

DNA Metabolism in Mycobacterial Pathogenesis

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Abstract Fundamental aspects of the lifestyle of *Mycobacterium tuberculosis* implicate DNA metabolism in bacillary survival and adaptive evolution. The environments encountered by *M. tuberculosis* during successive cycles of infection and transmission are genotoxic. Moreover, as an obligate pathogen, *M. tuberculosis* has the ability to persist for extended periods in a subclinical state, suggesting that active DNA repair is critical to maintain genome integrity and bacterial viability during prolonged infection. In this chapter, we provide an overview of the major DNA metabolic pathways identified in *M. tuberculosis*, and situate key recent findings within the context of mycobacterial pathogenesis. Unlike many other bacterial pathogens, *M. tuberculosis* is genetically secluded, and appears to rely solely on chromosomal mutagenesis to drive its microevolution within the human host. In turn, this implies that a balance between high versus relaxed fidelity mechanisms of DNA metabolism ensures the maintenance of genome integrity, while accommodating the evolutionary imperative to adapt to hostile and fluctuating environments. The inferred relationship between mycobacterial DNA repair and genome dynamics is considered in the light of emerging data from whole-genome sequencing studies of clinical *M. tuberculosis* isolates which have revealed the potential for considerable heterogeneity within and between different bacterial and host populations.

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1 Introduction

Mycobacterium tuberculosis is characterized by a number of features that are inextricably linked to DNA replication and repair. First, as a slow-growing intracellular pathogen that preferentially targets host phagocytic cells, *M. tuberculosis* is exposed to host-derived reactive oxygen and nitrogen species which exacerbate the oxidative and nitrosative stresses imposed by normal cellular metabolism (Nathan and Shiloh 2000; Zahrt and Deretic 2002; Russell 2007). Consistent with this idea, insights from animal models of tuberculosis infection (Sasseti and Rubin 2003; Darwin and Nathan 2005; Dutta et al. 2010) suggest that the environments encountered by *M. tuberculosis* during residence in the human host are inherently genotoxic and pose a major threat to the integrity of the genome. Moreover, analyses of *M. tuberculosis* gene expression in various infection models (Talaat et al. 2004), and in clinical samples (Rachman et al. 2006), suggest that DNA repair mechanisms are active throughout the course of infection (Gorna et al. 2010). Second, *M. tuberculosis* bacilli are able to persist for extended periods within the human host in a poorly understood subclinical state, in some cases reactivating decades later to cause post-primary tuberculosis (Lillebaek et al. 2002). This implies a continual role for DNA repair mechanisms in the maintenance of genome integrity—and bacterial viability—throughout the course of prolonged infection. Finally, there is strong evidence that *M. tuberculosis* is secluded genetically, and might even be regarded as a monomorph (Achtman 2012): single nucleotide polymorphisms (SNPs) and large sequence polymorphisms offer the only means to infer phylogenies reliably among circulating strains that can be characterized by limited diversity (Comas et al. 2009). Furthermore, resistance to anti-tubercular drugs arises exclusively from mutations in

Table 1 Notable findings from mycobacterial DNA metabolism research

Discovery	Significance	Reference
Mycobacterial genomes lack identifiable MMR components	Absence of a mutator phenotype suggests alternative mechanism to ensure replication fidelity	(Mizrahi and Andersen 1998)
Novel mechanisms of regulation of RecA-dependent and RecA-independent DNA damage (SOS) responses	RecA processing via splicing of intein Simultaneous operation of alternate SOS responses Regulation of LexA/RecA-independent response by ClpR ClpR implicated in genomic stability	(Davis et al. 1992), (Rand et al. 2003), (Wang et al. 2011)
Mycobacteria employ a novel, dual-polymerase damage tolerance system	TLS by a C-family DNA polymerase, and adaptor role defined for a pseudo Y-family polymerase Implicated in the evolution of rifampicin resistance in vivo	(Boshoff et al. 2003), (Warner et al. 2010)
Mycobacteria possess a NHEJ system for DSB repair	First prokaryote in which NHEJ was identified	(Della et al. 2004), (Gong et al. 2005), (Shuman and Glickman 2007)
Mycobacteria encode novel helicase-nuclease complex	Heterodimeric AdnAB motor-nuclease limited to Actinobacteria and required for DSB repair	(Sinha et al. 2009b)

chromosomal genes or regulatory elements that are associated in some way with drug action (Sandgren et al. 2009; Almeida Da Silva and Palomino 2011), providing compelling support for the idea that chromosomal mutagenesis drives the microevolution of *M. tuberculosis* within the human host. In combination, these three features imply competing roles for DNA repair and damage tolerance pathways in ensuring the maintenance of genome integrity while accommodating the evolutionary imperative to adapt to variable environments and changing selection pressures. Moreover, the ability of *M. tuberculosis* to respond rapidly at the transcriptional level to environmental cues (Rachman et al. 2006; Rohde et al. 2012) suggests that functional transcription-coupled DNA repair could be critical for mycobacterial adaptation and survival (Prabha et al. 2011), particularly under infectious conditions which might require the bacillus to make maximum use of limited transcriptional and translational resources.

Significant advances have been made in the last decade toward elucidating mechanisms of DNA metabolism in *M. tuberculosis* and the related non-pathogen, *M. smegmatis*, and in exploring the role of DNA metabolic enzymes or pathways in mycobacterial pathogenesis. In many cases, these studies have contributed novel insights to the field of DNA metabolism in general (Table 1), and have reinforced

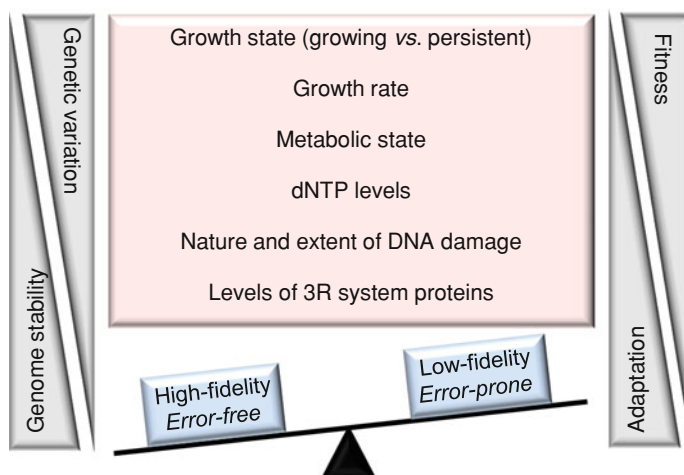


Fig. 1 The balance between high-fidelity genome maintenance and adaptation. Multiple factors are expected to influence the fidelity of replication and repair during host colonization (highlighted in box). Adapted from Warner (2010)

the idea that current models of bacterial DNA repair should be expanded to include alternatives to the well characterized *E. coli* system. Different aspects of mycobacterial DNA metabolism have been reviewed in a number of recent articles, and the reader is referred to these for additional mechanistic and biological insight (Mizrahi et al. 2000; Warner and Mizrahi 2006; Davis and Forse 2009; Dos Vultos et al. 2009; Gorna et al. 2010; Warner 2010; Kurthkoti and Varshney 2011; Kurthkoti and Varshney 2012). In this chapter, we provide an overview of DNA metabolism in *M. tuberculosis*, focusing particularly on recent findings in the area of DNA repair, and discuss the role of specific DNA metabolic pathways in pathogenesis. We then consider insights from whole-genome sequencing projects on the inferred relationship between mycobacterial DNA metabolic pathways and genome dynamics, and suggest possible areas for future study.

2 Fidelity of the “3R” System: Striking the Right Balance

The need for *M. tuberculosis* to maintain genome stability in order to remain fit—that is, able to establish an infection, grow and persist within a host, and transmit between hosts—must be balanced against the need for the organism to adapt genetically to the stresses encountered during infection, including those imposed by anti-tubercular drug administration. This balance is set by the fidelity of the so-called “3R” system of DNA replication, repair, and recombination (Fig. 1). The high-fidelity operation of the system favors genome stability, whereas relaxed fidelity—or loss of specific 3R functions (Dos Vultos et al. 2008)—might facilitate

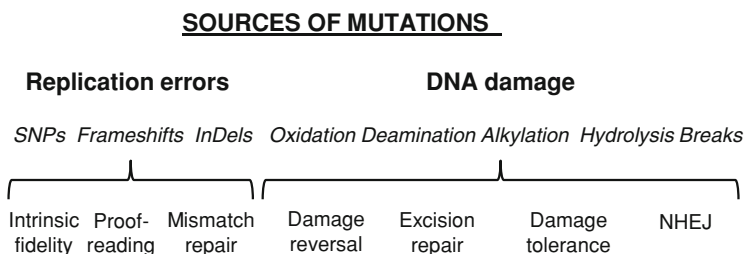


Fig. 2 DNA damage lesions and the repair mechanisms restricting their fixation

genetic adaptation (Warner 2010). In this context, it is interesting to note that genomic analyses have implicated DNA repair in the evolution of *M. tuberculosis* pathogenesis (McGuire et al. 2012). Moreover, comparatively high numbers of polymorphisms are found in genes of the 3R system of *M. tuberculosis* compared to housekeeping genes, identifying strong selection pressure on 3R genes as a common component in the modern evolutionary history of different strain lineages (Dos Vultos et al. 2008). It is tempting, therefore, to speculate that the identified polymorphisms result in a relaxation of 3R fidelity that facilitates the adaptation of this clonal pathogen to conditions of stress. To date, however, in vitro mutation rate analyses by fluctuation assay have failed to associate a hypermutable (or mutator) phenotype with a specific strain genotype (Werngren and Hoffner 2003).

2.1 Replication Fidelity

Mutations can arise through replication errors or as a consequence of DNA damage (Fig. 2). Based on fluctuation analyses that utilized *rpoB* as the target for rifampicin resistance, and which were corrected for the mutational target size, the in vitro mutation rate of *M. tuberculosis* was estimated at $\sim 2 \times 10^{-10}$ per base pair per round of replication (Ford et al. 2011). This is comparable to *E. coli*, in which intrinsic replication fidelity, proof-reading, and post-replicative mismatch repair (MMR) contribute 10^{-5} , 10^{-2} , and 10^{-3} , respectively, to the overall error rate of 10^{-10} (Mizrahi et al. 2000). Given the lack of a canonical MMR system in *M. tuberculosis*, this result is notable since it suggests that intrinsic fidelity and/or proofreading makes a proportionally greater contribution to mycobacterial replication fidelity, or that alternative mechanisms exist for the correction of replication errors, perhaps including a non-orthologous system for MMR (Mizrahi and Andersen 1998). While the formal possibility exists that the organism possesses an alternative MMR pathway, multiple lines of evidence instead suggest that *M. tuberculosis* has adapted to the lack of MMR through the activity of alternative repair components (Springer et al. 2004; Machowski et al. 2007; Wanner et al. 2008). This idea has been reinforced by the recent observation (Guthlein et al. 2009) that nucleotide excision repair (NER) might mitigate the absence of MMR

via UvrD1-dependent processing of recombination-associated mismatches (see Sect. 2.3). As for replication fidelity and proof-reading, these have not been studied at all in *M. tuberculosis* or other mycobacteria and so represent an important area for future research.

2.2 Excision Repair and DNA Damage Reversal

In contrast to the paucity of information on replication fidelity, considerable progress has been made in characterizing the DNA damage repair and reversal systems that operate in *M. tuberculosis* and the model organism, *M. smegmatis*, and in elucidating the contribution of these systems to growth, survival, mutation avoidance, and mutation induction under conditions of genotoxic stress. Damage repair and reversal in mycobacteria have been investigated using genetic and biochemical approaches; however, there are relatively few examples of studies which have utilized a combination of both (Warner and Mizrahi 2011). Moreover, as a result of the functional redundancy and/or overlap that exists within and between certain DNA repair systems, definitive phenotypes have been difficult to establish for some individual and even multiple gene knockout mutants (Guo et al. 2010). Nevertheless, the phenotypes in animal models of infection of *M. tuberculosis* strains deficient in specific DNA damage repair or reversal components have proved very useful in inferring the nature and extent of genotoxicity of the in vivo environment. These derive in particular from screens of pools of transposon mutants by Transposon Site Hybridization (TraSH) (Sasseti and Rubin 2003) or Designer Arrays for Defined Mutant Analysis (DeADMAN) methodology (Dutta et al. 2010), both of which have elucidated mutants that are comparatively disadvantaged for growth and/or survival as a result of impaired DNA repair or reversal function. So, the base excision repair (BER) components *ung*, *xthA*, and *nfo* are critical for growth of *M. tuberculosis* in mice (Sasseti et al. 2003) while *fpgI* and *nei2* are required for bacillary viability in non-human primates (Dutta et al. 2010); only *uvrB*, the gene encoding the key NER excinuclease, is essential in both models.

In addition to reinforcing the idea that the in vivo environment encountered by *M. tuberculosis* is genotoxic, the genes identified in these studies specifically implicate deaminated cytosine, abasic sites, oxidative base damage, and bulky adducts as the major lesions requiring repair in order for the organism to retain full replicative fitness during infection. These findings are consistent with the type of damage expected for an intracellular organism (Fig. 3) with a genome of high G + C content (O'Sullivan et al. 2005). However, an important caveat is that, of the five genes listed above, *uvrB* is the only repair component which has been independently validated by in vivo phenotyping of a defined allelic exchange mutant (Darwin and Nathan 2005).

The finding that *fpgI* is required for growth and survival of *M. tuberculosis* in the lungs of non-human primates (Dutta et al. 2010) underscores the risk posed to *M. tuberculosis* by persisting oxidative base damage, and is consistent with recent

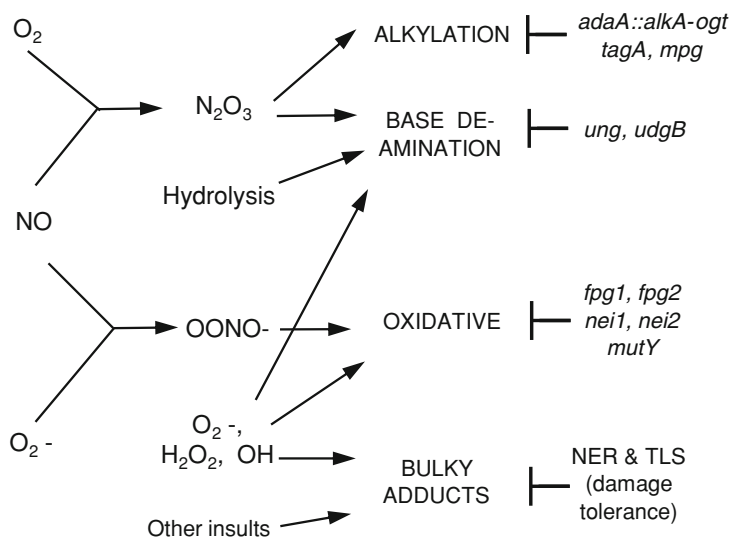


Fig. 3 The type of DNA damage expected to be sustained in *M. tuberculosis* and the genes implicated in damage repair and reversal (deRojas-Walker et al. 1995; Durbach et al. 2003)

evidence from whole-genome sequencing analyses of bacilli recovered from the lungs of non-human primates which identified polymorphisms that are potentially associated with oxidative DNA damage (Ford et al. 2011). At a functional level, the importance of *fpg1* is also consistent with the fact that a second predicted *fpg* homolog in the *M. tuberculosis* genome, *fpg2*, is truncated at its 5' terminus and so non-functional (Olsen et al. 2009; Guo et al. 2010). In contrast, the identification in the same model of *ung*, which encodes a class 1 uracil DNA glycosylase, is unexpected as *M. tuberculosis* also possesses a class 5 uracil DNA glycosylase, UdgB (Srinath et al. 2007; Wanner et al. 2009), which exhibits broader substrate specificity than Ung, but is also able to excise uracil. Similarly intriguing is the classification of *nei2* as essential for growth of *M. tuberculosis* in the non-human primate lung: the *M. tuberculosis* genome contains a second *nei* homolog, *nei1*, which encodes a DNA glycosylase that appears to be specific for oxidized pyrimidines (Guo et al. 2010). It is notable that a recombinant form of Nei2 did not show any biochemical activity in vitro; however, heterologous expression of *M. tuberculosis nei2* in *E. coli* decreased the spontaneous mutation frequency of a triple *fpg mutY nei* mutant as well as a double *nei nth* mutant, confirming that Nei2 is functional in vivo and is able to recognize both oxidized guanine and cytosine products (Guo et al. 2010).

DNA damage also results from exposure to alkylating compounds that are generated from endogenous metabolism as well as the host environment, including sources of nitrosative stress. Alkylation damage is both genotoxic and mutagenic; consistent with this dual threat, the *M. tuberculosis* genome encodes repair systems that are specifically directed to the repair of these lesions. Loss of the alkylation

damage repair and reversal operon, *adaA::alkA-ogt*, had no effect on the growth of *M. tuberculosis* in mouse organs even though the mutant strain was hypersensitive in vitro to the cytotoxic and mutagenic effects of the alkylating agent, N-methyl-N'-nitro-N-nitrosoguanidine (Durbach et al. 2003). This result suggests that *M. tuberculosis* does not sustain significant alkylation damage in vivo, or that the alternate DNA glycosylases, TagA and/or Mpg, could substitute for the DNA glycosylase function of AlkA in recognizing and excising bases damaged by alkylation. More recent work has established that the adaptive response to alkylation damage is mediated exclusively by the DNA methyltransferases, AdaA, and Ogt (Yang et al. 2011a), which are restricted to the suppression of alkylation-induced mutagenesis. In light of these observations, the failure to detect an in vivo phenotype in the *adaA::alkA-ogt* knockout mutant (Durbach et al. 2003) appears to be even more significant since it implies that any hypermutability that might arise as a consequence of loss of this response could be tolerated without adversely affecting virulence. Interestingly, several studies have identified polymorphisms in the *adaA::alkA-ogt* operon in both susceptible and multidrug-resistant clinical strains (Ebrahimi-Rad et al. 2003; Nouvel et al. 2007), perhaps indicating a link between impaired alkylation damage repair and mutability. This possibility raises an important question: how might hypermutability be detected utilizing in vivo infection models where colony counts—and, perhaps, histopathology—are the standard measures of bacterial viability and “fitness”?

2.3 Recombination and End-Joining

Double-strand breaks (DSBs) in chromosomal DNA are caused by a variety of genotoxic agents from endogenous and exogenous sources, and constitute a major threat to bacillary viability. It is not surprising, therefore, that multiple mechanisms exist for the detection, processing, and repair of these potentially cytotoxic lesions. Until very recently, non-homologous end-joining (NHEJ) and homologous recombination (HR) were thought to constitute the major systems in *M. tuberculosis* for DSB repair (Shuman and Glickman 2007). However, the application in *M. smegmatis* of an elegant reporter assay that distinguishes DSB repair outcomes has expanded the mycobacterial DSB repair pathway complement to include single-strand annealing (SSA), simultaneously demonstrating that these pathways can be distinguished according to their differential requirements for the DSB-resecting, helicase-nuclease machines, AdnAB, and RecBCD (Gupta et al. 2011). That is, mycobacterial RecBCD is a dedicated SSA nuclease, while AdnAB is required for RecA-dependent HR. This represents a key distinction between mycobacteria and *E. coli*, in which the absence of an AdnAB homolog requires that RecBCD functions as the dominant helicase–nuclease and drives HR. Moreover, as Gupta and colleagues (2011) proposed, the identification of mycobacterial SSA might imply a key role in the repair of an *M. tuberculosis* genome that contains multiple repeat loci (Cole et al. 1998). The observation that alternative DSB

pathways operate in *M. tuberculosis* and appear to be regulated asymmetrically provides another example of a mycobacterial function that has required a re-evaluation of prevailing models of bacterial DNA repair. This assessment is further supported by the recent biochemical characterization of a mycobacterial XPB helicase, which raises the additional possibility that *M. tuberculosis* is able to repair DSBs via synthesis-dependent strand annealing (Balasingham et al. 2012).

Although it was originally thought to be limited to eukaryotes, the first evidence for prokaryotic NHEJ was provided by the characterization of the mycobacterial system, which involves the activity of two proteins, Ku and LigD (Della et al. 2004). Mycobacterial Ku is a small protein comprising a core domain which is required for dimerization and enables binding of free DNA ends via the sugar-phosphate backbone. Recognition and binding of DNA ends by Ku is critical since it limits their degradation, and ensures the recruitment of LigD, a much larger protein that is responsible for end-processing and ligation and contains polymerase, nuclease, and ligase activities within a single polypeptide (Della et al. 2004). The *M. tuberculosis* genome also contains a number of other predicted ligases; however, with the exception of the essential replicative ligase, LigA (Gong et al. 2004), the potential role of these other proteins in processes such as NHEJ remains unclear (Aniukwu et al. 2008). Moreover, recent data have shown that a Sir2-like NAD-dependent deacetylase functionally interacts with Ku during NHEJ (Li et al. 2011), suggesting that multiple interacting components might mediate NHEJ activity and function.

Owing to the processing involved, NHEJ is inherently error-prone (Gong et al. 2005). However, the sensitivity of *M. smegmatis* *ku* and *ligD* mutants to DNA damage during stationary phase and in response to desiccation (Pitcher et al. 2007) suggests that NHEJ might fulfil a key repair function under special circumstances, including long-term survival and during transmission (Davis and Forse 2009). Importantly, the presence of multiple DSB repair mechanisms with different intrinsic fidelities reinforces the need to balance the activity of (error-free) DNA repair mechanisms in maintaining genomic integrity, and the operation of mutagenic pathways that might generate diversity during host infection (Fig. 1). It also suggests the possibility that DNA repair functions might have evolved specifically to cope with the high G + C content of the mycobacterial genome that is likely to influence both the type and frequency of mutational events (O'Sullivan et al. 2005).

DNA unwinding by helicases and gyrases is a prerequisite for repair and replicative polymerases to access the nucleic acid strands during replication, repair, recombination, transcription, and RNA processing (Schmid and Linder 1992; Matson et al. 1994). Like other mycobacteria, *M. tuberculosis* encodes two superfamily I DNA helicases, UvrD1, and UvrD2 (Davis and Forse 2009), which display 3'–5' polarity (Sinha et al. 2007). The differential essentialities of these two helicases—*uvrD2* is predicted to be essential (Sinha et al. 2008; Williams et al. 2011), whereas *uvrD1* is not—suggests non-redundant helicase function. Interestingly, recent evidence has shown that although *uvrD2* is essential, expression of a mutant form of UvrD2 lacking helicase activity was able to rescue the lethal effect of *uvrD2* deletion (Williams et al. 2011). UvrD1 depends on an

interaction at its C-terminus with the key DNA-binding NHEJ protein, Ku, to stimulate its DNA unwinding activity (Sinha et al. 2007). In contrast, the in vitro helicase activity of a truncated UvrD2 mutant can be restored in trans by Ku (Sinha et al. 2007). UvrD1 and UvrD2 are structurally distinct: although UvrD1 resembles UvrD-like helicases, UvrD2 possesses an unusual, superfamily II-like C-terminal domain that appears to be limited to *Actinomycetales* and is not required for helicase function (Sinha et al. 2008). Instead, genetic analyses suggest that the inferred essentiality vests in the N-terminally encoded ATPase activity, implying a potential role for UvrD2 in DNA translocation, and/or protein displacement. As noted above, UvrD1 has also been shown to inhibit RecA-mediated strand exchange (Singh et al. 2010) and, together with the other NER component UvrB, has been implicated in ensuring the fidelity of HR in the absence of functional MMR (Guthlein et al. 2009).

In addition to UvrD1/2, the *M. tuberculosis* genome encodes another superfamily I helicase, the heterodimeric helicase-nuclease, AdnAB (discussed above), that is required for HR and appears to be limited to the actinomycetes (Sinha et al. 2009a). Added to the list of helicases in *M. tuberculosis* are a number of superfamily II helicases, including homologs of the eukaryotic basal transcription factor II (TFII) helicases, XPB, and XPD, which together with other TFII complex proteins, are required for NER and proofreading of transcription initiation (Coin et al. 2007). Whereas the function and role of mycobacterial XPD (DinG) is unknown, in vitro work has shown that XPB is an ATP-dependent 3'–5' DNA helicase (Biswas et al. 2009) which, in addition to DNA unwinding, is able to catalyze the ATP-independent annealing of complementary DNA strands in vitro (Balasingham et al. 2012). The catalog of superfamily II helicases has recently been extended with the characterization of a structurally distinct clade of DNA-dependent ATPases which are well-represented in mycobacteria (Yakovleva and Shuman 2012). It is notable that SftH provides yet another example of a DNA repair function that is elucidated in the mycobacterial model; however, additional studies combining biochemical and genetic analyses will be required to elucidate the relative contributions of the different helicases to specific mycobacterial DNA repair functions, as well as transcription-coupled repair (TCR) processes.

2.4 Transcription-Coupled Repair

In addition to the threat posed to genomic integrity, DNA lesions which hinder the function of RNA polymerase can have serious real-time consequences for cellular homeostasis and survival, especially where these occur in genes undergoing active transcription. Stalling of RNA polymerase triggers the TCR pathway, an important subclass of NER which functions to ensure that actively transcribed genes are repaired more rapidly than inactive regions of the genome. In bacterial organisms including *M. tuberculosis*, TCR is mediated by the *mfd* (mutation frequency decline)-encoded protein (Prabha et al. 2011), a homolog of the eukaryotic

transcription-repair coupling factor (TRCF). As in other bacteria, *M. tuberculosis* Mfd does not recognize DNA damage directly, but through its interaction with the N-terminus of the RNA polymerase β subunit (Westblade et al. 2010). Binding of RNA polymerase by Mfd initiates a cascade of events which ensure the removal of the stalled RNA polymerase from the site of damage, and the recruitment of the NER components to effect the necessary repair. Unlike the *E. coli* protein, which is prevalent as a monomer, *M. tuberculosis* Mfd appears to exist as both monomers and hexamers which form via a self-interaction that requires the C-terminal region (Prabha et al. 2011). Although it is tempting to speculate that this might indicate the adaptation of this repair factor to a specific mycobacterial function(s) or property, that possibility requires further investigation.

Recently, the model for bacterial TCR was expanded with the demonstration that, in *E. coli*, the transcription elongation factor, NusA, directs NER to transcribed genes through a pathway that is independent of Mfd, and so offers an alternative to “classic” TCR (Cohen et al. 2010). NusA was previously shown to interact with the *dinB*-encoded *E. coli* DNA polymerase IV (Cohen et al. 2009), and the recent observations of Cohen and colleagues implicate this interaction in the recruitment of the TLS polymerase for bypass of transcription-blocking DNA lesions in an additional process dubbed “transcription-coupled TLS” (Cohen et al. 2010). Although similar processes have not been described in *M. tuberculosis*, it seems likely that multiple mechanisms exist to ensure the maintenance and operation of cellular information pathways and, in the context of obligate pathogen, to maximize the use of limited transcriptional and translational resources during host infection. This conclusion is supported by several other lines of evidence which indicate a role for DNA repair components in regulating cellular functions that are extensions of—or even distinct from—their “traditional” repair activities: for example, the DNA glycosylase, Tag, appears to modulate mycobacterial growth and morphology through a direct interaction with the chromosome partitioning factor, ParA (Huang and He 2012). Similarly, in *M. smegmatis*, disruption of the *uvrA*-encoded NER subunit results in an impaired ability to survive under nutrient- and oxygen-limited conditions (Cordone et al. 2011). The contribution of these and other DNA repair functions to mycobacterial physiology and pathogenesis therefore represents an important area for future research.

3 DNA Damage Tolerance

Persisting DNA lesions that have escaped detection and repair present a threat to the organism. Uracil, 5-hydroxyuracil, 8-oxo-guanine and O⁶-methylguanine have strong miscoding potential, and can result in mutation induction and fixation through normal DNA replication. Because they do not cause distortions in the DNA helix, these lesions are difficult to detect, and so present the major threat to genome integrity. If replication-blocking lesions such as cyclobutane dimers and other bulky adducts persist, they can result in replication fork collapse and cell

death. Specialized translesion synthesis (TLS) polymerases offer a solution to this problem: by allowing bypass of replication-blocking lesions, these enzymes provide a mechanism for tolerating DNA damage (Yang and Woodgate 2007). The importance of this function is suggested by the fact that TLS polymerases are conserved across all kingdoms of life (Ohmori et al. 2001). However, while these polymerases can display a high fidelity of replication across cognate lesions, their replication fidelities on non-cognate lesions or on undamaged templates are often reduced (Yang and Woodgate 2007). For this reason, TLS function is often associated with mutagenesis (Andersson et al. 2010).

3.1 The Y-Family of Specialist DNA Polymerases

Although it now seems obvious that specialized DNA polymerases might have evolved to replicate across persisting lesions, the discovery that the replicative flexibility they provide can incur error rates two to four orders of magnitude greater than those of replicative polymerases demanded a redefinition of traditional concepts of DNA synthesis fidelity (Yang and Woodgate 2007). Moreover, the fact that many of these polymerases are upregulated in response to stress suggested a potential role in induced mutagenesis (Andersson et al. 2010). Most TLS polymerases fall into the Y polymerase superfamily comprising a wide range of structurally related proteins present in bacteria, archaea, and eukaryotes (Ohmori et al. 2001). Of these, the DinB subfamily—represented by *E. coli* Pol IV—is the most widespread. Members of the Y-family catalyze low-fidelity synthesis on non-substrate templates such as undamaged DNA or non-cognate lesions, lack intrinsic 3' → 5' proofreading exonuclease activity, adopt a distributive mode of DNA replication, and are able to support TLS across DNA lesions which might block replication by members of the other polymerase families. The structural relation of members of this family to the other main DNA polymerases, the features enabling faithful bypass of DNA lesions but low-fidelity synthesis of undamaged or non-cognate DNA and, finally, the factors that co-ordinate multiple different polymerase classes at a replication fork, have all been subjects of active research (Yang and Woodgate 2007). These studies have revealed that, while Y-family polymerases share little sequence homology with the other polymerase families, they exhibit a similar overall architecture. Critically, the active site of Y-family polymerases is sufficiently flexible to allow bypass of lesions that might distort replicative polymerase geometry, but with a consequent reduction in stringency that has implicated members of this family in mutagenesis. However, while it has been suggested that TLS polymerases might have been selected equally for their ability to ensure continued replication as for their inherent capacity for mutagenesis, it seems increasingly likely that mutagenesis is a secondary consequence of the reduced fidelity that tolerance necessarily demands (reviewed in Andersson et al. (2010)).

3.2 Damage Tolerance and Mutagenesis in *Mycobacteria*

The *E. coli* genome contains three DNA polymerases which function in TLS—the B-family polymerase Pol II, and the Y-family polymerases Pol IV and Pol V which are encoded by *dinB* and *umuDC*, respectively—all of which are upregulated in the DNA damage or SOS response (Goodman 2002). In contrast to *E. coli*, there is no B-family DNA polymerase in *M. tuberculosis*. Therefore, it was originally assumed that all specialist bypass function in *M. tuberculosis* would depend on the two canonical Pol IV polymerase homologs, originally annotated as DinP and DinX (Mizrahi and Andersen 1998). The DNA damage response in *M. tuberculosis* is unusual (Rand et al. 2003) in that it comprises both a classic RecA-dependent SOS regulon (Smollett et al. 2012) as well an alternate, RecA-independent, damage regulon (Gamulin et al. 2004) that is controlled by a mycobacterial ClpR-like regulator (Wang et al. 2011) (Table 1). Surprisingly, neither Pol IV homolog is included in the mycobacterial DNA damage response (Boshoff et al. 2003), an additional departure from the *E. coli* model. Instead, the *dnaE1* and *dnaE2*-encoded catalytic (α) subunits of DNA polymerase III represent the only *bona fide* DNA polymerases that are induced as part of the mycobacterial SOS regulon (Boshoff et al. 2003; Warner et al. 2010). Furthermore, DnaE2 has been shown to be the central player in damage-induced base substitution mutagenesis in *M. tuberculosis*, identifying the mycobacterial polymerase as the founder member of a novel family of DnaE-type family C polymerases from Gram-positive bacteria that catalyse TLS. Loss of DnaE2 activity renders *M. tuberculosis* hypersensitive to DNA damage and eliminates induced mutagenesis. Moreover, functional inactivation of *dnaE2* attenuates virulence and reduces the frequency of drug resistance in vivo. Coupled with the induction of *dnaE2* during stationary infection, these observations implicate DnaE2-mediated DNA repair in the virulence of *M. tuberculosis* and in the adaptive evolution of drug resistance during persistent infection (Boshoff et al. 2003).

Subsequent efforts to understand the role of TLS-mediated DNA damage tolerance in mutation induction in *M. tuberculosis* led to the functional characterization of a novel damage tolerance system that is widely distributed across other bacterial genera (Boshoff et al. 2003; Warner et al. 2010). The mycobacterial version comprises the C-family DNA polymerase, DnaE2 (Boshoff et al. 2003) [more recently referred to as “ImuC” (McHenry 2011b)], and two “accessory factors”, ImuA’, and ImuB, (Warner et al. 2010) which are encoded by genes carried on a split, mutagenesis cassette that forms part of the SOS regulon controlled by RecA and LexA (Smollett et al. 2012). ImuB is one of three putative Y-family polymerase homologs in the *M. tuberculosis* genome. However, it is distinguished from the canonical Y-family homologs, DinB1 (DinX), and DinB2 (DinP), by two key features: in contrast to *bona fide* DNA polymerases, ImuB lacks the invariant active site acidic residues that are necessary for catalysis and, in addition, possesses an extended C-terminal domain comprising stretches of predicted structural disorder that are reminiscent of protein–protein interaction sites.

The features of the *imuA'-imuB/dnaE2* system were elucidated by genetic studies in *M. smegmatis* and *M. tuberculosis* using DNA damage survival and induced mutagenesis as phenotypic readouts, and yeast-two hybrid analysis to probe the protein interaction network. Consistent with the predicted inability of ImuB to catalyze nucleotidyl transfer, DnaE2 was identified as the TLS polymerase with ImuB apparently acting as hub protein that interacts with both ImuA' and DnaE2 via the C-terminal domain, and with the β -clamp via a canonical hexapeptide motif. Although the function of ImuA' remains cryptic, homology modeling suggests a RecA-type structure, and so reinforces the notion that the *imuA'-imuB/dnaE2*-encoded system constitutes a non-orthologous replacement of the PolV mutasome, UmuD'₂C•RecA•ATP, which has been extensively characterized in *E. coli* (Jiang et al. 2009). As noted elsewhere, the elucidation of this system represents an important example of the contribution of mycobacterial research to the DNA repair field in general (McHenry 2011a, b) (Table 1).

In contrast to the demonstrated role for ImuB in damage tolerance, the functions of the two other Y-family polymerases in *M. tuberculosis* remain poorly understood (Kana et al. 2010). These proteins are homologous to *E. coli* Pol IV which has been implicated in TLS across N^2 -dG adducts (Jarosz et al. 2006) and cytotoxic alkylation damage (Bjedov et al. 2007). To date, extensive phenotypic characterization of mutants of *M. tuberculosis* lacking *dinB1* and/or *dinB2* has failed to yield discernible phenotypes in vitro and in vivo (Kana et al. 2010). As noted above, in contrast to the *E. coli* model in which three specialist DNA polymerases, Pol II (B-family), Pol IV, and Pol V (both Y-family) are induced as part of the SOS regulon (reviewed in (Goodman 2002)), the *M. tuberculosis* DNA damage response does not include *dinB1* or *dinB2* and is instead limited to the two α subunits, DnaE1 and DnaE2 (Warner et al. 2010). Elucidating the function of the mycobacterial DinB proteins will therefore require the combined application of biochemical assays as well as phenotypic analyses in DNA repair-defective strain backgrounds that offer the potential to uncover defects in damage tolerance by enabling lesions to persist (Bjedov et al. 2007).

4 DNA Metabolism in Bacterial Pathogenesis

In general, DNA repair pathways in different organisms have been cataloged with reference to the *E. coli* model. For the most part, this has provided a useful starting point; however, the identification of repair pathways and systems that are present in other organisms but do not possess homologs in *E. coli* constitutes a major limitation of this approach. For example, although NHEJ was originally thought to be limited to eukaryotes owing to its apparent absence in *E. coli*, homologs of the bacterial NHEJ system were described in multiple organisms following its first identification in *M. tuberculosis* (Shuman and Glickman 2007) (Table 1). As an aside, it is interesting to note that an alternative end-joining pathway has subsequently been characterized in *E. coli* that differs from conventional NHEJ in its

dependence on RecBCD (Chayot et al. 2010), again highlighting the potential existence of as-yet unrecognized repair pathways and/or alternative functions for known repair proteins.

Even where specific pathways or repair components are present in multiple organisms, there is a need to establish whether they fulfil identical functions. Bacterial genomes are characterized by significant differences in size, structure (including the presence of episomal genetic content), organization (e.g., the distribution of essential versus non-essential genes, the relative proportion of operons and intergenic regions), and nucleotide composition (e.g., G + C content, repeat regions, and homopolymeric runs) and, in the case of pathogens, are located in organisms with very specific host and tissue tropisms. The selective pressures determining genome architecture remain poorly understood (Koonin 2009); however, it seems likely that the composition and function of DNA replication and repair components have co-evolved with intrinsic features to ensure the propagation of specific properties (Zhao et al. 2007).

Notwithstanding this complexity, comparative genomics of the 3R complements of selected bacterial pathogens revealed some common themes (Ambur et al. 2009): for example, only a limited number of pathways are conserved across all species and, for many organisms, a reduced number of genes characterizes each repair class relative to the *E. coli* model. Notably, these analyses identified DNA replication, NER, and recombinational repair pathways as common to the pathogen genomes analyzed (Ambur et al. 2009). While this seems to contradict the very limited DNA metabolic complement of the inferred “minimal bacterial gene set” (Gil et al. 2004), it perhaps attests to the fact that the ability to sustain genotoxic stress represents a major component of the pathogen arsenal. Although untested, this interpretation is consistent with recent evidence associating DNA repair with the evolution of *M. tuberculosis* pathogenesis (McGuire et al. 2012), as well as previous work implicating selection on 3R genes as a major force in the differentiation of modern *M. tuberculosis* lineages (Dos Vultos et al. 2008).

As noted above, there are relatively few examples of the contribution of a single DNA repair pathway to mycobacterial pathogenesis. This is not unique to *M. tuberculosis*: direct evidence of a dominant role for specific DNA repair pathways has proven difficult to obtain for many pathogens, probably owing in part to the operation in most organisms of multiple mechanisms for the repair and/or tolerance of a specific DNA lesion (Ambur et al. 2009). That said, the need for active DNA repair has been cogently demonstrated in a number of infection models, providing insight into the functions that enable continued survival within—and adaptation to—disparate host environments, as well as the nature of the genotoxic stresses encountered. For example, *Salmonella enterica* requires active recombination repair (Buchmeier et al. 1993, 1995) and BER pathways for infection of mice (Richardson et al. 2009). Functional recombination repair (Loughlin et al. 2003; Amundsen et al. 2008, 2009) and BER (O’Rourke et al. 2003) enzymes are also essential for *Helicobacter pylori* to colonize mouse stomachs, while loss of BER limits *Neisseria meningitidis* bacteraemia in rats (Carpenter et al. 2007). Similarly, disruption of key BER and MMR components impairs the ability of

Vibrio cholerae to survive passage through the mouse gastrointestinal tract (Davies et al. 2011). While these examples highlight the deleterious consequences of impaired DNA repair function, there is also evidence that, in some cases, loss of specific repair components can result in improved pathogen outcomes. For example, targeted deletion of selected BER genes increases the virulence of *Streptococcus mutans* in a larval model of infection, probably, as a result of a hypermutator phenotype (Gonzalez et al. 2012). In most cases, however, this represents a short-term benefit: even where virulence is not negatively affected, susceptibility to extinction as a result of genetic bottlenecks, and a reduced capacity to adapt to secondary environments, can limit the ability of mutator populations to persist through successive infectious cycles (Merino et al. 2002).

In general, detectable phenotypes are limited to gross changes in bacterial cell number (CFU counts) or effects on host survival—the standard measures of virulence. Genetic complexity and a shortage of suitable assays to measure repair function (or the consequences of its loss) have even hindered in vitro approaches to resolving questions of redundancy for a number of repair pathways. The application of a sensitive genetic reporter assay to elucidate the roles of different mycobacterial recombination and end-joining pathways to DSB repair (Gupta et al. 2011) might provide an instructive example in this regard: although the DSBs induced in the experimental system were lethal (fewer than ~0.03 % cells survived), this provided a pool of surviving mutants which was sufficiently large to enable the detection and quantification of discrete repair events. In *M. tuberculosis*, the increasing appreciation that bacilli can occupy discrete lesions (Russell et al. 2010)—and, perhaps, distinct environments (Barrios-Payan et al. 2012)—within a single host, raises the additional complication that different genotoxic stresses might be encountered depending on the site and stage of infection. In turn, this suggests that loss of a specific DNA repair function might be associated with subtler—and, perhaps, longer term—effects that could demand a more sensitive measure of genotoxicity. It is possible, for example, that consequences for adaptability and fitness, albeit significant for global strain prevalence and disease epidemiology (Gagneux 2012), cannot be assessed within the lifespan of a single, experimental infection.

5 Targeting DNA Metabolism for New TB Drug Discovery?

The predicted role of DNA metabolism in pathogenesis suggests the possibility of targeting selected mycobacterial pathways with novel chemotherapeutic agents. Antibiotic drug development prioritizes pathways or functions that are essential for bacterial growth and survival; for this reason, components of the DNA replication machinery represent the preferred 3R targets. To date, however, the targeting of bacterial “replisome” proteins has failed to delivered candidate drugs, a shortfall that has prompted a call for the more vigorous application of new

technologies—including structure-based drug design and fragment-based lead generation—to assess the tractability of replication components for chemical inhibition (Sanyal and Doig 2012). In contrast to the essential DNA metabolic enzymes, repair components which are dispensable for normal growth *in vitro* but critical for pathogenesis *in vivo* offer an alternative class of drug target. This strategy is attractive for several reasons: repair components are required only in response to DNA damage and, although untested, there is an idea that for conditionally essential functions, the selective pressure to mutate to antibiotic resistance might not be as great given that the pathway is essential for pathogenesis but not survival.

For *M. tuberculosis*, candidate DNA repair targets in this category would include UvrB, the Fpg/Nei-family formamidopyrimidine-DNA glycosylase (*fpg*), and endonuclease VIII (*nei*), based on the phenotypes of knockout mutants in animal models of infection (Sassetti and Rubin 2003; Darwin and Nathan 2005; Dutta et al. 2010). In support of the attractiveness of these components and the pathways in which they function, a micromolar inhibitor of the mycobacterial NER pathway was recently described which selectively targets the UvrABC complex in damaged cells, preventing the recovery of *M. smegmatis* following UV-induced DNA damage (Mazloum et al. 2011). Although the efficacy of this compound remains to be validated *in vivo*, the demonstration that a high-throughput phenotypic screen can be successfully applied to identify a candidate inhibitor of DNA repair holds promise for the targeting of other mycobacterial 3R functions. There is also some evidence to suggest that DNA repair components might be profitably targeted by “co-drugs” designed to potentiate existing compounds by inhibiting proteins/pathways whose operation undermines the efficacy of frontline therapies. In a clever screen of the *E. coli* Keio collection, knockout mutants were identified which exhibited increased susceptibility to specific compounds relative to the wild-type strain (Liu et al. 2010). Notably, many of the genes that were implicated in the intrinsic “resistome” for many antibiotics would not have been predicted based on current knowledge of that drug’s mode of action. Moreover, a number of those fell within the category of DNA repair, which suggests the utility of this approach in identifying unexpected target combinations and, simultaneously, its potential to reveal unknown functions of classic 3R components. Very recent evidence implicating the lethal incorporation of oxidized guanine into DNA as a major cause of antibiotic-induced bacterial cell death (Foti et al. 2012) provides further support for the idea that DNA replication and repair pathways contribute significantly to intrinsic drug resistance and, for that reason, should be considered as viable targets for novel therapies.

The role of damage tolerance in bacterial survival and, possibly, adaptation was discussed in a preceding section. Consistent with their inferred importance for mycobacterial pathogenesis, tolerance pathways might offer an additional option for novel antibacterial therapies. As discussed previously (Warner 2010), the possibility of inhibiting tolerance mechanisms—particularly inducible mutagenesis pathways—represents a subtle deviation from the concept of designing co-drugs to potentiate existing antibiotics, in this case by developing compounds

that can be added to existing therapies to protect current drugs by targeting the mechanisms that underlie the evolution of resistance (Smith and Romesberg 2007). In some respects, this approach can be considered analogous to inhibiting efflux pathways (Adams et al. 2011): on its own, a specific efflux pump(s) represents a useless 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. While there is some evidence to suggest the feasibility of this approach (Georgescu et al. 2008; Wigle et al. 2009), the utility of “anti-evolution” compounds remains to be demonstrated in the context of an infectious disease such as tuberculosis.

6 Genome Dynamics in *M. tuberculosis*

The increasing availability of relatively cheap platforms for the generation of whole-genome sequence (WGS) data has enabled significant advances in the understanding of *M. tuberculosis* evolution, epidemiology, population structure, and strain diversity (Ford et al. 2012). It seems likely, therefore, that the application of WGS to in vivo infection models (Ford et al. 2011) and clinical isolates from strain collections (Hershberg et al. 2008; Ioerger et al. 2010; Casali et al. 2012; Namouchi et al. 2012) as well as individuals presenting with active disease (Saunders et al. 2011) might similarly enable an unprecedented glimpse into mycobacterial genome dynamics and the role of DNA repair during host infection.

The motivation for genome-wide characterization of clinical isolates is multifaceted: as noted above, *M. tuberculosis* is non-transformable and non-conjugative yet, despite (or because of) its relative genetic isolation, is a very successful pathogen (Russell 2001). At the levels of both species and strains (lineages), the application of comparative genomics has revealed unexpected genetic diversity in *M. tuberculosis* and, therefore, might be applied to correlate particular strain lineages (phylogenies) with disease outcomes (virulence) (Parwati et al. 2010) and disease type (eg., pulmonary versus extrapulmonary; Click et al. 2012). So, what can WGS reveal about the microevolution of *M. tuberculosis* strains within the human host? In the most compelling example to date of the application of these techniques to investigate in vivo genome dynamics, Sarah Fortune and colleagues analyzed the mutational events that occurred during both active and latent infection in a non-human primate model (Ford et al. 2011). In accordance with the idea that oxidative stress constitutes a major source of DNA damage in *M. tuberculosis* during host infection, their analysis revealed that the few mutations detected were indeed consistent with lesions caused by oxidative damage. Moreover, by applying a flexible range of predicted in vivo replication rates, they were able to estimate the rate at which the mutations arose. Unexpectedly, the calculated mutation rate was very similar to rates inferred from in vitro analyses. Furthermore, the mutation rate did not seem to differ between active and latently infected animals. Although this may indicate that bacilli replicate throughout latent infection, these results

might be contrasted with evidence of apparent genomic stasis in sub-clinical human tuberculosis infections (Yang et al. 2011b).

Is there any evidence to support a role for mutators in mycobacterial evolution? For many bacterial pathogens, the absence of key DNA repair functions might facilitate adaptation during specific stages of the life cycle; however, the loss of repair machinery can impact long-term colonization and transmission. To some extent, this balance can be offset by the ability to re-acquire genes encoding DNA repair functions through mechanisms that enable horizontal gene transfer. However, evidence from comparative genomic studies seems to eliminate horizontal gene transfer as a contributor to the modern evolution of *M. tuberculosis*, at least in the case of drug-resistant isolates (Casali et al. 2012). The potential contribution of inducible mutagenesis mechanisms to bacterial pathogenesis is less clear, and might be difficult to infer (or measure) given the predicted role of mutations in providing the genetic diversity that enables the adaptive evolution of the invading organism to its host through multiple infection stages. Moreover, recent evidence suggesting the ability of *M. tuberculosis* to reside in multiple extrapulmonary sites and cell types during subclinical infection (Barrios-Payan et al. 2012) implies that the operation of maintenance versus adaptive functions might be separated spatially and temporally. That is, genome diversification might be favored during active disease (and transmission), whereas genome maintenance might be paramount during subclinical persistence.

7 Conclusions

There is increasing evidence that the DNA metabolic pathways in *M. tuberculosis* have evolved to ensure *M. tuberculosis* survival in the face of the metabolic and immune-mediated stresses encountered through multiple cycles of infection, replication, transmission, and persistence. Understanding the emergence and propagation of *M. tuberculosis* strains better adapted to host pathogenesis, including isolates resistant to one or more frontline anti-tubercular drugs, will require the elucidation of the specific molecular mechanisms governing mycobacterial DNA replication fidelity and maintenance. For that reason, additional work will be needed to give detail to the existing models of mycobacterial DNA replication and repair. Future research questions might therefore include the following: In the absence of MMR, what are the mechanisms that ensure replication fidelity in *M. tuberculosis*? Does *M. tuberculosis* modulate its mutation rate in response to specific environmental cues? What is the role of the DinB homologs in damage tolerance or repair? Does the presence of multiple homologs and/or analogs of specific repair components indicate redundant function or is it indicative of the specialist adaptation of specific repair proteins?

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