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Controlling Mutation: Intervening in Evolution as a Therapeutic Strategy

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ABSTRACT Mutation is the driving force behind many processes linked to human disease, including cancer, aging, and the evolution of drug resistance. Mutations have traditionally been considered the inevitable consequence of replicating large genomes with polymerases of finite fidelity. Observations over the past several decades, however, have led to a new perspective on the process of mutagenesis. It has become clear that, under some circumstances, mutagenesis is a regulated process that requires the induction of pro-mutagenic enzymes and that, at least in bacteria, this induction may facilitate evolution. Herein, we review what is known about induced mutagenesis in bacteria as well as evidence that it contributes to the evolution of antibiotic resistance. Finally, we discuss the possibility that components of induced mutation pathways might be targeted for inhibition as a novel therapeutic strategy to prevent the evolution of antibiotic resistance.

KEYWORDS induced mutation, antibiotic resistance, SOS response

INTRODUCTION

Evolution is the most fundamental force in biology. Mutation creates genetic diversity that through natural selection allows organisms to adapt to constantly changing environments and new selection pressures. However, from the perspective of an individual organism, mutation is generally considered detrimental, as it is more likely to decrease fitness than increase it (Mayr, 1985; Roth *et al.*, 2003). For example, mutation of the human genome has many detrimental outcomes, such as aging and cancer. Thus, traditionally it was thought that organisms evolved to replicate their genomes with high fidelity, generating mutations only when the polymerases and repair systems failed to function perfectly. If true, there would be little possibility for therapeutic intervention to reduce mutation, as any approach would require the design of molecules that increase the fidelity of already extremely high-fidelity enzymes–an unlikely prospect.

According to conventional models of evolution, the forces that generate diversity are independent from those that select for novel properties. However, in contrast to this idea, as well as to the notion that cells have evolved to always replicate DNA with high fidelity, observations made over the past several decades suggest that cells respond to certain selection pressures by actively increasing the rate atwhich mutations are introduced (Cairns & Foster, 1991; Lombardo *et al.*, 1999; Radman, 1974;

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Radman *et al.*, 1999; Ryan, 1955). In bacteria, this process has been shown to be induced by, regulated by, and dependent on the function of specific enzymes, including nonessential, error-prone DNA polymerases (Friedberg *et al.*, 2002; McKenzie *et al.*, 2000; Tompkins *et al.*, 2003). The dependence of induced mutation on distinct biochemical pathways suggests that it may be possible to inhibit the associated proteins and actually intervene in evolution. Small molecule inhibitors with the ability to stop mutation, used as drugs themselves or as co-drugs with other chemotherapeutic agents, would have a tremendous impact on human health. This review focuses on the observations of induced/regulated mutagenesis in bacteria and discusses these results in the context of the important human health issue of antibiotic resistance.

EARLY OBSERVATIONS OF INDUCIBLE MUTAGENESIS IN *E. Coli*

In 1953, Weigle made what was perhaps the earliest observation that mutagenesis is an inducible and regulated process. At the time, it was known that direct damage to DNA by agents such as ultraviolet (UV) light causes an increase in mutation rate, presumably by generating 'mis-instructive' lesions in the DNA. However, Weigle infected *Escherichia coli* with UV-damaged phage and observed no increase in mutation of the damaged DNA, demonstrating that the DNA lesions were repaired in an error-free manner. Conversely, irradiation of the *E. coli* cells prior to the addition of irradiated phage, resulted in mutagenesis of the phage DNA (Weigle, 1953). These results suggest that, in addition to the state of the DNA (*i.e.*, damaged or undamaged), the state of the cell itself determines whether mutagenesis will occur. It was later shown by Defais *et al.* that this process, termed Weigle or W-mutagenesis, does not occur in cells treated with translational inhibitors following UV treatment (Defais *et al.*, 1971), suggesting that mutagenesis requires the synthesis of new proteins. It was also shown that mutation does not occur in bacterial strains lacking the *recA* gene or in strains containing a mutation that prevents autoproteolysis of the LexA repressor protein (Defais *et al.*, 1971); both RecA and LexA are involved in the induction of what was later coined the 'SOS response' (Radman, 1974) (Figure 1). The work by Defais *et al.* (1971) provided some clues as to how the induced mutation process is regulated and linked its regulation to the SOS response. Later studies showed that error-prone translesion synthesis through damaged DNA is the cause of the increase in mutation rates in Weigle's system (Bridges & Woodgate, 1985; Caillet-Fauquet *et al.*, 1977), though the detailed mechanism remained unknown.

The origin of induced mutation became more clear in the late 1990s, when it was shown that the SOS-response in *E. coli* regulates the expression of three nonessential DNA polymerases (Friedberg *et al.*, 2005). These polymerases are induced when single-stranded DNA accumulates, for example during problematic DNA replication. Single-stranded DNA templates the formation of RecA filaments, which activate the autoproteolytic activity of the repressor protein LexA (Cox, 2007). Once cleaved, LexA no longer represses the SOS genes, including the genes encoding the nonessential DNA polymerases. Characterization of two of these polymerases, DNA Polymerase IV (encoded by *dinB*) and DNA Polymerase V (encoded by *umuDC*) revealed

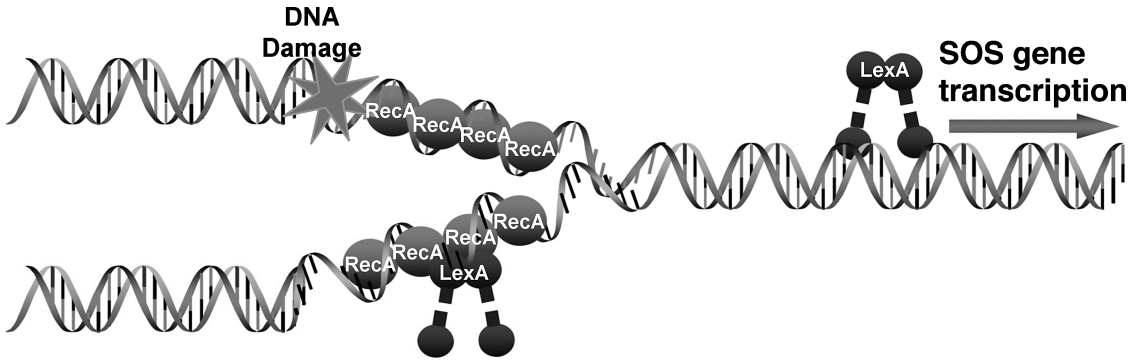

FIGURE 1 **The SOS response is initiated when RecA protein forms filaments on single-stranded DNA that accumulates in the presence of DNA damage or stalled DNA replication. These RecA-single-stranded DNA filaments facilitate repair of strand breaks or function to restart stalled replication complexes. However, the filaments also bind to and induce the auto-cleavage of the SOS transcriptional repressor protein, LexA. Cleavage of LexA causes it to dissociate from its DNA operator site (right), and the SOS genes are subsequently transcribed.**

that they lack an exonuclease domain, have reduced fidelity on undamaged DNA templates, and have the ability to replicate through lesions that stall the replicative polymerases (Tang *et al.*, 1999; Wagner *et al.*, 1999). Both Pol IV and Pol V are members of the Y-family of DNA polymerases, which are found in bacteria as well as yeast and higher eukaryotes, including humans (Goodman, 2002). Structural studies of the Pol IV homolog from *Sulfolobus solfataricus* bound to a DNA template containing an abasic site suggest that the ability of the Y-family polymerases to replicate through lesions follows from their ability to accommodate a bulge in the DNA near the templating base (Boudsocq *et al.*, 2004; Ling *et al.*, 2004).

The LexA regulons of four bacterial species have been fully characterized, including the regulons from *E. coli* (Courcelle *et al.*, 2001), *B. subtilis* (Au *et al.*, 2005; Goranov *et al.*, 2006), *P. aeruginosa* (Cirz *et al.*, 2006), and *S. aureus* (Cirz *et al.*, 2007). Comparison of these LexA regulons reveals only three completely conserved components: the 'on' and 'off' switches (RecA and LexA, respectively), and at least one tightly regulated, error-prone DNA polymerase (Cirz *et al.*, 2007). Generally, Gram-negative bacteria appear to have multiple LexA-regulated polymerases, including the Y-family polymerases Pol IV and Pol V (as with *E. coli*) or an operon encoding Y-family polymerase and an alternate alpha subunit of DNA Pol III, encoded by *dnaE2* (as with *P. aeruginosa* (Erill *et al.*, 2006)). The presence of the LexA-regulated operon appears to correlate with the lack of a two-subunit Pol V homolog, suggesting that the encoded proteins may perform similar functions as Pol V (Erill *et al.*, 2006). Gram-positive organisms, such as *B. subtilis* and *S. aureus* appear to have two single-subunit Y-family polymerases, of which one (Cirz *et al.*, 2007) or both (Au *et al.*, 2005) are LexA-regulated. Regardless of the specific gene organization, most bacteria appear to have a least one non-essential Y-family or DnaE2-like polymerase that is regulated by LexA.

Once it was established that the SOS response regulates the production of error-prone polymerases, a model for Weigle mutagenesis became clear (Figure 2). The DNA damage caused by irradiating the host cells prior to phage infection induces the RecA/LexA-regulated SOS response and production of the error-prone polymerases, DNA Pol IV and Pol V. When the UV-damaged phage DNA enters the host cell, DNA Pol V-mediated synthesis through pyrimidine dimers results in increased mutagenesis. If production of Pol V is prevented by translational inhibition or mutation of RecA or LexA, the damaged DNA is repaired in an error-free manner and no increase in mutation rate is observed.

UV-induced mutation of the *E. coli* chromosome also depends on RecA, LexA, and Pol V (Friedberg *et al.*, 2005; Witkin, 1969). Mutation is also induced by other DNA-damaging agents such as methylmethane

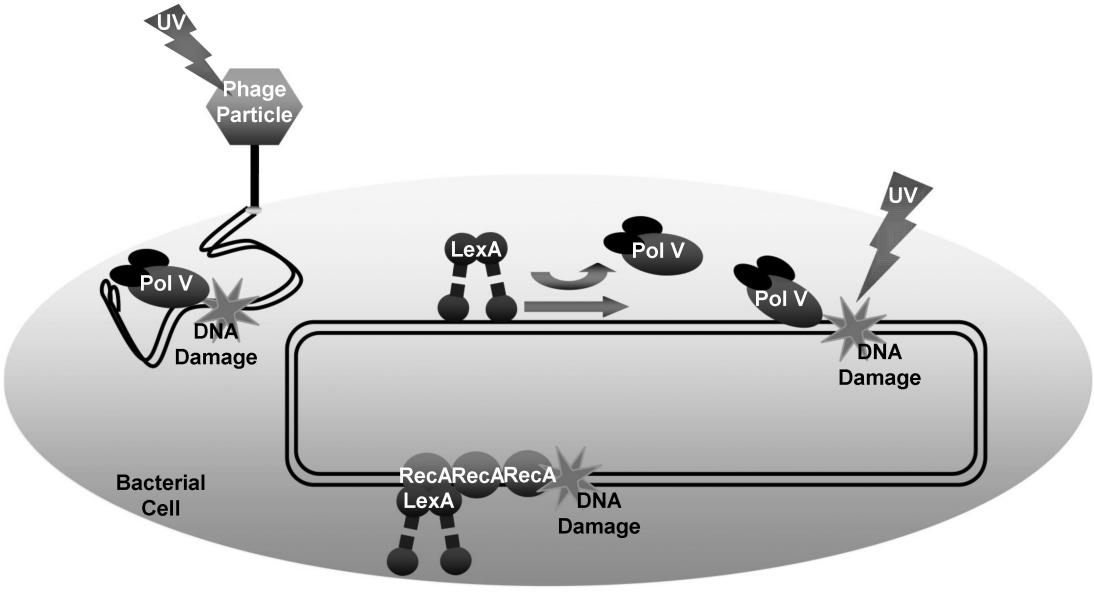

FIGURE 2 **Mechanism of Weigle mutagenesis. Irradiation of *E. coli* induces the SOS response, which includes the formation of RecA filaments that bind to, and cause the cleavage of LexA (*bottom*). Cleavage of LexA causes the de-repression of Pol V (*top*). After de-repression, Pol V replicates across damaged phage (*left*) or chromosomal DNA (*right*) resulting in mutations. When the cell is not irradiated, Pol V is not produced and any damage to the phage DNA is repaired in an error-free manner.**

sulfonate (Schendel and Defais, 1980) or hydrogen peroxide (Imlay & Linn, 1987). However, the exclusive dependence on Pol V for mutagenesis is limited to UV-induced lesions; other mutagens show a more complex dependency on the LexA-regulated polymerases. For example, mutations induced by the mutagen *N*-2-acetylaminofluorene depend predominantly on Pol II (the third LexA-regulated polymerase in *E. coli*) or Pol II and Pol V, depending on the specific DNA sequence context (Wagner *et al.*, 2002). These results indicate that the role of the polymerases in processing different lesions may be variable, depending on the type of damage, sequence context, or even the state of the cell.

Endogenous oxidative DNA damage also induces mutagenesis in a process that depends on Pol IV and Pol V (Yamada *et al.*, 2006). Yamada *et al.* (2006) created a mutant strain of *E. coli* that accumulates intracellular reactive oxygen species and observed an increase in substitution mutations that was dependent on Pol IV and Pol V (Yamada *et al.*, 2006). Deletion of both polymerases results in a mutation rate similar to deletion of either polymerase alone, suggesting that in this system, Pol IV and Pol V cooperate to introduce the required mutations. These results demonstrate that the SOS polymerases play a critical role in the mutagenicity of different DNA damaging agents, whether of exogenous or endogenous origin.

All of the observations described above suggest that a regulated increase in mutation rate is induced by DNA damage. However, induced mutation has also been observed in the absence of DNA damage (Brotcorne-Lannoye & Maenhaut-Michel, 1986; Wood & Hutchinson, 1984). In several cases in *E. coli*, this 'untargeted mutagenesis' has been linked directly to the production of DNA Pol IV and Pol V (Brotcorne-Lannoye & Maenhaut-Michel, 1986; Maor-Shoshani *et al.*, 2000). It has been postulated that when Pol IV and Pol V are present in sufficient concentrations, they are able to compete effectively with the replicative polymerases for replication termini *in vivo*. This model is supported by studies of Lopez de Saro *et al.* (2003) who showed that Pol II, Pol IV, Pol V, and the replicative polymerases Pol I and Pol III, all directly compete for binding of the β-clamp processivity subunit of the replication fork (Lopez de Saro *et al.*, 2003). It has also been shown that the β-clamp can bind both Pol III and Pol IV simultaneously, perhaps facilitating polymerase switching when the replicative polymerase stalls (Indiani *et al.*, 2005). Thus, induction of a low-fidelity polymerase (Kim *et al.*, 1997) and replication fork stalling (Strauss *et al.*, 2000) may be sufficient to induce mutation in the absence of damage.

STRESS-INDUCED MUTATION IN *E. Coli*

In the early 1990s, Cairns and Foster developed an experimental system to examine mutation in *E. coli* under starvation conditions (Cairns & Foster, 1991). An *E. coli* strain containing a frameshift mutation in the lactose catabolism (*lac*) operon carried on the F episome is plated onto media containing only lactose as a carbon source. Without a functional *lac* gene, no growth occurs; however, after several days on the selective media, mutant colonies begin to appear. Formation of these *lac*-revertant colonies requires the function of RecA and the cleavage of LexA, suggesting that starvation induces mutation.

Later work by the Rosenberg and Foster groups showed that the mechanism for starvation-induced mutation involves a host of proteins, including RecA, RuvC, the RuvAB complex, the RecBCD complex, and DNA Pol IV (Bull *et al.*, 2001; Foster & Rosche, 1999; He *et al.*, 2006; Lombardo *et al.*, 1999; Ponder *et al.*, 2005; Tompkins *et al.*, 2003). The current model for this induction proposes that DNA double-strand breaks (DSBs) formed during DNA replication are converted by RecBCD to single-stranded 3′-overhangs that template the formation of RecA filaments, which catalyze recombinational repair and increase the rate of deletion mutation by inducing LexA cleavage and the derepression of Pol IV (Figure 3). Reversion at the *lac* gene relies on Pol IV but not Pol V (Lombardo *et al.*, 1999), likely because the required mutation is a frameshift. We (Cirz *et al.*, 2005), and others (Yamada *et al.*, 2006) have suggested that Pol IV may be specialized for the extension of aberrant primer termini, for example, termini that are slipped or mispaired. This activity would facilitate the introduction of deletion or insertion mutations and is consistent with the structure of the *S. solfataricus* Pol IV homolog, described above. In contrast, Pol V may be more efficient at catalyzing incorrect triphosphate insertion (Cirz *et al.*, 2005; Yamada *et al.*, 2006), which is consistent with its ability to insert a nucleoside triphosphate opposite a pyrimidine dimer (Friedberg *et al.*, 2005). This separation of function provides a simple explanation for why the *lac* reversion system requires only Pol IV while other systems are dependent on

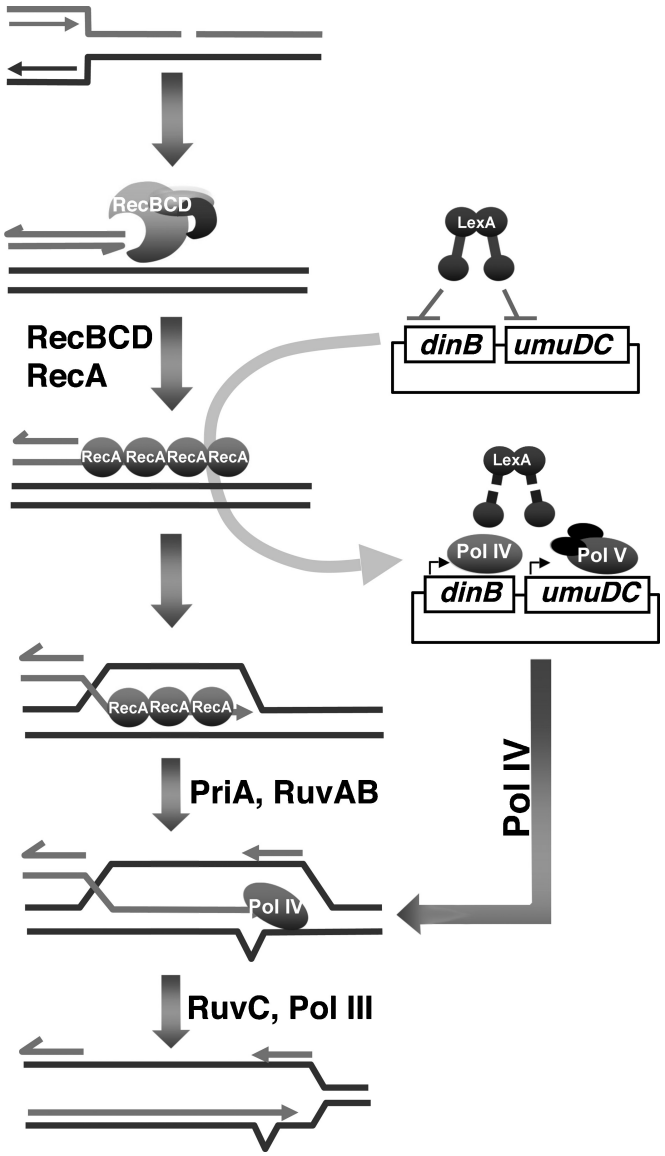

FIGURE 3 **Model of induced mutation in the Lac system. A single-strand nick in the DNA template leads to a double-strand end as the replication fork progresses through the lesion. The resulting double-strand end is a substrate for RecBCD processing and RecA filament formation. This leads to repair of the DNA through recombination, which is followed by RuvAB- and RuvC-mediated processing and resolution of the recombination as well as PriA-dependent reinitation of replication. However, the RecA filaments also cause cleavage of LexA and expression of the error-prone DNA polymerases Pol IV (and Pol V). Pol IV activity introduces frameshifts into the DNA during the reinitiation of replication.**

multiple polymerases (Cirz *et al.*, 2005; Napolitano *et al.*, 2000; Prakash & Prakash, 2002; Yamada *et al.*, 2006).

Interestingly, Ponder *et al.* (2005) have shown that the mutations in the *lac* system are proximal to sites where DSBs were repaired. This finding suggests that the recombination proteins required in the *lac* system may function beyond simply inducing the SOS response. Subsequently, genetic studies by He *et al.* (2006) suggest that recombination proteins are required beyond inducing the SOS response, perhaps by producing DNA structures that are substrates for the induced polymerases or by more directly regulating the polymerases themselves (He *et al.*, 2006). In addition, elegant work from Goodman & coworkers (2002) has demonstrated that RecA filaments act *in trans* to stimulate the activity of Pol V (Schlacher *et al.*, 2006), an activity that seems perfectly suited to focus mutations to recombination intermediates formed during the repair of DSBs or stalled replication forks. These observations hint at a potential mechanism for the tolerance of high mutation rates–limiting mutagenesis to specific regions of the DNA. While such 'directed mutation' is well known in the case of immunoglobulin gene somatic hypermutation (Steele, 1991), evidence that it contributes to bacterial evolution is only now beginning to emerge (Caporale, 2003; Schmidt *et al.*, 2006).

Induced mutation in the *lac* system is not without controversy. The frameshifted *lac* operon actually encodes a protein with low but measurable activity, and thus growth on the selective lactose media may also result from gene amplification (Hastings *et al.*, 2004; Hendrickson *et al.*, 2002; Roth *et al.*, 2003; Slechta *et al.*, 2003). Indeed, repeats of the mutant *lac* gene of up to ~100 copies have been observed and are sufficient to allow growth (Hastings *et al.*, 2004). The presence of multiple copies of the frameshifted *lac* allele increases the probability that a mutation will produce a wild-type sequence, without requiring any increase in the mutation rate. It has been proposed that after one copy of the *lac* gene acquires the requisite mutation, the remaining mutant genes may be deleted, removing the evidence of the amplification mechanism and making it appear as though a single gene acquired the mutation at an elevated rate (Hendrickson *et al.*, 2002) (Figure 4). However, it appears that gene amplification can only account for a portion of the total mutants observed (Hastings *et al.*, 2004), and it cannot easily explain the dependence on Pol IV (Foster, 2004; Hastings *et al.*, 2004; McKenzie *et al.*, 2001; Rosenberg & Hastings, 2004; Slack *et al.*, 2006; Stumpf *et al.*, 2007). While gene amplification may account for some *lac* reversion, it is less clear how it would contribute to other systems where the target allele provides no benefit prior to mutation. For example, many antibiotics, including the fluoroquinolones and rifamycins (see below), act by making the activity of their target protein toxic, and thus amplification of

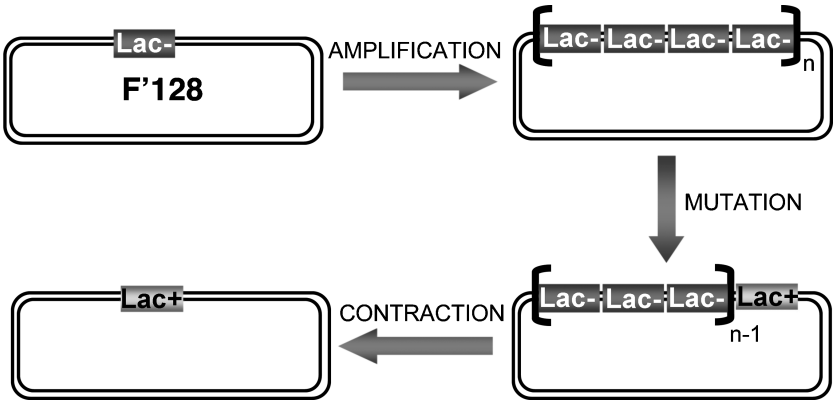

FIGURE 4 **Adaptive amplification in the lac system. Amplification of the frameshifted *lac* gene (which still retains approximately 1% activity) on the F'128 plasmid leads to some growth. This in turn leads to further amplification until full growth is permitted on media containing lactose as the sole carbon source. In addition, the increase in the copy number of the *lac* gene increases the probability of acquiring a point mutation that fully restores function. Once the mutation is acquired, the selective pressure for maintaining multiple copies is removed and contraction of the loci occurs.**

the corresponding gene prior to mutation would not be advantageous.

Work outside the *lac* reversion system suggests that stress-induced mutagenesis is a general phenomenon. The requirements for mutagenesis differ slightly in each system examined (Bjedov *et al.*, 2003; Humayun, 1998; Rahman & Humayun, 1999; Taddei *et al.*, 1995; Yeiser *et al.*, 2002; Zambrano *et al.*, 1993), but most report the involvement of the SOS response. For example, when *E. coli* cells are incubated for an extended period of time on antibiotic-free rich media, the SOS response is induced and the frequency of mutations conferring rifampicin resistance increases nine-fold from day 1 to day 7 (Taddei *et al.*, 1995). This process, termed mutagenesis in resting organisms in a structured environment, depends on RecA and LexA cleavage (Taddei *et al.*, 1995). Interestingly, the SOS induction observed in this system is dependent on cAMP (Taddei *et al.*, 1995), which may represent an important mechanism for inducing mutagenesis in response to a stress that does not cause direct DNA damage.

In addition, it has been shown that stationary phase cultures of *E. coli* that have been aged for 10 days are more fit and outcompete cells from a fresh culture (Finkel *et al.*, 2000; Zambrano *et al.*, 1993). This phenomenon, termed growth advantage in stationary phase, or GASP, appears to rely on mutations generated during the extended incubation in stationary phase (Zambrano *et al.*, 1993) and has been shown to require the SOS polymerases Pol II, Pol IV, and Pol V (Yeiser *et al.*, 2002). In total, these observations suggest that mutation can be induced by a variety of different types of stress.

INDUCED MUTATION IN OTHER BACTERIA

Although the majority of studies demonstrating induced mutagenesis have focused on *E. coli*, this phenomenon has also been observed in other bacteria. UV-induced mutagenesis has been observed in *B. subtilis* (Sung *et al.*, 2003), *C. crescentus* (Galhardo *et al.*, 2005), *P. aeruginosa* (Kokjohn & Miller, 1994), and *S. aureus* (Thompson & Hart, 1981). Moreover, in *B. subtilis* (Sung *et al.*, 2003), *C. crescentus* (Galhardo *et al.*, 2005), and *S. aureus* (Cirz *et al.*, 2007), this UV-induced mutation has been shown to require at least one LexA-regulated DNA polymerase.

Starvation-induced mutagenesis has been observed in *B. subtilis* (Sung & Yasbin, 2002; Sung *et al.*, 2003) and *Pseudomonas putida* (Tegova *et al.*, 2004). Using a strain of *B. subtilis* containing a mutation in the *hisC* gene that prevents growth in the absence of histidine, it was shown that reversion mutants accumulate over 9 days on minimal media lacking histidine (Sung & Yasbin, 2002) and their appearance depends on YqjH, a LexA-regulated Y-family polymerase (Sung *et al.*, 2003). In *P. putida*, a similar experiment was performed with cells carrying different mutations in the phenol monooxygenase gene, *pheA*. *pheA* mutant strains were plated on media containing phenol as the only carbon source and reversion mutants appeared over the course of 15 days (Tegova *et al.*, 2004). By using different mutations in the *pheA* gene it was shown that, as with *E. coli*, Pol IV is required to induce frameshift mutations but not substitution mutations.

Finally, GASP mutation has been observed in the Gram-negative bacteria *Shigella dysenteriae*, *Enterobacter cloacae*, *P. putida*, and *Bacillus abortus*; the Gram-positive bacteria *S. aureus*, *Entererococcus faecalis*, and *Bacillus globigii*; and the mycobacterium *Mycobacterium smegmatis* (reviewed in (Finkel *et al.*, 2000). However, the role of LexA and the error-prone polymerases in the acquisition of the GASP phenotype in these organisms has not yet been investigated.

More work is required to understand the process of induced mutation in these other bacteria at the level that it is understood in *E. coli*. However, it is clear that in most bacteria, induced mutagenesis is a conserved process and that it involves at least one LexA-regulated error-prone polymerase (Cirz *et al.*, 2007; Erill *et al.*, 2003).

ANTIBIOTIC-INDUCED MUTAGENESIS IN BACTERIA

Throughout history, people have died from bacterial infections. The introduction and widespread use of antibiotics in the first half of the 20th century promised to finally rid humanity of its oldest and greatest threat. However, the remarkable ability of bacteria to evolve resistance to these antibiotics now threatens to end the 'antibiotic era.' For example, when the first fluoroquinolone antibiotic (norfloxacin) was introduced in the 1980s, it was effective against virtually all Gram-negative bacteria, and derivatives were soon found that were also potent against Gram-positive pathogens. However, as with all antibiotics, resistance to the fluoroquinolones increased and continues to rise at an alarming rate. For example, the proportion of ciprofloxacin-resistant *Helicobacter pylori* isolates in Germany doubled between 2003 and 2005 from 11% to 22% (Glocker *et al.*, 2007). Interestingly, clinically relevant levels of resistance to the fluoroquinolones requires several substitution mutations in the bacterial chromosome (Lindgren *et al.*, 2003). The acquisition of mutations also plays a central role in the evolution of resistance to other important antibiotics such as rifampicin, extended spectrum β-lactams, dihydrofolate reductase inhibitors, and in the evolution of the most common forms of resistance to the glycopeptides vancomycin and teicoplanin (Bischoff & Berger-Bachi, 2001; Bischoff *et al.*, 2001; Finch *et al.*, 2003; Schaaff *et al.*, 2002; Tenover *et al.*, 1998; Walsh & Howe, 2002).

By their very nature, antibiotics provide a source of stress for bacteria. Therefore, it is perhaps not surprising that many antibiotics induce mutagenesis. This has been most intensively studied with the fluoroquinolones (Gocke, 1991; Mamber *et al.*, 1993; Ysern *et al.*, 1990). The mutagenicity of fluoroquinolones was first demonstrated by Ysern *et al.* (1990) with *Salmonella typhimurium* (Mamber *et al.*, 1993; Ysern *et al.*, 1990), and later with *E. coli* (Drlica & Zhao, 1997), *S. pneumoniae* (Henderson-Begg *et al.*, 2006), *P. aeruginosa* (Fung-Tomc *et al.*, 1993), *S. aureus* (Fung-Tomc *et al.*, 1993) and *Mycobacterium fortuitum* (Gillespie *et al.*, 2005).

The induction of mutation by fluoroquinolones is likely a result of their effect on DNA and its replication. This class of drug functions by binding to either one or both of the type II topoisomerases in bacteria, topoisomerase IV (encoded by *parC* and *parE*) and gyrase (encoded by *gyrA* and *gyrB*). These topoisomerases function by forming a protein-bridged DNA DSB, introducing or relaxing supercoils, and finally rejoining the DNA ends. Fluoroquinolones bind to the protein-bridged DSB intermediate and inhibit rejoining of the DNA ends. This produces either a free DSB when the topoisomerase dissociates from the DNA, or a potent block of DNA replication when the topoisomerase remains bound to the DNA (Drlica & Zhao, 1997; Khodursky & Cozzarelli, 1998) (Figure 5). Either outcome provides a direct mechanism for the induction of the SOS response.

Work by Hall and colleagues (Riesenfeld *et al.*, 1997), Blázquez (2003), and our group (Cirz *et al.*, 2005) has shown that ciprofloxacin also induces the generation of mutations that confer resistance to the drug itself. The mechanism of this induction is likely the activation of the SOS response (Drlica & Zhao, 1997; Phillips *et al.*, 1987; Power & Phillips, 1993) and the associated production of the error-prone DNA polymerases (Cirz *et al.*, 2005; Henderson-Begg *et al.*, 2006; Ysern *et al.*, 1990). Indeed, ciprofloxacin has been shown to induce the production of error-prone DNA polymerases in several species including *E. coli* (Power & Phillips, 1992), *B. subtilis* (Au *et al.*, 2005), *P. aeruginosa* (Cirz *et al.*, 2006), and *S. aureus* (Cirz *et al.*, 2007), and a correlation between production of these polymerases and an increase in mutation rate has been shown in *E. coli* (Wagner *et al.*, 1999) and *P. aeruginosa* (Sanders *et al.*, 2006).

In addition to the fluoroquinolones, other antibiotics have been shown to induce the SOS response. The ribonucleotide reductase inhibitors, trimethoprim and

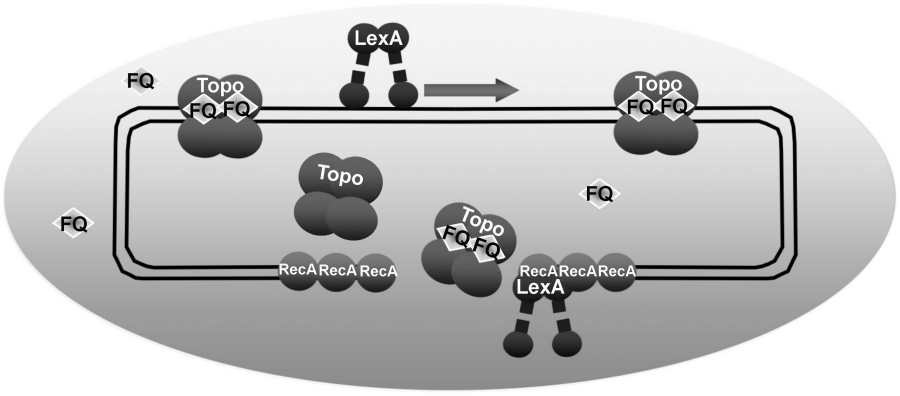

FIGURE 5 **Model of fluoroquinolone-induced SOS response. Binding of fluoroquinolones to the type II topoisomerases ('*Topo*') stabilizes the protein-DNA complex which leads to replication blocks (not shown) and dissociation of the topoisomerase from the DNA without re-ligating the DNA ends (shown). Both lead to DNA double-strand ends and therefore filamentation of RecA on single-stranded DNA and subsequent cleavage of LexA which induces the SOS response.**

sulfamethoxazole, which act by depleting cellular levels of deoxyribonucleotides and by inhibiting DNA replication (Finch *et al.*, 2003; Gleckman *et al.*, 1979), also induce the SOS response (Goerke *et al.*, 2006; Lewin & Amyes, 1991). Recent work has shown that the SOS response is also induced by antibiotics that do not directly damage DNA or affect its replication. For example, β-lactam antibiotics have been shown to induce the SOS response in *E. coli* (Miller *et al.*, 2004). These antibiotics function by inhibiting cell wall biosynthesis, but a subclass of β-lactams that target the transpeptidase PBP3 also activate the two-component response regulator DpiBA (Miller *et al.*, 2004). DpiA induces the SOS response by competing with the replication proteins DnaA and DnaB for binding to AT-rich sequences (Figure 6). In addition, the antibiotic ceftazidime (a PBP3-specific inhibitor) has been shown by T. Pérez-Capilla *et al.* (2005) and J. Blázquez *et al.* (2006) to induce the SOS response, the expression of DNA Pol IV, and an increase in mutation rate in *E. coli* (Perez-Capilla *et al.*, 2005) and *P. aeruginosa* (Blazquez *et al.*, 2006). Finally, the inhibition of cell wall biosynthesis at steps other than PBP3 activity has been shown to specifically induce DNA Pol IV expression in *E. coli*, however this induction does not appear to require LexA cleavage. The indirect mechanism coupling the SOS response and/or error-prone polymerase production to cellular perturbations caused by β-lactam antibiotics may be a harbinger of undiscovered mechanisms linking induced mutation to other antibiotics.

The β-lactam antibiotics, including the widely used antibiotics ceftriaxone and cloxacillin, also induce the SOS response in *S. aureus* (Maiques *et al.*, 2006). While a β-lactam-mediated increase in *S. aureus* mutation rates has not been demonstrated, induction of the SOS response in this pathogen has been shown to result in

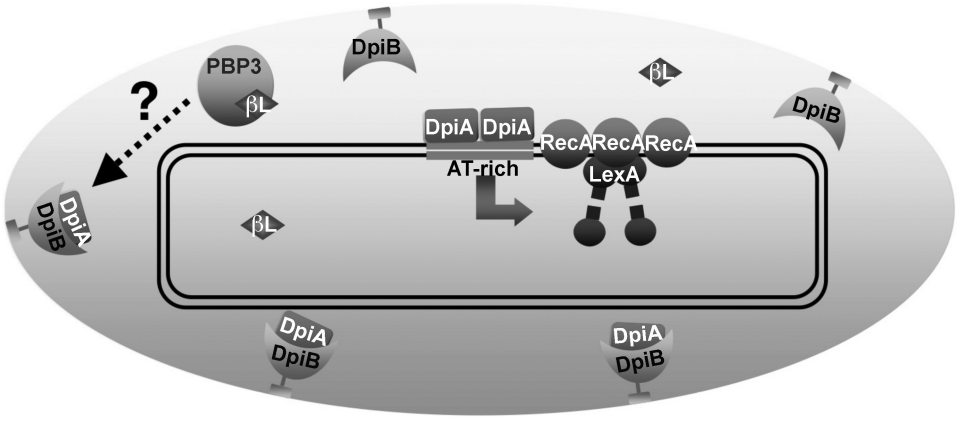

FIGURE 6 **Mechanism of β-lactam induced SOS response. β-lactam drugs (labeled 'βL') bind and inhibit PBP3 which, through an incompletely understood mechanism (*question mark*) leads to activation of DpiBA. DpiA then binds to the AT-rich sequences in the DNA which causes replication blocks. This leads to RecA filamentation on single-stranded DNA and therefore LexA cleavage and induction of the SOS response.**

the de-repression of the LexA-regulated Y-family polymerase, SACOL1400 (Cirz *et al.*, 2007). SACOL1400 has been directly linked to the induction of the mutations that facilitate long-term adaptation (Watson *et al.*, 1998) and UV-induced mutability (Cirz *et al.*, 2007) in this organism. Therefore, it seems likely that β-lactam antibiotics also induce mutation in *S. aureus*.

THE POTENTIAL FOR INHIBITING INDUCED MUTAGENESIS

It is clear that different stresses, including exposure to antibiotics, can induce mutation in bacteria. It is also clear that, at least in some cases, mutation is required for the evolution of antibiotic resistance. It is therefore possible that the remarkable ability of bacteria to evolve antibiotic resistance relies on induced mutation. This suggests that drugs targeting the proteins required for induced mutation may act to slow the development of resistance (Cirz *et al.*, 2005; Lee & Singleton, 2005). We now review data suggesting that the inhibition of LexA cleavage, proteins that act upstream of LexA cleavage (*i.e.*, RecBCD, RecA, *etc.*), or the induced polymerases themselves would indeed slow the evolution of resistance during therapy.

In the early 1990s, Ysern *et al.* provided the first evidence that the evolution of antibiotic resistance might be subject to inhibition (Ysern *et al.*, 1990). They observed that in a *S. typhimurium* strain that contains *muckA-B*, a Pol V homolog, fluoroquinolones induce mutations that revert a nonfunctional *his* gene. They also characterized a variety of other *S. typhimurium* strains and found that the drugs do not induce mutation in strains lacking a Pol V homolog. This data demonstrated that, like other types of stress-induced mutations discussed above, fluoroquinolone-induced mutation requires the activity of Pol V.

More recently, several studies have expanded our understanding of the link between antibiotics, induced mutation, and resistance. In 2003, Barry and coworkers showed that, in *Mycobacterium tuberculosis*, the LexA-regulated DNA polymerase DnaE2 is required for the efficient evolution of rifampicin resistance in a mouse lung infection model (Boshoff *et al.*, 2003). Rifampicin is an antibiotic that inhibits RNA polymerase, which produces protein-DNA complexes that block DNA replication. It is thus likely that rifampicin induces LexA cleavage and the upregulation of *dnaE2*, which is also induced by UV irradiation, mitomycin C, and hydrogen peroxide in a RecA- and LexA-dependent manner (Boshoff *et al.*, 2003). In addition, the long-term *in vivo* persistence of a mutant *dnaE2* strain of *M. tuberculosis* in the mouse model was significantly lower than that of the wild-type strain (Boshoff *et al.*, 2003). Finally, no mortality was observed in D2B6F1 mice (a lineage that is particularly susceptible to *M. tuberculosis* infection) 4 months after infection with the *dnaE2* mutant strain, while 20% mortality was observed after infection with the wild-type strain (Boshoff *et al.*, 2003). These observations suggest that the DnaE2 polymerase is required to introduce mutations that facilitate *in vivo* persistence (Cardona & Ruiz-Manzano, 2004; Schnappinger *et al.*, 2003; Shi *et al.*, 2005) and resistance (Boshoff *et al.*, 2003). They also suggest that the inhibition of DnaE2 (directly or indirectly through the inhibition of LexA cleavage or any protein required for LexA cleavage) would significantly improve the treatment of *M. tuberculosis* infections.

Work by our group has shown that in *E. coli*, the evolution of ciprofloxacin resistance in the presence of the drug requires LexA cleavage and SOS polymerase de-repression (Cirz *et al.*, 2005). Disrupting any of the SOS polymerases, alone or in combination, does not confer significant sensitivity to ciprofloxacin, suggesting that the polymerases are not essential for tolerating the drug. However, preventing LexA autoproteolysis or disrupting any of the SOS polymerases dramatically decreases the evolution of resistance. The fact that all three SOS polymerases are required for the acquisition of resistance-conferring substitution mutations suggests that the mechanism involves Pol II-mediated replication restart of a collapsed fork, Pol V-mediated synthesis of a mispair, and finally, Pol IV-mediated extension of the mispaired primer terminus (Figure 7) (Cirz *et al.*, 2005).

Disabling the mismatch repair system in bacteria has also been evoked as a potential mechanism to elevate mutation rates and facilitate the evolution of antibiotic resistance (Blázquez, 2003). Indeed, hypermutability has been linked to drug resistance, and disruption of the *mutS* gene (which encodes a central component of the bacterial mismatch repair system) is commonly observed in hypermutator bacteria isolated from human infections (Hall and Henderson-Begg, 2006; Oliver *et al.*, 2000). However, disabling mismatch repair can only be an *independent* mechanism of increasing mutation rates if the mutations that it repairs are unavoidably present as a result of DNA replication

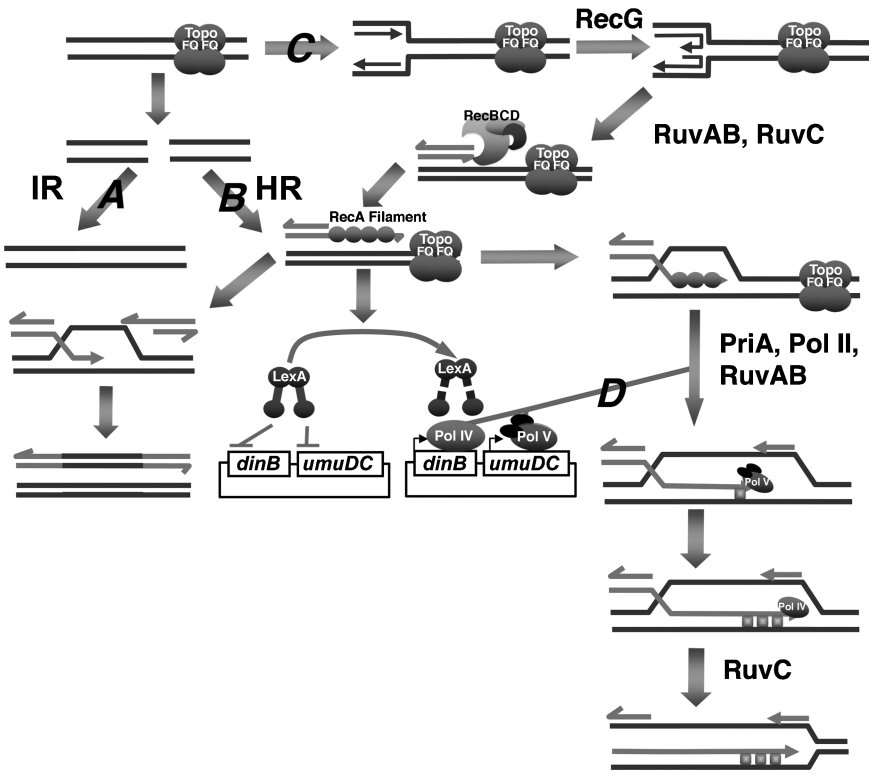

FIGURE 7 **Model of ciprofloxacin-induced mutation and resistance. In the absence of homologous sequences, free ciprofloxacin induced DSBs are repaired by nuclease and polymerase-dependent illegitimate recombination leading to small deletions (*Pathway A*). In the presence of a suitable homologous sequence, free DSBs may be repaired by recombination-dependent replication (*Pathway B*). Finally, replication forks that encounter topoisomerases that are covalently-bound to the DNA are repaired by recombination-dependent fork repair (*Pathway C*). This involves RecG-mediated fork regression and RuvC cleavage to produce double-stranded ends where RecBCD mediates RecA-single-stranded DNA filament formation. These filaments catalyze strand invasion of a homologous sequence where PriA, and possibly Pol II, help to reestablish a processive replication fork. With sufficient accumulation of DSBs and collapsed forks, persistent RecA-single-stranded DNA filaments induce LexA cleavage to levels sufficient to de-repress the error prone polymerases, Pol IV and Pol V, which cooperate to induce mutations (*Pathway D*). Once resistance-conferring mutations are made, DSBs, collapsed replication forks, and RecA-single-stranded DNA filaments are no longer present and the cellular concentration of LexA increases, shutting down expression of the pro-mutagenic polymerases. For more details, see Cirz *et al.* (2005).**

(*i.e.*, spontaneous and not requiring induction). We have demonstrated that the ciprofloxacin-induced hypermutability observed in *mutS* mutants of *E. coli* is virtually completely dependent on LexA cleavage and SOS polymerase de-repression (Cirz and Romesberg, 2006). Thus, preventing cleavage of LexA or induction of the regulated polymerases is likely to inhibit the evolution of antibiotic resistance even in hypermutable strains of bacteria.

LexA activity is required for the evolution of resistance to ciprofloxacin not only *in vitro* but also *in vivo*. We demonstrated that a pathogenic strain of *E. coli* with a mutation in the *lexA* gene that prevents induction of the SOS response is unable to evolve resistance to ciprofloxacin or rifampicin in a mouse thigh infection model (Cirz *et al.*, 2005). Interestingly, similar numbers of bacteria were isolated from mouse thighs infected with the wild-type and *lexA* mutant strains in these experiments–the difference was the complete lack of resistance observed in the mutant strain (Cirz *et al.*, 2005).

These results suggest that the induction of the SOS response may be required for the most efficient generation of resistance-conferring mutations during an infection. Further testing this hypothesis will require experiments designed at deconvoluting the contribution of induced mutation to clinical resistance versus the contribution of preexisting resistance or of resistance generated spontaneously during repeated cycles of bacterial clearance and regrowth. If the evolution of resistance during therapy is facilitated by the induction of mutation, and if this resistance complicates therapy, then the inhibition of error-prone polymerase activity, their LexA cleavage-mediated de-repression, or any protein required for LexA cleavage (*i.e.*, RecBCD or RecA), may be a promising new strategy in the battle against the emergence of antibiotic-resistant pathogens. While the search for such inhibitors has just begun, a small

molecule that inhibits RecA filamentation has already been identified from a screen of small molecules (Lee and Singleton, 2005).

THE PURPOSE OF INDUCIBLE MUTAGENESIS

While these systems may have evolved to tolerate high levels of DNA damage, it has been suggested that inducible mutation may have evolved to facilitate evolution in times of stress (Earl & Deem, 2004; Hersh *et al.*, 2004; Radman *et al.*, 1999). It has also been suggested that the cost of elevating mutation rates may outweigh the benefits, as mutations may accumulate throughout the genome (Roth *et al.*, 2003). However, one potential advantage of elevating mutation via inducible polymerases, as opposed to disabling mismatch repair (Harris *et al.*, 1997; Modrich, 1989; Tsui *et al.*, 1997) or the proofreading subunit of the replicative polymerase, DNA Pol III (Fijalkowska & Schaaper, 1996), is the potential for regulation (Caporale, 2003). For example, as already discussed, recent work has shown that error-prone polymerase-mediated mutations may be localized to sites of DSB repair (He *et al.*, 2006; Ponder *et al.*, 2005). Recombination intermediates involved in DSB repair or the restart of stalled replication forks (Colloquium, 2001; Indiani *et al.*, 2005) might provide a scaffold for RecA-ssDNA filaments to direct error-prone DNA Pol IV (He *et al.*, 2006) or Pol V synthesis (Schlacher *et al.*, 2006). From this perspective it is interesting to note that a potential gyrase binding site (Morrison & Cozzarelli, 1981), where DSBs are likely to accumulate, exists in the immediate vicinity of the fluoroquinolone resistance determining region of *gyrA*.

In principle, these mechanisms could direct induced polymerase-mediated mutations to DNA sites that are damaged or that are difficult to replicate, or to genes that are being intensively transcribed (Schmidt *et al.*, 2006), with replication occurring normally throughout the rest of the genome. Other mechanisms for elevating mutation rates, such as the disablement of mismatch repair proteins or the proofreading subunits of the replicative polymerases do not offer the possibly of such control. While these suggestions are both provocative and controversial, it is important to emphasize that regardless of why bacteria induce mutation, various of data now suggest that induced mutation is a general phenomenon and that its inhibition would prevent the evolution of antibiotic resistance.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the hundreds of millions of dollars required to develop an antibiotic, it is now clear that within a few years of widespread use, resistance will evolve and erode the efficacy of any antibiotic. Bacteria will do what all living things have always done–evolve. With the revelation that this evolution requires the activity of specialized pro-mutagenic proteins (*i.e.* RecA, RecBCD, LexA, Pol IV, Pol V), it seems that intervention in the process will be possible with properly designed molecules. Such molecules would have a profound effect on the long-term efficacy of different antibiotics, and they might represent a much needed new approach to combating bacterial pathogens. We end this review by reiterating that induced mutation also seems to be conserved in human cells (Goodman, 2002; Li *et al.*, 2002) where it may play an essential role in different human diseases such as aging, cancer, and the evolution of resistance to chemotherapeutic drugs. Expanding our understanding of induced mutation in both bacterial and human cells promises to radically change our understanding and treatment of a host of human diseases.

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