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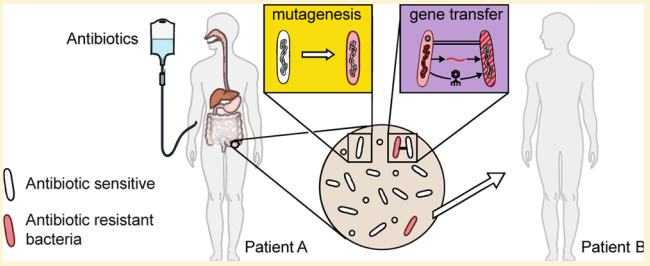
Targets for Combating the Evolution of Acquired Antibiotic Resistance

Matthew J. Culyba,[†] Charlie Y. Mo,[‡] and Rahul M. Kohli*[,†,§]

[†]Department of Medicine, [‡]Biochemistry & Molecular Biophysics Graduate Group, and [§]Department of Biochemistry & Biophysics, Perelman School of Medicine, University of Pennsylvania, 3610 Hamilton Walk, Philadelphia, Pennsylvania 19014, United States

ABSTRACT: Bacteria possess a remarkable ability to rapidly adapt and evolve in response to antibiotics. Acquired antibiotic resistance can arise by multiple mechanisms but commonly involves altering the target site of the drug, enzymatically inactivating the drug, or preventing the drug from accessing its target. These mechanisms involve new genetic changes in the pathogen leading to heritable resistance. This recognition underscores the importance of understanding *how* such genetic changes can arise. Here, we review recent advances in our understanding of the processes that contribute to the evolution of antibiotic resistance, with a particular focus on hypermutation mediated by the SOS pathway and horizontal gene transfer. We explore the molecular mechanisms involved in acquired resistance and discuss their viability as potential targets. We propose that additional studies into these adaptive mechanisms not only can provide insights into evolution but also can offer a strategy for potentiating our current antibiotic arsenal.



"We are continually faced with a series of great opportunities brilliantly disguised as insoluble problems." - John W. Gardner

The use of penicillin and sulfonamides in the 1940s marked the start of a new era in the management of human health and disease.[1] The success of these drugs led to the enthusiastic discovery of several new classes of antibiotics peaking during the 1950s−1970s, a time now often termed the "golden era" of antibiotic discovery. Unfortunately, for each new antibiotic class discovered, reports of resistant microbes emerged within only a few years, heralding the impending challenge of antibiotic resistance.[2,3]

The rising tide of multi-drug-resistant organisms (MDROs) is increasingly diminishing the efficacy of our antibiotic arsenal.[4] This trend is in part due to the selection of resistant bacteria via widespread use of antibiotics and the dissemination of resistance genes in bacterial populations across the globe.[5−7] Additionally, the unique pharmacological challenges of targeting bacteria, coupled with economic disincentives to developing antibiotics, have conspired to slow the rate of discovery.[8,9] Despite infection control efforts, resistance continues to outpace drug discovery, raising the specter of a "post-antibiotic era", a time in the future when high mortality caused by MDROs cannot be easily prevented.[10]

While innovative new approaches are underway to discover antimicrobials with different mechanisms of action,[11−13] the most conventional approach to overcoming resistance has involved the chemical modification of existing antibiotic scaffolds.[14] Our antibiotic arsenal has undergone a stepwise tailoring of core structures, akin to evolution, to both increase their spectrum of activity and overcome resistance mechanisms. For example, antibiotics that maintain the β-lactam core started with penicillins, moved forward through "generations" of cephalosporins, and onward to carbapenems. While these "next-generation" antibiotics could overcome some existing resistance mechanisms, many bacteria, in turn, have rapidly adapted to counteract these drugs (Figure 1).

These cycles of antibiotic discovery and resistance illustrate the importance of understanding *how* drug resistance evolves, which is the focus of this review. Although seemingly an intractable problem, the evolution of antibiotic resistance may represent a great opportunity. Indeed, efforts to understand the evolution of drug resistance could serve a dual purpose: providing a window into how bacteria adapt to harsh environments while simultaneously elucidating novel targets to potentiate our current antibiotic arsenal. Prior reviews have introduced the concept of targeting evolution,[15] and developments since have provided new insights into the mechanisms by which genetic changes lead to heritable acquired resistance. In this review, we focus on the biochemistry that mediates genomic mutation by the bacterial SOS pathway or via horizontal gene transfer (HGT). We conclude with a discussion of the feasibility, challenges, and opportunities of targeting these pathways.

■ ACQUIRED ANTIBIOTIC RESISTANCE

Antibiotic resistance can be classified as either intrinsic or acquired, and by whether the mechanism involves a genetic change. Intrinsic resistance refers to a generalizable trait that

Received: February 3, 2015
Revised: May 25, 2015
Published: May 27, 2015

DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582

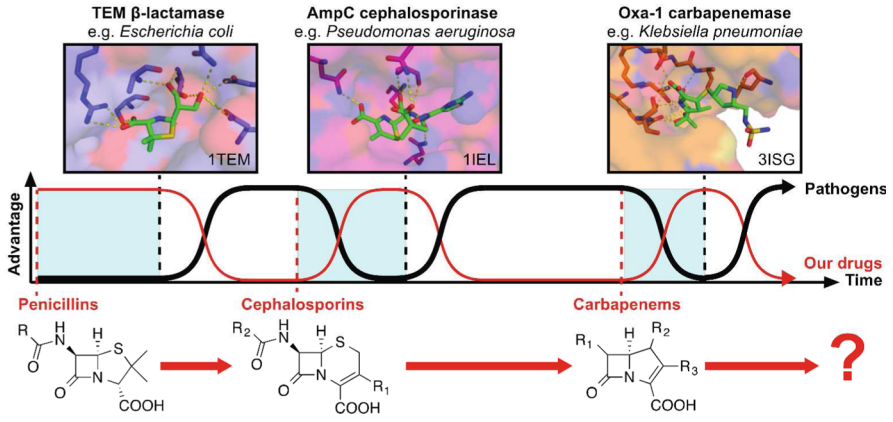


Figure 1. Cycles of drug discovery and antimicrobial resistance. An illustrative schematic is shown presenting several generations of β-lactam antibiotics chronologically coupled to the β-lactamases that have emerged in clinical pathogens to counteract these "next-generation" antibiotics.

does not change regardless of antibiotic selective pressure. For example, resistance to vancomycin for Gram-negative bacteria is due to differences in their cell wall architecture relative to Gram-positive bacteria and not a specific resistance mechanism. By contrast, acquired resistance develops when a new trait is expressed, often because of a genetic change that has been selected for in the setting of antibiotic exposure. Bacteria can also mediate tolerance to antibiotics independent of genetic change, such as with persister states or biofilm formation.[16]

Genetic changes can confer resistance to antibiotics through a diverse set of mechanisms. Though other mechanisms are known, common and prominent examples include altering the target site of the drug, enzymatically inactivating the drug, and preventing the drug from accessing the target. Many of these resistance mechanisms result either from a small number of specific genomic mutations or, alternatively, from HGT (Figure 2). Point mutations can alter the interactions between a drug

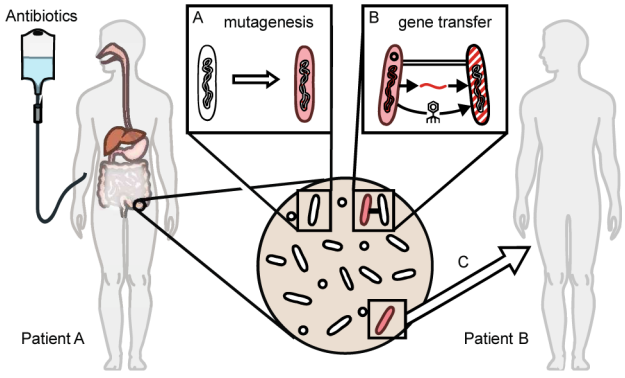


Figure 2. Acquisition and spread of antimicrobial resistance. Stress, including treatment with antibiotics, promotes acquired resistance in an initially sensitive strain by driving (A) mutagenesis or (B) horizontal gene transfer. Strains with preexisting resistance can (C) then spread by transmission between people.

and its target, as evidenced by mutations in RNA polymerase that mediate resistance to rifampin. Point mutations can also affect nontarget genes, as illustrated by promoter mutations resulting in the overexpression of drug efflux pumps. Unlike point mutations, HGT can result in the acquisition of genes with entirely novel functions for the cell. For example, some acquired genes can inactivate drugs, such as plasmid-encoded β-lactamases. Others can even alter cellular metabolic or structural products, as in the case with vancomycin-resistant enterococci, where a cassette of genes mediates changes to a peptidoglycan motif that dramatically weakens vancomycin binding.[17] While the genetic elements that directly confer resistance have been well reviewed,[18,19] the biochemical mechanisms by which these genetic changes arise within bacteria have been less scrutinized.

FIXED AND TRANSIENT HYPERMUTATION

Mutation is a major contributor to the evolution of drug resistance. Some important pathogens, such as *Mycobacterium tuberculosis* (*Mtb*), rely almost exclusively on mutagenesis, rather than gene transfer, to evolve resistance.[20] Similarly, for certain classes of antibiotics, such as fluoroquinolones, point mutations are the primary mechanism of acquired resistance.[21] Furthermore, although mobile resistance genes largely account for the high prevalence of MDROs, the evolution of these genes against "next-generation" antibiotics also occurs at the level of mutation.

Bacteria can acquire mutations spontaneously and at a relatively constant rate because of the inherent mutational frequency associated with genomic replication. However, under various conditions, mutation rates can increase, in some cases as high as 100-fold above the basal rates.[22−24] Two basic mechanisms are known to accelerate mutation in bacterial strains: a loss of DNA repair or proofreading systems and the induction of pro-mutagenic pathways.

The best-studied disruption in DNA repair involves loss of mismatch repair (MMR). MMR deficiency can result in a *fixed hypermutator* phenotype where the organism's mutation rate is rendered constitutively high.[24] The clinical implications of this phenotype are evident in cystic fibrosis patients, where hypermutator *Pseudomonas aeruginosa* strains with MMR deficiency are frequently isolated.[25] Interestingly, although MMR deficiency is typically the result of a fixed loss of function, bacterial strains that exhibit transient inactivation via excision and reintegration of a cryptic prophage at a gene locus critical for MMR function have also been isolated.[26]

While fixed hypermutators are important to acquired resistance, the induction of transient pro-mutagenic pathways is another important driving force for acquired antibiotic resistance. *Transient hypermutation* has been linked to conserved stress responses within bacteria.[27,28] These stress responses are mediated by tightly regulated genetic pathways that poise bacteria to respond to a wide range of stressful

DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582

environments, from host immune systems to ultraviolet radiation to toxic biomolecules, including antibiotics.[29] Different stress responses have been shown to contribute to accelerated mutagenesis, including the starvation response and envelope stress response;[27,28] however, the majority of studies on induced mutagenesis have focused on the bacterial SOS pathway, where the biochemistry of the key players in the pathway has been well-delineated.[30,31] To this end, we next turn our attention to the biochemistry of the SOS response and opportunities for slowing acquired drug resistance by targeting the sensor, regulator, or effector enzymes in the pathway.

TARGETING THE SOS RESPONSE

The SOS pathway is a widely conserved DNA damage response pathway that, upon detection of DNA damage, responds by expressing genes involved in DNA repair and damage tolerance (Figure 3).[30,31] SOS genes lie under the control of the transcriptional repressor LexA. In the basal, unstressed state, LexA binds to specific operator DNA (SOS box) sequences in SOS gene promoters.[32] In the setting of DNA damage, single-stranded DNA (ssDNA) accumulates at stalled replication forks and serves to activate the DNA damage sensor of the system, RecA. Activated RecA stimulates LexA to undergo a self-cleavage reaction, which promotes LexA dissociation and derepression of SOS genes. The induced genes follow an interesting chronology that implies a transition from high- to low-fidelity repair, based on damage severity. Initially, repair genes, including those for nucleotide excision repair, are expressed; however, later in the SOS response, error-prone translesion DNA polymerases are induced.[33] Notably, LexA is self-regulated, and re-accumulation of full-length LexA upon rescue from damage can halt the SOS response. As a result, DNA damage can cause a transient hypermutator phenotype, known as SOS mutagenesis, which occurs for the duration of the genotoxic stress.

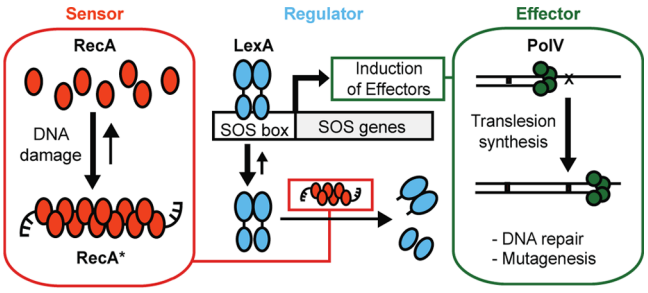


Figure 3. The SOS response is a key regulator of transient hypermutation in bacteria. Activation of the stress sensor, RecA (red ovals), promotes self-cleavage of the SOS regulator, LexA (blue ovals). LexA cleavage results in induction of the SOS effectors, which include error-prone DNA polymerases (green circles) that can bypass DNA lesions leading to mutations during error-prone repair.

Genetic experiments have validated the SOS pathway as an important target for combating the evolution of antibiotic resistance. Experimentally inactivating the SOS regulators, either by deletion of *recA* or by engineering a noncleavable LexA into the bacteria, renders the bacteria unable to initiate the SOS response. These mutant bacteria are hypersensitive to genotoxic antimicrobials and exhibit decreased mutation rates.[34−36] In a particularly revealing experiment, the Romesberg group infected mice with either wild-type *Escherichia coli* or *E. coli* harboring a noncleavable mutant of LexA. Upon treatment with either rifampin or ciprofloxacin, the wild-type infection showed an initial response to therapy but then rebounded with drug-resistant bacteria. By contrast, infection with the strain containing noncleavable LexA continued on a trajectory toward eradication with no evidence of detectable resistance.[34] In a different experiment by the Collins group, infecting drug-resistant *E. coli* with a phage overexpressing a noncleavable LexA exerted a dominant-negative effect that prevented SOS activation and resensitized the *E. coli* to antibiotics.[37] Preventing SOS activation has also been reported to antagonize other mechanisms that mediate survival in response to antibiotic stress, including integron-mediated gene transfer, biofilm formation, and bacterial persistence.[34,38,39]

With regard to SOS effectors, deletion of the SOS-induced translesion polymerases decreases bacterial fitness, lowers their mutation rate, and slows acquisition of drug resistance.[40,41] Some of the most compelling evidence comes from studies in *Mtb*. While the *Mtb* SOS operon contains fewer genes than other pathogens, the key effector in the pathway is DnaE2, a translesion DNA polymerase.[42] Deletion of *dnaE2* is associated with decreased *Mtb* virulence in infection models and suppresses the emergence of resistance to rifampin, a key first-line anti-tuberculosis agent.[40] Together, these genetic studies suggest the potential therapeutic benefits of perturbing the regulators or effectors of the SOS pathway.

The Damage Sensor, RecA. RecA is a highly conserved ∼38 kDa protein that plays a critical role in homologous recombination and also acts to stimulate LexA self-cleavage.[43] Structurally, monomeric RecA consists of three domains with a central core RecA fold that is flanked by smaller regulatory domains.[44] These monomers can form large nucleoprotein filaments on ssDNA (Figure 4A), which can extend across thousands of base pairs via cooperative oligomerization mediated by the core RecA fold.[45] Filamentous RecA has a deep helical groove that envelopes, stretches, and unwinds the bound DNA, preparing it for homology searching and subsequent DNA strand exchange. The core RecA fold binds ATP at the monomer−monomer interface (Figure 4A).[44] While only binding of ATP is required for filament formation and simple DNA strand exchange reactions, RecA also catalyzes ATP hydrolysis, which is important for filament depolymerization as well as some specific types of recombination activities.[43]

Filamentous RecA acts as a co-protease to stimulate self-cleavage of LexA (discussed below), as well as other related members of the LexA/signal peptidase superfamily, such as phage λ repressor and UmuD in *E. coli*. In the case of phage repressor, cleavage stimulates the prophage to enter the lytic cycle.[46] Interestingly, RecA serves two roles in association with UmuD: it stimulates self-cleavage of UmuD to UmuD′ and is also, itself, an essential component of the associated Pol V mutasome.[47] The LexA binding site on the RecA filament has not been fully elucidated, but current models suggest that LexA may span adjacent RecA monomers across the deep helical groove.[48] ATP binding, but not hydrolysis, is required for the co-protease activity.

As Δ*recA* strains are hypersensitive to antibiotics and less prone to acquired resistance,[35,36] RecA has been proposed as a novel target for slowing the evolution of antibiotic resistance. The feasibility of targeting RecA is further supported by the existence of biological protein modulators of RecA, including RecX and DinI, which both can antagonize SOS induction.[49] In an effort to discover small molecule RecA inhibitors, Singleton

DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582

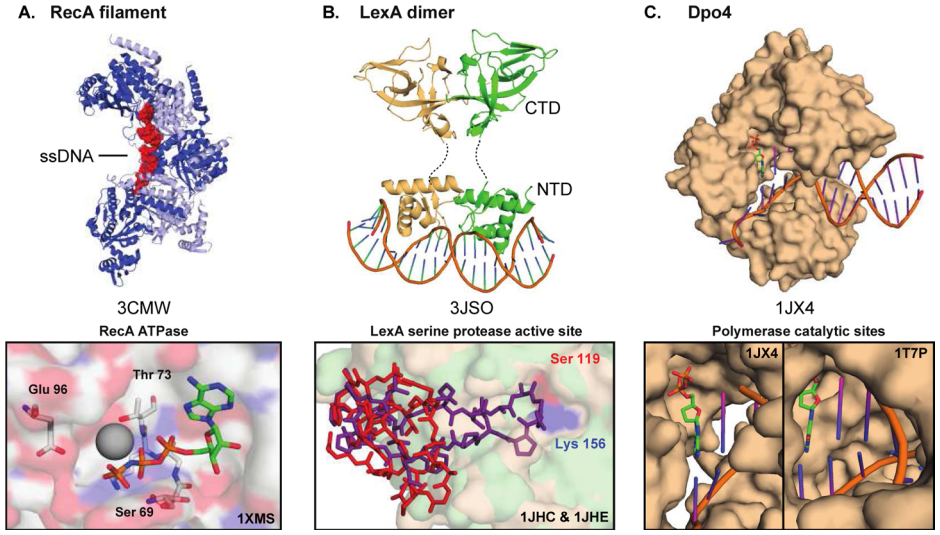


Figure 4. Targets of the SOS pathway. (A) Structure of the SOS sensor, RecA, shown as a filament (PDB entry 3CMV), with alternating monomers colored dark or light blue. The ssDNA is shown as red spheres. The panel below is a close-up of the ATP binding pocket (PDB entry 1XMS), a site that could be targeted. (B) Shown is dimeric LexA, bound to SOS box DNA (PDB entry 3JSO), with individual monomers colored green and yellow. The C-terminal protease domain (CTD) is connected to the N-terminal DNA binding domain (NTD) by a structurally unresolved linker (dashed line). In the self-cleavage mechanism, LexA undergoes a large conformational change in its C-terminal domain between inactive (red sticks, PDB entry 1JHC) and active states (purple sticks, PDB entry 1JHE) that positions the cleavage loop within the active site, adjacent to the Ser/Lys dyad. The overlaid active and inactive conformations are shown in the bottom panel. (C) Shown is a representative Y-family polymerase, Dpo4, an error-prone polymerase, bound to DNA (PDB entry 1JX4). Unlike high-fidelity T7 polymerase, shown for comparison (PDB entry 1T7P), Dpo4 possesses a more open, exposed catalytic site, which reduces the selectivity for the incoming nucleotide, colored green.

and colleagues have designed several high-throughput screens largely focused on *E. coli* RecA ATPase activity and identified potential inhibitors.[50−53] While antimicrobial activity against *E. coli* has not yet been described, one lead RecA probe, suramin, was characterized against *Mtb*, where the inhibitor was suggested to potentiate the activity of the fluoroquinolone ciprofloxacin.[54] Although studies aimed at inhibiting RecA are promising, specificity is one important consideration that needs to be explored. In mammals, RecA has up to seven important homologues (Rad51 family).[55] In this context, rational approaches using nucleotide analogues to target RecA's ATP binding site have been examined to a limited extent.[56] These offer a potential starting point for applying strategies that have yielded analogous protein kinase inhibitors that are ATP competitive and selective.[57]

The Regulator, LexA. The ∼22 kDa LexA molecule consists of two domains separated by a short flexible linker and exists as a homodimer in solution. The N-terminal domain (NTD) contains specific DNA binding activity, and the C-terminal domain (CTD) contains protease activity (Figure 4B).[58,59] Dimeric LexA binds to SOS box DNA through a winged helix−turn−helix motif in the NTD with dimerization mediated by the CTDs.[59] The CTD contains a protease active site, with a serine-lysine catalytic dyad. Self-cleavage occurs at a protein loop in the same monomer, located near the linker between the two domains.[58] Crystal structures of well-characterized LexA mutants show that this cleavage loop can exist in two distinct states. In the "non-cleavable" state, the loop is far removed from the active site. In the "cleavable" state, it undergoes a large ∼20 Å conformational change, positioning the scissile peptide bond adjacent to the active site serine.[58] Interestingly, in the "cleavable" state, LexA binds its peptide substrate in a sharp β-turn, rather than the extended β-sheet peptide conformation common to canonical proteases.[58,60]

Given the promising genetic studies on bacteria with noncleavable LexA discussed above, small molecule inhibition of LexA's protease domain has been proposed.[15,34] To this end, while LexA's distinct active site architecture offers potential advantages, it also poses two major challenges. First, as the substrate is tethered in *cis*, any competitive inhibitor will have to overcome the high local substrate concentration of the internal cleavage loop. Indeed, LexA shows inhibition only under large excesses of nonspecific protease inhibitors, such as diisopropyl fluorophosphates.[61] Second, given self-cleavage, classical high-throughput protease assays, such as using fluorophore quencher-containing peptides in *trans*, cannot be readily translated to LexA. Despite these challenges, rational or screening-based approaches to the discovery of LexA inhibitors are well-justified.

To help inform rational inhibitor discovery efforts, we have performed extensive mutagenesis of LexA to elucidate the substrate specificity determinants.[62] These experiments suggested that several residues within the cleavage loop make essential recognition contacts, while other specificity determinants are likely involved in facilitating LexA's conformational change. Interestingly, stabilization of the β-turn within the cleavage loop accelerates self-cleavage, suggesting that small cyclic peptides may be tractable rational inhibitor starting points.[62] Alternatively, allosteric inhibitors that prevent LexA's conformational change or small molecules that could disrupt the LexA−RecA interface are viable strategies for LexA inhibition; however, our understanding of the biochemical mechanisms involved is incomplete. Despite the available structural snapshots, the basis for LexA's conformational dynamics has not been elucidated and, in particular, the LexA−RecA interface remains poorly characterized despite dedicated efforts.[63,64] Further studies are required to understand these essential elements of RecA-induced LexA catalysis to help drive the discovery of potential SOS inhibitors.

DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582

The Effector, Error-Prone Polymerases. Foremost among the effectors in SOS mutagenesis are DNA polymerases (Pol II, IV, and V in *E. coli*).[33] These polymerases catalyze translesion synthesis (TLS) by replacing the replicative Pol III, which stalls when encountering a damaged DNA template.[65] The ability of these polymerases to catalyze TLS, however, is associated with an increased frequency of mutation because of the lack of 3′−5′ exonuclease proofreading activity, weak processivity, and low fidelity.[66,67]

Several of the critical enzymes involved in TLS, including Pol IV and Pol V in *E. coli*, are dissimilar enough from replicative polymerases that their identity as DNA polymerases came long only after the discovery of their role in mutagenesis.[24,68] Crystal structures of several of these "Y-family" DNA polymerases have yielded insight into their function and fidelity (Figure 4C). Despite a low level of sequence identity, the error-prone polymerases share the palm, finger, and thumb domains characteristic of their high-fidelity relatives (Figure 4C);[69−71] however, a detailed comparison shows structural differences that likely account for their lower fidelity.[24,72,73] The finger and thumb domains of the error-prone polymerases are in general shorter and appended with an additional domain known as the little finger domain, which has specialized function in Y-family polymerases. Further, O-helices, which typically play a role in proper Watson−Crick base pairing, are absent from the finger domains. Overall, these modifications result in a more flexible and open active site that may facilitate TLS over DNA damage due to bulky adducts or strand cross-links. Notably, elegant studies using FRET or time-resolved crystallography have demonstrated that, despite the appearance of a more static open active site, enzyme dynamics are critical to lesion bypass and catalysis.[74,75]

The importance of these error-prone polymerases in generating mutation and resistance, combined with their relaxed fidelity, suggests the opportunity to inhibit these enzymes with small molecules. While to the best of our knowledge no specific inhibitors of bacterial Y-family polymerases have been discovered, there is a rich precedent for use of specific nucleotide drugs to combat viral infection or cancer. The structural features of Y-family polymerases could potentially be exploited to achieve the required specificity needed for a polymerase inhibitor. For example, the more open active site and lack of requirement for canonical Watson−Crick base pairing may permit incorporation of bulky chain-terminating nucleotides that would be discriminated against by high-fidelity polymerases. Because genotoxic antibiotics induce the expression of these error-prone polymerases, Y-family polymerase inhibitors would be predicted to synergize with SOS-inducing antibiotics.

TARGETING GENE TRANSFER

While hypermutation results in the production of novel antibiotic resistance determinants by small, relatively random mutations, which are not subjected to selection until after their inception, HGT involves DNA that has already survived selective forces. Gene transfer is therefore a highly efficient mechanism for bacteria to evolve and adapt, and the process has resulted in the massive dissemination of antibiotic resistance genes among, and between, different bacterial species. HGT has been shown to occur in highly diverse environments, ranging from the soil to intensive care units in hospitals to the human microbiome.[76−78] The clinical importance of HGT is highlighted by examples such as the emergence and recent dissemination of carbapenem-resistant Enterobacteriaceae.[79] For example, several reports on individual patients harboring *Klebsiella pneumoniae* with plasmid-encoded resistance to carbapenems have shown that other species, such as *E. coli* or *Serratia marcescens*, could be isolated from the same patient containing the identical resistance plasmid, suggesting the occurrence of HGT within the patient's microbiome.[79,80] Notably, the exchange of genetic information appears to be dependent on time. As a recent example, genomic studies of the gut microbiota of more than 100 healthy individuals revealed the presence of numerous antibiotic resistance genes in the human gut and showed that the diversity of these resistance genes increased with an individual's age.[81]

Despite the prominent role of HGT in the spread of antibiotic resistance, critical aspects of these processes remain poorly understood and present challenges to the idea of therapeutically targeting HGT. For instance, although the frequency of bacteria harboring antibiotic resistance determinants on mobile DNA (i.e., the end products of HGT) within certain environments is becoming better appreciated, the timing and location of HGT within and between different clinically relevant ecosystems remain to be elucidated. Without this information, it is difficult to predict the impact of active HGT in clinical models, especially when considering specific environments (e.g., intensive care units vs the microbiome of an individual patient). Furthermore, unlike the conserved SOS response, HGT mechanisms are highly diverse, making the targeting of HGT conceptually more difficult. Despite these clear challenges, the dire need for novel therapeutic paradigms highlights the importance of seeking out commonalities in HGT mechanisms and exploring the plausibility of targeting these pathways.

The movement of large DNA blocks can be broken into two general steps: DNA recombination and transport (Figure 5). Both recombination and transport are subject to regulation by stress responses. As an example, lytic gene expression of many temperate phages has long been known to be triggered by bacterial stress, in particular the SOS response (described above). More recent insights into the regulation of gene transfer, however, suggest this may be a broad theme. For instance, functional SOS boxes have been found within integrons and shown to regulate activation of the gene transfer by controlling expression of the integrase gene.[38] Likewise, in pathogens such as *Vibrio cholerae*, a large integrative and conjugative element encoding resistance to multiple antibiotics is under the control of a LexA homologue, SetR, which also requires SOS activation for mobilization.[82] Additionally, stress-linked mobile elements are also critical to causing disease, including the superantigens encoded by pathogenicity island genes in *Staphylococcus aureus* or Shiga-toxin production by some enteric pathogens.[83,84] Stress responses have further been linked to natural competency in *Streptococcus pneumoniae* and can enhance conjugational recombination rates.[85,86] Thus, like SOS mutagenesis, gene transfer events that lead to acquired antibiotic resistance are closely linked to stress.

Specialized DNA Recombination and Mobile DNA. HGT is mediated by natural competency and transformation, transduction, and conjugation, with the latter mechanisms being more common in clinical isolates.[87] Whereas plasmid DNA can be maintained outside the chromosome, the life cycle of other types of mobile DNA relies on recombination with the host chromosome (Figure 5). These mobile DNA elements vary greatly in size and complexity, but all encode a specialized

DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582

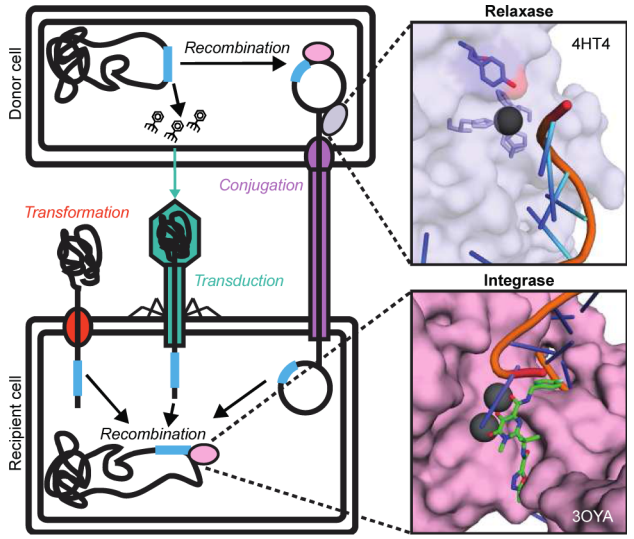


Figure 5. DNA recombination and transport are targets of horizontal gene transfer. Within the donor cell, site-specific recombination and transposition reactions (black arrows) can mobilize antibiotic resistance genes (blue rectangles) to the DNA transport machinery of a bacteriophage (transduction) or a type IV secretion system (conjugation). Environmental naked DNA can be taken up by natural competency (transformation). Once inside the recipient cell, the antibiotic resistance gene may be maintained within a plasmid or recombine with the recipient genome. DNA recombination could be targeted by inhibiting the DDE/integrase family of transposases: a related retroviral integrase is shown in complex with the small molecule raltegravir (PDB entry 3OYA). DNA transport could be targeted by inhibiting relaxase enzymes: the nicking enzyme of *S. aureus* is shown in complex with *oriT* DNA (PDB entry 4HT4).

recombination enzyme. These enzymes catalyze DNA breaking–joining reactions at the element termini, thus allowing for mobilization of the DNA element from its chromosomal site to distant sites. The simplest mobile element, the insertion sequence (IS), encompasses only a transposase enzyme and a pair of short inverted repeat sequences that flank the transposase gene and function as recognition sites where the DNA breaking–joining reactions occur. Transposons are more complex, with a set of inverted repeats that capture additional genes between them. Finally, bacteriophage elements may be more complex yet, encoding all the proteins necessary to conduct the infectious life cycle. Thus, unlike a simple IS, a transposon or phage is capable of mobilizing accessory genes that aid in its own dissemination, often by accumulating and evolving genes that endow its host with an increased rate of survival or pathogenicity such as virulence factors and antibiotic resistance determinants.[88]

Integrons make up a related class of DNA elements that function as gene assembly platforms to direct the expression of exogenous genes.[89] As with transposons, integrons may similarly be enriched with antibiotic resistance and virulence genes. Whereas transposons "hop" from location to location, occasionally capturing functionality along the way, integrons are stationary elements that collect exogenous genes and insert them into their DNA locus. Remarkably, once a set of genes is a part of the integron, the integrase enzyme can catalyze the rearrangement of those genes with respect to the promoter, thus changing which genes are expressed. In this way, integrons can serve as a cache of antibiotic-resistant genes to be deployed for future use if needed.

The two major classes of specialized recombination enzymes are site-specific recombinases and transposases. Both conduct transesterification reactions of the DNA phosphodiester backbone without a requirement for high-energy cofactors such as ATP.[90,91] Site-specific recombinases contain an active site Tyr (or Ser) residue, which forms a covalent intermediate with the DNA backbone. This allows them to conduct a conservative two-step reaction that catalyzes strand exchange between two different pieces of double-stranded DNA (dsDNA), resulting in an intact, ligated dsDNA product. Bacterial transposases generally fall into the DDE/integrase superfamily of enzymes, containing protein folds remarkably similar to integrases encoded by retroviruses.[92] The protein folds of these enzymes are topologically similar and bring at least three acidic residues (typically DDE) in the proximity of one another.[92] The active site acidic residues bind divalent metal cations, required cofactors for catalysis, which promote transesterification reactions via a two-metal ion mechanism without any protein–DNA covalent intermediate.[93,94]

Although there have been only a few studies identifying small molecule inhibitors of specialized bacterial recombination enzymes, the targeting of the retroviral integrase of HIV-1, a prominent integrase/DDE superfamily member, has been a significant clinical achievement and serves as a model for efforts to inhibit other specialized recombinases. The crystal structure of the Prototype Foamy Virus (PFV) retroviral integrase bound to donor DNA and different strand transfer inhibitors has been determined (Figure 5).[93] The structure supports a model in which the "diketo acid-like" pharmacophore of the inhibitors binds to the two active site divalent cations of the activated enzyme–donor DNA intasome complex. Inhibitor binding displaces the reactive 3′-OH of the donor DNA, thus deactivating the complex and inhibiting strand transfer. Diketo acid-like inhibitors have also been found for the Holliday junction (HJ) resolving enzyme encoded by poxviruses and for the Tn5 transposase,[95,96] suggesting that the motif may represent a general scheme for inhibiting members of this enzyme family. Small peptide inhibitors of site-specific Tyr recombinases that specifically bind to HJ DNA to interrupt enzymatic DNA transactions have also been identified.[97] One candidate peptide was able to block prophage excision by trapping such an intermediate, which also additionally resulted in antimicrobial activity, perhaps because of interference with DNA replication and repair.[98]

DNA Transport. For a DNA molecule to transfer between bacteria, it must cross lipid membranes, which is energetically disfavored. Large multiprotein molecular machines for natural competency, conjugation, and transduction allow for DNA shuttling and therefore represent potential targets for preventing the spread of resistance determinants (Figure 5). We refer our readers to recent reviews covering the molecular mechanisms and structural biology of each of these topics, noting that targeting of these processes with small molecules is virtually unexplored.[99−101] However, here we will highlight recent discoveries regarding the inhibition of a bacterial conjugation system with small molecules developed through rational design.

In bacterial conjugation, one strand (T-strand) of a dsDNA plasmid is transferred between organisms, ultimately resulting in the complete transfer of the genetic information encoded on the plasmid. The major components of conjugation are a relaxase enzyme, a type IV coupling protein (T4CP), and the membrane pore and pilus of a type IV secretion system (T4SS).

DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582

The relaxase enzyme, often as part of a multiprotein "relaxasome", recognizes the origin of transfer (*oriT*) sequence on the plasmid and nicks the T-strand to form a covalent 5′-phosphotyrosine linkage, thus creating a free DNA end to be transported through the membrane pore of the T4SS. After DNA transport and synthesis of the complementary strand, relaxase again nicks *oriT*, this time resulting in release and recircularization of the T-strand.

Recently, the Redinbo laboratory has discovered inhibitors for two types of relaxase enzymes using rational design based on structural insights. First, they determined the structure of an F-plasmid relaxase, whose catalytic cycle includes two simultaneous phosphotyrosine linkages to *oriT*. In the structure, they observed that a single divalent metal ion stabilized the formation of both phosphotyrosine linkages. On this basis, they reasoned that bisphosphonates could serve as functional mimics and potential inhibitors. After a directed screen against a small library of bisphosphonates, they found several that inhibited relaxase *in vitro* and inhibited conjugation in a relaxase and F-plasmid-dependent manner in cell-based assays.[102] In a different study, the group also determined the crystal structure of the nicking enzyme of *S. aureus* (NES) in complex with *oriT* DNA (Figure 5). NES is present on clinically important conjugative plasmids known to result in vancomycin-resistant *S. aureus*. Unlike the F-plasmid relaxase, NES forms only one phosphotyrosine linkage. In this case, catalysis could be disrupted using a small polyamide designed to bind specifically to a five-nucleotide region of *oriT* that makes critical contacts with NES.[103]

These examples, although preliminary, suggest specific targeting of the relaxasome is feasible and highlights the potential clinical utility of inhibitors of bacterial conjugation. Interestingly, in the F-plasmid case, inhibition of the relaxasome led to cell death.[102] Because gene transfer mechanisms are highly activated during antibiotic stress, this finding raises the interesting possibility that inhibiting effector pathways in creative ways could not only curtail gene transfer but also lead to cell death by poisoning the cell with trapped intermediates.

OPPORTUNITIES AND CHALLENGES FOR TARGETING THE EVOLUTION OF RESISTANCE

We have provided an overview of two mechanisms for the evolution and spread of antibiotic resistance within and between organisms: stress-induced mutagenesis caused by the SOS pathway and acquisition of resistance genes by HGT. Studying these and related pathways could not only provide insight into how bacteria evolve and adapt but also expose weaknesses that we are hopeful can be exploited in the form of new, "anti-evolutionary" therapeutics.

Pursuit of such therapies will pose a unique set of challenges and open up new areas of inquiry. As one notable challenge, purely anti-evolutionary drugs will not reverse preexisting genetic resistance in either nature or patients. Indeed, this problem is ancient and embedded in natural history, as the analyses of bacteria present before the rise of civilization have shown that antibiotic resistance genes far predate our use of antibiotics.[104] However, rather than focusing on the existing pool of resistance, we posit that anti-evolutionary agents could prevent, or at least delay, the *de novo* generation and acquisition of resistance in pathogens. Clinical cases in which pathogens are repeatedly exposed to antimicrobial therapy, as in the case of cystic fibrosis, severely immunosuppressed patients, complicated medical device infections, or mycobacterial infections, are the main areas in which such novel therapies might be employed. Additionally, the examples of the SOS response, prophage excision, and relaxase function suggest targeting of these processes can potentially lead to the accumulation of toxic intermediates and cell death.[37,98,102] Further, as stress responses are linked to bacterial persistence, biofilm formation, and the expression of virulence factors, targeting evolutionary processes may decrease pathogenicity.[11,105,106] In the case of HGT-mediated antibiotic resistance, while there is an assumption that all clinically relevant gene transfer events have already occurred by the time a patient shows signs of an infection, we do not know the timing of such events and, at a minimum, preventative strategies for patients at high risk of acquiring new resistant pathogens via HGT could be entertained. Despite challenges, the impetus for pursuing anti-evolutionary drugs comes from their potential to offer a one−two punch, potentiating the action of existing antibiotics that trigger stress responses and blocking the development of resistance.

Our overview also highlights the gaps in basic science knowledge regarding how to assess the clinical viability of targeting evolution. With genomic advances, we are learning more about the relative frequencies of clinically relevant resistance determinants. Many studies are also beginning to look at transmission events between patients within hospital settings, and even the molecular relationship between resistance genes found in human pathogens and the environment.[107−109] However, insight into the relative rates, timing, and location of each of these events remains lacking. Does antibiotic stress enhance the mutation rate to a degree that has biological consequences within an individual patient? What is the rate of clinically relevant HGT within microbial ecosystems such as the human gut? A similar set of questions applies to understanding the clinical relevance of nongenetic mechanisms for antibiotic tolerance, such as bacterial persistence and biofilm formation. This type of knowledge, broken down by relevant ecosystem and pathogen, and coupled to the kinetics of the biochemical steps, would offer enormous insight into which mechanisms to target, as it would tell us what the "rate-limiting" steps are in evolution and adaptation to antibiotics. We are hopeful further technological advances in DNA sequencing and microbiome research will lead to answers to these questions.

On the surface, the problem of antibiotic resistance appears insurmountable, but bridging the gaps in our knowledge holds the promise of unmasking great opportunities to intervene. The scale of the clinical problem suggests the need for innovative new approaches to antibacterials. It is our hope that these efforts will take many forms: from augmenting natural product discovery by accessing the uncharted molecular diversity present within "unculturable" organisms to counteracting nongenetic mechanisms that mediate antibiotic tolerance and, finally, targeting the very mechanisms that underpin the evolution of genetic resistance.[12,13,105]

AUTHOR INFORMATION

Corresponding Author

*E-mail: rkohli@upenn.edu. Telephone: (215) 573-7523.

Author Contributions

M.J.C. and C.Y.M. contributed equally to this work.

DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582

Funding

We acknowledge funding from the National Institutes of Health (NIH Director's New Innovator Award DP2-GM105444 to R.M.K., Training Grant T32 GM7229 for C.Y.M., and Grant T32 AR007442 for M.J.C.), the Doris Duke Charitable Foundation, and the Edward J. Mallinckrodt, Jr. Foundation.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge Mary Leonard for assistance with Figure 2 and Jeff Kubiak and Mandie Samuels for editorial assistance.

■ ABBREVIATIONS

MDROs, multi-drug-resistant organisms; HGT, horizontal gene transfer; MMR, mismatch repair; ssDNA, single-stranded DNA; TLS, translesion synthesis; IS, insertion sequence; dsDNA, double-stranded DNA; HJ, Holliday junction; PDB, Protein Data Bank.

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DOI: 10.1021/acs.biochem.5b00109
Biochemistry 2015, 54, 3573−3582