HHS Public Access

Author manuscript

Trends Microbiol. Author manuscript; available in PMC 2019 August 01.

Published in final edited form as:

Trends Microbiol. 2018 August ; 26(8): 715–726. doi:10.1016/j.tim.2018.01.008.

Phasevarions of bacterial pathogens: methylomics sheds new light on old enemies

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Abstract

A wide variety of bacterial pathogens express phase-variable DNA methyltransferases that control expression of multiple genes via epigenetic mechanisms. These randomly switching regulons – phasevarions – regulate genes involved in pathogenesis, host-adaptation and antibiotic resistance. Individual phase-variable genes can be identified *in silico* as they contain easily recognised features such as simple sequence repeats (SSR) or inverted repeats (IR) that mediate the random switching of expression. Conversely, phasevarion-controlled genes do not contain any easily identifiable features. The study of DNA methyltransferase specificity using Single-Molecule, Real-Time (SMRT) sequencing and methylome analysis has rapidly advanced the analysis of phasevarions by allowing methylomics to be combined with whole transcriptome/proteome analysis to comprehensively characterise these systems in a number of important bacterial pathogens.

Keywords

phasevarion; DNA methyltransferase; SMRT sequencing; methylome analysis; phase-variation

Bacterial epigenetics, phase-variation, and 'phasevarions'

Epigenetics is the study of heritable changes in gene expression that occur without changes in DNA sequence [1]. Many mechanisms exist by which these changes are mediated, including DNA methylation, histone modification, and genomic imprinting [1]. DNA methylation is one of the best-studied epigenetic mechanisms, and several well-characterised systems exist within bacteria whereby DNA-methylation leads to changes in gene expression. For example, variable expression of the Pap pilus and antigen 43 in *Escherichia coli* is mediated by Dam (DNA adenine methyltransferase) methylation of sites in the gene's

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promoter region, which alters the ability of the LRP and OxyR regulatory proteins to bind [2]. Loss of Dam leads to decreased virulence in a number of human pathogens, such as *Salmonella enterica* and *Haemophilus influenzae* [3]. As another example of methyltransferase action regulating phenotype, the cell cycle of *Caulobacter crescentus* is regulated by the methyltransferase CcrM (Cell cycle regulated methyltransferase). The functions of solitary DNA methyltransferases, such as Dam and CcrM, have been reviewed in detail previously [2–4].

Many bacterial DNA **methyltransferases** (see Glossary) exist as part of a restriction-modification (R-M) systems. Four main classes of R-M system exist - types I, II, III & IV, which differ in their subunit composition, cofactor requirements, and DNA cleavage position and sequence specificity [5] (BOX 1). R-M systems are classically considered to confer protection to the bacterial cell against bacteriophages and other horizontal DNA transfers [6], with the cognate methyltransferase protecting 'self' DNA from 'non-self' foreign DNA, which is degraded by the restriction enzyme component. However, diverse roles of R-M systems have been described, such as genomic island stabilization, genome evolution, and co-factor utilization [7], in addition to their role in epigenetic regulation of gene expression [8].

Box 1

Restriction-Modification (R-M) systems

Four major classes of restriction-modification (R-M) systems have been characterized in bacteria, differing in their subunit composition, cleavage position, sequence specificity, and cofactor requirements [5]. R-M systems consist of restriction enzymes, which cleave DNA in a sequence specific manner, and cognate methyltransferase enzymes, which methylate the sequences recognized by the restriction components, protecting DNA from cleavage.

Type I systems consist of co-transcribed *hsdM*, *hsdR* and *hsdS* genes, encoding Methyltransferase (M), Restriction (R) and Specificity (S) subunits, respectively [62]. The M and R subunits are highly conserved, whereas the S subunits are highly variable. Each specificity protein is made up of two separate target recognition domains (TRDs). These domains recognize two separate 3 or 4bp sequences separated by a central spanning domain. Shuffling of the DNA sequences encoding the separate TRDs generates variability within the resulting S subunits, leading to restriction/methylation at different sequences [62]. The M_2S trimers are active, stand-alone methyltransferases; the R_2M_2S pentamer is required for DNA cleavage.

Type II systems consist of two independent enzymes: a methyltransferase (Mod) and a restriction endonuclease (Res). Recognition sequences are typically 4–8nt long and palindromic. Eleven distinct subtypes of type II class Res exist, characterised by their behavior and cleavage properties [63]. Mod and Res are both active, stand-alone enzymes.

Type III systems consist of a methyltransferase (encoded by *mod*) and a restriction endonuclease (encoded by *res*). These genes are transcribed together and form a two-

subunit complex [64]. Mod catalyses the methylation of a single strand of DNA at a specific 4–6 bp asymmetrical recognition sequence, independently of the Res subunit [65]. Mod (M_2) is active as a methyltransferase alone, whereas Res requires a complex with the Mod subunit in an R_2M_2 stoichiometry in order to cleave DNA, usually 25–27 bp downstream of the sequence recognized by Mod [66]. REBASE contains over 1500 type III systems [40], although only ~60 of these have a defined specificity [65].

Type IV systems are methylation dependent restriction systems, and although they are useful tools for epigenetic research [67] they are not associated with cognate methyltransferases [5, 67].

Intriguingly, the genomes of many bacterial pathogens contain DNA methyltransferase genes, associated with R-M systems, that are phase-variable [9–16]. **Phase-variation** is the random and reversible switching of gene expression (BOX 2), and is typically associated with bacterial surface structures [17]. Phase-variation of DNA methyltransferases can occur due to either (1) hypermutation of **simple sequence repeats** (SSRs) in the open reading frame (ORF), resulting in ON-OFF switching of gene expression, or (2) through genetic 'shuffling' of expressed and silent genes via **inverted repeats** (IRs), which results in multiple allelic variants of a single protein (BOX 2). Phase-variation of DNA methyltransferase expression results in differential DNA methylation throughout the genome, leading to variable expression of *multiple* genes via epigenetic mechanisms (Figure 1; Key Figure). These systems are called **phasevarions** (*phase-vari*able regul*ons*) [8, 18]. The concept of the phasevarion was first described in *H. influenzae* strain Rd [16]. In this system, phase-variable ON/OFF switching of a type III DNA methyltransferase gene, *modA1*, occurs as a result of reversible changes in the number of simple sequence repeats located in the *modA1* open reading frame. Comparison of the *modA1* ON vs. OFF variants revealed that fifteen genes are differentially expressed, including heat-shock proteins *dnaK* and *dnaJ*, and outer-membrane opacity protein *opa* [16]. Since this initial characterization, phase-variable DNA methyltransferases have been identified and shown to control phasevarions in many human-adapted pathogens including the pathogenic *Neisseria* [14], *Helicobacter pylori* [15] *Moraxella catarrhalis* [10] and *Streptococcus pneumoniae* [11]. All these phasevarions regulate expression of genes that are involved in host colonisation, survival, and pathogenesis, and many regulate expression of putative vaccine candidates.

Box 2

Phase-variation

Phase-variation is the random and reversible switching of gene expression. It is traditionally associated with genes encoding bacterial surface features, such as adhesins [68], pili [69], iron acquisition proteins [70, 71], and lipo-oligosaccharide (LOS) [72, 73]. *Phase-variation allows a population of organisms to generate a phenotypically diverse population*. These mixed populations may contain individuals that are, for example, primed to evade an immune response, or better equipped to colonise certain host niches. This random switching of expression means that proteins encoded by phase-variable genes are not ideal vaccine candidates, as their expression is not stable. Phase-variable

genes contain sequence features that are easily identified *in silico*, meaning the proteins they encode can be discounted from development as vaccine candidates. These easily identifiable features are inverted repeats (IRs), and simple-sequence repeats (SSRs) (Figure 1) [8, 17]. Recombination between homologous IRs results in gene shuffling between expressed and silent variants of particular loci. Therefore, the protein encoded by a gene containing IRs is always expressed, but shuffles between a number of allelic variants. SSR tracts are unstable, and vary in length through DNA polymerase slippage during replication. Depending on the number of SSRs present in the tract, genes containing SSRs are in-frame, and expressed (ON), or are out-of-frame, resulting in a premature stop codon, and not expressed (OFF).

This review aims to detail the current state of phasevarion research, and highlight the role of phase-variable DNA methyltransferases in several major human pathogens.

Detection of DNA methylation and the advent of SMRT sequencing/ methylome analysis

The epigenetic nature of phasevarions complicates the *in silico* identification of stably expressed proteins, as the regulated genes do not contain any identifiable features [8]. The only way to identify genes in a phasevarion is by detailed study of the organisms containing these systems, using gene and/or protein expression analysis techniques. Although epigenetic gene regulation has been studied for many years, the actual characterization of the DNA methyltransferases themselves, in particular the sequences methylated and their genomic context, has been difficult and time consuming. This is especially true for bacteria, in which adenine methylation is the most common form of DNA methylation [19]. Many methods have been developed for eukaryotic CpG methylation, which is important in a variety of processes, including X-chromosome inactivation, carcinogenesis, and chromatin structure [20]. Specific methods to study CpG methylation, such as bisulphite sequencing [21], are not applicable to other forms of methylation. Other methods based on bisulphite sequencing require knowledge of sequence context within which methylations occur, such as methylation specific PCR [22] or methylation specific co-immunoprecipitation [23]. Methods for monitoring adenine methylation are rare, with those developed requiring extensive experimentation, such as chemical modification and bond formation using modified oligonucleotides and chemical crosslinking [24], or the use of radio-labelled AdoMet [25]. Restriction-inhibition assays using methylation sensitive restriction enzymes can be used [14, 26], but these are time consuming and not guaranteed to be successful as restriction enzyme sites may not overlap the particular DNA sequence that is methylated. Mass spectrometry can be used to detect the methyl group itself, but this technique gives no information on the actual sequence context. These limitations therefore made study of adenine methyltransferases particularly difficult, as no high-throughput method was available to rapidly detect the motifs methylated by these systems. Knowledge of DNA methyltransferase sequence context facilitates the identification and study of genes controlled by differential methylation. Recently, Oxford Nanopore MinION DNA sequencing technology has been used to map methylated adenine and cytosine residues using bacterial genomic DNA [27], and of methylated cytosine residues using human

genomic DNA [28]. However, this technology has not yet been used to discover the specificity of uncharacterized methyltransferases, although these recent advances are an excellent development for the field of methylomics.

Single Molecule Real Time (SMRT) sequencing was developed as a new DNA sequencing technology in 2010 by Pacific Biosciences (PacBio), and was applied to the study of genome wide methylation patterns [29, 30]. During SMRT sequencing, analysis of the kinetics of DNA synthesis allow a sequence to be generated and the position of modifications such as methylation to be identified [29, 31] (BOX 3). SMRT sequencing/methylome analysis therefore provides a complete, closed genome sequence, and reveals the position of every DNA modification in that genome [32]. A thorough review of SMRT methylome analysis has been published previously [33] and provides significant detail about this technique.

Box 3

Single-Molecule, Real-Time (SMRT) DNA sequencing and methylome analysis

SMRT sequencing technology uses fluorescently labeled nucleotides, and directly synthesizes DNA from the input template in order to generate a sequence by monitoring the pulse from each nucleotide as it is incorporated into the nascent polynucleotide chain [29, 31]. It is possible to monitor the time between pluses using this system, with the time between the incorporation of two adjacent bases known as the inter-pulse duration (IPD). When bases are modified on the template strand, e.g., a methyl group is present on an adenine residue, the IPD is increased, as this modification delays incorporation of the complementary thymidine base into the nascent daughter strand. Through thousands of reads, the average IPD of every position can be calculated, and that base called as modified or unmodified based on the average IPD for that context in known unmodified samples [29, 30]. Therefore, SMRT sequencing coupled to whole genome methylome analysis not only gives complete, closed genomes for the organism under study, but also shows exactly which residues are modified, and their position in the genome.

Over the last ~5 years, SMRT sequencing/methylome analysis has been used to verify existing DNA methyltransferase specificities [30] and to identify new, previously uncharacterized methyltransferases in a variety of bacterial species [30, 34]. SMRT sequencing/methylome analysis has also been used to characterize the complete methylomes of a number of important bacterial pathogens, including *Campylobacter jejuni* strains 11168 and 81–176 [34], *E. coli* ST131 [35] and several strains of *H. pylori* [36, 37]. Knowledge of the methylome will be invaluable in further understanding the pathobiology of these organisms. Methylome studies provide the opportunity to investigate the roles of DNA methyltransferases in bacterial physiology; for example, the role of DNA methyltransferases during the cell cycle has been characterized in *C. crescentus* using SMRT sequencing/methylome analysis [38]. The power of SMRT sequencing/methylome analysis has also been demonstrated while analyzing phase-variable DNA methyltransferases that control phasevarions. Comparison of genomic DNA from a pair of isolates containing a phase-variable DNA methyltransferase gene, with the gene expressed in one sample (i.e., phase-

varied ON) and not-expressed in the other (i.e., phase-varied OFF, or a knock-out mutant; Figure 2), has been used in several different bacteria to identify the exact sequence recognized and methylated by that particular DNA methyltransferase [9, 10, 39]. In addition, knowledge of methylation differences can be correlated with gene expression profiles, facilitating studies of the exact mechanism responsible for differential gene expression. There are currently over 4,000 PacBio SMRT records in the restriction enzyme database REBASE [40], with almost half of the identified methylation motifs assigned to known methyltransferases.

Phase-variable type III *mod* genes are the most well-studied phasevarion-controlling methyltransferases

Since the first description of a type III *mod* gene in *H. influenzae* strain Rd that controlled a phasevarion [16], a number of phase-variable type III *mod* genes encoding a phase-variable methyltransferase have been identified in human-adapted bacterial pathogens. In every case studied to date, phase-variable ON/OFF switching of the type III DNA methyltransferase, mediated by SSRs, results in differential regulation of multiple genes. Currently, well characterized phasevarions and *mod* genes include *modA* in non-typeable *Haemophilus* influenzae (NTHi), *Neisseria meningitidis* and *Neisseria gonorrhoeae; modB* in *N. meningitidis* and *N. gonorrhoeae* [14, 41]; *modD* in *N. meningitidis* [13]; *modH* in *H. pylori* [15]; and *modM* in *Moraxella catarrhalis* [12].

The *mod* family is highly variable, with SSR tract unit and length, and tract location varying considerably between *mod* genes (Figure 3). There is very little sequence identity between *mod* genes [42]. Thus phase-variation of *mod* genes, and consequently phasevarions, appear to have evolved independently several times in different bacterial species. This implies that the phenotypic diversity resulting from *mod* phase-variation provides a considerable advantage. Each individual *mod* gene (*modA*, *modB*, etc) is highly conserved in the N- and C- terminal regions, with only the central DNA recognition domain (DRD) showing significant allelic variation [14, 15, 42] (Figure 3). The DRD dictates the specificity of the enzyme. Therefore, different Mod alleles with distinct DRDs methylate different DNA sequences, and therefore regulate expression of different genes. For *modA*, different alleles evolve from shuffling and transfer of different *modA* sub-sequences, leading to new DRDs, and therefore new alleles with different methylation specificities [42]. This process has led to the evolution of twenty-one different *modA* alleles in *H. influenzae* and *Neisseria* spp. [9, 42–44]. A recent study describing the phasevarions controlled by the five most prevalent *modA* alleles (*modA2, 4, 5, 9, 10*) in NTHi isolated from children with middle ear infection used SMRT sequencing and methylome analysis to rapidly define the methylation specificities of these alleles, as well as generate closed, annotated genomes for prototypical strains containing each of these alleles [9]. This analysis also showed differential regulation of the putative NTHi vaccine candidates HMW, OMP P5, and OMP P6 by *modA* phase variation in NTHi [9]. Multiple allelic variants of other *mod* genes have also evolved: six *modB* and seven *modD* alleles have been identified in the pathogenic *Neisseria* [39, 44]; *H. pylori* strains contain one of seventeen different *modH* alleles [15]; and at least three *modM* alleles have been identified in *M. catarrhalis* [10, 12]. The study of *modM* in *M. catarrhalis*

used SMRT sequencing/methylome analysis to define the methylation specificity of the most prevalent *modM* allele, *modM2* [10], and was also used recently to determine the methylation specificity of the *modM3* allele [45].

Some strains of *N. meningitidis* can contain up to three separate phase-variable *mod* genes - *modA, modB* and *modD*. Each individual *mod* gene has a different methyltransferase specificity, and all have been shown to control individual phasevarions [13, 14, 25, 39, 44]. Even though the pathogenic Neisseria contain multiple *mod* genes, SMRT sequencing/ methylome analysis allowed the specificity of the most common *mod* alleles present in these organisms to be rapidly identified [39, 44]: *modA11* (5′-CGY**m6A**G-3′), *modA12* (5′-AC**m6A**CC-3′) and *modD1* (5′-CC**m6A**GC-3′). SMRT sequencing technology was particularly powerful in determining the ModA11 recognition sequence, which has a highly relaxed specificity around the core recognition motif of CGY^{m6}**A**G. The level of methylation was also dependent on the bases flanking this core regions, ranging from 4.6% methylation at GCGC**m6A**GG sites, to 100% methylation at ACGT**m6A**GG sites (core sequence underlined) [39]. Determination of this specificity would have been almost impossible without the power of SMRT sequencing/methylome analysis.

There is significant evidence that particular *mod* alleles, meaning particular phasevarions, are associated with virulence and pathogenesis, and this was reviewed in detail recently [8]. The selection for NTHi containing *modA2* ON has been demonstrated to occur in the middle ear during experimental otitis media [9], and the switch from the *modA2* OFF to ON state within the middle ear is associated with increased disease severity [46]. Phase-variation of *modA2* also leads to differential responses to oxidative stress and neutrophil killing [47]. Phase variation of the *modA10* allele in NTHi leads to increased cellular adhesion and invasion, and in increased host death when it is switched OFF compared to ON [48]. A preference for the *modM3* allele in *M. catarrhalis* has been suggested during middle ear infection, with a significant number of middle ear isolates containing this allele when compared to strains isolated from the nasopharynx [10]. A recently identified phasevarion in the paediatric pathogen *Kingella kingae* modulates the host immune response, and increased bacterial toxin production is seen when the *modK1* allele is ON, relative to *modK1* OFF [49].

The most prevalent *modH* allele in *H. pylori*, *modH5*, has been shown recently to control expression of the flagellum of this organism [50]. SMRT sequencing and methylome analysis revealed that ModH5 methylates the sequence 5′-G^{m6}**A**CC-3′. This sequence was found to be over-represented in a number of virulence associated genes, including the major flagellar component, *flaA* [50]. Determination of the recognition sequence of ModH5 by SMRT sequencing and methylome analysis subsequently allowed the demonstration that differential methylation of a 5′-GACC-3′ motif in the promoter of *flaA* leads directly to expression differences in this gene. This is the first demonstration of methyltransferase phase-variation directly controlling the gene expression of a member of a phasevarion [50], with rapid elucidation of the methylation specificity of ModH5 key to this demonstration.

Phase-variable type I R–M systems switch their expression and specificity through a variety of methods

Type I R-M systems have been shown to phase-vary through changes in length of SSRs, and by genetic shuffling of sequences via IRs (BOX 2). Changes in the length of SSRs leads to ON/OFF switching of DNA methyltransferase activity, akin to the type III *mod* systems, and also changes the specificity of some type I systems. Shuffling of sequences leads to multiple methyltransferase activities by producing a variety of HsdS proteins.

Variation in SSR length can occur in both *hsdM* and *hsdS* subunits. For example, ON/OFF switching of a type I *hsdM* gene in *H. influenzae* occurs due to changes in length of a pentanucleotide $GACGA_{(n)}$ SSR located in the *hsdM* open reading frame. This ON/OFF switching results in differences in resistance to phage infection [51]. Phase-variation of an *hsdM* gene in the bovine pathogen *Mannheimia haemolytica* results in variable production of the leukotoxin produced by this organism [52]. This *hsdM* gene also contains a pentanucleotide SSR, but the repeating unit is $CAGCA_{(n)}$.

N. gonorrhoeae contains an unusual phase-variable type I system, with the *hsdS* gene of this loci split into two different open reading frames ($hsdS_{NgoAV1}$ and $hsdS_{NgoAV2}$), due to changes in length of a poly-guanidine tract in the 3′ end of the gene. This *hsdS* gene produces a truncated ($hsdS_{NgoAV1}$ only) or full length ($hsdS_{NgoAV1}$ and $hsdS_{NgoAV2}$ fused as a single polypeptide) HsdS specificity protein, dependent on the length of this poly-guanidine tract [26]. The truncated HsdS protein designated NgoAV, results in methylation of the sequence 5′-$GC^{m6}A(N_8)$TGC-3′/3′-GC $^{m6}A(N_8)$TGC-5′. whereas the full length HsdS protein, NgoAVΔ, results in methylation of 5′-$GC\mathbf{^{m6}A}(N_7)$GTCA-3′/3′-$TG\mathbf{^{m6}A}C(N_7)$TGC-5′ and 5′-$GCA(N_7)$CTCA-3′/3′-$TG\mathbf{^{m6}A}G(N_7)$TGC-5′, although the latter sequence is methylated only on the complementary strand [26]. Thus, rather than variation in SSRs leading to ON/OFF methyltransferase switching, two distinct methyltransferase activities result from SSR changes in this gene. Since this phase variation would result in distinct genomic methylation patterns, distinct phasevarions may be controlled, although this remains to be investigated. The specificity of these enzymes was determined by time-consuming restriction-inhibition assays [26], which are dependent on restriction enzymes that cut at the same site as the methyltransferase acts and are inhibited by this methylation.

A third type of phase variable type I system has been identified that contains multiple variable *hsdS* genes, which recombine through shuffling of different *hsdS* genes to produce methyltransferases with distinct specificities. An excellent review has recently been published describing the role and variety of these ‘locus inverting’ phase-variable type I methyltransferases [53]. Rearrangements occur between distinct IRs located in the *hsdS* loci, and may be facilitated by a locus-associated recombinase. The first example of a ‘locus inverting’ phase-variable type I system controlling phasevarions was described in the human pathogen *Streptococcus pneumoniae* [11, 54]. Shuffling between the variable *hsdS* genes in the SpnD39III locus results in six different HsdS proteins, termed SpnIIID39A-F, that have six different methylation specificities, and result in six distinct gene expression patterns [11]. Several genes involved in capsule biosynthesis are downregulated when the SpnD39III-B

variant (specificity of 5′-CRA**m6**AN$_9$TTC-3′/3′-GYTTN$_9$**m6**AAG-5′) is expressed, adding a further layer to the complexity of capsule regulation in the pneumococcus. A distinct phasevarion was shown to be regulated when the SpnD39III-A variant is expressed (specificity of 5′-CRA**m6**AN$_8$CTG-3′/3′-GYTTN8**m6**G**A**C-5′), with genes involved in the stress response (*dnaK, gpx*) and nutrient acquisition (*psaABC*, *fucA, K, U*) differentially regulated compared to other SpnD39III alleles. The six DNA methyltransferase activities where rapidly defined by PacBio SMRT sequencing/methylome analysis by using strains where each methyltransferase was ‘locked’ into a single *hsdS* allele and unable to switch [11]. The unusual motifs recognized and methylated by type I enzymes would have made elucidation extremely difficult and time-consumingusing conventional methods.

A similar system with duplicated, variable *hsdS* loci (*hsdS* and *hsdS*’) containing IRs has been identified in the zoonotic pathogen *Streptococcus suis* [55]. This system is associated with human invasive disease [56], although the methylation specificity and demonstration of methyltransferase phase-variation has yet to be demonstrated.

Phase-variable type II methyltransferases have been identified in closely related gastric pathogens

In *H. pylori,* the genes encoding DNA methyltransferases associated with several type II R–M systems contain SSRs [57], and many are associated with colonization and virulence [58, 59]. For example, a survey of the gene encoding the M.HpyAIV methyltransferase in clinical isolates of *H. pylori* found changes in a poly-adenine tract correlated with ON/OFF switching of this methyltransferase [60]. Methylation by M.HpyAIV, at 5′-G**m6**ANTC-3′ sites, was also shown to influence expression of catalase (*katA*), and was demonstrated to induce a more robust host response in mice, suggesting it controls a phasevarion [60]. In another DNA methyltransferase of *H. pylori*, Hpy99XXII, changes in the length of a poly-guanidine tract located in the gene resulted in expression of this methyltransferase from an inactive form with a different poly-guanidine tract length [36]. SMRT sequencing/methylome analysis showed that the recognition sequence of this methyltransferase is 5′-TC**m6**AN$_6$TRG-3′. Analysis of the genome sequences of multiple strains of *H. pylori* show variation in the length of the poly-guanidine tract found in the gene encoding this methyltransferase [36], indicating phase-variable expression; however, control of a phasevarion needs to be experimentally confirmed.

C. jejuni contains a phase-variable type II restriction modification system, Cj0031 [61]. ON/OFF switching of this R-M system resulted from variation in the length of a poly-guanidine tract located in the open reading frame of *cj0031.* A number of clinically significant phenotypes such as biofilm formation and cellular invasion were significantly altered by ON/OFF switching of this methyltransferase [61]. Genes such as the autotransporter *capA*, the adhesin *cadF*, and the periplasmic binding protein *peb1A* were all regulated by phase-variable ON/OFF switching of the *cj0031* gene. The specificity of the Cj0031 methyltransferase enzyme was determined to be 5′-CCYG**m6**A-3′ using SMRT sequencing/methylome analysis [61]. The variability in this site would have been almost impossible to determine using standard restriction-inhibition assays, providing another

example of the power of SMRT sequencing/methylome analysis to determine the specificity of previously uncharacterised systems.

To our knowledge, no other phase-variable type II R-M systems have been identified to date, which leads to the intriguing possibility that they have only evolved in closely related pathogens that cause disease in a specific niche, i.e., the human digestive tract.

Concluding remarks and future perspectives

The list of phase-variable DNA methyltransferases controlling phasevarions is ever expanding, with many new systems characterised within the last five years. The identification of a variety of phase-variable methyltransferases, that switch their expression via distinct mechanisms, implies that phasevarions have evolved independently in different species and suggests that this type of variable epigenetic regulation provides a strong selective advantage. The random and reversible switching of phase-variable DNA methyltransferases leads to multiple distinct phenotypes in a population that are subject to periodic selection and counter-selection in different environments. Improvements in DNA sequencing technology has identified many new R-M systems in a variety of bacterial species, with SMRT sequencing/methylome analysis allowing the specificity of these newly identified systems to be rapidly identified. Although extensive effort is still required to elucidate the genes regulated by phase-variable methyltransferase expression, ongoing advances in transcriptomic and proteomic technologies have made this process much easier. Identification of the sites of methyltransferase activity by SMRT sequencing/methylome analysis allows coupling of phenotypic analysis with gene expression studies to comprehensively identify all members of a phasevarion

A thorough understanding of phase-variable methyltransferases, and the phasevarions they control, is required for a better understanding of bacterial pathogenesis as well as the development of new and novel vaccines and treatments (see Outstanding Questions). Due to the complexity and variability of epigenetic regulation in bacterial pathogens, *in silico* methods to determine if a gene is phase-variable may no longer be adequate; even targets that contain no identifiable features associated with phase-variation may be subject to variable expression as they can be regulated in a phasevarion. Therefore, rapid methods to identify the sequences modified by phase-variable DNA methyltransferases (such as SMRT sequencing/methylome analysis) and to determine the genes within the phasevarions (such as RNA-Seq and/or SWATH proteomics), should be widely employed during bacterial studies. A comprehensive characterization of phasevarions is a necessity to direct and inform future vaccine development and treatments for the ever-growing list of pathogenic bacteria containing these systems.

OUTSTANDING QUESTIONS

- The exact molecular mechanism by which differential methylation affects gene expression needs to be investigated, and is a major area of work currently underway in this field.

  - Does differential methylation directly affect gene expression as a result of methylation in promoter regions that affects binding by regulators/transcription factors/RNA polymerase, etc?
  - Does methylation indirectly affect gene expression due to regulation of an unlinked locus, for example an activator of the gene in the phasevarion?
- Most phasevarion characterization to date has been carried out *in vitro*. While this provides invaluable information about the genes controlled by each phasevarion, many genes may not be identified as they may be regulated only during *in vivo* conditions. Therefore, what are expression profiles from bacteria isolated *in vivo*?
- What is the combined effect of multiple phase-variable methyltransferases in single strains? For example, pathogenic *Neisseria* can contain up to three phase-variable *mod* genes (*modA*, *modB* and *modD*), all switching their expression independently, and all controlling different phasevarions.
- Several phase-variable methyltransferases have been highlighted in this review, but do they control phasevarions?

Acknowledgments

This study was funded by Australian National Health and Medical Research Council (NHMRC) (Project Grant 1021631 and Career Development Fellowship to KLS; Project Grant 1099279 to KLS and JMA; Program Grant 1071659 to MPJ); Garnett Passe & Rodney Williams Memorial Foundation (GPRWMF) Research Training Fellowship to AT, and National Institute of Health (NIH) USA grant R01 DC015688 to LOB and MPJ.

GLOSSARY

Inverted repeat; IR
a short sequence, typically ~10–80 bases long, that is duplicated and inverted a number of base pairs downstream allowing recombination, or shuffling, of the DNA between the two repeated sequences

Modification enzyme/Methyltransferase
enzymes that add a methyl (CH3) group to a specific base in DNA, usually in a sequence specific manner. They can protect ‘self’ DNA from degradation by a cognate restriction enzyme

Phase-variation
the rapid and reversible switching of gene expression

Phasevarion
phase-variable regulon. The suite of genes regulated by phase-variation of a single methyltransferase

Simple sequence repeat; SSR

a short simple genetic sequence (e.g., G(n), TA(n), AGCC(n)) repeated a number of times within or associated with an open reading frame

Restriction enzyme

bacterial enzymes that degrade DNA in a sequence specific manner

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TRENDS BOX

- Phase variable DNA methyltransferases mediate epigenetic regulation in many human pathogens
- Phase variable regulons, phasevarions, play important roles in bacterial virulence and pathobiology
- In all characterised phasevarions, methyltransferase phase variation controls genes involved in pathobiology, and contain current and putative vaccine candidates
- SMRT DNA sequencing and methylome analysis has revolutionised the field of bacterial epigenetics
- Understanding phasevarions is key to the development of effective treatments and vaccines

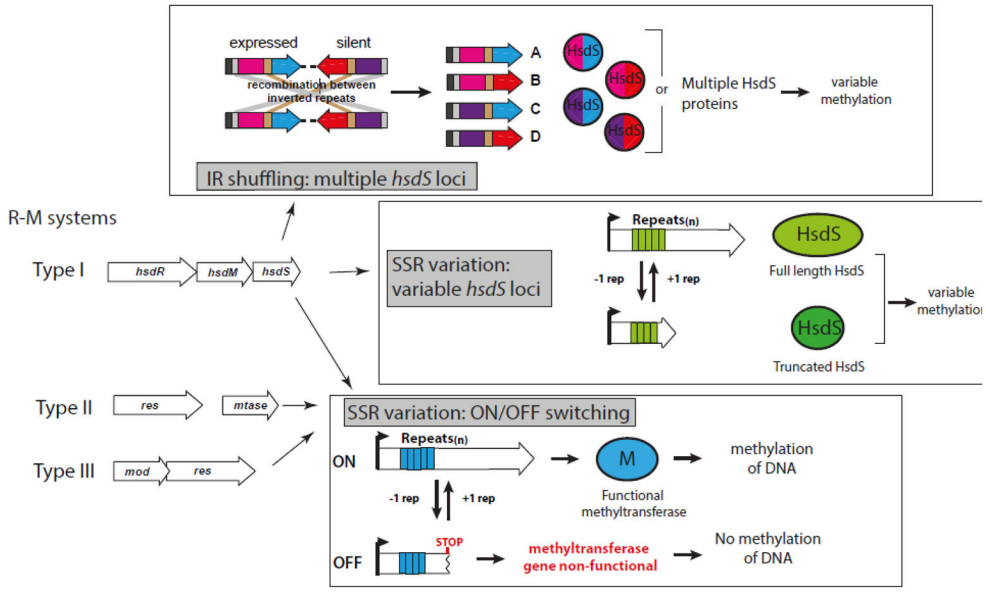
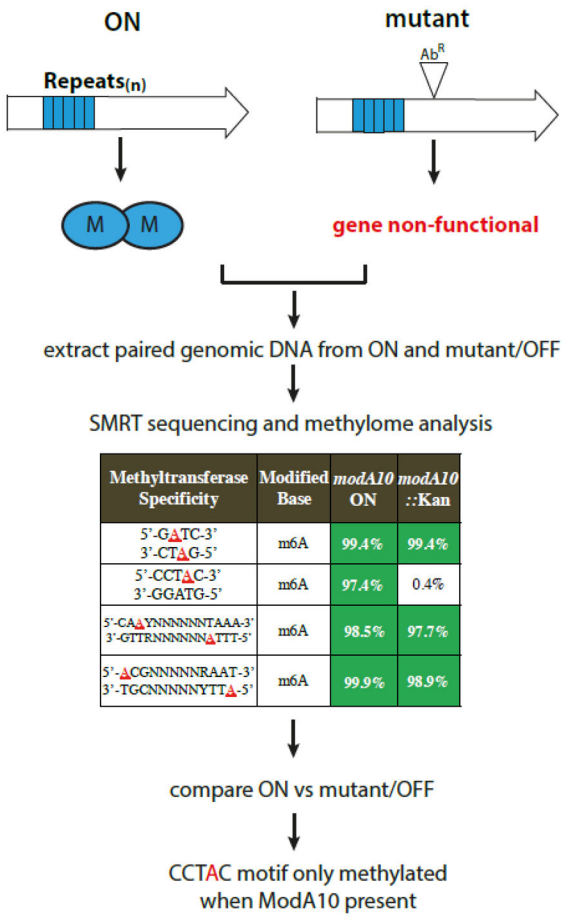


Figure 1. An illustration of the ways methyltransferase loci (R-M) phase-vary

Type I R-M systems can produce multiple methyltransferase variants via shuffling of variable, duplicated *hsdS* loci through the presence of inverted repeats (IRs) in the ORF of these *hsdS* genes. Type I loci can also generate multiple HsdS proteins through variation in simple sequence repeats (SSR). Variation in SSR length leads to ON/OFF switching of all three types of R-M loci.



Methyltransferase Specificity	Modified Base	*modA10* ON	*modA10* ::Kan
5'-GATC-3' 3'-CTAG-5'	m6A	99.4%	99.4%
5'-CCTAC-3' 3'-GGATG-5'	m6A	97.4%	0.4%
5'-CAAYNNNNNNTAAA-3' 3'-GTTRNNNNNNATTT-5'	m6A	98.5%	97.7%
5'-ACGNNNNNRAAT-3' 3'-TGCNNNNNYTTA-5'	m6A	99.9%	98.9%

Figure 2. Determination of phase-variable methyltransferase specificity using SMRT sequencing/ methylome analysis

By using genomic DNA preparations from paired isolates that express the methyltransferase (ON variant) or do not express the methyltransferase (OFF variant or a knock-out mutant), it is possible to determine the specificity of the methyltransferase under investigation. Paired genomic DNA samples are subjected to SMRT sequencing, the resulting methylomes are compared, and the phase-variable methyltransferase specificity is identified as the motif that is absent from the OFF variant/knock-out. This example is actual SMRT data generated to decipher ModA10 specificity in NTHi [9].

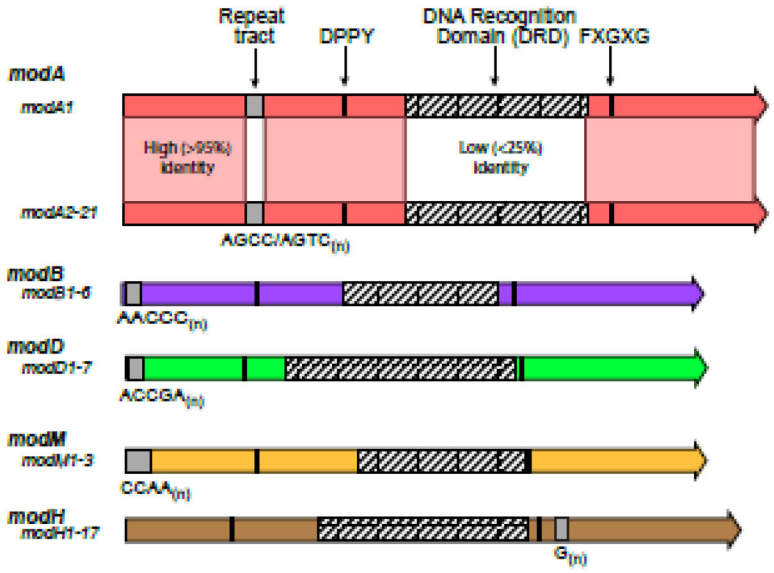


Figure 3. Type III *mod* genes have evolved phase-variable expression multiple times

There are currently five well characterized phase-variable type III *mod* genes, all of which control phasevarions – *modA*, *modB*, *modD*, *modH*, and *modM*. Each of these *mod* genes is distinct, with low sequence homology between each class of *mod* gene. Within each class of *mod* gene there are multiple allelic variants, which are highly conserved except for their central DNA recognition domain (DRD). Diversity of the DRD means that each *mod* allele methylates a different DNA sequence, and therefore controls a different phasevarion.