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Antibiotic Resistance and Epigenetics: More to It than Meets the Eye

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ABSTRACT   The discovery of antibiotics in the last century is considered one of the most important achievements in the history of medicine. Antibiotic usage has significantly reduced morbidity and mortality associated with bacterial infections. However, inappropriate use of antibiotics has led to emergence of antibiotic resistance at an alarming rate. Antibiotic resistance is regarded as a major health care challenge of this century. Despite extensive research, well-documented biochemical mechanisms and genetic changes fail to fully explain mechanisms underlying antibiotic resistance. Several recent reports suggest a key role for epigenetics in the development of antibiotic resistance in bacteria. The intrinsic heterogeneity as well as transient nature of epigenetic inheritance provides a plausible backdrop for high-paced emergence of drug resistance in bacteria. The methylation of adenines and cytosines can influence mutation rates in bacterial genomes, thus modulating antibiotic susceptibility. In this review, we discuss a plethora of recently discovered epigenetic mechanisms and their emerging roles in antibiotic resistance. We also highlight specific epigenetic mechanisms that merit further investigation for their role in antibiotic resistance.

KEYWORDS adaptive resistance, antibiotic resistance, antibiotics, bacterial epigenetics, methylation

The discovery of antibiotics brought about a revolution in the field of medicine. Antibiotics have become the backbone of modern-day health care. Several classes of antibiotics are widely used today, and they target essential processes in bacteria, including cell wall synthesis, translation, transcription, etc. (1). However, bacteria are known to acquire drug resistance by various means. Mobilization of genetic elements from different strains and the environment allows horizontal transfer of resistance-conferring genes (2). Mutations which confer resistance can also negatively affect bacterial fitness as they have important roles in cellular processes. But off-site compensatory mutations which negate this cost of fitness can lead to the stable resistance status of bacterial strains. Resistance mutations that do not compromise fitness have also been reported (3). The genetic basis of antimicrobial resistance has been studied for several decades. In Fig. 1, we have summarized the genetic mechanisms underlying antimicrobial resistance; these mechanisms have been extensively reviewed elsewhere (4–7). In addition to genetic mechanisms, bacteria acquire resistance to antimicrobials by modulating the expression of chromosomally encoded proteins as well as plasmid-borne efflux pumps (8).

In recent years, there has been a spike in interest to explore nonclassical mechanisms, including bacterial epigenetics which may contribute to antibiotic resistance in bacteria. Here, we introduce the readers to the latest findings in the field of bacterial epigenetics and provide a comprehensive account summarizing the biological roles of

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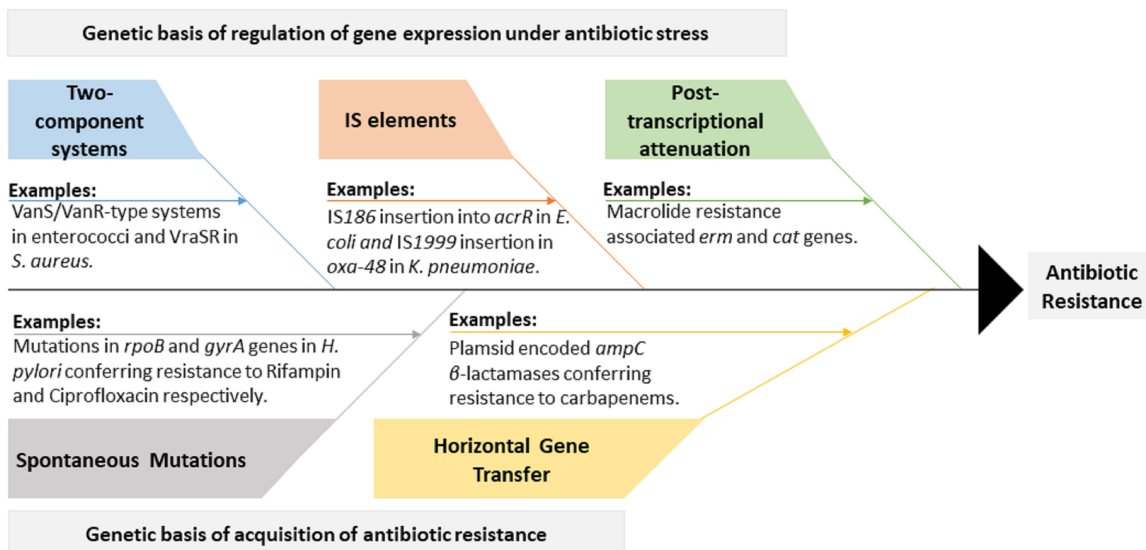
FIG 1 An outline of the genetic basis of antibiotic resistance. Bacteria can acquire resistance to antibiotics by spontaneous mutations in the target genes or their regulators. Interchange of mobile genetic elements among bacteria (horizontal gene transfer [HGT]) also contributes to the dissemination of antibiotic resistance genes. In addition to acquisition of genetic modifications, bacteria can also survive antibiotic stress by modulating their gene expression. Well-known mediators of gene expression changes in response to antibiotic stress include two-component systems, insertion sequence (IS) elements, and posttranscriptional attenuation of gene expression.

epigenetics and epigenetic modulators in the development of antibacterial resistance. In addition, we discuss multiple facets of bacterial epigenetics which have not been specifically explored in the context of antibiotic resistance. As genetic mechanisms cannot entirely explain the rapidity of resistance development or its transient nature (particularly in adaptive resistance), we believe that bacterial epigenetics may provide new answers.

BACTERIAL EPIGENETICS

In eukaryotic systems, an epigenetic trait has been defined as a "stably heritable phenotype resulting from changes in chromosomes without alterations in the DNA sequence" (9). Epigenetic mechanisms in eukaryotes, such as nucleotide modifications and histone modifications, have been studied extensively for decades. In contrast, bacterial DNA is not packaged in histones. Therefore, bacterial epigenetics is limited to modifications of bacterial DNA and RNA. In particular, methylation of bacterial DNA has been well studied. Methyl groups are transferred from S-adenosyl-L-methionine to adenine or cytosine by a group of enzymes called DNA methyltransferases (Mtases). The most commonly known DNA methyltransferases are those associated with the widely known defense mechanism in bacteria, the restriction-modification (R-M) system. As these methyltransferases add methyl groups only to specific sequences on the host bacterial DNA, unmethylated foreign DNA is recognized and degraded by endonucleases of these R-M systems. There are different groups of R-M systems depending on the proximity of the restriction site to the site of methylation and on the nature of polypeptides for restriction activity and modification activity (10). Bacterial DNA methyltransferases which do not belong to any R-M system are collectively called orphan methyltransferases. These orphan methyltransferases can regulate multiple cellular processes in bacteria, including cell cycle regulation, DNA mismatch repair, and regulation of gene expression (11).

Deoxyadenosine methylase (Dam) is an extensively studied orphan methyltransferase found in *Escherichia coli*; it is an adenine methyltransferase which adds a methyl group to the N^6 position of the adenine residue in the palindromic sequence GATC. The Dam methyltransferase has been shown to play a crucial role in methyl-directed mismatch repair in *E. coli* and related gammaproteobacteria (12). The mismatch repair

protein, MutH, recognizes unmethylated GATC and cleaves it. This ensures that only the newly synthesized DNA strand is cleaved; the parental strand is spared as the GATC sites in the parental strand are methylated (13). Dam-mediated methylation is also essential for regulation of replication initiation. The majority of the GATC sites in *E. coli* are completely methylated except for a transient period immediately after replication, during which they are present in a hemimethylated state. SeqA protein, which prevents reinitiation from hemimethylated *oriC*, allows only Dam to methylate the daughter strand after the removal of DnaA activity (responsible for initiation of replication) (14, 15). It has been postulated that Dam-mediated methylation is not a common mechanism of regulation of gene expression within the same generation. However, due to the ability of Dam to control methylation states transgenerationally, it can control phase variation (11, 16). Homologs of Dam with high sequence similarity have been found in several Gram-negative bacteria including *Salmonella* (17), *Vibrio cholerae* (18), etc. These homologs also methylate the adenine residue in the palindromic GATC sequence. YhdJ is a nonessential orphan methyltransferase, found in both *E. coli* and *Salmonella enterica*; this enzyme recognizes the sequence 5′-ATGCAT-3′ and adds a methyl group to the second adenine from the 5′ direction (19). Another well-studied orphan methyltransferase is CcrM; it recognizes and methylates the adenine residue in the sequence GANTC. CcrM utilizes hemimethylated DNA as a substrate, and its expression is cell cycle dependent. CcrM-mediated methylation is required for the functioning of some cell cycle regulators (such as GcrA and ctrA) (20, 21). CcrM is also reported in *Agrobacterium tumefaciens* (22) and other *Alphaproteobacteria* (23). In addition to adenines, orphan methyltransferases also methylate cytosines similar to their R-M counterparts. DNA cytosine methyltransferase (Dcm) found in *E. coli* can add a methyl group to the internal cytosine residue in the sequence 5′-CC(A/T)GG-3′ at the C^5 position. Cytosine methylation mediated by Dcm is not essential for survival, but it can regulate gene expression of sigma factor RpoS (which is a major regulator of genes expressed in stationary phase) and some of its targets (24). VchM, an orphan methyltransferase found in *Vibrio cholerae*, methylates the sequence 5′-RCCGGY-3′ at the first cytosine, but it is not necessary for survival (25). There are several other orphan methyltransferases that have been discovered over the years apart from the ones mentioned in this review; due to limited space, discussing them is beyond the scope of this review.

R-M systems, which involve host DNA modifications, represent one of the mechanisms by which bacteria protect themselves (26). The phosphorothioate system is another lesser known defense mechanism that works in a way similar to that of the R-M system. It involves sequence-specific modification of the sugar-phosphate backbone of host DNA, wherein a sulfur replaces a nonbridging oxygen. This modification is mediated by the products of the *dndABCDE* gene cluster. Unmodified backbone of foreign DNA is recognized and cleaved by DndFGH proteins (27–29). In many bacterial strains, it is found that the phosphorothioate modification exists without the presence of the cognate restriction component (29).

In addition to DNA modification, nucleotide modifications in RNA are also widely found in bacteria. rRNA and tRNA harbor the majority of RNA modifications. Since bacterial rRNA and tRNA modifications have been extensively studied and reviewed in the past (30–32), we will not be discussing these modifications in this review. The presence of RNA modifications in bacterial mRNA has been discovered only recently. Methylation of adenine residue at the N^6 position in the sequence 5′-GCCAG-3′ has been found to occur in the transcripts of *E. coli* and *Pseudomonas aeruginosa*. The m^{6}A modification is found to be enriched in the open reading frames (ORFs) of the transcripts (33). The enzyme(s) which brings about m^{6}A modification of mRNA in bacteria is yet to be identified. Another newly discovered modification of bacterial mRNA, a 5′ NAD cap, has been shown to prevent degradation of mRNA *in vitro* although the physiological significance is yet to be identified (30, 34). Modifications involved in bacterial epigenetics have been summarized in Fig. 2.
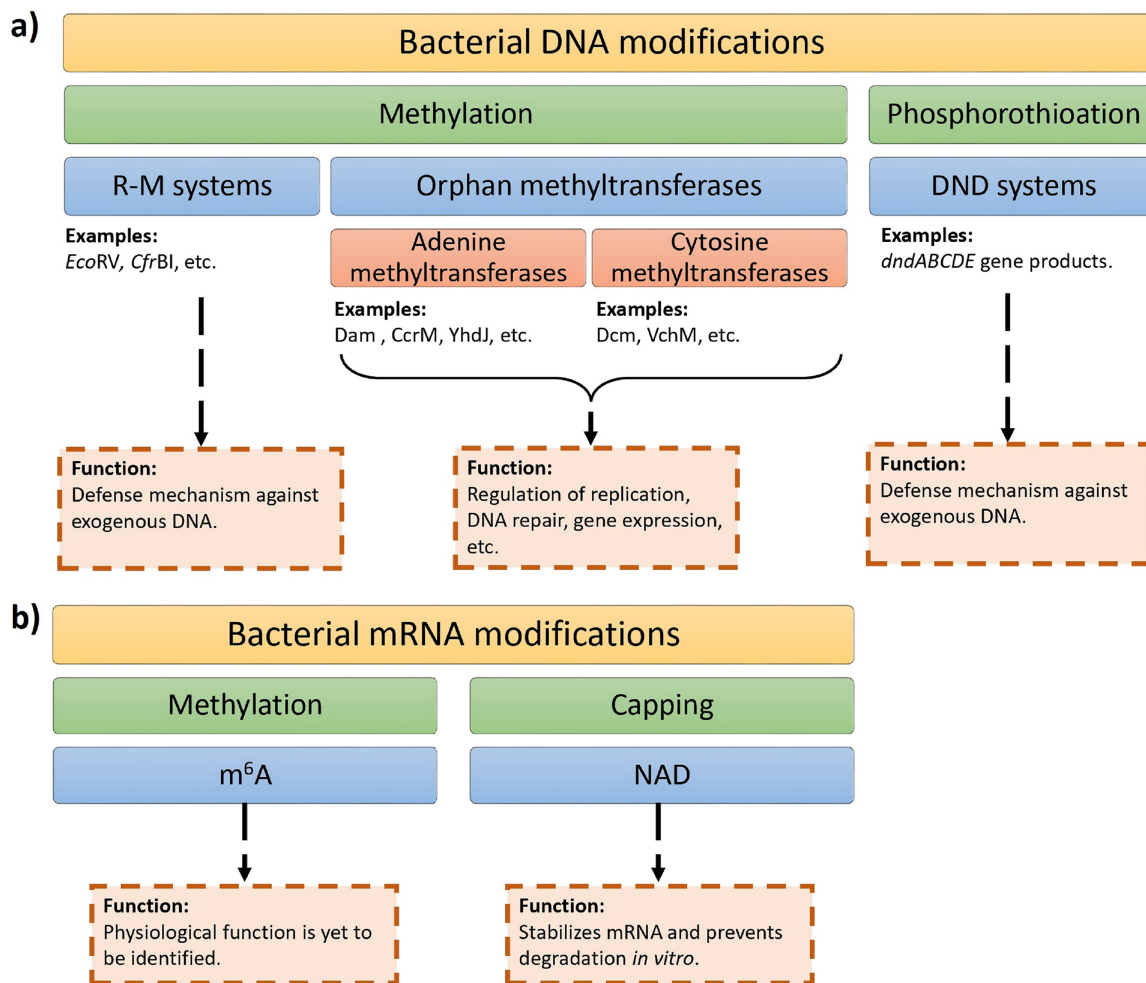
a)

Bacterial DNA modifications

Methylation		Phosphorothioation

R-M systems	Orphan methyltransferases		DND systems

Examples:
*Eco*RV, *Cfr*BI, etc.

Adenine methyltransferases	Cytosine methyltransferases

Examples:
Dam , CcrM, YhdJ, etc.

Examples:
Dcm, VchM, etc.

Examples:
dndABCDE gene products.

Function:
Defense mechanism against exogenous DNA.

Function:
Regulation of replication, DNA repair, gene expression, etc.

Function:
Defense mechanism against exogenous DNA.

b)

Bacterial mRNA modifications

Methylation	Capping

m^6A	NAD

Function:
Physiological function is yet to be identified.

Function:
Stabilizes mRNA and prevents degradation *in vitro*.

FIG 2 Overview of bacterial epigenetics. (a) There are two broad classes of bacterial DNA modifications: methylation of adenines and cytosines and phosphorothioation of the DNA backbone, where a nonbridging oxygen gets replaced by sulfur. Bacterial DNA methylation is mediated by enzymes belonging to the restriction-modification (R-M) systems or orphan methyltransferases. Phosphorothioation is facilitated by the gene products of a DNA degradation (DND) system, *dndABCDE*. (b) Two classes of epigenetic modifications of bacterial mRNA have been discovered: methylation of adenine at N^6 and NAD capping at the 5′ end.

EPIGENETIC TAGS AND BACTERIAL GENE EXPRESSION

Methylation brought about by DNA methyltransferases from R-M systems as well as orphan methyltransferases can alter gene expression by directly modulating the binding of RNA polymerase to promoters. For example, RNA polymerase cannot bind to the transposon Tn*10* promoter with fully methylated GATC sites. RNA polymerase can bind only hemimethylated DNA, and, thus, Tn*10* gets expressed only transiently following replication (35). Dam-mediated GATC methylation also represses the transcription of many genes including *trpR* and *trpS* (which control tryptophan operon in *E. coli*) due to decreased binding of RNA polymerase (10, 36). Dam-mediated regulation of gene expression during phase variation will be discussed later in this review with specific examples. Methyltransferase-mediated feedback repression is common in many bacteria (37–40). For example, in *Shigella sonnei*, a methyltransferase of the R-M system, SsoII, mediates its own repression through a negative-feedback loop (40). Negative regulation of the methyltransferases in R-M systems is crucial to ensure that the phage DNA does not get methylated along with that of the host.

Methylation can also promote gene expression. The essential cell cycle regulator in *Caulobacter crescentus*, GcrA, is a $\sigma70$ cofactor which binds to the majority of $\sigma70$-dependent promoters, but it is postulated that it can activate transcription only if a

nearby methylated adenine stabilizes it (41). Methylation of the gene promoter of the phase-variable outer membrane protein antigen 43, which plays a role in biofilm formation (42), auto-aggregation, and colony morphology (43), prevents the binding of its repressor OxyR in *E. coli*. The repressor can bind to the promoter only after replication, when the DNA is present in a hemimethylated state (44). The *tra* (transfer) operon in the virulence plasmid pSLT in *Salmonella* is transcriptionally activated by the protein encoded by *traJ*. Dam methylation negatively regulates the expression of *traJ*. A bacterial small RNA (sRNA) encoded by *finP*, which represses TraJ expression, gets activated by Dam methylation (10, 45). As horizontal gene transfer (HGT) contributes significantly to acquisition of drug resistance, epigenetic control of transfer operons which can mediate conjugal gene transfer can perhaps indirectly aid in dissemination of antibiotic resistance-associated genes.

Methylation of cytosines is primarily repressive. In *Vibrio cholerae*, DNA-protein interactions, such as transcription factor binding, occur in regions which exhibit underrepresentation of cytosine methylation by VchM (an orphan Mtase) (46). Even in *E. coli*, expression of genes encoding ribosomal proteins is negatively regulated by Dcm-mediated methylation (24, 47).

EVIDENCE LINKING EPIGENETICS AND ANTIBIOTIC RESISTANCE

Over the years, literature has primarily focused on the genetic basis for the development of antimicrobial resistance in bacteria. But some findings suggest that changes in the DNA sequence alone cannot fully explain neither the rapidity of the development of resistance nor the reversibility to susceptible phenotype. There is evidence that suggests that epigenetic mechanisms may contribute to resistance development. They are discussed in the following sections.

Adaptive resistance. When bacteria are subjected to increasing subinhibitory concentrations of an antibiotic, they develop adaptive resistance to the antibiotic (48–50). The characteristic feature of adaptive resistance is the rapidity with which the resistance phenotype emerges and reverts to a susceptible phenotype upon the withdrawal of the antibiotic. The survival rate of bacteria in subinhibitory concentrations of antibiotics cannot be explained by genetic mutations as expected mutation rates that lead to resistance are lower than the observed survival rates (48). Genetic changes also cannot explain the high reversion rates because for the bacteria to revert to susceptible phenotype, compensatory or back mutations are needed, and these are known to occur at a very low rate (51, 52). Adaptive resistance is the consequence of the presence of heritable phenotypic variations even in genetically identical bacteria from isogenic populations. Culture-based evolution studies, investigation of genetic knockout and transcriptomes, and modeling-based studies suggest that epigenetic inheritance and stochastic heterogeneity in gene expression patterns are responsible for this phenomenon (48, 51, 53–55).

As discussed in the previous section, epigenetic tags are known to influence the expression of genes in bacteria by modulating the binding of RNA polymerase or other factors such as transcription factors, repressors, etc. Figure 3 is a putative representation of the findings, which suggest that epigenetics can contribute to the development of adaptive resistance. Epigenetic inheritance is dynamic in nature and thus can explain the transience of adaptive resistance. The resistance which develops when bacteria grow in the presence of nonlethal concentrations of antibiotics could be the consequence of changes in the epigenetic landscape of the bacterial genome. As soon as the antibiotic stress is removed from the environment, the epigenetic changes are no longer sustained on the genome of the subsequent generations, and, thus, they retrogress to a susceptible phenotype.

Phase variation. Bacteria can survive highly dynamic environments by rapidly modulating the expression of certain genes in a switch-on/switch-off manner, and this reversible switch is called phase variation. There are several ways by which phase variation is brought about, such as DNA inversion, methylation/demethylation of gene promoters or other regulatory sequences, slipped-strand mispairing, homologous re-
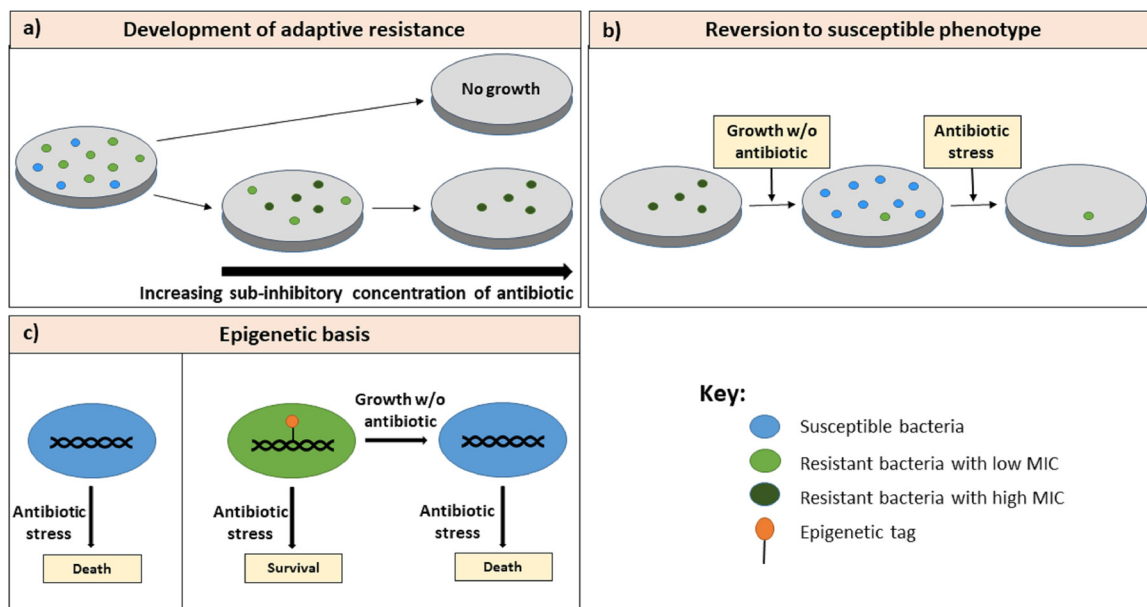
FIG 3 Epigenetic basis of adaptive resistance. (a) When bacteria are exposed to subinhibitory concentrations of an antibiotic, they acquire adaptive resistance and are able to survive in increasing concentrations of the antibiotic. (b) When the antibiotic is withdrawn, the bacteria that have acquired adaptive resistance revert to the susceptible phenotype (48). (c) The instability of the resistance phenotype can be explained by the dynamic nature of epigenetic inheritance that governs gene expression. In the presence of the antibiotic, the epigenetic landscape of the resistant bacteria is passed on to subsequent generations, whereas in the absence of the antibiotic, the epigenetic tags are lost. Epigenetic changes thus modulate gene expression patterns, allowing the bacteria to switch between susceptible and resistant phenotypes.

combination, and transposition (56, 57). Changes in methylation profiles of DNA regulate phase variation in bacteria, but the reverse is true as well, wherein phase variation leads to regulation of the expression of methyltransferases or changes in their target specificities (58–66).

Expression of the pyelonephritis-associated pilus (*pap*) operon in uropathogenic *E. coli* (67) and alteration of the length of lipopolysaccharide (LPS) O antigen in *Salmonella enterica* (which provides resistance to bacteriophages that use O antigen as receptor) (60) are well-studied examples of GATC methylation-mediated control of phase-variable gene expression. In *Streptococcus pneumoniae*, random switching that leads to genetic rearrangements in the *hsdS* gene of a type I restriction-modification system, called SpnD39III, generates six different variants of the gene, with each variant having a different target specificity. The six subpopulations that arise out of these rearrangements are phenotypically dissimilar due to global changes in methylation profiles (58).

Different DNA methyltransferases which exhibit phase-variable expression have been discovered in bacteria, including *Helicobacter pylori* (64), *Neisseria* (63, 68), and *Haemophilus influenzae* (66). Interestingly, phase-variable adenine DNA methyltransferases (Mod proteins) can influence susceptibility to certain antibiotics in *Neisseria meningitidis*. Common phase-variable methyltransferases found in *N. meningitidis*, ModA11 and ModA12, increase susceptibility to cloxacillin, ciprofloxacin, etc., which are widely used antibiotics for treatment of meningococcal disease. Absence of ModA11 has been associated with reduced susceptibility to ciprofloxacin and ceftazidime by 4-fold (62). It is unclear why bacteria retain methyltransferases that may increase drug susceptibility during their evolution. It is possible that these methyltransferases modulate the expression of other genes during phase variation to provide a hitherto unrecognized survival advantage to bacteria in a rapidly changing environment. Although the exact mechanisms of how these Mod proteins affect resistance or susceptible phenotype are not known, these results do indicate that epigenetic regulation of gene expression plays a role in antimicrobial resistance.
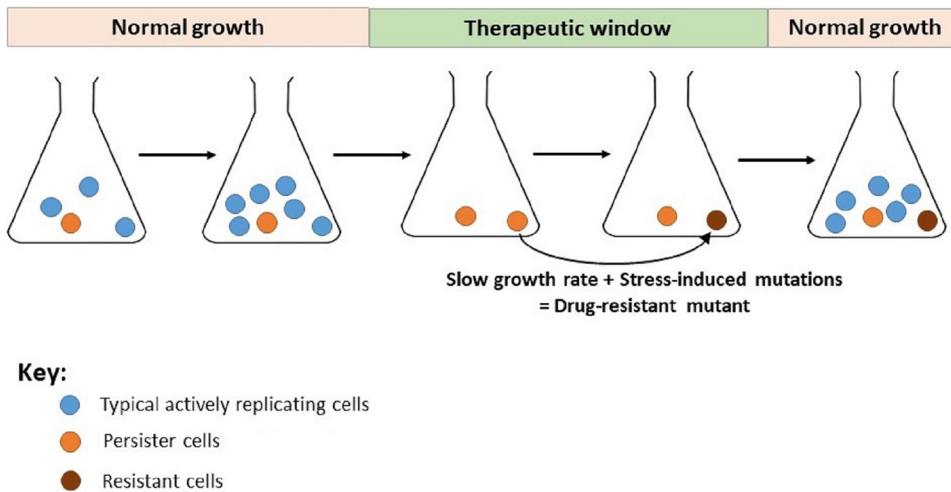
FIG 4 Bacterial persistence can contribute to development of resistance-conferring mutations. Persister cells represent a small proportion of the bacterial population that are growth arrested or slow growing. Persister cells are neither defective nor have specific genetic changes; they are present in bacterial populations as a seed bank to survive rapidly changing environments. When the bacterial population is subjected to antibiotic therapy, typical bacterial cells rapidly decline in numbers, but the persister cells are able to survive in the presence of antibiotics. Persister cells can even be induced by environmental stresses or other factors. Epigenetic inheritance has been predicted to be a key player contributing to phenotypic drug tolerance in persister cells. Persister cells can lead to adaptive evolution of drug-resistant mutants due to the low rate of growth and mutations induced by stress conditions.

Persistence and heteroresistance. A heterogenous response by members of an isogenic population to the same environmental cues resulting in varying fitness and survival under stress is called phenotypic heterogeneity (69, 70). This can sometimes lead to the generation of two distinct subpopulations, a phenomenon known as bistability (70, 71). Phenotypic heterogeneity and the resulting bistability can contribute to adaptive resistance (72) and formation of persister cells. Persister cells are typically described as slow-growing or growth-arrested cells in a bacterial population which can survive antibiotic treatment although recent evidence suggests that persister cells need not have altered growth rates (73). Upon the withdrawal of therapy, the persister cells can lead to a relapse of infection and thus are a major cause of recurrent infections. The presence of persister cells in a bacterial population is considered to be a bet-hedging strategy to ensure survival under changing milieus. But persister cells can also arise in response to environmental stresses. There are several mechanisms by which persisters arise, including the following: (i) spontaneous persistence via toxin/antitoxin systems and stochastic variability in expression of stress-related genes, (ii) environmental induction such as heat shock, nutrient deprivation, etc., (iii) inactivation of drug targets, (iv) reduction in drug uptake, (v) biofilm formation, (vi) quorum sensing from other persister cells, and (vii) a host immune response such as phagocytosis (74–78). Interestingly, epigenetic inheritance has been linked to the evolution of the persister phenotype. The plasticity of epigenetic inheritance along with within-generation heterogeneity (especially in stationary phase) can explain the rapid evolution of persister cells in changing environments (79). Genetic changes resulting in resistance to antibiotics has been suggested to follow persistence as a low rate of cell division coupled with stress-induced mutations provides a stable groundwork for adaptive evolution (74, 80, 81). The contribution of bacterial persistence to eventual development of resistance-conferring mutations has been illustrated in Fig. 4.

In an isogenic bacterial population, subpopulations can exhibit heterogeneous susceptibilities to an antibiotic, and this phenomenon is termed heteroresistance. Heteroresistance has been reported in both Gram-positive and Gram-negative bacteria (82–84), and it is a cause for concern as it can lead to antibiotic treatment failures (85). Epigenetic regulation of gene expression has been suggested to be one of the potential

mechanisms that explain heteroresistance (86–88). Heteroresistance can increase the likelihood of the generation of genetic resistance to antibiotics (88).

Contrasting roles of Dam- and Dcm-mediated methylation in *E. coli*. An inverse correlation exists between resistance to a fluoroquinolone (ciprofloxacin) and global m^5C levels in genomic DNA of resistant *E. coli*. An analysis of 40 clinical isolates of *E. coli*, including 30 ciprofloxacin-resistant isolates, suggests that the global levels of m^5C in genomic DNA inversely correlated to the MIC of ciprofloxacin. Ciprofloxacin-sensitive *E. coli* isolates had higher levels of m^5C than ciprofloxacin-resistant isolates (89).

SugE is a well-studied multidrug efflux system protein belonging to the small multidrug resistance (SMR) protein family. Overexpression of SugE in *E. coli* has been linked to increased resistance to quaternary ammonium compounds (90). The promoter region and the gene body of the SugE gene in *E. coli* have one Dcm site [i.e., 5′-CC(A/T)GG-3′] and three Dcm sites, respectively. Interestingly, *E. coli dcm* mutants expressed SugE mRNA at 7-times-higher levels than *dcm*-expressing wild-type *E. coli*. Importantly, increased SugE expression led to increased resistance to ethidium bromide. Furthermore, *dcm* mutants of *E. coli* expressed much higher levels of a stationary-phase sigma factor, RpoS. Robust SugE expression in the early stationary phase of *E. coli* is dependent on RpoS expression. In other words, Dcm may (i) directly repress SugE expression by promoter/gene body methylation and (ii) indirectly repress SugE expression by promoter/gene body methylation of a sigma factor (RpoS) that modulates the transcription of SugE (91). This example highlights a multilayered control of efflux pump proteins by *dcm*-mediated methylation. Another study has linked the antimicrobial resistance phenotype in *Enterobacter cloacae* to SugE expression (92). It would be interesting to study the role of epigenetic players in modulation of SugE expression in *E. cloacae*.

Dam influences virulence of uropathogenic *E. coli* by regulating expression of the *pap* operon. The results of a recent study (93) indicate that Dam also influences resistance to several antibiotics. In *dam* mutants, a severalfold decrease in resistance was observed for ciprofloxacin, gentamicin, etc. This suggests that Dam-mediated N^6-adenosine methylation facilitates resistance development in *E. coli*. An earlier study has also shown similar results wherein a 5-fold increase in survival rate in the presence of nalidixic acid was observed when *dam* mutants were complemented with expression plasmids carrying a functional *dam* gene (48). Investigation of the *E. coli* genome using single-molecule real-time (SMRT) sequencing has revealed that Dam-mediated GATC methylation is crucial for prevention of deleterious mutations and survival of bacteria under antibiotic stress and that *dam* strains exhibit hypersensitivity toward quinolone and β-lactam antibiotics (55).

These studies clearly point toward the opposing roles of adenine and cytosine methylation in *E. coli*. While Dcm-mediated cytosine methylation increases susceptibility to antibiotics, GATC methylation by Dam gives a survival advantage to *E. coli* under antibiotic stress (Fig. 5).

INTERPLAY BETWEEN EPIGENETICS AND GENETICS

Genetic changes or rearrangements in methyltransferases can change target specificities (59, 94), thus altering the epigenetic landscape of bacterial genomes. Conversely, methylation of adenine and cytosine residues can alter the genetic makeup by either facilitating or preventing mutations.

Methylated cytosines act as mutational hot spots. Cytosine undergoes spontaneous deamination to uracil. Since the presence of uracil is atypical in genomic DNA, it gets excised by uracil-DNA glycosylase, initiating the base excision repair pathway. But spontaneous deamination of 5-methylcytosine (m^5C) yields thymine, which is not easily recognized by the DNA repair machinery and thus may not get excised or repaired (95). This results in fixing of the mutation in the genome (Fig. 6, left box). The frequency of deamination of m^5C is 2 to 4 times higher than that of cytosine (96). Methylated cytosines are regarded as hot spots for cytosine to thymine mutations in
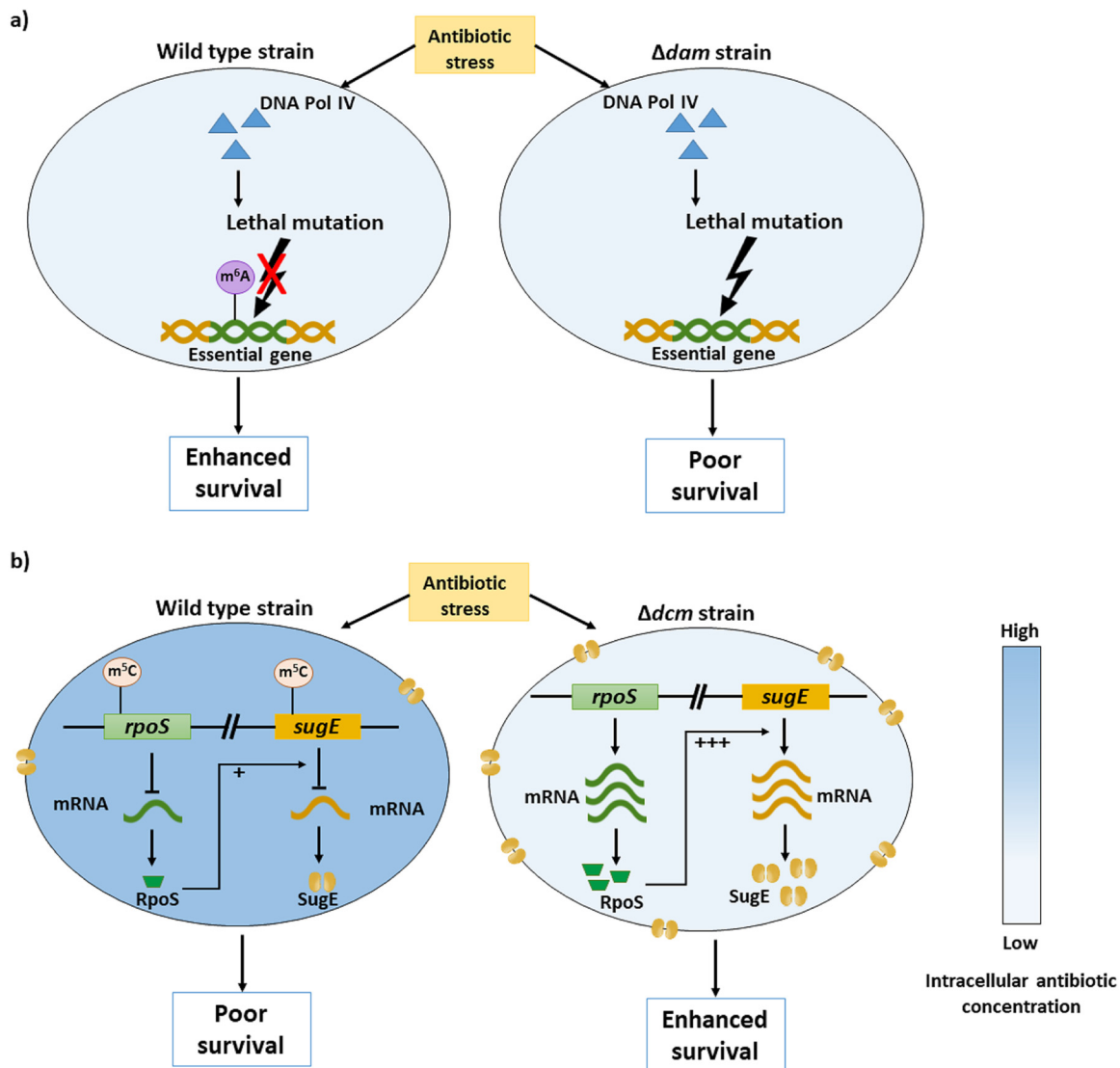
FIG 5 Contrasting roles of Dam- and Dcm-mediated methylation in antibiotic stress. Global methylation profiling identified increased adenine methylation and reduced cytosine methylation in antibiotic-resistant bacteria compared to levels in bacteria susceptible to antibiotics. (a) Dam-mediated adenine methylation facilitates DNA repair, reducing deleterious mutations in the bacterial genome and enhancing bacterial survival under antibiotic stress (55). (b) On the other hand, cytosine methylation has been linked to reduced expression of resistance-conferring genes such as *sugE* (a multidrug efflux pump) and *rpoS* (a sigma factor which modulates *sugE* expression), leading to poor survival under antibiotic stress. Robust *rpoS* expression from the Δ*dcm* strain can enhance transcription of the *sugE* gene, representing a second layer of control or downstream effects linked to epigenetic control of gene expression in bacteria. In other words, *sugE* transcript levels are controlled by epigenetic mechanisms at two levels, as follows. (i) The presence of methylated cytosines in the *sugE* promoter or gene body is associated with reduced levels of *sugE* transcripts. (ii) The extent of inhibition of *sugE* transcription is further augmented by the lack of *rpoS* (a transcription factor) expression (91).

bacteria (97) although very-short-patch (VSP) mismatch repair may reduce the frequency of such mutations (98).

Overexpression of the methyltransferases, Dcm and EcoRII methylase, in *E. coli* resulted in almost a 50-fold increase in C-to-T transitions at the canonical site of methylation (i.e., 5′-CC(A/T)GG-3′; the substitution is underlined). Interestingly, the overexpression of these methyltransferases also led to a 10-fold increase in the frequency of C-to-T transitions at a noncanonical site (i.e., 5′-CCGGG-3′). This finding suggests that promiscuous methylation at noncanonical sites may significantly increase the extent of methylation-mediated genetic changes than previously predicted (99). In addition, methylated cytosine derivatives, including 5-hydroxymethylcytosine, have been shown to induce C-to-T transitions in *E. coli* (100). A recent report analyzing
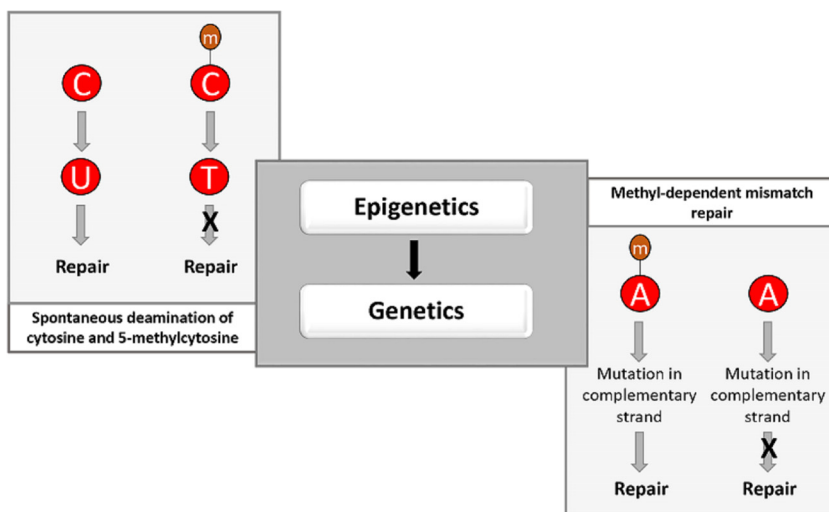
FIG 6 The epigenetic landscape influences bacterial genetics. Spontaneous deamination of cytosine yields uracil that is recognized by the DNA repair machinery, which corrects it back to cytosine. However, in case of methylated cytosines, deamination yields thymines which are not recognized as aberrant nucleotides in DNA, making an irreparable lesion; during subsequent DNA replication the thymine base pairs with adenine, and the lesion becomes permanent or fixed (left box). Methyl-dependent mismatch repair mechanisms correct mutations in the complementary strand during replication; this mode of repair is dependent on the presence of GATC sites with methylated adenines on the parental strand in the vicinity of the lesion on the complementary (nascent) strand. In the absence of methylated adenine in the parent strand, the mutation in the complementary (nascent) strand is not repaired (right box).

whole-genome sequences of thousands of bacteria suggests that cytosine methylation may increase the rate of C-to-T transitions by over 50-fold (101).

Environmental stresses, including exposure to antibiotics, induce the expression of error-prone polymerases to increase genetic variability of bacterial genomes (102). Occurrence of stable mutations that facilitate antibiotic resistance requires a high frequency of mutations. Methylation of cytosine provides a stable framework for the emergence of mutations which can potentially be either deleterious or beneficial for bacteria under antibiotic stress, depending on the site of mutation. Studies investigating the synergy between antibiotic stress-induced error-prone polymerases and C-to-T transitions of methylated cytosines may help us better understand the specific role of methylation-mediated genetic changes in regulating antimicrobial resistance.

Methyl-dependent mismatch repair requires GATC methylation. In *E. coli*, a methyl-dependent mismatch repair system (also called MutHLS system) corrects insertion/deletion loops (which cause frameshifts) as well as base pair mismatches (103). These mismatches can arise due to multiple reasons, including spontaneous deamination of C/m^{5}C, replication errors, etc. But their frequency increases under stress conditions due to the induction of error-prone DNA polymerases such as Pol IV (104) and Pol V (105). The MutHLS system requires Dam-mediated GATC methylation to differentiate between a newly synthesized strand and a parental strand; the GATC sites on the parental strands are methylated while the new strand lacks methylated GATC sites. MutH is the endonuclease which preferentially nicks the new strand at an unmethylated GATC site nearest to the mismatch. The repair machinery gets recruited to the site of the nick, and helicase displaces the new strand and is followed by exonuclease-mediated cleavage, resynthesis, and ligation (103). In the absence of methylated GATC sites, the MutHLS system generates lethal double-stranded breaks in the DNA (106) (Fig. 6, right box).

Poor survival rates of *E. coli dam* mutants in the presence of antibiotics has been reported (55). Antibiotic stress induces the expression of *dinB* which encodes the mutagenic DNA Pol IV; as a result, higher survival rates were documented for *dam dinB* mutants under antibiotic stress. However, as mismatch repair by the MutHLS system is
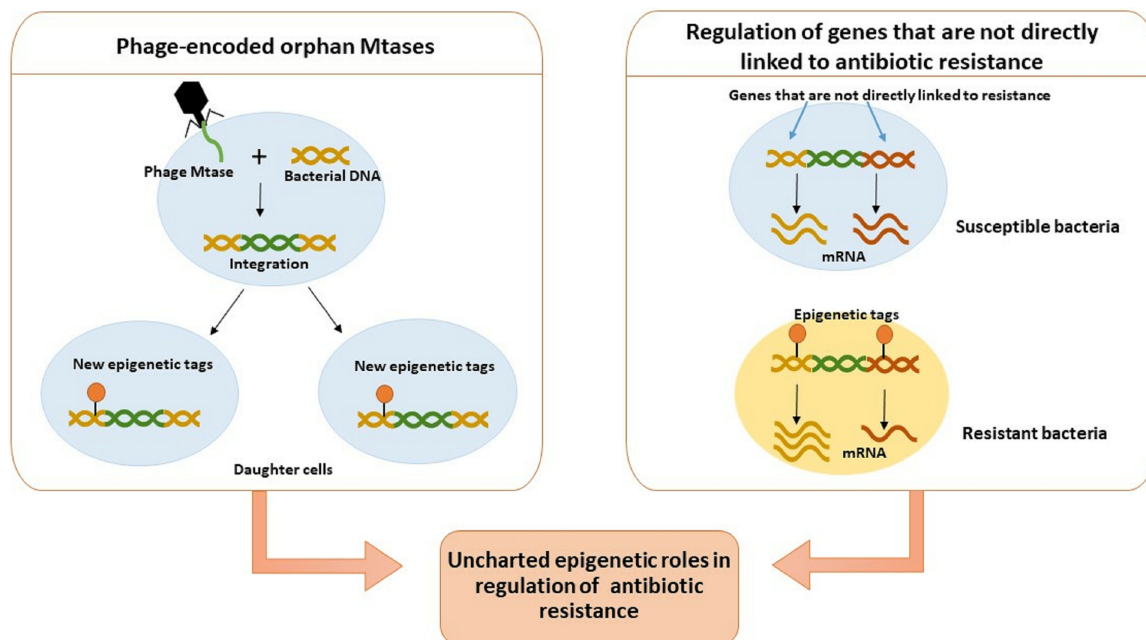
FIG 7 Uncharted epigenetic mechanisms and their putative role in antibiotic resistance. Some epigenetic mechanisms are putatively associated with antibiotic resistance but remain poorly documented or partially understood. When methyltransferase-encoding phages infect bacteria, the phage DNA encoding methyltransferases (Mtases) can get integrated into the bacterial genome and influence the methylomes of the daughter cells. The link between altered methylomes and antibiotic resistance has been proposed by several groups, but direct evidence for this hypothesis is still lacking (left box). Epigenetic regulation of expression of genes that are not directly associated with antibiotic resistance has been predicted to contribute to the resistant phenotype, but the underlying mechanisms are poorly understood (right box).

dependent on Dam-mediated GATC methylation and as *dam* mutants lack methylated GATC sites, survival of *dam* mutants is compromised due to increased mutations generated by DNA Pol IV. Better survival of *dam* mutants in the presence of ampicillin after deletion of MutH- or MutS-encoding genes also clearly indicate that MutHLS-mediated double-stranded DNA breaks are fatal in the absence of GATC methylation.

AN EPIGENETIC LINK TO UNCHARTED MECHANISMS IN ANTIBIOTIC RESISTANCE

There are several gray areas in bacterial epigenetics which need to be explored further to understand if they have any influence on the rapidity and high frequency of antimicrobial resistance development. A potential role for epigenetic mechanisms will be discussed in two nonoverlapping themes (Fig. 7) in the following sections.

Phage-encoded orphan methyltransferases. Analysis of full-length bacterial genomes suggests that a sizable portion of their genome is made up of prophages or phage DNA that has integrated into bacterial genomes (107). Bacteriophages can incorporate bacterial genes into their own genome, including DNA methyltransferases. Not only does this facilitate HGT among bacteria, but it also helps these phages overcome the R-M systems of newer hosts, thus broadening their host range (108). Over 800 putative orphan methyltransferases are present in currently available annotated phage genomes, which account for almost 20% of these genomes, and they include m^6A, m^5C, and m^4C classes of methyltransferases (108). Orphan adenine methyltransferases homologous to Dam are present in several bacteriophages such as phage T4, phage P1, phage *Vibrio harveyi* myovirus-like (VHML), 936-type phages, etc. (10, 108–112). The phage T4 adenine methyltransferase has the same target site (i.e., GATC) as Dam of *E. coli*, and it protects the viral DNA from cleavage by restriction endonucleases which recognize this site (109, 113). The methyltransferase of phage P1 also methylates the adenine residue in the sequence GATC (111), and it plays a role in the packaging of DNA in addition to protection from host endonuclease (108, 114). It has been proposed that in the bacteriophage VHML, GATC methylation in the ORF of the *rha*

antirepressor gene mediates the switch from the lytic to lysogenic cycle (110). Evidently, methyltransferases encoded by phages have functions other than protection from host R-M defense systems. Furthermore, orphan methyltransferases in bacteriophages may methylate cytosines or adenines at multiple recognition sites (115). We speculate that since phages can integrate into host genomes and also mediate HGT among bacteria, genes which encode phage methyltransferases may integrate into host chromosomal DNA and potentially influence the bacterial methylome of subsequent generations as shown in Fig. 7 (left box). While the contribution of prophages to genomic diversification in bacteria (107), modulation of fitness (116), and virulence of bacteria (107) is known, their specific roles in contributing to antimicrobial resistance is not well studied.

Regulation of genes that are not directly associated with antibiotic resistance. In several bacterial species, resistance-associated intrinsic genes such as efflux pumps are upregulated in the presence of antibiotic stress. Bacterial sRNAs, which can be regulated by DNA methylation (45), can influence the expression of efflux pumps under antibiotic stress. Recently, a genome-wide interrogation using single-molecule real-time (SMRT) sequencing was used to identify differentially methylated genes in laboratory-generated rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains (117). The investigators found that in both rifampin- and isoniazid-resistant strains, 175 genes were hypermethylated while 160 genes exhibited hypomethylation compared to levels in sensitive strains. Interestingly, it was observed that the nitrogen metabolism pathway was enriched for differentially methylated genes in both rifampin- and isoniazid-resistant strains. Also, rifampin, which inhibits transcription by targeting RNA polymerase, induces differential methylation in genes of the ribosome pathway. Clearly, antibiotic stress influences the methylation profile of genes involved in essential pathways, but further investigation is required to understand the significance or the role of the differential methylation status of these genes in antibiotic-resistant bacteria. Additionally, we reflect that since conditionally essential genes (such as enzymes responsible for utilizing carbon sources, biosynthesis of membrane proteins, amino acids, etc.) aid in the survival of bacteria under nutrient deficit or other stress conditions (118), this group of genes should be scrutinized to discern if epigenetic regulation plays a role in their expression, leading to better survival under stress conditions, including antibiotic stress (Fig. 7, left box).

CHALLENGES IN UNDERSTANDING THE EPIGENETICS OF ANTIBIOTIC RESISTANCE

Our current understanding of bacterial epigenetics is still elementary. For example, the role of methylation in bacterial genomes is not well understood beyond R-M systems. Modifications in eukaryotic mRNAs regulate cellular processes such as translation, mRNA decay, etc. (119, 120). As nucleotide modifications in bacterial mRNAs are being increasingly reported (33), understanding the role of epigenetics in bacteria has become crucial. The need to understand the role of epigenetics in antimicrobial resistance in bacteria, genome- and transcriptome-wide interrogation and functional analyses of differentially methylated genes or regulatory elements are increasingly recognized. Genome-wide high-resolution mapping of epigenetic landscapes have started to emerge with the advent of single molecule real-time (SMRT) sequencing technology and nanopore-based sequencing technology. These technologies allow the detection of various types of nucleotide modifications in the same run, such as m^6A, m^5C, m^4C, etc., without the need for prior chemical treatment to identify modified nucleotides (121–125). In addition, new sequencing technologies allow single-base resolution of bacterial transcriptomes, thus enabling direct identification of nucleotide modifications in mRNAs. But a major drawback of these sequencing technologies is the dependence on populations to achieve a consensus sequence which provides no information on the epigenetic heterogeneity which exists within different cells of isogenic populations. As discussed earlier in this review, epigenetic heterogeneity is a major driver of adaptive resistance. Researchers are now attempting to overcome this

limitation by developing newer pipelines which allow better resolution and detection of epigenetic heterogeneity (122). Although curated databases for bacterial gene regulation are now available for a few bacteria (126, 127), epigenetic regulatory networks in bacteria remain poorly understood to be cataloged.

CONCLUSION AND FUTURE PERSPECTIVES

Since well-documented biochemical or genetic changes fail to fully explain mechanisms underlying antibiotic resistance, it is becoming increasingly evident that we need to shift our focus to newer, nonclassical mechanisms such as epigenetic regulation. The biological role of epigenetic modifications in modulating gene expression and other cellular processes is being increasingly recognized. New epigenetic modifications are being discovered in both prokaryotes and eukaryotes, including phosphorothioation in bacterial DNA backbone (27, 29) and acetylation of cytidine in eukaryal mRNA (120). Since it has been established that modifications in eukaryotic mRNAs can modulate a variety of cellular processes (119, 120), it may not be too speculative to suggest that such modifications in bacterial transcripts may be linked to critical functions in the bacterial life cycle. Even though m^{6}A modification in bacterial mRNAs has been identified, the epigenetic writers (the enzymes that add the epigenetic tag to the nucleotide), readers, etc., for this modification are yet to be identified. The influence of epigenetics on the heterogeneity among members of isogenic bacterial populations merits detailed studies.

While the literature from the last decade reviewed here unambiguously indicates a role for epigenetics in antibiotic resistance among bacteria, we believe that precise mapping of epigenetic tags on bacterial genomes and their functional analysis will help create a paradigm shift in our understanding of this field. Furthermore, epigenetic modifications on bacterial genomes represent new diagnostic markers as well as novel drug targets.

ACKNOWLEDGMENTS

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