase the spontaneous mutation rate (99).

These observations, which supported the dispensability of the mycobacterial Y-family polymerases for damage tolerance and induced mutagenesis, presented a genuine puzzle until recently. Then, two elegant biochemical studies by Stewart Shuman and colleagues elucidated potential specialist function differentiating DinB1 as a *bona fide* DNA-dependent DNA polymerase, whereas DinB2 was shown to possess the additional capacity to catalyze the incorporation of ribonucleotides (105, 106). As the authors demonstrated, an aromatic steric gate side chain in DinB1 and most other DNA polymerases enables rNTP discrimination; this is notably absent in DinB2, which carries a leucine residue in the corresponding position and so lacks the ability to differentiate between dNTPs and rNTPs during template-directed synthesis. In addition to a surprising capacity to synthesize long stretches of RNA from a DNA template (106), these studies also revealed that *M. smegmatis* DinB2 is a low-fidelity enzyme, displaying characteristic signatures of misincorporation opposite undamaged DNA during both DNA and RNA synthesis *in vitro* (105). Moreover, DinB2 is promiscuous in its incorporation of both 8-oxo-dGMP and oxo-rGTP, as well as misincorporation of rNTPs opposite 8-oxo-dG lesions. It seems likely, therefore, that DinB2 might function as “ribo patch” DNA repair polymerase during dNTP starvation or under conditions of oxidative stress (105); however, this prediction needs to be tested.

THE MYCOBACTERIAL REPLICATION RATE

The term “replication rate” is often applied to mycobacteria to denote the rate at which an individual bacillus divides; that is, the time taken for one (parent) cell to generate two (daughter) cells. Although convenient, this is not strictly accurate, for two principal reasons. In the first place, the stem “replica” demands that the products of the reproduction process are near-identical. For chromosomes, this mostly holds true: replication of the genome is accomplished with high fidelity by a replisome complex with a very low error rate. In contrast, for whole organisms, the products of cell division—the daughter cells—will generally fail to satisfy this criterion owing to unequal cellular division and/or distribution of cellular constituents including macromolecules, metabolites, and cofactors. Accumulating evidence reinforces this interpretation and its implications for understanding *M. tuberculosis* population dynamics in the infected host: mycobacteria divide asymmetrically, producing daughter cells that are morphologically similar but vary in length (one cell is often longer), composition (the distribution of the “old” and “new” cell poles means that some cells are much older than others), and cellular content (macromolecules are not evenly distributed during division) (107, 108). A number of single-cell analyses have shown that all these factors can have significant physiological and survival consequences, most notably in the form of differential susceptibility to antibiotics (28, 30, 107, 109) (discussed below).

The second reason for caution is that the chromosomal replication rate is not strictly concordant with cell division: although mycobacteria complete only one round of

chromosomal replication per cell division cycle (29, 31), there are instances in which these processes appear to be uncoupled. For example, mycobacteria in hypoxia-induced nonreplicating persistence are reported to be diploid, and initiate RNA synthesis, cell division, and then DNA replication in an ordered sequence following reexposure to oxygen (110). Similarly, although it has not been directly investigated, it is tempting to speculate that the filamentous mycobacteria that develop during infection of macrophages (111, 112) might also be polyploid. It is worth noting, too, that separating concepts of cell division from chromosomal replication appears consistent with the idea that it may be simplistic to assume that mycobacterial metabolism (including DNA metabolism) has been selected to generate maximal bacillary numbers within a given microenvironment during host infection (23). As discussed previously, bacillary numbers determined from individual pulmonary lesions in a non-human primate model of TB suggest that the infecting population maintains a fairly consistent bacterial load throughout active disease (14), and that maximal growth may even be detrimental to the immediate fate of the infecting bacillus (individual host outcome) or to its long-term survival (evolutionary persistence within the human population). In turn, this reinforces the likelihood that *M. tuberculosis* infection is characterized by a spectrum of disease that extends from nonreplicating persisting organisms, to replicating but asymptomatic infections, to low-level disease with higher numbers of actively replicating bacteria, to full-blown disease pathology and transmission (14, 113, 114). It seems inevitable, therefore, that both the replication and cell division rates will vary within specific lesions, and over time, as a function of metabolic and environmental pressures. The growing appreciation that low metabolic activity and limited to no growth represent the dominant states of most bacteria in their natural environments (115) further underscores the need to move away from the more experimentally tractable laboratory-based studies that are predicated on maximal growth rates in nutrient-replete conditions.

M. tuberculosis divides every ~18 to 24 hours during optimal growth *in vitro*, a period in which the bacillus undergoes a single round of chromosomal replication (29) with an accompanying mutation rate of $\sim 10^{-10}$ errors per base pair per round of replication (70). As discussed previously (20), there are limited to no data on the rates of chromosomal replication, cell division, and mutagenesis in *M. tuberculosis* bacilli during host infection, especially through periods of clinical latency. What is now known, however, is that the replication rate achieved by the recombinant DnaE1 polymerase in biochemical assays is at least as fast as (if not faster than) *E. coli* PolIII α (43), which contradicts any notion that intrinsic replicative (in)capacity necessarily limits the mycobacterial growth rate. This observation must be contrasted with evidence from single-cell analyses of mycobacterial growth and division by time-lapse fluorescence microscopy which indicate that chromosomal replication in *M. smegmatis* accounts for approximately 70% of the bacterial cell cycle (that is, approximately 140 min of the standard 180- to 200-min period between successive division events) (29,31). As noted by Trojanowski and colleagues (29), this corresponds to a DNA synthesis rate of ~400 bases (b)/s, which is approximately 8 times faster than that estimated for *M. tuberculosis* (44, 116), but 1.5 to 2.5 times slower than the fast-growing *E. coli*, which is capable of multifork replication at rates approaching 600 to 1,000 bp/s. The disconnect between the rate of DNA synthesis estimated from the activity of recombinant replicase proteins *in vitro* and that inferred from whole bacterial cells

implicates other factors in determining the *in vivo* replication rate. These are likely to include the supply of dNTPs for incorporation into DNA, as well as the need to coordinate chromosomal replication with cell division; in addition, it must be remembered that, where DNA replication occurs in the context of a living cell, numerous other DNA transactions (including DNA repair, RNA transcription, dsDNA folding and packaging, and binding and unbinding of transcriptional regulators) are taking place at the same time. It seems certain that intracellular dNTP concentrations must also play a critical role in determining the rate and fidelity of DNA replication in *M. tuberculosis*. However, while numerous studies have investigated the ribonucleotide reductase and thymidylate synthase enzymes responsible for the provision of dATP/dCTP/dGTP and dTTP, respectively (117–130), the measurement of dNTP pool sizes remains an elusive (23), but high-priority, research focus.

The Evidence from In Vivo Infection Models and Clinical *M. tuberculosis* Strains

Molecular epidemiological studies have established the capacity for endogenous reactivation of *M. tuberculosis* after three decades of latent infection (12) and this risk increases 10% per year in HIV-infected patients relative to immune-competent individuals (131). Previous models of latent TB infection (LTBI) suggested that, during the latent phase, *M. tuberculosis* enters a very slowly replicating or nonreplicating (but probably metabolically active) state in which bacilli are insensitive to killing by host immune effector molecules and anti-TB drugs (110). In contrast, alternative models by Sherman and colleagues, which are based on the use of a “clock” plasmid that is lost from daughter cells during division, instead propose a stable balance *in vivo* between bacillary replication and death, probably as a consequence of active immune surveillance (132).

To gain insight into the mutational capacity of *M. tuberculosis* during different stages of infection, Ford and colleagues used WGS to measure the mutation rate of *M. tuberculosis* isolates from cynomolgus macaques with active, latent, and reactivated disease (70). Given that the *in vivo* generation time of *M. tuberculosis* in this model is unknown, the mutation rate was calculated allowing for a broad range of generation times (between 18 and 240 h). Interestingly, the authors observed similar replication and mutation rates in *M. tuberculosis* isolates from latent, reactivated, and actively infected macaques. In addition, they demonstrated that bacilli isolated from macaques with clinically latent infection had acquired mutations at rates similar to those of rapidly replicating bacteria *in vitro* (70)—an intriguing observation since these would be expected to differ on the basis that mutation rates determined *in vitro* often involve large mycobacterial populations either at exponential or stationary phases of growth (133) that do not represent *in vivo* conditions. Instead, the findings from this study suggest that *M. tuberculosis* continues to divide actively during the entire course of prolonged clinical latency, and that active replication is balanced by robust killing. The authors further concluded that the mutation rates observed during latency are likely attributable to oxidative DNA damage rather than replicative errors. In summary, these observations were interpreted as suggesting that the mutational capacity of *M. tuberculosis* during latent infection might be determined primarily by the length of time the organism spends in the host environment rather than the replicative capacity and replicative errors of the organism during infection (70).

In contrast, findings from clinical *M. tuberculosis* samples suggest that the non-human primate model might not appropriately recapitulate latent TB in humans (134). Again using WGS, Alland and colleagues calculated the replication and mutation rates of latent *M. tuberculosis* by comparing the genome sequence of a single strain that had been transmitted from a single, incident TB case that subsequently resulted in TB disease in close contacts over a period of 20 years. Unlike the findings in macaques, these analyses yielded lower mutation rates during latency, for any given generation time, even after adjusting for the predicted higher mutation rate that was considered likely to occur during the final stage of infection as the individual progressed to active TB (134). Moreover, the mutation spectrum in the human LTBI model did not reveal a higher proportion of mutations associated with oxidative damage (GC>AT or GC>TA mutations) as observed in macaques; instead, there were fewer mutations associated with oxidative damage, suggesting that, during latent infection in humans, mutations are driven by replicative error rather than oxidative stress. By analyzing four strains derived from the same index case, two of which were classified as latent for more than 20 years, these authors further showed that the mutation rate was likely to be 10 times lower during latency than active disease, possibly indicating less selective pressure on the organism during latency, and therefore, a reduced likelihood that adaptive mutations would become fixed in the infecting population.

A very recent study has tested this notion by applying WGS to matched pairs of clinical *M. tuberculosis* isolates obtained from individuals thought to have harbored latent TB infection for prolonged periods of as much as 3 decades (13). The estimated mutation rate from these analyses was 0.2 to 0.3 single nucleotide polymorphisms (SNPs) per genome per year over 33 years, which again places the rate at which *M. tuberculosis* accumulates mutations during clinical latency in a range that is very similar to those calculated for active TB disease in infection models (70) and from outbreak studies (135–137). In summary, therefore, it appears that further analyses of this nature are required, but will require the careful selection of clinical *M. tuberculosis* isolates that have been obtained and archived sequentially during disease progression.

COORDINATING CHROMOSOMAL REPLICATION AND CELL DIVISION

In eukaryotic cells, the genetic material is encapsulated within a defined intracellular organelle, the nucleus. For bacteria, however, chromosomal DNA is located in an undefined region of the cytoplasm called the nucleoid that often consumes a major portion of the total cell volume (138, 139). Therefore, to avoid damaging the genetic material, DNA replication and repair must be coordinated with mycobacterial cell division. In turn, this implies that these pathways are tightly regulated to ensure successful completion of the growth and division cycle: once initiated, sustained interruptions to cell division, or chromosomal replication and segregation, are likely to be lethal. The mechanisms governing these processes in mycobacteria have been the subject of intense recent research, enabled largely through the increasing availability of advanced single-cell imaging techniques. A series of comprehensive reviews has documented the significant progress made (107, 108); therefore, in the context of this review, it is considered sufficient to provide a brief overview, highlighting those aspects of special relevance to chromosomal replication.

All bacteria, including *M. tuberculosis*, have evolved rigorous control mechanisms to regulate the initiation of DNA replication, and to ensure that it does not occur at random sequences throughout the chromosome (140). In some bacteria, including *E. coli*, replication is initiated at a single site (the origin, *ori*) in the mid-cell region, with sister replisomes proceeding bidirectionally around the chromosome until the two replication forks meet in the replication terminus (*ter*), a region located approximately opposite *ori*. For organisms such as *Caulobacter crescentus* and *Helicobacter pylori*, the *ori* is located at the old cell pole, with the replisomes moving toward the mid-cell before terminating. In contrast, in *B. subtilis* and *P. aeruginosa*, the chromosome is spooled through a “replication factory” comprising sister replisomes colocalized at the mid-cell (141, 142). Largely irrespective of the location of the replisomes, chromosomal replication requires that the two strands of the template DNA are separated at the origin, yielding two fork structures. Replicative DNA polymerases and accessory proteins are assembled onto each of these forks, and synthesize new DNA bidirectionally around the circular chromosome until the two replication forks meet in the *ter*, yielding two copies of the bacterial chromosome, each containing one strand from the parental chromosome and one nascent strand. Moreover, since this must occur only once during the cell cycle, a diverse array of regulatory mechanisms ensures that the assembly of the replication machinery is triggered at the appropriate stage (22, 25).

As noted elsewhere (107), the application of advanced live-cell imaging techniques has facilitated key insights into mycobacterial growth and division. However, the results have not always been consistent across different groups, especially on the question of the (a)symmetry of mycobacterial cell division: this has necessitated an attempt to rationalize the different experimental observations (107). It seems that further work is required in order to establish definitely the timing and location of cell division in *M. tuberculosis*, particularly under conditions that prevail during host infection. Recent observations (29,32), however, appear to support a general model which holds that, in *M. smegmatis*, the replisome is positioned near mid-cell, with the two replication forks remaining colocalized (or, at least, in very close proximity to each other) (29) throughout the DNA replication cycle, broadly consistent with the “replication factory” model of *B. subtilis* and *P. aeruginosa* (32). These studies also agree on the critical role played by ParB in chromosome segregation and positioning of the *oriC* region, as well as in the localization of newly assembled replisomes.

Bacterial chromosomes are folded and functionally organized according to a hierarchy of organizational units of different nucleobase lengths so that individual chromosomal loci occupy specific subcellular locations within the cell (139, 141, 142). Together with the structural maintenance of chromosome (SMC) protein, ParB functions as part of the ParABS chromosome partitioning system to ensure that the spatial arrangement of the chromosome is restored in the daughter cells after completion of chromosome replication and segregation. In recent years, advanced methods for determining chromosomal architecture and topography have been developed that have been profitably applied in some bacteria (143). This suggests the need to obtain equivalent insight into the *M. tuberculosis* chromosome to understand the physical/structural properties of the genome that affect (or are affected by) fundamental processes such as DNA replication and RNA transcription (144) and, in turn, that might impact the propensity for mutagenesis.

MUTAGENESIS IN *M. TUBERCULOSIS*

As noted above, *M. tuberculosis* is not a natural mutator (70). However, since evolution in this organism (including the emergence of drug resistance) occurs exclusively through chromosomal rearrangements and point mutations, determining the rate and cause of these mutagenic events is critical to understand evolutionary dynamics during host infection. Numerous studies have provided both experimental and clinical evidence demonstrating that *M. tuberculosis* strains from different lineages vary in their capacity to cause disease (145–148) and to acquire drug resistance (149–152). However, the evidence for an association between specific *M. tuberculosis* strains and an elevated mutation rate is mixed. Gicquel and colleagues demonstrated that *M. tuberculosis* strains from the Beijing family contain mutations in genes whose disruption in other bacteria confers a mutator phenotype (153). Moreover, strains from this lineage have also been shown to have polymorphisms in DNA replication, recombination, and repair genes, raising the possibility that they have higher mutation rates (154). Also consistent with these observations, Fortune and colleagues used WGS to demonstrate strain-based differences in mutation rates between lineage 2 (East Asian) and lineage 4 (Euro-American) strains (155). Specifically, the authors reported that *M. tuberculosis* lineage 2 strains acquire drug resistance *in vitro* more rapidly than lineage 4 strains and, further, that the observed differences were not due to the enhanced ability of lineage 2 strains to adapt to antibiotic pressure but rather due to a higher basal mutation rate in the presence of the drug. Nevertheless, the mechanism underlying the inferred difference in mutation rates between the selected lineages remains to be determined (155).

In contrast to these studies, previous *in vitro* analyses observed no differences between the mutation rates of the Beijing versus non-Beijing strains (156), consistent with the idea that multiple factors other than the mutation rate contribute to the apparent success of the Beijing clade (157). It is possible that the apparent discrepancies reflect the representative Beijing versus non-Beijing strains that were used in each case. Therefore, while these findings offer compelling evidence of strainbased differences in mutation rates, it is worth noting that the CDC155 strain employed as an exemplar lineage 4 strain in the Fortune study is a minor branch within this lineage, with its own mode of evolution (158); similarly, HN878, which was used as representative lineage 2 strain, has also separated from other Beijing family members in constructed phylogenies (158). Whether these are genuine caveats is not clear; however, it does appear that further studies are required to address this important question adequately.

TARGETING THE REPLISOME FOR NEW TB DRUG DEVELOPMENT

The essential role of DNA replication in survival and pathogenesis suggests the possibility of targeting the mycobacterial replication and repair machinery with novel chemotherapeutic agents (25). Until recently, however, the replisome has largely failed to yield candidate drugs—with the exception of DNA gyrase inhibitors, for which several antibiotic classes exist (15, 22,25). There are some encouraging results, however: for example, 6-anilinouracils and their derivatives have been shown to inhibit DNA PolIII and have antibacterial activity against low GC Gram-positive bacteria (159, 160). Moreover, a recent study identified a panel of novel imidazoline compounds that disrupt replication by displacing the replisome from the

nucleoid, and are bactericidal against both replicating and nonreplicating *M. tuberculosis* and Gram-positive cocci (161). The bacterial β_2 -sliding clamp has also emerged as a potentially vulnerable target owing to its central role in DNA replication as a protein-protein interaction hub (162, 163): small molecule inhibitors (163) and tetrahydrocarbazole derivatives have been shown to inhibit DNA replication in both Gram-positive and Gram-negative organisms (162), suggesting the need to test these compounds in *M. tuberculosis*.

New technologies, including structure-based drug design and fragment-based lead generation, might be usefully applied to identify replication components suitable for chemical inhibition (164), but these are dependent on the availability of high-quality structural data that currently do not exist for the majority of *M. tuberculosis* replisome components. In the meantime, natural products seem likeliest to provide the best leads based on very recent evidence: specifically, the exciting identification of two unrelated natural product classes targeting the bacterial replisome. The first of these is nargenicin, a macrolide produced by *Nocardia argentinensis*, which has recently been shown by researchers at Merck Research Laboratories to target the DnaE subunit in both Gram-positive and Gram-negative bacteria (165). A related patent application from the same group claims that nargenicin is bactericidal against *M. tuberculosis* (International Patent Number WO2016/061772A1), but there are no additional reports describing its antimycobacterial activity. In addition, even though resistance in *Staphylococcus aureus* mapped to a single SNP in *dnaE*, that mutation was distal to the DnaE active site, suggesting that further work is required to elucidate the exact mechanism of action (and resistance) against different bacterial pathogens.

The second natural product targeting the replisome, the griselimycins, are cyclic peptides isolated from *Streptomyces griseus* (166). Although described over 50 years ago, the key pharmacological liabilities of these compounds were considered insurmountable until recently, when a revived program generated a series of fully synthetic griselimycin analogs with superior pharmacokinetic properties (167). Griselimycins are potently active against *M. tuberculosis*, binding to the *dnaN*-encoded β -sliding clamp with high affinity and so disrupting DNA replication. Spontaneous resistance to griselimycin results from amplification of the *dnaN* gene—a novel mechanism that, although it incurs a severe fitness cost (167), simultaneously highlights the daunting number of routes to drug resistance that are seemingly available to bacterial mutants (168).

Consistent with their inferred importance for mycobacterial pathogenesis, DNA damage tolerance pathways might offer an additional option for novel antibacterial therapies. There has been considerable discussion of the possibility of inhibiting tolerance mechanisms, particularly inducible mutagenesis pathways, in order to protect current drugs by targeting the mechanisms that underlie the evolution of resistance (169). In some respects, this approach can be considered analogous to inhibiting efflux pathways (170): on its own, a specific efflux pump(s) is not an attractive target but, in combination with the appropriate frontline drug, its inhibition might be critical to efficacy by ensuring that the active compound is maintained at an elevated intracellular concentration (170–172). *M. tuberculosis* DnaE2 represents a good candidate for this approach since it is not essential for normal growth *in vitro*, yet loss of DnaE2 activity attenuates virulence *in vivo* and reduces the frequency of drug-resistance mutations during chronic infection (69). Furthermore,

DnaE2 has been demonstrated to function in association with other DNA damage response proteins as a split “mutagenic cassette” (67), suggesting an alternative strategy of targeting the other pathway components, for example, by disrupting the protein-protein interactions that are essential to mutasome function (162, 163).

Recent evidence implicating the lethal incorporation of oxidized guanine into DNA as a major cause of antibiotic-induced bacterial cell death (173) suggests that DNA replication and repair pathways might contribute significantly to intrinsic drug resistance and, for that reason, further supports the call for DNA metabolic pathways to be targeted aggressively as potentially novel antimicrobial therapies.

DNA REPLICATION AND MYCOBACTERIAL PERSISTENCE

The fixation of chromosomal mutations through spontaneous replicative error, or via the operation of error-prone DNA repair or damage tolerance pathways, provides a clear route to the generation of drug-resistant isolates. Less certain, however, are the roles that DNA replication and repair might play in the ability of a genetically susceptible mycobacterial population to exhibit phenotypic tolerance of an applied drug. The terminology relating to “antibiotic tolerance” (a phrase often used interchangeably with “persistence”) can be confusing (174, 175), and a thorough discussion of the various functional definitions is beyond the scope of this review. However, it does seem intuitive that, by altering the replicative status of all bacilli exposed to a genotoxic stress, a general, regulated response—such as the LexA/RecA-dependent DNA damage (or SOS) response—might impact the susceptibility of the *entire* population to an applied antibiotic. In contrast, spontaneous blocks to replication—whether mediated by errors in cellular function, or temporary halts to the replication cycle to enable repair of endogenous DNA damage—will occur in individual cells (or even a very small subpopulation of cells) within a population, where the effect will not be generalized; this effect is captured in the Persistence as Stuff Happens (or PaSH) model of Johnson and Levin (176).

The framework developed recently by Balaban and colleagues (177) for classifying the drug response of bacteria according to specific definitions of “resistance,” “tolerance,” and “persistence” is instructive in this context. Using the combined parameters of antibiotic susceptibility (minimum inhibitory concentration, MIC), and kill dynamics (minimum duration for killing, MDK), the authors proposed a practical algorithm to distinguish tolerance and persistence phenotypes. Critically, the Balaban framework defines tolerance as a population effect—whether by slow growth, or by extended lag phase—whereas persistence is a property of a subpopulation of cells. So, for a tolerant population, the MDK₉₉ value (minimum duration for killing 99% of the population) will be greater than for a susceptible population; in contrast, a persistence phenotype (often manifest as a biphasic kill curve) is revealed only in the MDK_{99.99} value (denoting the MDK for 99.99% of the population) which will be much greater for the minority persister subpopulation than for the remainder of the (susceptible) population.

In the context of mycobacterial replication, this suggests that a general, population-wide effect on replication (e.g., slow growth as a result of dNTP starvation, or SOS-induced

replication arrest) will give rise to a tolerant state (Fig. 4). On the other hand, where replication is arrested in only a small fraction (or subpopulation) of mycobacterial cells owing to transient disruptions to cell function (as proposed in the PaSH model [176]), the effect will be of persistence. This is key, since it in turn implies a general framework (Fig. 4) in which the capacity for population heterogeneity is minimized at theoretical extremes: where there is limited (or no) stress—such as under logarithmic growth in nutrient-rich medium or under minimal host immune pressure—or under high stress, in which every cell in the population responds similarly—such as by triggering expression of a regulatory program like the SOS response. It is perhaps worth noting that the bulk of the mycobacterial phenotypes modeled *in vitro* (including in TB drug discovery) are located in either of these extremes (logarithmic growth, or under a generalized stress such as low pH or hypoxia)—largely for practical reasons. Yet, it seems intuitive to predict that the majority of environments encountered in the host during natural infection are more likely to impose conditions that occur somewhere between those experimentally tractable extremes (depicted inside the theoretical dotted lines in Fig. 4), thereby increasing the propensity for phenotypic heterogeneity (a mixture of tolerant and persistent phenotypes). In turn, this suggests that the true impact of population heterogeneity on drug susceptibility (and other relevant pathogenic phenotypes) remains largely unknown.

Is There a Link Between Persistence and Resistance?

It has been argued that the quiescent metabolic state of tolerant (or persistent) cells necessarily precludes the *de novo* generation and fixation of resistance mutations (a process that requires active replication); that is, (nongenetic) tolerance/persistence and (genetic) resistance cannot be linked since growth and replication are halted. However, recent evidence indicating that persister cells *can* replicate in the presence of a lethal antibiotic (109, 178) does suggest the possibility for tolerant (or persister) cells to be implicated in the emergence of genetic drug resistance. Consistent with the predictions of the PaSH model (176), spontaneous DNA damage and breakage events occur at detectable frequencies in *E. coli* cells during normal growth *in vitro* (179, 180) and induce the bacterial SOS response (181), which regulates the coordinated expression of multiple genes and operons to minimize the effects of genotoxic stress (182). Since the SOS regulon includes elements involved in transiently halting the progression of cellular division (78–80, 183) to enable DNA repair and, as described above (Fig. 4), can be induced by endogenous (metabolic) (179–181) and exogenous (host immune-mediated) damage, as well as applied (antibiotic-mediated) (184–186) stress, activation of the SOS response in bacteria including *M. tuberculosis* might invoke a physiological state analogous to persistence in a single organism or subpopulation of bacilli, or tolerance at a population level (187). That is, the elements necessary for both phenotypic heterogeneity (through growth arrest, damage repair, and detoxification) and genetic heterogeneity (through mutagenesis) are united in a single regulon.

Some support for this possibility is provided in a set of studies by Robert Austin and colleagues describing the accelerated emergence of drug resistance in *E. coli* cells exposed to the genotoxic antibiotic, ciprofloxacin (188, 189). Using microfluidics and time-lapse imaging, the authors first demonstrated that resistant mutants could emerge in a fully

susceptible population, provided the bacteria were located in connected microenvironments that created an antibiotic concentration gradient (188). Importantly, these studies established that, for a resistant population to emerge, the total bacterial numbers in the antibiotic-treated population need not be especially high: resistant mutants were obtained from a starting (antibiotic-treated) population of as few as ~100 bacteria. However, essential to the emergence of resistance under treatment was the SOS-dependent induction of a filamentation phenotype, in which cells exposed to sublethal antibiotic concentrations elongated without division. The resulting polyploid filaments occasionally generated a viable “bud,” which possessed a chromosome containing a drug-resistance mutation that consequently enabled resumption of normal cell division. Although the precise mutagenic mechanism was not determined (it seems intuitive that any of the SOS-inducible TLS polymerases in *E. coli* might be involved, for example), these studies nevertheless hinted at the potential for a tolerance phenotype (SOS-induced division arrest and bacterial filamentation) to create the transient opportunity for the generation (and fixation) of a resistance mutation(s) under strong selective pressure. Additional research will be required to test this notion, which seems to be of special relevance to *M. tuberculosis*, an obligate human pathogen that is increasingly associated with genetic diversity and microvariation, including the development of drug resistance under chemotherapy (19).

Acknowledgments

A portion of the review presented here was submitted by Z.D. in partial fulfilment of the requirements for a Doctor of Philosophy in Medical Microbiology at the University of Cape Town. We thank Valerie Mizrahi and members of the MMRU for many helpful discussions and critical review of this manuscript. Work in the MMRU on mycobacterial DNA replication and repair is funded by grants from the US National Institute of Child Health and Human Development (NICHD) U01HD085531-02 (to D.F.W.); the Department of Science and Technology (DST) of South Africa; the South African Medical Research Council; and the National Research Foundation of South Africa (to D.F.W.). We gratefully acknowledge the support of the Carnegie Corporation of New York (to Z.D.); the National Research Foundation of South Africa (to Z.D.); and the German Academic Exchange Service (DAAD), in partnership with the National Research Foundation of South Africa (to Z.D.). We thank Herman de Klerk and Anastasia Koch for technical assistance with the preparation of the figures.

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FIGURE 1.

A working model of the mycobacterial replisome. Schematic representation of the model replisome consisting of the PolIII core polymerase, the homodimeric β_2 -sliding clamp, the $\tau_3\delta\delta'$ clamp-loader complex, DnaB helicase (red hexamer), DnaG primase (blue), PolI (pink) DNA ligase (purple), and SSB (orange). Recent biochemical evidence suggests that, in *M. tuberculosis*, the ϵ proofreader forms part of the core replicase together with the β_2 and α subunits (42). As noted in the main text, the precise stoichiometry and architecture of the mycobacterial replisome remain to be established; similarly, it is not known whether the mycobacterial replisome functions as a di- or tripolymerase system, nor whether DnaE2 is able to access the replisome under non-DNA-damaging conditions in the absence of ImuB and ImuA' accessory factors.

FIGURE 2.

Subcomplex division in the bacterial replisome. The replisome contains three catalytic centers: core, clamp loader, and helicase-primase. The core complex and clamp-loader complex assemble into a larger, stable complex termed Pol III*. Together with the β clamp, they form the Pol III holoenzyme. The DnaB helicase and DnaG primase form a transient complex to synthesize primers on the lagging strand. Modified with permission from the Annual Review of Biochemistry, Volume 74 © 2005 by Annual Reviews, <http://www.annualreviews.org>

FIGURE 3.

Structure of the C-family polymerases. (A) Computational model of *M. tuberculosis* DnaE1 based on the crystal structure of *T. aquaticus* PolIII. Different domains indicated in separate colors (C-terminal domains not shown). (B) Domain organization in the different polymerase families. The DnaE families are defined by the presence of the C-terminal domains, whereas PolC forms a distinct class where an e-like exonuclease domain is inserted into the PHP domain.

FIGURE 4.

Population heterogeneity as a function of the applied stress. The cartoon summarizes the notion that the degree (or strength) of applied stress might determine the extent of phenotypic heterogeneity within a specific (myco)bacterial population. So, as the applied stress (e.g., genotoxin, antibiotic, nutrient deprivation, pH, oxygen starvation) increases toward a critical point or concentration (which will differ for each stress), the degree of heterogeneity within the population increases. Beyond that critical point (the vertex of the parabola), the result is more likely to be manifest as a general, regulated response at the population level; this has the effect of reducing the extent of heterogeneity within the population. At each extreme (low/absent stress versus high/severe stress), the degree of heterogeneity approaches a minimum. Importantly, for conditions under which both the applied stress and the degree of heterogeneity are low, a small subpopulation of persister cells might enable survival, consistent with the framework proposed by Balaban and colleagues (177). At the other extreme—high/severe stress, low heterogeneity—any observed tolerance will exist at the population level, and will be mediated by a dominant regulatory mechanism(s), such as the LexA/RecA-dependent SOS response.

TABLE 1

Components of the bacterial replisome

<i>E. coli</i> protein	<i>E. coli</i> gene	<i>M. tuberculosis</i> <i>M. smegmatis</i> <i>M. leprae</i>	Function	Catalytic activity	Essentiality in <i>M. tuberculosis</i> in vitro ^{a/} comments	Reference(s)
Primosome (loading of replicative helicase DnaB)						
DnaA	<i>dnaA</i>	<i>Rv0001</i> MSMEG_6947 ML0001	Replication initiator	ATPase, DNA unwinding	Essential; required for regulation of DNA replication	46, 190
DnaC	<i>dnaC</i>	Absent Absent Absent	Helicase loader, replication restart	ATPase		
DnaT	<i>dnaT</i>	Absent Absent Absent	Helicase loader, replication restart			
PriA	<i>priA</i>	<i>Rv1402</i> MSMEG_3061 ML0548	Helicase loader, replication restart	ATPase, helicase	Essential	
PriB	<i>priB</i>	Absent Absent Absent	Helicase loader, replication restart			
PriC	<i>priC</i>	Absent Absent Absent	Helicase loader, replication restart			
Helicase-primase (DNA unwinding, RNA primer synthesis, lagging-strand protection)						
DnaB helicase	<i>dnaB</i>	<i>Rv0058</i> MSMEG_6892 ML2680	Replicative helicase	ATPase, helicase	Essential; controls DnaA complex formation and interaction with <i>oriC</i>	46
DnaG primase	<i>dnaG</i>	<i>Rv2343c</i> MSMEG_4482 ML0833	RNA primase	RNA primase	Essential; required for regulation of DNA replication	191
SSB	<i>ssb</i>	<i>Rv0054</i> MSMEG_6896 ML2684	Single-stranded DNA binding protein		Essential ^b	32
Core-clamp (leading- and lagging-strand DNA synthesis)						
PollIII	<i>dnaE</i>	<i>Rv1547</i> MSMEG_3178 ML1207	α subunit, polymerase activity	DNA polymerase	Essential; high-fidelity replicative polymerase	42, 43, 69
ϵ -Exonuclease	<i>dnaQ</i>	<i>Rv3711c</i> MSMEG_6275 Absent	ϵ subunit, proofreading activity	Exonuclease	Nonessential; component of <i>M. tuberculosis</i> α $\beta_2\epsilon$ core replicase, interacts with β but not DnaE1; deletion does not	42, 43, 77

<i>E. coli</i> protein	<i>E. coli</i> gene	<i>M. tuberculosis</i> <i>M. smegmatis</i> <i>M. leprae</i>	Function	Catalytic activity	Essentiality in <i>M. tuberculosis in vitro</i> ^{a/} comments	Reference(s)
θ	<i>holE</i>	Absent Absent Absent	e stabilizer		result in increased mutation rate phenotype <i>in vitro</i>	
β clamp	<i>dnaN</i>	<i>Rv0002</i> MSMEG_0001 ML0002	β ₂ -sliding clamp		Essential; processivity factor, component of <i>M. tuberculosis</i> α β ₂ e core replicase, interacting separately with α and e subunits	42, 43
Clamp loader (clamp loading, SSB loading, polymerase connection)						
τ/γ	<i>dnaX</i>	<i>Rv3721c</i> MSMEG_6285 ML2335	Clamp loading, connects leading- and lagging-strand polymerase	ATPase	Essential; the alternative gene product γ has not been observed in mycobacteria	
δ	<i>holA</i>	<i>Rv2413c</i> MSMEG_4572 ML0603	Clamp loading	Inactive ATPase	Essential	
δ'	<i>holB</i>	<i>Rv3644c</i> MSMEG_6153 ML0202	Clamp loading	ATPase	Essential	
X	<i>holC</i>	Absent Absent Absent	SSB loading			
Y	<i>holD</i>	Absent Absent Absent	SSB loading			
Okazaki maturation: removal of RNA primer, synthesis and ligation of single-stranded gap						
Pol I	<i>polA</i>	<i>Rv1629</i> MSMEG_3839 ML1381	Removal of RNA primer	DNA polymerase	Essential; lacks a proofreading 3'-5' exonuclease activity, <i>polA</i> mutant displayed a DNA damage phenotype following UV irradiation and hydrogen peroxide treatment	94, 95
DNA ligase I	<i>ligA</i>	<i>Rv3014c</i> MSMEG_2362 ML1705	Closing of nicks on lagging strand	NAD-dependent DNA ligase	Essential	192
Topoisomerases, gyrases						
Topoisomerase I	<i>topA</i>	<i>Rv3646c</i> MSMEG_6157 ML0200	DNA topoisomerase I	Relax supercoiling	Essential; role in DNA repair	193
GyrA	<i>gyrA</i>	<i>Rv0006</i> MSMEG_0006 ML0006	DNA gyrase, subunit A (DNA topoisomerase II)	Negative supercoiling, ATPase	Essential	194

<i>E. coli</i> protein	<i>E. coli</i> gene	<i>M. tuberculosis</i> <i>M. smegmatis</i> <i>M. leprae</i>	Function	Catalytic activity	Essentiality in <i>M. tuberculosis</i> in vitro ^{a/} comments	Reference(s)
GyrB	<i>gyrB</i>	<i>Rv0005</i> MSMEG_0005 ML0005	DNA gyrase, subunit B (DNA topoisomerase II)	Negative supercoiling, ATPase	Essential	194
Translesion polymerases and associated proteins						
Pol II	<i>polB</i>	Absent Absent Absent	Translesion DNA polymerase	DNA polymerase		
Pol IV	<i>dinB</i>	<i>Rv1537</i> MSMEG_3172 Absent	Translesion DNA polymerase	DNA polymerase	Nonessential; capacity for ribonucleotide discrimination during DNA synthesis	99, 105, 106
Pol IV	<i>dinB</i>	<i>Rv3056</i> MSMEG_2294 Absent	Translesion DNA polymerase	DNA polymerase	Nonessential; dispensable for DNA damage tolerance; capacity for ribonucleotide incorporation	99, 105, 106
Pol V (UmuC)	<i>umuC</i>	Absent Absent Absent	Translesion DNA polymerase	DNA polymerase		
Pol V (UmuD)	<i>umuD</i>	Absent Absent Absent	Translesion DNA polymerase subunit			

^a*In vitro* essentiality, as determined by transposon site hybridization (TraSH) (201, 202).

^b*Rv0054* did not satisfy the strict criterion for essentiality in the study by Griffin et al. (201); however, no transposon (Tn) insertions were identified in any of the five possible TA dinucleotides in the open reading frame, suggesting that the gene is likely to be essential.

TABLE 2

Unique components of the mycobacterial replisome/repair—not present in *E. coli*

<i>M. tuberculosis</i> protein	<i>M. tuberculosis</i> gene	<i>M. tuberculosis</i> <i>M. smegmatis</i> <i>M. leprae</i>	Function	DNA polymerase	Catalytic activity	Essentiality in <i>M. tuberculosis</i> in vitro ^a /Comments	References
DnaE2	<i>dnaE2</i>	<i>Rv3370c</i> MSMEG_1633 pseudogene	Translesion DNA polymerase		DNA polymerase	Nonessential; required for DNA damage-induced mutagenesis; implicated in the emergence of drug resistance <i>in vivo</i>	67, 69
ImuA'	<i>imuA'</i>	<i>Rv3395c</i> MSMEG_1620 Absent	Not known		Not determined	Nonessential; required for DNA damage-induced mutagenesis	67
ImuB	<i>imuB</i>	<i>Rv3394c</i> MSMEG_1622 Absent	Predicted translesion DNA polymerase		Not determined	Nonessential; required for DNA damage-induced mutagenesis	67
PolX	<i>polX</i>	<i>Rv3856c</i> MSMEG_6445 Absent	DNA PolX		Unknown ^b	Nonessential	
LigB	<i>ligB</i>	<i>Rv3062</i> MSMEG_2277 Absent	DNA ligase		ATP-dependent DNA ligase	Nonessential; role in DNA repair	195
LigC	<i>ligC</i>	<i>Rv3731</i> MSMEG_6304 Absent	DNA ligase		ATP-dependent DNA ligase	Nonessential; role in Ku-dependent nonhomologous end-joining (NHEJ) DSB repair pathway	195
RnhB	<i>rnhB</i>	<i>Rv2902c</i> MSMEG_2442 ML1611	RNase HII		RNase	Nonessential	196
LigD	<i>ligD</i>	<i>Rv0938</i> MSMEG_5570 Absent	DNA ligase, DSB repair		ATP-dependent DNA ligase	Nonessential; plays a central role in the mutagenic NHEJ pathway of DSB repair	197, 198
RnhA-CobC		<i>Rv2228c</i> MSMEG_4305 ML1637	Chimeric protein; N-terminal RNase HI domain, C-terminal CobC-like α-ribazole phosphatase domain		RNase/α-ribazole phosphatase	Essential; deletion of <i>rnhB</i> in <i>M. smegmatis</i> does not alter genome stability	199
DnaQ-UvrC		<i>Rv2191</i> MSMEG_4259 Absent	Fusion protein; N-terminal 3'-5' exonuclease domain, C-terminal UvrC-like endonuclease domain		Exonuclease/Endonuclease	Nonessential; RecA-independent induction of expression in response to mitomycin C treatment; expressed during chronic infection in rabbits <i>in vivo</i>	43, 81, 200

^a *In vitro* essentiality, as determined by transposon site hybridization (TraSH) (201, 202).^b A natural truncation in the polymerase domain of Rv3856c is predicted to eliminate catalytic activity, suggesting that Rv3856c does not function as *de facto* PolX.