A Positive Perspective on DNA Methylation: Regulatory Functions of DNA Methylation Outside of Host Defense in Gram-Positive Bacteria

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Abstract

The presence of post-replicative DNA methylation is pervasive among both prokaryotic and eukaryotic organisms. In bacteria, the study of DNA methylation has largely been in the context of restriction-modification systems, where DNA methylation serves to safeguard the chromosome against restriction endonuclease cleavage intended for invading DNA. There has been a growing recognition that the methyltransferase component of restriction-modification systems can also regulate gene expression, with important contributions to virulence factor gene expression in bacterial pathogens. Outside of restriction-modification systems, DNA methylation from orphan methyltransferases, which lack cognate restriction endonucleases, has been shown to regulate important processes, including DNA replication, DNA mismatch repair, and the regulation of gene expression. The majority of research and review articles have focused on the epigenetic regulatory contribution of bacterial DNA methylation in the context of Gram-negative bacteria, with emphasis towards *Escherichia coli*, *Caulobacter crescentus*, and related Proteobacteria. Here we summarize the epigenetic functions of DNA methylation outside of host defense in Gram-positive bacteria, with a focus on the regulatory effects of both phase variable methyltransferases and DNA methyltransferases from traditional restriction-modification systems.

Introduction

The occurrence of genomic DNA methylation is ubiquitous across all three domains of life, where modification events function in diverse and critical cellular processes. In eukaryotes, the predominant type of DNA methylation is 5-methylcytidine (m5C) and the presence of these modifications is necessary for the regulation of gene expression and development (Jones, 2012; Chen and Zhang, 2019). In humans, aberrant DNA methylation events are implicated in numerous disease states, including cancer (Jones, 2012; Smith et al., 2017; Zhou et al., 2018). In addition to m5C, the genomes of bacteria are known to include N4-methycytidine (m4C) and N6-methyladenine (m6A) modifications [(Blow et al., 2016) and references therein]. A recent survey of prokaryotic genomes demonstrates the widespread occurrence of m5C, m4C, and m6A, where at least one type of modification was detected in 93% of the ~230 genomes analyzed (Blow et al., 2016). For all of the prokaryotes included in the study, DNA methylation was detected using Pacific Biosciences (PacBio) Single-Molecule Real-Time (SMRT) sequencing platform (Flusberg et al., 2010). PacBio SMRT sequencing uses inferences from DNA polymerase kinetics during sequencing reactions to detect the presence of DNA base modifications without prior knowledge of the presence of genomic methylation or the sequence contexts in which modifications occur (Flusberg et al., 2010). In the survey, 75% of the modifications detected were m6A, which is likely an overrepresentation of m6A relative to cytosine methylation because PacBio SMRT sequencing is more robust for detection of m6A and m4C modifications but is not well suited for the detection of m5C modifications (Blow et al., 2016; Flusberg et al., 2010). In addition to the Blow *et al.* study, New England Biolabs (NEB) maintains a free database, REBASE, that serves as a repository for bacterial genome methylomics results as well as information about predicted MTases, REases, and their putative recognition sites (http://rebase.neb.com). This resource is available to scientists interested in understanding if DNA methylation is detected or predicted in a genome of interest.

The importance of DNA methylation in bacterial genomes can also be highlighted by the diverse processes in which they function, including protection from the invasion of

foreign DNA (Loenen et al., 2014; Loenen et al., 2014), phase variation (Atack et al., 2018; Hernday, Braaten, and Low, 2003), the regulation of DNA replication (Han et al., 2004; Nievera et al., 2006), strand discrimination during DNA mismatch repair (Bale, d'Alarcao, and Marinus, 1979), and the regulation of gene expression (Casadesus and Low, 2013). The majority of the methylation-dependent processes listed above have been extensively studied and reviewed for Gram-negative bacteria (Sanchez-Romero, Cota, and Casadesus, 2015; Marinus and Lobner-Olesen, 2014; Mouammine and Collier, 2018; Adhikari and Curtis, 2016). This bias in study towards Gram-negative bacteria is reflected in the organisms included in the survey of prokaryotic DNA methylation, where 57% of the prokaryotes included were Gram-negative organisms, 33% were Gram-positive, and 10% were undefined or belonged to the domain Archaea (Blow et al., 2016) **(Fig 1)**. Gram-positive bacteria include members of the high GC content phylum Actinobacteria and the low GC content Firmicutes, accounting for 6.6% and 26.3% of surveyed genomes, respectively (Blow et al., 2016) **(Fig 1)**. Actinobacteria include the genus *Streptomyces*, which are responsible for the production of two thirds of clinically relevant antibiotics, while Firmicutes includes several important human pathogens from the genera *Staphylococcus, Streptococcus, Enterococcus,* and *Clostridia.* Despite the importance of Gram-positive bacteria to human health and industry, the functions of DNA methylation outside of host defense have been understudied **(Fig 2)**. Here we summarize the current knowledge of the presence and known biological functions of DNA methylation in Gram-positive bacteria with the goal of opening new and important areas of study within this important field.

DNA Methyltransferases: Origins in orphan methyltransferases and host defense systems. Enzymes called DNA methyltransferases (MTases) catalyze post-replicative modifications in DNA by transferring a methyl group from the donor S-adenosylmethionine (SAM) to adenosine or cytidine bases in DNA (Jurkowska and Jeltsch, 2016). DNA MTases can function as part of a host defense system, such as the well-studied restriction-modification (RM) systems and the newly discovered bacterial exclusion (BREX) systems, or as stand-alone “orphan” MTases **(Fig 3)**. RM systems are minimally comprised of an MTase component and a restriction endonuclease

(REase) partner. RM systems are hypothesized to predominately function as bacterial defense systems against the invasion of foreign DNA, however they have also been shown to function in phase variation and the regulation of gene expression (Ershova et al., 2015). Similar to RM systems, BREX systems also function as bacterial defense systems and use DNA methylation to distinguish between self and foreign DNA (Barrangou and van der Oost, 2015; Goldfarb et al., 2015). However, as opposed to the cleavage of foreign DNA observed in RM systems, BREX systems function by blocking replication of phage DNA (Barrangou and van der Oost, 2015; Goldfarb et al., 2015). Orphan MTases, as the name suggests, only have MTase activity and orphan MTases contribute to a variety of DNA processes, including DNA mismatch repair, origin sequestration, and the regulation of gene expression with the majority of orphan MTase characterization occurring in Gram-negative bacteria (Sanchez-Romero, Cota, and Casadesus, 2015; Marinus and Lobner-Olesen, 2014; Mouammine and Collier, 2018; Adhikari and Curtis, 2016).

Regulatory functions of methylation from orphan MTases. Orphan MTases are hypothesized to be the products of RM systems that have lost their REase component (Seshasayee, Singh, and Krishna, 2012) **(Fig 3)**. The most well studied orphan MTases are Dam and CcrM from Gram-negative *Escherichia coli* and *Caulobacter crescentus*, respectively. Dam methylates G**A**TC sites throughout the *E. coli* genome and functions in origin sequestration, strand discrimination during DNA mismatch repair, and the regulation of gene expression (Sanchez-Romero, Cota, and Casadesus, 2015; Adhikari and Curtis, 2016; Marinus and Casadesus, 2009). CcrM methylates G**A**NTC sites and regulates cell cycle progression in *C. crescentus* (Mouammine and Collier, 2018; Marczynski and Shapiro, 2002). While CcrM homologs are only conserved through α-Proteobacteria, Dam homologs are conserved throughout Proteobacteria and even occur in several strains of Gram-positive bacteria (Mouammine and Collier, 2018; Marinus and Casadesus, 2009). Notably, in many Gram-positive systems the Dam homolog is typically paired with a cognate endonuclease as part of an active Type II RM system as characterized in *Streptococcus mutans*, a dental pathogen (Banas, Biswas, and Zhu, 2011).

The Blow *et al.* survey of DNA methylation in prokaryotic genomes identified 165 candidate orphan MTases, a subset of which were identified in the Gram-positive genera *Clostridia*, *Nocardia,* and *Arthrobacter*. In agreement with previous studies, the authors found that orphan MTases tend to be far more conserved than MTases that belong to an RM system, with 57% and 9% conservation at the genus level, respectively (Blow et al., 2016; Seshasayee, Singh, and Krishna, 2012). A candidate orphan MTase from two *Arthrobacter* species, which are Gram-positive bacteria belonging to the Actinobacteria phylum, was also conserved in 93% (39/42) of the available *Arthrobacter* genome sequences for which PacBio SMRT sequencing data is not available. The strong conservation of this orphan MTase in *Arthrobacter* highlights the potential biological significance to this genus (Blow et al., 2016). For both *Arthrobacter* and *Nocardia* species, another Gram-positive Actinobacteria, the recognition site for the candidate orphan MTase was enriched in the putative origin of replication (Blow et al., 2016). Recognition sites for Dam MTase are also enriched in the *E. coli* origin, where they function in origin sequestration to limit the frequency of DNA replication initiation, suggesting that the orphan MTases from *Arthrobacter* and *Nocardia* species may also contribute to the regulation of origin firing (Han et al., 2004; Nievera et al., 2006).

It is worth noting that both *Arthrobacter* and *Nocardia* species also have conserved unmethylated recognition sites upstream of putative transcriptional regulators. In *Nocardia*, unmethylated recognition motifs from the orphan MTase are enriched up to 20-fold in regions upstream of transcriptional regulators (Blow et al., 2016). In *E. coli*, although the majority (99.9%) of Dam recognition sites are fully methylated, there is a small subset of unmethylated sites on both strands of DNA that have important functions in gene regulation (Blow et al., 2016; Hernday, Braaten, and Low, 2003; Wallecha et al., 2002). The presence and conservation of unmethylated motifs suggests that the orphan MTases from *Arthrobacter* and *Nocardia* may also function in the regulation of gene expression.

A Type II RM system MTase lacking a cognate endonuclease has also been identified across 36 clinical isolates of the Gram-positive pathogen *Clostridioides difficile*. Oliveira

et al. identified the CamA MTase, which methylates CAAAA**A** motifs at an average of 7,721 sites across *Clostridioides difficile* genomes (Oliveira et al., 2020). Unlike the enrichment of recognition motifs for the putative orphan MTase observed in the origin of *Arthrobacter* species, CamA recognition motifs were not enriched in the origin but were present upstream of genes involved in transcriptional regulation, cell wall protein production, membrane transport, and sporulation (Oliveira et al., 2020). Consistent with a regulatory role for CamA-dependent methylation, deletion of *camA* resulted in global transcriptome changes and defects in both sporulation and colonization and infection of animal models (Oliveira et al., 2020). It is worth noting that, unlike the conservation of the putative orphan MTases across the genera *Arthrobacter* and *Nocardia*, CamA is not well conserved across *Clostridiales* and is instead fairly unique to *C. difficile* (Oliveira et al., 2020). Because a direct role in host defense has not been tested one possibility is that CamA functions both as part of a host defense system and in the regulation of gene expression. Given the important roles of orphan MTases in Gram-negative bacteria, the conservation of orphan MTases in Gram-positive bacteria, and the contribution of CamA to gene expression, more studies are necessary to understand the role of putative orphan MTases to the regulation of gene expression and chromosome dynamics in Gram-positive bacteria.

DNA methylation from BREX defense systems. Relative to the study of orphan MTases and RM systems, the discovery of the BREX family of defense systems is new. The term BREX (bacterial exclusion) was coined in a 2015 paper characterizing the system from *Bacillus cereus*, a Gram-positive Firmicute (Barrangou and van der Oost, 2015; Goldfarb et al., 2015). BREX systems were identified based on conservation of a putative alkaline phosphatase gene, *pglZ*, which is commonly found on genomic defense islands surrounded by 4-8 conserved BREX systems genes (Goldfarb et al., 2015). The majority of putative systems identified contain six genes, which include *pglZ*, the putative alkaline phosphatase, *pglX*, which contains a methyltransferase domain, a gene encoding a Lon-like protease domain, a putative RNA binding protein, a gene of unknown function, and a gene containing an ATP binding motif (Goldfarb et al., 2015). A previous study in the Gram-positive Actinobacteria *Streptomyces coelicolor* showed that

the *pgl* gene, along with three surrounding genes, conferred resistance to phage infection following an initial round of infection (Chinenova, Mkrtumian, and Lomovskaia, 1982; Sumby and Smith, 2002). In the Goldfarb *et al.* study researchers found that the six-gene BREX system from *B. cereus* was sufficient to provide protection from both temperate and virulent phages when expressed in *B. subtilis* (Goldfarb et al., 2015). The PglX protein, containing the MTase domain, was found to catalyze the formation of m6A at TAGG**A**G sites throughout the host chromosome **(Fig 3)**. Interestingly, none of the 43 TAGGAG sites in the phage DNA were methylated during infection (Goldfarb et al., 2015). While the MTase activity is necessary to confer protection against the invasion of foreign DNA, in the *B. cereus* system there is no decrease in cell viability in the absence of the MTase or observable cleavage of foreign DNA, suggesting BREX systems do not achieve protection through the cleavage mechanism of a canonical RM system (Goldfarb et al., 2015). Further, although the mechanism(s) of protection remain unclear, it is evident that BREX systems allow for adsorption of phage but not replication of phage DNA. Of the 1,500 bacterial genomes surveyed in Goldfarb *et al.*, 10% contained a putative BREX system across both Gram-positive and Gram-negative bacteria (Goldfarb et al., 2015). More work will be necessary to understand the mechanism(s) of BREX defense systems and to determine if DNA methylation from BREX MTases has additional regulatory roles outside of conferring protection to the host by blocking phage replication **(Fig 3)**.

DNA methylation from RM systems. While MTases from RM systems methylate the bacterial chromosome subsequent to replication, invading double-stranded foreign DNA from phages often enters the cell unmethylated, which allows for cleavage of the foreign DNA by the cognate REase activity. There are several different types of RM systems that vary in subunit composition, cofactor requirement, recognition site, and cleavage pattern that are reviewed extensively elsewhere (Ershova et al., 2015; Roberts et al., 2003; Wilson and Murray, 1991). Types I-III all have MTase and REase activities and are reviewed briefly here while Type IV systems, which lack MTase activity and instead cleave methylated DNA, are not discussed further and are reviewed elsewhere (Loenen et al., 2014).

Type I RM systems consist of *hsdM*, *hsdS*, and *hsdR* genes which encode the MTase, specificity, and REase subunits, respectively (Ershova et al., 2015; Murray, 2000). The specificity subunit is composed of two target recognition domains that recognize specific bipartite recognition sites in DNA **(Fig 3)** (Murray, 2000; Fuller-Pace et al., 1984; Nagaraja, Shepherd, and Bickle, 1985). The bipartite recognition sites, which are characteristic of Type I RM systems, consist of conserved DNA sequences at the 5' and 3' ends with 6-8 base pairs of degenerate sequence in the middle (Murray, 2000). Methylation is achieved at hemi-methylated bipartite motifs through the complex of two MTase subunits and one specificity subunit, resulting in methylation of both DNA strands (Suri and Bickle, 1985; Taylor et al., 1992). Restriction activity requires complex formation of two MTase subunits, two REase subunits, and one specificity subunit. The REase complex recognizes fully unmethylated bipartite recognition sequences and collision of the complex with a DNA binding protein is required for cleavage events, which can occur several kilobases away from the original recognition site (Dryden et al., 1997).

Type II RM systems are the most well recognized and commonly used for biotechnology applications (Pingoud, Wilson, and Wende, 2014). Type II RM systems typically consist of stand-alone MTase and REase genes. A notable exception is the Type IIG family, which consists of a single polypeptide with both MTase and REase activities (Roberts et al., 2003; Pingoud, Wilson, and Wende, 2014). Type II REase enzymes, which bind to and cleave unmethylated DNA independent of the MTase, are incredibly diverse and exhibit very low sequence identity (Pingoud, Wilson, and Wende, 2014). The Type II systems generally have 4-8 base pair palindromic recognition motifs, methylate both DNA strands, and cleave unmethyated sites within or near the recognition site **(Fig 3)** (Pingoud, Wilson, and Wende, 2014). The defined cleavage within the recognition sites from REases of Type II RM systems as well as the independent activities of the MTase and REase proteins make them well-suited for applications in biotechnology (Pingoud, Wilson, and Wende, 2014).

Type III systems are comprised of *mod* and *res* genes that encode components for the MTase and REase activities (Rao, Dryden, and Bheemanaik, 2014). The complex of two Mod subunits is necessary to bind and methylate one strand of DNA at 5-6 base pair non-palindromic motifs **(Fig 3)**(Rao, Dryden, and Bheemanaik, 2014; Brockes, 1973). Restriction activity requires the complex of one or two Res subunits with two Mod subunits, because the DNA binding activity is intrinsic to the Mod subunits and not the Res subunit (Janscak et al., 2001). Cleavage by the REase complex requires two recognition motifs oriented in opposite directions that results in cleavage 25-27 base pairs downstream of the recognition site (Rao, Dryden, and Bheemanaik, 2014; Piekarowicz and Brzezinski, 1980; Hadi et al., 1979; Meisel et al., 1992).

Type I-III RM systems are present across Gram-positive bacteria as a means of protection against the invasion of foreign DNA. Oftentimes, RM systems act as a barrier for horizontal gene transfer among closely related bacteria, resulting in clade separation between important pathogens (Huo et al., 2019; Waldron and Lindsay, 2006). Some Gram-positive species have overcome the restriction barrier to allow for the acquisition of pathogenicity islands in similar strains while maintaining the RM system for protection from phage predation (Johnston et al., 2013; Johnston, Polard, and Claverys, 2013). In addition to DNA restriction, these systems also provide underappreciated roles in the regulation of gene expression and virulence potential of Gram-positive pathogens (Manso et al., 2014; Nye et al., 2019; Li et al., 2016).

Balancing host protection and the benefits of genetic transformation. In addition to host defense, Type I RM systems have been shown to regulate strain separation in Gram-positive bacteria. *Enterococcus faecium* isolates are separated into clades, where clade A consists of multi-drug resistant isolates and clade B consists of drug susceptible fecal commensals (Lebreton et al., 2013). Clade A is further separated into subclades A1 and A2. Subclade A1 isolates are associated with hospital acquired infections and have a larger genome size and higher mutation rate relative to subclade A2 (Lebreton et al., 2013). Hou *et al.* identified multiple putative Type I RM systems across clades A and B and showed that the MTase and REase components of a Type I RM system shared

greater than 90% sequence identity between these subunits in subclade A1 and clade B strains (Huo et al., 2019). However, subclades A1 and B showed high variability in their S subunits, which are required for DNA recognition and binding (Huo et al., 2019). The S subunits were highly conserved between strains from subclade A1 but appeared to be strain-specific across clade B. The authors speculate that the divergence in S subunits and subsequent methylation patterns between the subclades act as a barrier to horizontal gene transfer between members of different clades (Huo et al., 2019). Type I systems in the human pathogen *Staphylococcus aureus* also mediate horizontal gene transfer by restricting exchange from strains possessing variable S subunits (Waldron and Lindsay, 2006).

While the *E. faecium* and *S. aureus* RM systems function to prevent horizontal gene transfer from between clades, other Gram-positive RM systems restrict phage DNA while maintaining mechanisms for acquisition of pathogenicity islands from related strains. Strains of the Gram-positive pathogen *Streptococcus pneumoniae* typically encode one of two Type II RM systems, DpnI or DpnII, which cleave at palindromic GATC sites throughout the genome (Lacks and Greenberg, 1975). DpnI represents an atypical system because it cleaves fully methylated sites while DpnII cleaves at fully unmethylated sites. Strains with DpnII encode two upstream DNA MTases, a Dam homolog, DpnM, and a single-stranded DNA MTase, DpnA (Cerritelli, Springhorn, and Lacks, 1989).

The occurrence of both RM systems across strains serves a mixed *S. pneumoniae* population in two ways. First, the occurrence of both systems protects against a broad range of phage predation, allowing for degradation of DNA independent of the methylation status at GATC sites. Second, the mixed population promotes preferential acquisition of DNA from kin. DpnI cells can acquire methylated genomic DNA from DpnII cells because the newly acquired DNA will exist in a hemi-methylated state that DpnI cannot cleave (Johnston et al., 2013; Johnston, Polard, and Claverys, 2013). Conversely, uptake of DpnI DNA in DpnII cells would also result in hemi-methylated DNA. If the newly acquired hemi-methylated DNA is not methylated prior to replication,

the DNA will exist in a complete unmethylated state and can be cleaved by DpnII. Cleavage of unmethylated DNA in DpnII cells is prevented via methylation of the new DNA from the unique single-stranded DNA MTase DpnA. DpnA is only expressed during genetic competence ensuring that the DpnII RM system remains active against incoming phage DNA but allows for the acquisition of beneficial pathogenicity islands from related DpnI strains (Johnston et al., 2013; Johnston, Polard, and Claverys, 2013).

Therefore, in addition to protecting against phage predation, RM systems function as barriers to horizontal gene transfer to maintain strain separation in Gram-positive bacteria such as *E. faecium* and *S. aureus.* Conversely, other Gram-positive species have adapted special mechanisms that use DNA methylation to acquire beneficial DNA (e.g. pathogenicity islands) while maintaining restriction activity to protect against phage predation. In the next sections we will review how RM system methylation functions in epigenetic regulation in bacteria.

Phasevarions: Epigenetic regulation by RM system MTases. Bacteria must have the ability to adapt to rapidly changing environmental conditions in order to survive. One mechanism bacteria use to cope with rapidly changing conditions is through phase variation. Phase variation occurs when certain genes, often those that encode cell surface proteins, undergo random differential expression in a reversible fashion among bacterial subpopulations (Henderson, Owen, and Nataro, 1999; Phillips et al., 2019). This variation can be achieved through the presence of simple sequence repeats within genes (e.g. tandem repeats or homopolymer runs), where DNA polymerase is prone to errors that can result in non-functional or non-expressed proteins, subsequently resulting in ON/OFF expression of the gene product within a subpopulation of cells (Phillips et al., 2019; van Belkum et al., 1998; Moxon, Bayliss, and Hood, 2006). The variation in expression can also occur as a result of genetic exchange of differentially expressed loci through homologous recombination, which typically occurs at inverted repeats within the exchanged loci (Phillips et al., 2019).

Phasevarions (phase variable regulons) consist of multiple genes that are differentially regulated within various subpopulations based on epigenetic control from phase-variable MTases (Srikhanta et al., 2005). In Gram-positive organisms, MTases from both Type I and Type III RM systems have been shown or predicted to be regulators of phasevarions (for review (Atack et al., 2018; De Ste Croix et al., 2017). In Type I systems, homologous recombination occurs at inverted repeats within the genes for multiple specificity subunits to generate unique methylation patterns throughout the genome **(Fig 4A)** (Manso et al., 2014; Li et al., 2016; Fagerlund et al., 2016; Claesson et al., 2006). The subspecies specific methylation patterns act as an epigenetic signal that gives rise to differential gene expression and subsequent phenotypic differences between the subpopulations (Manso et al., 2014; Li et al., 2016). In Type I and Type III RM systems, variation in simple sequence repeats can result in DNA polymerase errors that give rise to subpopulations with active and inactive MTases, resulting in loss of methylation and subsequent differential gene expression **(Fig 4B)** (for review (Srikhanta, Fox, and Jennings, 2010) and (Atack et al., 2018; Atack et al., 2020). This mechanism allows for gene expression heterogeneity within a population of cells.

Regulation from S subunit variation in Type I RM systems. In the Gram-positive pathogen *Streptococcus pneumoniae* Type I phasevarions have been shown to regulate virulence via global epigenetic changes (Manso et al., 2014; Li et al., 2016). In one system, three separate specificity subunit genes containing inverted repeats allow for six possible specificity subunit variants **(Fig 5)** (Manso et al., 2014). Manso *et al.* "locked" the strains into one epigenetic state by expressing only one of the six specificity subunits and then used PacBio SMRT sequencing to show that each variant methylated different motifs, with the frequency of the various motifs differing within the genome **(Fig 5)** (Manso et al., 2014). The locked strains showed differential gene expression relative to one another resulting in phenotypic consequences. Most notably, the different subtypes varied in colony opacity, which is a reversible morphological change between opaque and transparent colonies (Weiser et al., 1994). While some variants were 100% opaque other variants showed as low as 7% opaque colonies **(Fig 5)**. The colony opacity phenotypes correlated with invasive disease and carriage phenotypes, where a

variant with 100% opaque colonies had poor colonization ability but was highly virulent and the variant with the majority of transparent colonies was greatly attenuated for virulence but not colonization (Manso et al., 2014; Weiser et al., 1994). Moreover, the authors showed variant switching with the “unlocked” wild type strain during the course of invasive disease infection, where the cells had predominately switched to the highly virulent state with reduced colonization as early as 4 hours post-challenge (Manso et al., 2014).

A similar Type I RM system encoding two specificity subunits with inverted repeats has been shown to produce four specificity subunit variants in *S. suis*, a major veterinary pathogen, though no differential expression has been associated with the variants to date (Atack et al., 2018). In fact, an analysis of 393 *S. suis* genomes identified that 262 strains contained Type I RM systems with multiple *hsdS* specificity subunits containing inverted repeats, suggesting that the occurrence of phase variable Type I RM systems may be pervasive across this species (Atack et al., 2018). Additionally, the presence of phase variable Type I RM systems have been predicted or identified in strains of *Enterococcus faecalis, Listeria monocytogenes, Clostridium botulinum, and Lactobacillus salivarus* (De Ste Croix et al., 2017; Fagerlund et al., 2016; Claesson et al., 2006). More work is needed to understand how phase variable Type I RM systems affect virulence gene expression across Gram-positive pathogens.

Regulation from bi-phasic MTases in Type III RM systems. In various Gram-negative pathogens, including species of *Haemophilus*, *Neisseria*, *Kingella, Helicobacter,* and *Moraxella*, phase variable Type III *mod* alleles, encoding the Mod protein responsible for MTase activity, have been shown to regulate gene expression (for review (Srikhanta, Fox, and Jennings, 2010) and (Srikhanta et al., 2005; Srikhanta et al., 2009; Srikhanta et al., 2017; Srikhanta et al., 2011; Blakeway et al., 2014). The Mod proteins from these Type III systems exhibit ON/OFF expression within a population due to the presence of simple sequence repeats (SSRs) within the *mod* gene, which can cause DNA polymerase slippage at the SSRs (Phillips et al., 2019; van Belkum et al., 1998; Moxon, Bayliss, and Hood, 2006). While no studies, to our

knowledge, have demonstrated a phase variable Type III RM system regulating gene expression in Gram-positive bacteria, the presence of candidate phase variable Type III systems have been identified in *S. thermophiles*, *S. galactiae*, *S. mitis*, and *L. saerimneri* strains (Atack et al., 2018). These candidate phase variable Type III systems were identified based on the presence of SSRs within the *mod* allele (Atack et al., 2018). Putative epigenetic regulation by these novel systems remains an area of continued investigation.

In addition to the examples of the Type I and Type III systems discussed above, both SSRs and inverted repeats have been observed in the PglX MTase of BREX systems, resulting in phase variation for expression of the system (Goldfarb et al., 2015). Phase variable MTases represent an important mechanism of epigenetic regulation in Gram-positive bacteria, allowing for differential methylation patterns and subsequently differential gene expression within various bacterial subpopulations (Phillips et al., 2019). Few studies have investigated the regulatory effects of DNA methylation from active and inactive RM systems outside of Type I RM systems with multiple specificity subunits or Type III RM systems containing short sequence repeats within the *mod* allele. Below we will discuss our current understanding of the important regulatory functions of DNA methylation from non-phase variable RM systems across bacteria from the two Gram-positive phyla, Actinobacteria and Firmicutes.

DNA methylation-dependent mechanisms for the regulation of gene expression in Actinobacteria. The Actinobacteria comprise one of the largest and most diverse bacteria phyla, including Gram-positive filamentous bacteria with high GC content genomes (for review (Barka et al., 2016; Lewin et al., 2016)). Actinobacteria can be found in aquatic and terrestrial environments where they are important contributors to diverse ecosystems (Barka et al., 2016; Goodfellow and Williams, 1983). The impact of DNA methylation outside of RM systems on the cell physiology of Actinobacteria remains largely unexplored, with the first studies focusing on *Streptomyces* and *Mycobacterium.* The soil dwelling *Streptomyces* have been well studied for their multicellular behaviors and complex lifestyles (Barka et al., 2016; Yague et al., 2013).

Streptomyces are also of tremendous importance to biotechnology and human health as they are responsible for the production of 2/3 of clinically relevant antibiotics (Lewin et al., 2016; Newman and Cragg, 2007; Procopio et al., 2012). *Mycobacterium* species are well known for causing a broad range of human diseases, particularly in immunocompromised individuals, and represent significant burdens on healthcare systems across the world (Dorman and Chaisson, 2007; Tornheim and Dooley, 2019; Kim et al., 2008). Given the importance of *Streptomyces* and *Mycobacterium* to human health, as well as the impact of other Actinobacteria genera on terrestrial and aquatic ecosystems, the initial studies suggesting an important regulatory role for DNA methylation in the adaptive lifestyles of these bacteria is of particular importance for on-going and future research.

Mycobacterium tuberculosis is a Gram-positive pathogen that represents a significant worldwide public health burden, causing more than 1.5 million deaths in 2018 ([WHO] (Dorman and Chaisson, 2007). The antibiotics rifampin and isoniazid, among others, have been used to treat tuberculosis infections, however multi-drug resistant tuberculosis (MDR-TB) strains, which are resistant to both rifampin and isoniazid, have emerged (Tornheim and Dooley, 2019; Kim et al., 2008). Among the mechanisms for emerging antibiotic resistance, a study by Chen *et al.* suggests that the extent of methylation differs between rifampin and isoniazid treated *M. tuberculosis* H37Rv strains compared to the untreated wild type strain (Chen et al., 2018). A separate study of para-aminosalicylic acid (PAS) resistant Mycobacterium suggests differential methylation in PAS resistant H37Rv, with 1,161 hyper-methylated and 227 hypo-methylated genes relative to the susceptible parent strain (Li et al., 2020). These data suggest that DNA methylation contributes to antibiotic resistance of *Mycobacterium tuberculosis* with the strong potential to contribute to formation of persister cells.

Another study suggests that DNA methylation may play an important role in *M. tuberculosis* survival under hypoxic conditions (Shell et al., 2013). Latent infections with *M. tuberculosis* can last decades, requiring the bacteria to survive, persist, and adapt to a range of environmental conditions within the human host (Getahun et al., 2015). Shell

et al. discovered a Type II MTase, MamA, present in a subset of *M. tuberculosis* strains that catalyzes m6A formation at CTGG**A**G sites throughout the genome (Shell et al., 2013). MamA is also conserved in other Mycobacterium species including *M. smegmatis*, *M. bovis, M. avium,* and *M. leprae.* Upon loss of *mamA* in *M. tuberculosis*, a small but significant decrease in the expression of a subset of genes was observed where the MamA recognition site overlapped with putative sigma factor -10 binding boxes. Moreover, Shell *et al.* found that the *mamA* deficient cells had decreased viability in hypoxic conditions relative to wild type cells (Shell et al., 2013). These hypoxic conditions were used to simulate those of hypoxic granulomas formed in the human host (Tsai et al., 2006). A separate study of nineteen *Mycobacterium tuberculosis* complex strains found that MamA had 13 binding sites that overlapped with SigA and that strains with inactive MamA variants showed decreased expression of the downstream genes relative to strains with active MamA (Chiner-Oms et al., 2019). The same study showed that while methylation from a separate Type I RM system in *M. tuberculosis* strains did not directly influence the expression of genes through overlap with known sigma factor binding sites, loss of methylation indirectly affected expression of a small subset of genes in the absence of a recognition site near the affected genes (Chiner-Oms et al., 2019). Therefore, these results suggest both direct and indirect mechanisms for DNA methylation in the regulation of gene expression **(Fig 6)** highlighting the importance of DNA methylation beyond restriction-modification systems in clinically important Actinobacteria.

In addition to m6A-dependent regulation, m5C modifications have been shown to function in the regulation of antibiotic production and development in Actinobacteria. Streptomycetes are Gram-positive soil-dwelling bacteria that produce two thirds of all clinically relevant secondary metabolites (Newman and Cragg, 2007; Procopio et al., 2012). In addition to antibiotic production, *Streptomyces* species are known for their complex life cycles, which include differentiation and programmed cell death (PCD) (for review (Barka et al., 2016; Yague et al., 2013). Briefly, subsequent to uninucleoid spore germination, hyphae growth gives rise to a first/vegetative mycelium (MI) (Manteca, Fernandez, and Sanchez, 2005). Upon nutrient depletion, PCD occurs as the

multinucleated second/differentiated mycelium (MII) develops, which consists of multiple cell types including the aerial mycelium and sporulating mycelium (Manteca, Fernandez, and Sanchez, 2005). The sporulating mycelium undergoes PCD to form the uninucleoid spore (Manteca, Fernandez, and Sanchez, 2005). A recent study showed that both antimicrobial production in *Streptomyces* and development are affected by m5C methylation (Pisciotta, Manteca, and Alduina, 2018). DNA extracted from strains of *S. coelicolor, S. avermitilis, S. griseus,* and *S. lividans* showed less m5C in the MII stages compared to MI in all four species (Pisciotta, Manteca, and Alduina, 2018). Moreover, the researchers used a gene interruption in the putative MTase *SCO1731* (*SCO1731::Tn5062*) and found significant reduction in the genomic m5C signal in the *S. coelicolor* genome in MI but only a slight reduction in signal in MII (Pisciotta, Manteca, and Alduina, 2018). Phenotypically, the *SCO1731::Tn5062* strain displayed a substantial delay in differentiation on solid media, with aerial mycelium formation occurring at 96 hours relative to formation at 48 hours in wild type cells. The mutant was also severely impaired for production of the antibiotic actinorhodin (Pisciotta, Manteca, and Alduina, 2018). *S. coelicolor* encodes 37 putative DNA MTases in addition to *SCO1731*, a subset of which are differentially expressed in MI and MII stages of development (Yague et al., 2013; Pisciotta, Manteca, and Alduina, 2018). Further studies are necessary to determine the extent to which various methylation events regulate development and the expression of clinically relevant secondary metabolites across Streptomyces. Nevertheless, it appears that further studies will reveal an important regulatory contribution for DNA methylation in the complex life cycles of *Streptomyces*, potentially raising broadly conserved biological parallels with the developmental regulatory functions of DNA methylation in eukaryotes.

DNA methylation-dependent mechanisms for the regulation of gene expression in Firmicutes. The Firmicutes phylum includes Gram-positive bacteria with low GC content genomes. In addition to being one of the dominating phyla in the human gut microbiome, members of the Firmicutes also encompass several important human pathogens, including *Staphylococcus*, *Streptococcus, Enterococcus*, *Clostridium*, and *Listeria* species (Ley, Peterson, and Gordon, 2006). Despite the very limited research

available outside of regulation by phase variable MTases, RM system MTases have been shown to regulate gene expression in Firmicutes, prompting important possibilities for the functions of DNA methylation across this phylum.

Epigenetic regulation of virulence factors from a Type I RM system has been shown for the important human pathogen *Streptococcus pyogenes*. Loss of m6A from an active Type I RM system resulted in substantial down regulation of 20 genes that clustered into six distinct loci in a clinical isolate of *S. pyogenes* (Nye et al., 2019). Many of the differentially expressed genes were part of the core regulon for the stand-alone transcriptional regulator, Mga (Nye et al., 2019). The Mga core regulon consists of genes that encode cell surface proteins, including the M-protein, C5a peptidase, which cleaves host complement, and the Mga regulator itself, which are important for adhesion, internalization, and immune evasion phenotypes (McIver and Scott, 1997; Hondorp and McIver, 2007). The m6A-dependent decrease in expression of the Mga regulon resulted in decreased adhesion of *S. pyogenes* cells to host epithelial cells, a decreased ability of the bacteria to survive within host neutrophils, and a decreased ability to evade the host immune response. Interestingly, the *S. pyogenes* genome contains another putative Type I specificity unit (AWM59_04585), which is not surrounded by *hsdM* or *hsdR* genes. However, AWM59_04585 is located 691kb from the S subunit (AWM59_07900) of the active Type I RM system and REBASE annotates AWM59_04585 as unlikely to be a genuine S subunit (http://rebase.neb.com). Thus, more work is necessary to determine if S-subunit switching occurs in *S. pyogenes* as it does in *S. pneumonae* or if the epigenetic regulation described in Nye *et al.* represents a phase variation independent mechanism of regulation by a Type I RM system (Nye et al., 2019). Either biological mechanism would impart regulation of *S. pyogenes* virulence.

Gene regulation in *Streptococcus* is also governed by the presence of a Type II RM system. As previously discussed, in Gram-negative *E. coli* and related Proteobacteria, Dam MTase occurs as a stand-alone orphan MTase that functions in many important cellular processes, including origin sequestration (Han et al., 2004; Nievera et al.,

2006), DNA mismatch repair (Bale, d'Alarcao, and Marinus, 1979; Lahue, Au, and Modrich, 1989), and the regulation of gene expression (Casadesus and Low, 2013). Homologs of Dam MTase occur in a subset of Gram-positive bacteria, however they often exist as part of an active RM system, such as the DpnM-DpnA-DpnII system from *S. pneumoniae* discussed above (Banas, Biswas, and Zhu, 2011; Johnston et al., 2013). Homologs of the DpnM-DpnA-DpnII system occur in a subset of strains from other Gram-positive bacteria such as *Streptococcus mutans, Lactococcus lactis, Streptococcus sanguinis,* and *Streptococcus suis* (Banas, Biswas, and Zhu, 2011; Moineau et al., 1995; Xu et al., 2007; Sekizaki et al., 2001). In *S. mutans*, it was shown that deletion of the DpnM homolog, DamA, resulted in the differential expression of over 100 genes, of which 70 were up regulated and 30 were down regulated at least two fold in the *damA* mutant relative to wild type (Banas, Biswas, and Zhu, 2011). The differentially expressed genes included virulence factors, bacteriocins, and genes involved in sugar metabolism, which would contribute to the formation of dental caries and tooth decay (Banas, Biswas, and Zhu, 2011). Importantly, this study showed that the differences in gene expression had effects at the phenotypic level. The up regulation of the cell surface glucan receptor, GpbC, in the *damA* mutant resulted in increased clumping in dextran-dependent aggregation assays and the increases in bacteriocin gene expression resulted in larger zones of clearing in the *damA* mutant against *Streptococcus godonii* and *Lactococcus lactis* strains (Banas, Biswas, and Zhu, 2011). Thus, in addition to functioning as part of a RM system, the *S. mutans* DNA MTase DpnM also functions in the regulation of gene expression. It remains unknown if the DpnM homologs in other *Streptococcus* species have regulatory functions beyond host restriction.

Another example of DNA methylation regulating gene expression in Firmicutes was demonstrated in a recent study of the *Bacillus subtilis* MTase, DnmA. In Nye *et al.* researchers characterized the methylomes of the lab and ancestral strains of *B. subtilis* PY79 and NCIB 3610, respectively (Nye et al., 2020). They found that the DnmA MTase from a Type I-like RM system catalyzed the formation of m6A at non-palindromic GACG**A**G sites throughout the chromosome. The absence of DnmA did not affect

natural transformation efficiency, suggesting that DnmA either does not have activity as a canonical Type I RM-like system or the endonuclease activity cannot be measured during natural transformation (Nye et al., 2020). Moreover, deletion of *dnmA* resulted in a small, but significant decrease in expression for a subset of genes that are important for chromosome structure and maintenance. DnmA recognition sites were proximal to the -35 box for sigma factor SigA binding in the promoters of the differentially expressed genes. Further, this study found that the transition state transcriptional repressor ScoC, preferentially bound an unmethylated promoter, providing mechanistic insight into the MTase-dependent regulation of gene expression in Gram-positive bacteria (Nye et al., 2020). These data show that ScoC binding to a reporter promoter region is stronger for unmethylated relative to methylated DNA, demonstrating that ScoC repressor binding serves to repress gene expression when methylation is absent (Nye et al., 2020; Caldwell et al., 2001).

Conclusions and Future Perspectives

Methylation of genomic DNA is pervasive across bacterial genomes, where it has been most extensively studied as a self-recognition mechanism in host defense. The majority of the pioneering studies exploring the function of DNA methylation outside of host defense have been completed in Gram-negative bacteria (Mouammine and Collier, 2018; Adhikari and Curtis, 2016; Marinus and Casadesus, 2009; Marczynski and Shapiro, 2002; Sanchez-Romero and Casadesus, 2020). However, outside of the CamA MTase conserved only in specific species of *Clostridiales*, much less is known about the functions of orphan MTases in Gram-positive bacteria (Oliveira et al., 2020). A critical area of future investigation is understanding the biological contribution for enrichment of orphan MTase recognition sites in the putative origin of replication region for *Arthrobacter* species, which are used for commercial production of glutamic acid, and *Norcardia* species, a subset of which can cause opportunistic infections in susceptible populations (Blow et al., 2016). The over-representation of MTase sites in their predicted origin region suggests that orphan MTase methylation regulates origin firing in a subset of Gram-positive species. Additionally, unmethylated recognition sites from Gram-positive orphan MTases can be also be found in promoter regions for

transcriptional regulators, suggesting an additional contribution in regulated gene expression (Blow et al., 2016). Given the conservation of putative orphan MTases in Gram-positive bacteria it is tempting to speculate that MTase function is conserved across distantly related species. In our opinion, more experiments are necessary to determine the function of orphan MTase methylation in Gram-positive bacteria and how methylation regulates cell proliferation and gene expression.

In addition to orphan MTases, the regulatory functions of methylation from RM systems has also focused on Gram-negative bacteria. While phase variable Type I RM MTases have been found to be important for *Streptococcus* virulence (Manso et al., 2014; Li et al., 2016), as discussed here, most other studies of Type I and Type III phase variable RM systems have been completed in Gram-negative bacteria. Outside of epigenetic regulation from phase variable RM systems, few studies have explored the regulatory consequences of DNA methylation from non-phase variable RM systems in both Gram-positive and Gram-negative bacteria. Here we have discussed epigenetic regulation from non-phase variable RM systems in *Mycobacterium, Streptomyces, Streptococcus,* and *Bacillus* species. In some systems, such as MamA and DnmA from *M. tuberculosis* and *B. subtilis*, respectively, the mechanism of methylation-dependent regulation appears to be direct, where m6A modifications overlap with transcription factor binding sites in differentially expressed genes **(Fig 6)** (Shell et al., 2013; Nye et al., 2020). In *B. subtilis* researchers identified an m6A sensitive transcriptional regulator, ScoC, which bound near the sigma factor binding site, providing insight into the mechanism of m6A-dependent regulation in Gram-positive bacteria (Nye et al., 2020). It remains to be determined if m6A regulation of ScoC binding is a common mechanism for ScoC regulated genes or specific to a particular locus. In other systems, such as the Type I RM systems in *M. tuberculosis* and *S. pyogenes*, the mechanism of methylation-dependent regulation of gene expression appears to be indirect, with modified recognition motifs occurring distal to the differentially expressed genes **(Fig 6)** (Nye et al., 2019; Chiner-Oms et al., 2019). Both direct and indirect mechanisms of regulation from non-phase variable RM systems appear to have important consequences for cells, where they affect virulence potential, adaptability to environmental conditions, and

bacterial development. Given the widespread occurrence of DNA methylation in Gram-positive bacteria and the importance of Actinobacteria and Firmicutes to human health, industry, and the environment, further study of DNA methylation in Gram-positive bacteria is important for understanding regulatory and phenotypic variations among bacteria within populations.

Conflict of Interest

The authors have no conflict of interest to declare.

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Figure Legends

Figure 1. DNA methylation has been most intensely studied in Gram-negative bacteria. (A) Gram staining of bacteria included in the Blow *et al.* survey of prokaryotic genome methylation (Blow et al., 2016). Bacteria were grouped based on reported Gram stain. The percent of Gram-negative (pink), Gram-positive (purple), and Other (green) species is indicated on the y-axis. The number of species in each category out of the total surveyed is indicated as a fraction underneath each bar. The 'Other' category consisted of Archaea and bacterial species from *Chloroflexi*, *Plantomycetes,* and *Deinococcus-Thermus,* which exhibit atypical Gram stains based on cell wall structure. **(B)** The percent of representative bacteria from the major Gram-positive phyla

in the Blow *et al.* survey (Blow et al., 2016). The percent of Actinobacteria (gray) and Firmicutes (black) species is indicated on the y-axis with the number of species included out of the total surveyed indicated as a fraction underneath each bar.

Figure 2. The functions of DNA methylation in Gram-positive bacteria. Genomic DNA methylation in Gram-positive bacteria occurs from the activity of RM system MTases (brown), orphan MTases (blue), or BREX MTases (green). Methylation from both BREX and RM MTases has been shown to function in host defense. Both phase variable and non-phase variable MTases from RM systems have been shown to regulate gene expression in Gram-positive bacteria as well. To date, a regulatory function for DNA methylation from BREX system MTases has not been experimentally demonstrated.

Figure 3. DNA MTases in Gram-positive bacteria. DNA methylation in Gram-positive bacteria comes from DNA MTases that exist as part of RM systems (brown), BREX (green), and orphan MTases (blue). The composition of the MTase component from Types I-III RM systems is indicated as well as the typical recognition motifs and methylation patterns. The typical recognition motif and methylation pattern from BREX systems and orphan MTases is also included (Barrangou and van der Oost, 2015; Goldfarb et al., 2015).

Figure 4. Phase variable MTases from Type I and III RM systems. (A) Phase variable MTases from Type I RM systems occur through S-subunit switching. Random recombination of the TRDs from *hsdS* and *hsdS'* occurs at inverted repeats within the genes by the proximally encoded recombinase. The recombination events produce multiple S-subunits with different combinations of TRDs that target the MTase, comprised of HsdM and HsdS subunits, to different recognition sites throughout the genome resulting in bacterial subpopulations with various methylation patterns. The subpopulation specific methylation patterns can result in differential gene expression between subpopulations. **(B)** Phase variable MTases from Type III RM systems occur through DNA polymerase slippage at SSRs. Random DNA polymerase slippage at a

homopolymer track in the coding region of the *mod* allele results in subpopulations with truncated and full-length Mod proteins. The subpopulations with the truncated Mod protein lack the DNA methylation present in the population with the functional full-length Mod-protein, resulting in subpopulation specific DNA methylation patterns that can result in differential gene expression between the populations (Seib et al., 2015).

Figure 5. Phase variable MTase in *S. pneumoniae* regulates virulence in distinct subpopulations. Shown are the six different S-subunits produced from recombination of the TRDs from three *hsdS* genes to produce systems A-F as described in Manso *et al*. The distinct recognition site for each system is listed according to the color-coded TRDs in the S-subunit. The percent of colonies displaying the opaque phenotype for each subpopulation is also indicated (Manso et al., 2014). This figure is based on the following reference (Manso et al., 2014).

Figure 6. Mechanisms of DNA methylation-dependent regulation of gene expression in Gram-positive bacteria. Direct regulatory mechanisms result from the occurrence of methylation within a promoter region of a gene that affects binding of transcriptional regulators that influence RNA polymerase activity, subsequently affecting gene expression. Indirect regulation can occur through differential expression of a gene that is directly regulated by DNA methylation, such as transcription factors (TF). The methylation-dependent differential expression of the TF can result in downstream differential expression of many genes within the TF regulon. Indirect regulation can also occur at genes that are differentially expressed upon loss of DNA methylation but are not proximal to any methylated sites. Such indirect mechanisms are poorly understood but occur in a number of bacteria.

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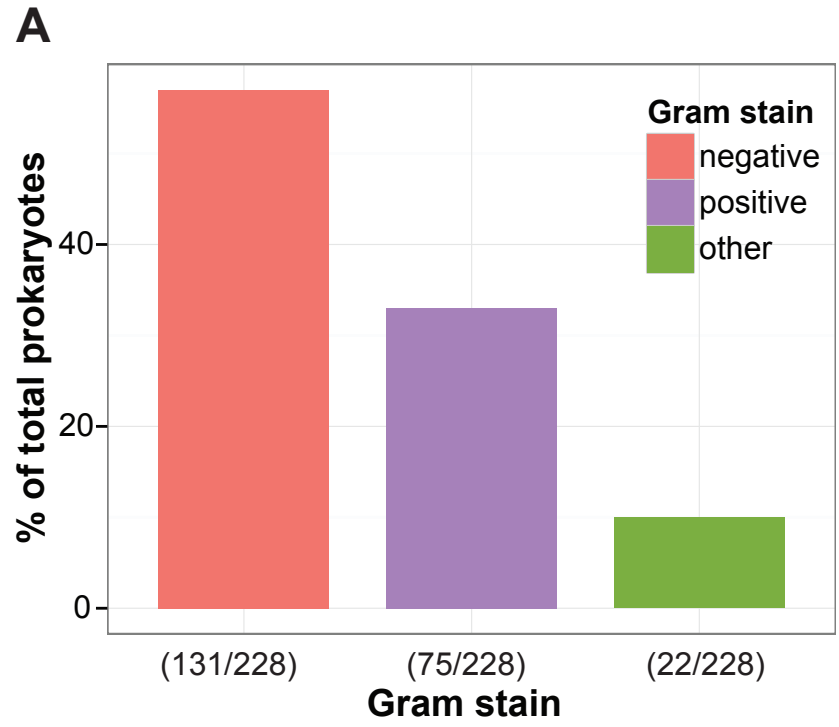
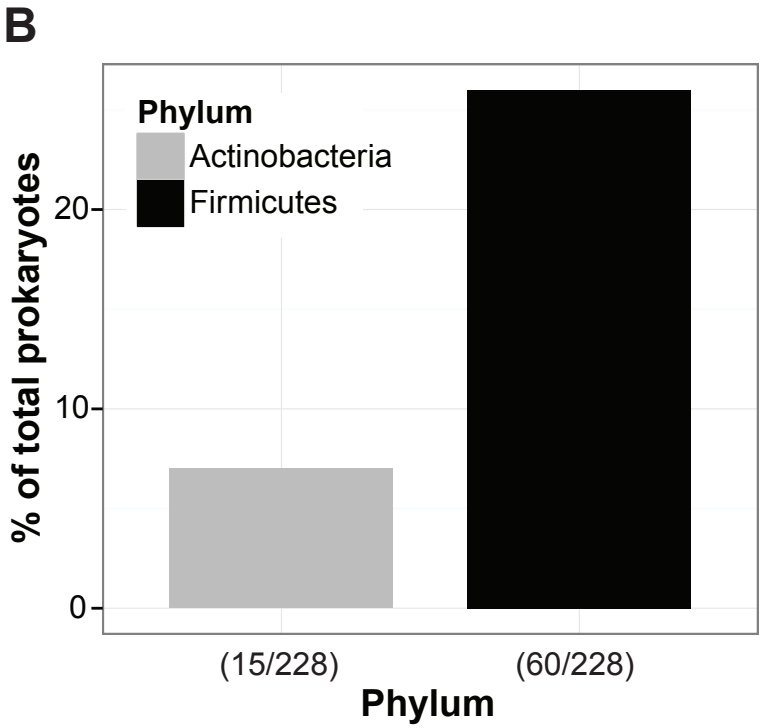
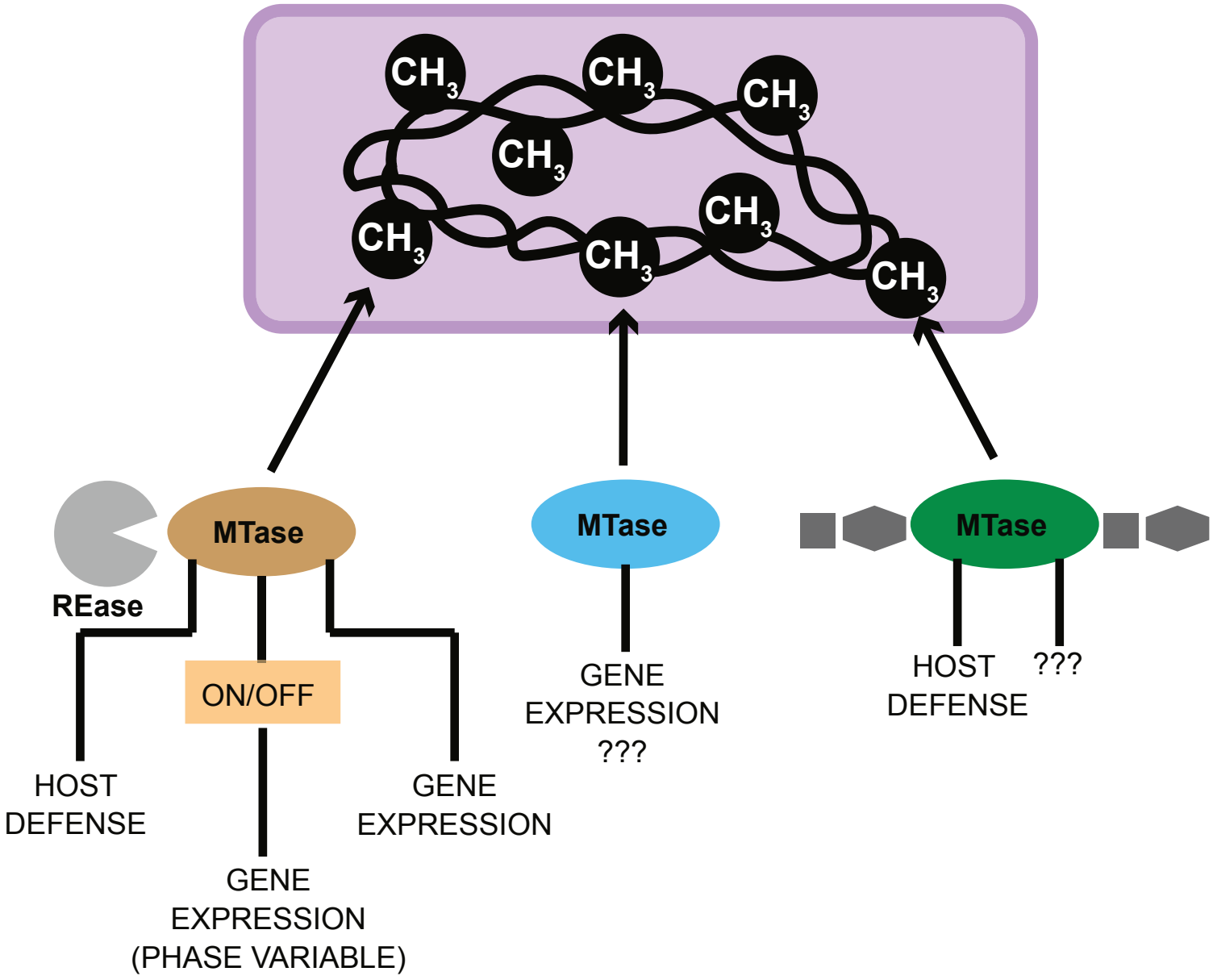

A
Gram stain
negative
positive
other
% of total prokaryotes
40
20
0
(131/228)
(75/228)
(22/228)
Gram stain


B
Phylum
Actinobacteria
Firmicutes
% of total prokaryotes
20
10
0
(15/228)
(60/228)
Phylum

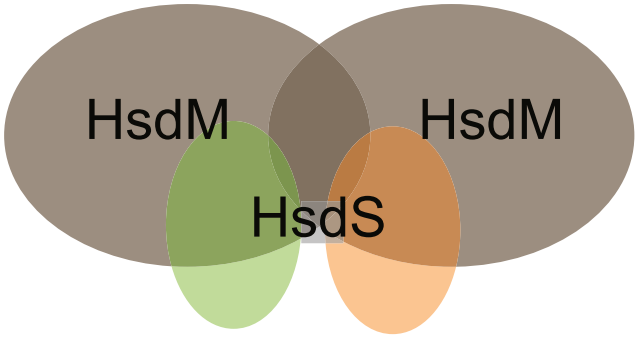

CH3
CH3
CH3
CH3
CH3
CH3
CH3
MTase
REase
ON/OFF
HOST
DEFENSE
GENE
EXPRESSION
(PHASE VARIABLE)
GENE
EXPRESSION
???
MTase
HOST
DEFENSE
???
MTase

Host defense

Orphan systems

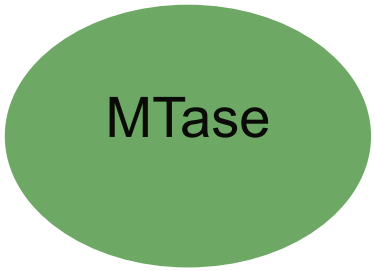
RM systems

I



Bipartite motif
(e.g. CRA**A**N_8CTG)
Methylates both DNA strands

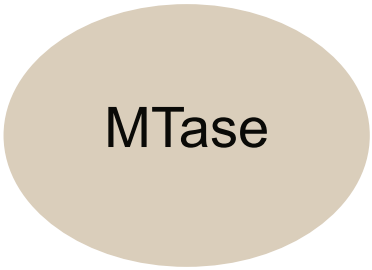
BREX systems



Non-palindromic
6 bp motif
m6A-specific
(e.g. TAGG**A**G)

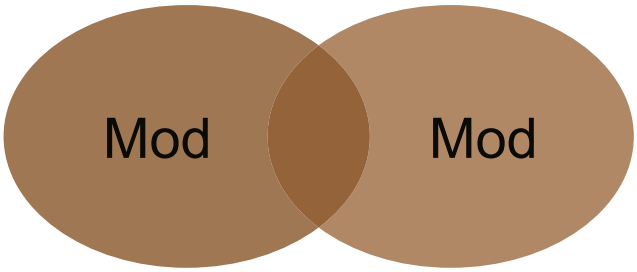
Palindromic
4-8 bp motif
(e.g. **C**TCGAG)

II

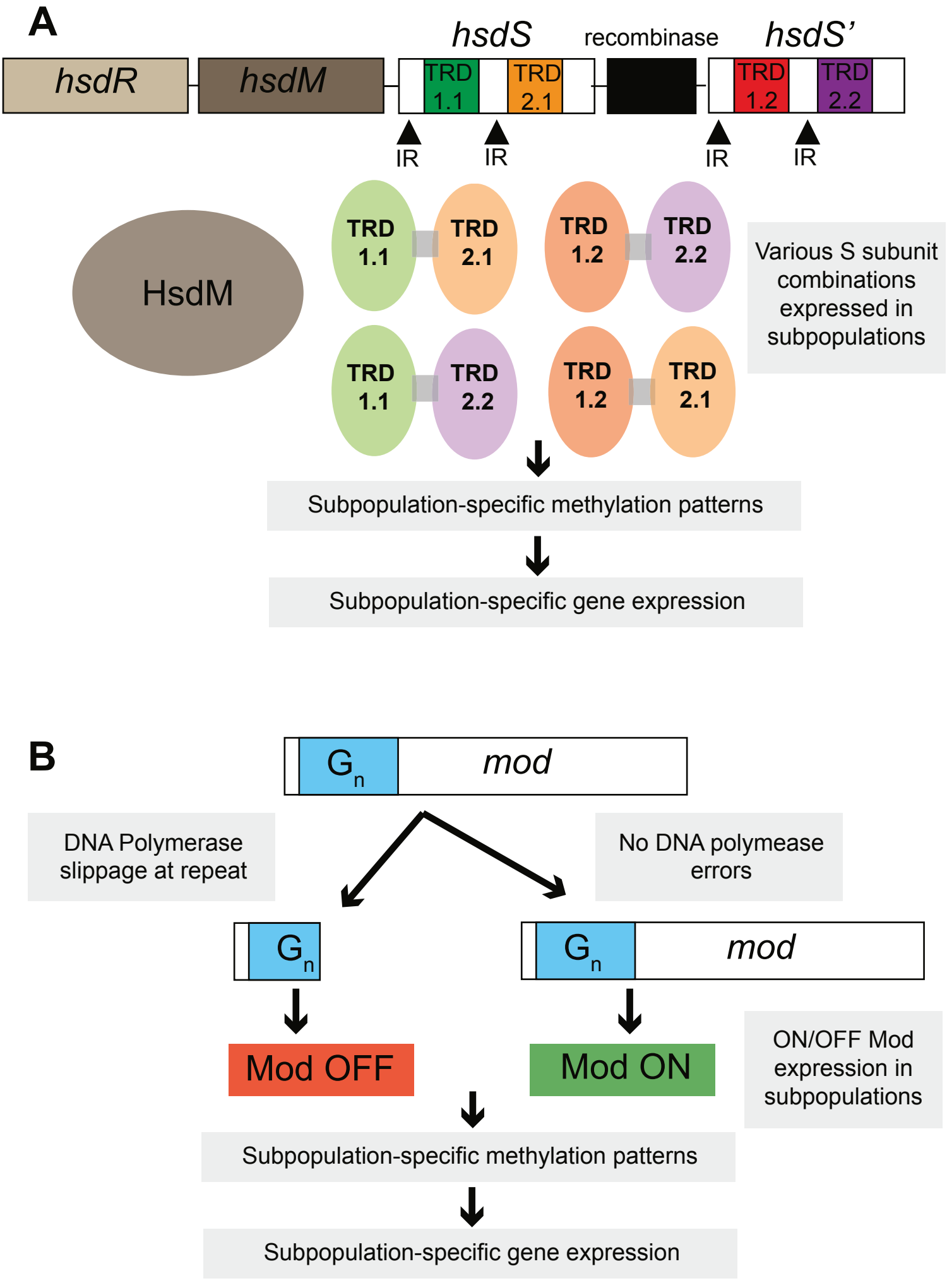


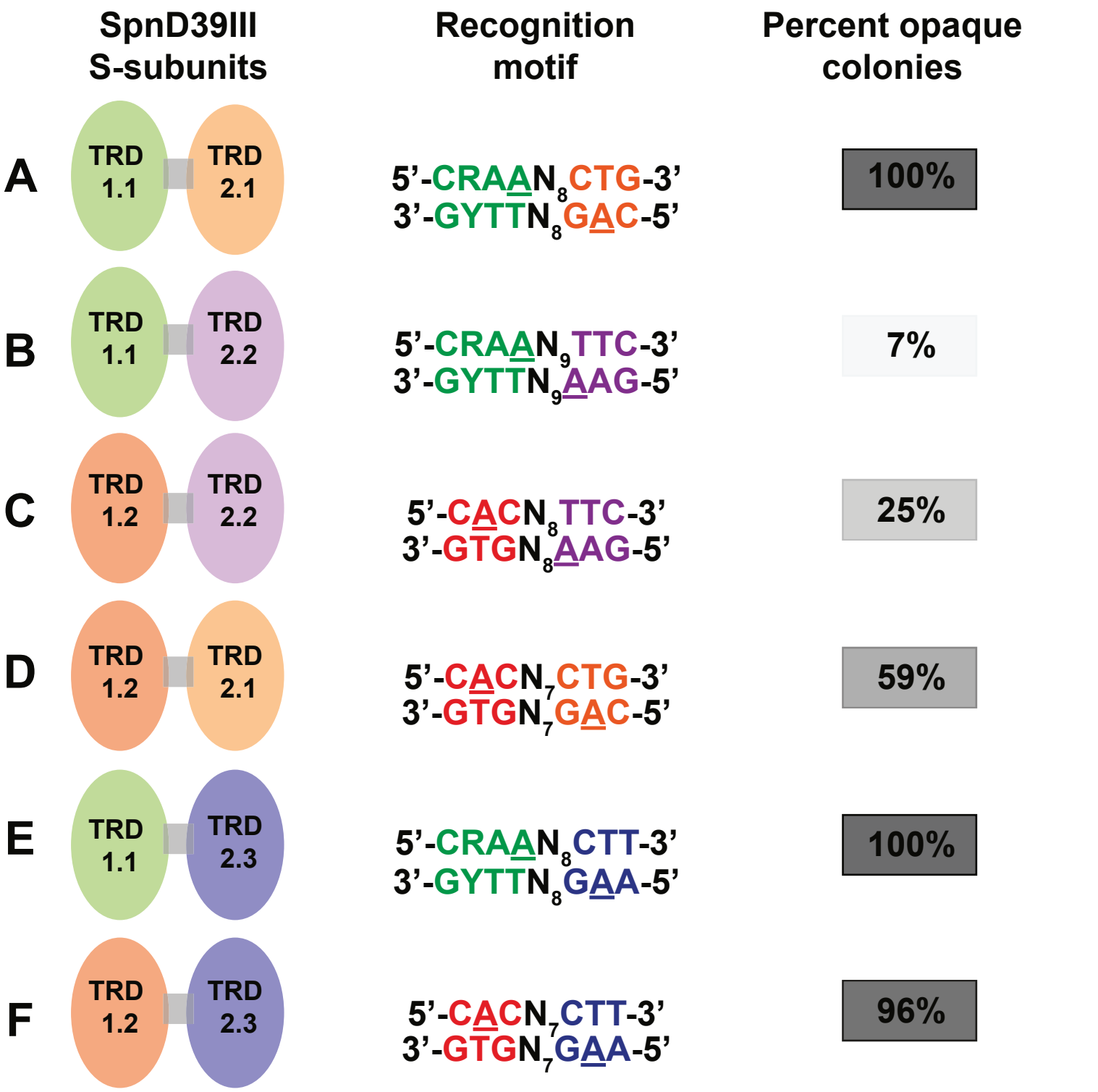
4-8 bp palindromic motif
Methylates both DNA strands
(e.g. G**A**TC)

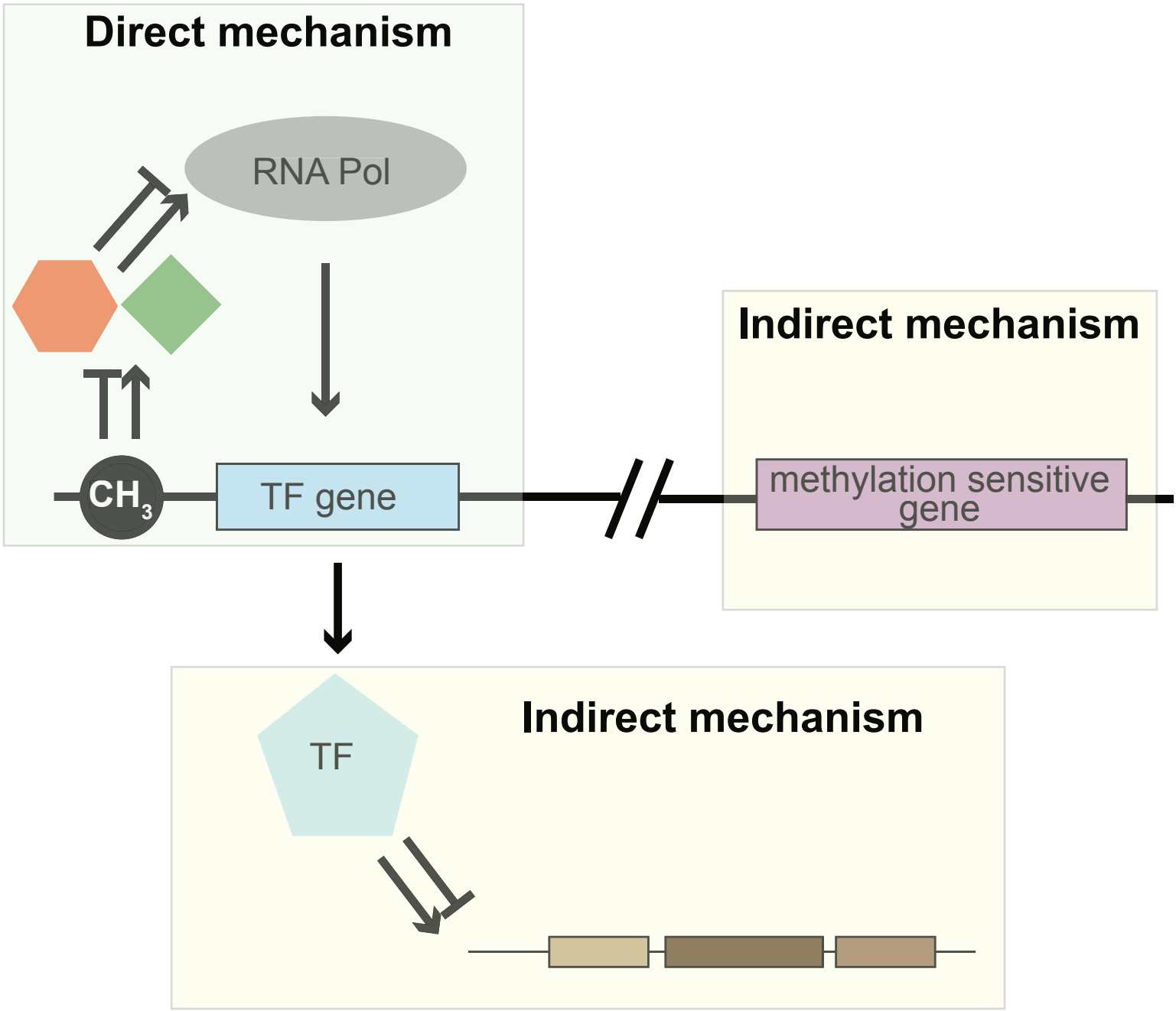
III



5-6 bp non-palindromic motif
Methylates one DNA strand
(e.g. CG**A**AT)


A
hsdS
recombinase
hsdS'
hsdR
hsdM
TRD 1.1
TRD 2.1
TRD 1.2
TRD 2.2
IR
IR
IR
IR
HsdM
TRD 1.1
TRD 2.1
TRD 1.2
TRD 2.2
TRD 1.1
TRD 2.2
TRD 1.2
TRD 2.1
Various S subunit combinations expressed in subpopulations
Subpopulation-specific methylation patterns
Subpopulation-specific gene expression
B
G n
mod
DNA Polymerase slippage at repeat
No DNA polymease errors
G n
G n
mod
Mod OFF
Mod ON
ON/OFF Mod expression in subpopulations
Subpopulation-specific methylation patterns
Subpopulation-specific gene expression


SpnD39III S-subunits
Recognition motif
Percent opaque colonies
A
TRD 1.1
TRD 2.1
5'-CRAAN8CTG-3'
3'-GYTTN8GAC-5'
100%
B
TRD 1.1
TRD 2.2
5'-CRAAN9TTC-3'
3'-GYTTN9AAG-5'
7%
C
TRD 1.2
TRD 2.2
5'-CACN8TTC-3'
3'-GTGN8AAG-5'
25%
D
TRD 1.2
TRD 2.1
5'-CACN7CTG-3'
3'-GTGN7GAC-5'
59%
E
TRD 1.1
TRD 2.3
5'-CRAAN8CTT-3'
3'-GYTTN8GAA-5'
100%
F
TRD 1.2
TRD 2.3
5'-CACN7CTT-3'
3'-GTGN7GAA-5'
96%


Direct mechanism
RNA Pol
CH3
TF gene
Indirect mechanism
methylation sensitive gene
Indirect mechanism
TF