

Exploring the Roles of DNA Methylation in the Metal-Reducing Bacterium *Shewanella oneidensis* MR-1

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We performed whole-genome analyses of DNA methylation in *Shewanella oneidensis* MR-1 to examine its possible role in regulating gene expression and other cellular processes. Single-molecule real-time (SMRT) sequencing revealed extensive methylation of adenine (N6mA) throughout the genome. These methylated bases were located in five sequence motifs, including three novel targets for type I restriction/modification enzymes. The sequence motifs targeted by putative methyltransferases were determined via SMRT sequencing of gene knockout mutants. In addition, we found that *S. oneidensis* MR-1 cultures grown under various culture conditions displayed different DNA methylation patterns. However, the small number of differentially methylated sites could not be directly linked to the much larger number of differentially expressed genes under these conditions, suggesting that DNA methylation is not a major regulator of gene expression in *S. oneidensis* MR-1. The enrichment of methylated GATC motifs in the origin of replication indicates that DNA methylation may regulate genome replication in a manner similar to that seen in *Escherichia coli*. Furthermore, comparative analyses suggest that many *Gammaproteobacteria*, including all members of the *Shewanellaceae* family, may also utilize DNA methylation to regulate genome replication.

DNA methylation plays a variety of functional roles in bacteria (1–3). For example, restriction-modification (R-M) systems use methylation patterns to identify and destroy foreign DNA during viral infections (4, 5). Bacteria also use DNA methylation to regulate genome replication (6), DNA mismatch repair (7), and gene expression (8–12). Methylation can even serve as an epigenetic modifier, influencing the expression patterns of daughter cells based on environmental conditions (13, 14). Because of these varied regulatory roles, DNA methylation should be incorporated into our emerging systems-level view of model microorganisms.

Despite the functional significance of DNA methylation, our understanding of its role in bacterial genetics and physiology remains incomplete due to methodological limitations. For example, bisulfite conversion can identify 5-methylcytosine modifications (15, 16), but there is no corresponding conversion assay for other common modifications in bacteria, such as N6-methyladenine (N6mA) or 4-methylcytosine (17). Methyl-sensitive restriction enzymes have been used to identify the methylation states of specific sequence motifs (18–20), but complete methylome analyses are not possible without prior knowledge of the entire set of methyltransferases and their sequence targets within a genome and access to methyl-sensitive restriction enzymes targeting these motifs. Single-molecule real-time (SMRT) sequencing overcomes these limitations and enables genome-wide analysis of DNA methylation with single base resolution (21). In this approach, modifications in the native-state DNA are revealed by deviations in the polymerase kinetics observed during sequencing. The specific type of DNA methylation can often be determined from the polymerase kinetics, e.g., N6-methyladenine or 4-methylcytosine. With SMRT sequencing, it is now possible to identify the complete set of methylated sequence motifs within a microbial genome and the methylation state for each instance of a motif (22–24). This represents a powerful tool for characterizing the functional roles of DNA methylation in a wide variety of bacteria.

Shewanella oneidensis MR-1 is a bacterial isolate belonging to the *Shewanellaceae*, a family distinguished by the wide variety of

electron acceptors they can utilize (e.g., iron, manganese, uranium, chromium, and plutonium) (25–28). Because of their flexible respiratory pathways, *Shewanella* species are recognized as potential agents for bioremediation at sites contaminated with heavy metals and radionuclides (29). To better exploit its metabolic potential, *S. oneidensis* MR-1 has been characterized extensively, including analysis of gene expression (30, 31), identification of regulatory regions (32), and the determination of fitness levels for thousands of gene knockout mutants (33). However, the developing systems-level view of *Shewanella* does not yet incorporate DNA methylation and its potential regulatory roles. Genomic analyses reveal multiple putative methyltransferases in *S. oneidensis* MR-1 (34, 35), including several apparent “orphans” that lack corresponding restriction enzymes. It remains unclear what role these orphan methyltransferases might play.

Here we used SMRT sequencing to provide the first look at DNA methylation in *S. oneidensis* MR-1. We identified methylated sites throughout the genome, as well as the sequence motifs targeted by predicted methyltransferases. To determine if DNA methylation regulates gene expression, we examined whether changes in the expression level correspond with changes in the DNA methylation state when cultures are transferred from one set of growth conditions to another. Finally, we examined the finished genomes of all *Gammaproteobacteria*, including the *Shewanellaceae*, to determine which groups appear to use DNA meth-

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ylation for regulating genome replication and DNA mismatch repair.

MATERIALS AND METHODS

Strains, culture conditions, and nucleic acid isolation. *S. oneidensis* MR-1 was obtained from the American Type Culture Collection (catalog number 700550). The aerobic minimal medium contained, per liter, 1.5 g NH₄Cl, 0.1 g KCl, 1.75 g NaCl, 0.61 g MgCl₂·6H₂O, 0.6 g NaH₂PO₄, 30 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer, 20 mM DL-lactate, Wolfe's vitamins, and Wolfe's minerals (pH 7). Fumarate was added as an electron acceptor to anaerobic minimal medium (30 mM, final concentration). Anaerobic minimal medium experiments were set up in an anaerobic chamber (Coy) with a gas mix of 5% H₂, 10% CO₂, and 85% N₂ and incubation in serum bottles closed with butyl rubber stopper at 200 rpm. Aerobic minimal medium experiments were conducted in 10-ml culture tubes or 250-ml culture flasks at 200 rpm. All cultures were incubated at 30°C.

To conduct the initial survey of DNA methylation, wild-type cells were collected during exponential growth in minimal medium (optical density at 600 nm [OD₆₀₀] = 0.32). DNA and RNA were isolated with the DNeasy Blood & Tissue kit (Qiagen) and RNeasy minikit (Qiagen), respectively. Cells were treated with RNeasy Protect reagent (Qiagen) according to the manufacturer's instructions prior to RNA extraction, and total RNA was isolated after an on-column DNase treatment. To examine changes in methylation and gene expression in wild-type cells, we inoculated exponentially growing cells from aerobic minimal medium (OD₆₀₀ = 0.32) into either rich medium (Luria-Bertani broth) or anaerobic minimal medium. DNA and RNA were collected after cultures reached exponential phase in the new medium (OD₆₀₀ of 0.45 for rich medium, ~5 population doublings post transfer; OD₆₀₀ of 0.16 for anaerobic medium, ~3 population doublings post transfer).

DNA methylation patterns were also determined for mutant strains whose putative methyltransferases were inactivated by transposon insertions (33). DNA from transposon mutants was collected from stationary-phase cultures growing in aerobic minimal medium (OD₆₀₀ of ~0.80). All mutants grew to the same density and did not display any substantial growth differences from the wild type.

DNA methylation detection. Libraries of replicates of wild-type *S. oneidensis* MR-1 cultures were prepared for SMRT sequencing using a library construction protocol described previously (36). These libraries were sequenced to a mean genome coverage depth of 118× to 222× on the Pacific Biosciences RS instrument using C2 chemistry. One replicate grown in anaerobic minimal medium was excluded from further analysis due to poor coverage (<70×). Methyltransferase transposon mutants were sequenced to a depth of 72× to 113×. Average read lengths ranged from 2,296 to 2,889 bp in all libraries. Reads were mapped to the reference genome (RefSeq NC_004347.1) using the software program BLASR (37). Base modification and motif detection were performed using the Modification and Motif Detection protocol in the software program SMRTPipe v.1.3.3. Positions with coverage of >25× and kinetic scores of ≥QV 40 were considered modified. The QV is the $-10 * \log(P \text{ value})$, where the *P* value was determined from a *t* test between the sample and the *in silico* model (http://pacb.com/applications/base_modification/index.html). Positions with coverage of <25× were excluded from analysis.

Gene expression analyses. Total RNA collected from biological replicates was sequenced on the Illumina platform to determine gene expression levels of *S. oneidensis* MR-1 grown under the three experimental conditions described above. rRNA was removed using the Ribo-Zero Meta-Bacteria kit (Epicentre) prior to creation of a strand-specific RNAseq library (38). Libraries were sequenced on the Illumina HiSeq 2000 platform. Ten million mRNA reads randomly selected from each library were analyzed for differential expression using the DESeq (39), edgeR (40), and RankProd (41) software tools. Differentially expressed genes were those confidently identified by all three tests (*Q* < 0.005; false discovery rate [FDR] < 0.01) as having a fold change of >2.

Detecting *dam*, *seqA*, *mutH*, and GATC enrichment at the origin of replication. We analyzed all 448 finished *Gammaproteobacteria* genomes available in the Joint Genome Institute's Integrated Microbial Genomes database (<http://img.jgi.doe.gov/>) (42) as of 6 March 2013. The numbers of genes assigned to COGs (clusters of orthologous groups) 0338, 3057, and 3066 were determined in order to quantify the number of *dam*, *seqA*, and *mutH* genes, respectively. The origin of replication was determined by *in silico* and/or *in vivo* predictions retrieved from the DoriC database v6.5 (<http://tubic.tju.edu.cn/doric/index.php>) (43, 44). For the small number of genomes missing from DoriC, the origin was found using the program Ori-Finder (<http://tubic.tju.edu.cn/Ori-Finder>) (45). The origin of replication was considered to be enriched in GATC motifs if the observed number of motifs with the origin was significantly greater than would be expected if GATC motifs were uniformly distributed throughout the genome (i.e., the total number of GATC sites divided by genome size). Significance was calculated using a binomial test with a *P* value threshold of 0.01.

In four of the genomes examined, we found that the origin annotated in DoriC was not enriched for GATC despite the presence of *dam* and *seqA*. The annotated origins shared the following characteristics: (i) the origin was predicted by *in silico* analysis, which considers both *dnaA* and *gidA* to be "indicator genes" of the origin; (ii) the proposed origin was located upstream of *dnaA*, whereas the predicted origin in other closely related strains was located upstream of *gidA*; and (iii) the region upstream of *gidA* was significantly enriched with GATC motifs. This suggested that the apparent anomalies were due to misannotation of the origin or replication. The origin was redefined around *gidA* in these cases.

Phylogeny of gammaproteobacteria. 16S rRNA sequences from finished *Gammaproteobacteria* genomes were aligned using the software program MAFFT v6.864b (46) and used to construct a maximum-likelihood phylogeny using the program RAxML v7.2.6 (47), using the GTRGAMMA model and rapid bootstrap algorithm (1,000 iterations).

RESULTS AND DISCUSSION

Methylation profile of *S. oneidensis* MR-1. To identify methylated sites within the genome of *S. oneidensis* MR-1, we performed SMRT sequencing on DNA extracted from triplicate exponential-phase cultures grown aerobically on minimal medium. Our analysis revealed 42,965 nucleotides (nt) that exhibited significant variations in polymerase kinetics that were diagnostic of DNA modification (21) (Fig. 1). Of those modified nucleotides, 41,853 were identified as N6-methyladenine (N6mA) based on their distinct kinetic fingerprint. The remaining kinetic variants included 396 cytosine, 301 guanine, and 415 thymine bases (see Table S1 in the supplemental material). While some of these were likely analytical artifacts, the agreement among all three biological replicates suggests additional, unidentified mechanisms for DNA modification at work in *S. oneidensis* MR-1, e.g., glucosylation, putrescinylation, and glutamylation (48, 49). The nature of these putative modifications could not be determined in this study but represent an interesting avenue for future investigation.

Next, we analyzed the local sequence context of these N6mA bases to determine if they were located within specific sequence motifs. We identified two palindromic motifs (GATC and ATCGAT) and three bipartite motifs (GCAN₄GTC/GACN₄TGC, TACN₆GTNGT/ACN₆GTA, and TGA_NY₆TGAC/GTCAN₆RTCA) enriched with N6mA bases (methylated bases are underlined) (Table 1). More than 99% of all N6mA residues could be assigned to one of these five sequence motifs, suggesting that methylation was performed by DNA methyltransferases targeting specific DNA sequences. Genome annotations of *S. oneidensis* MR-1 predicted three type I R-M systems with unknown target sequences (34). The discovery of three bipartite motifs is consistent with type I R-M systems (50), but the specific motif

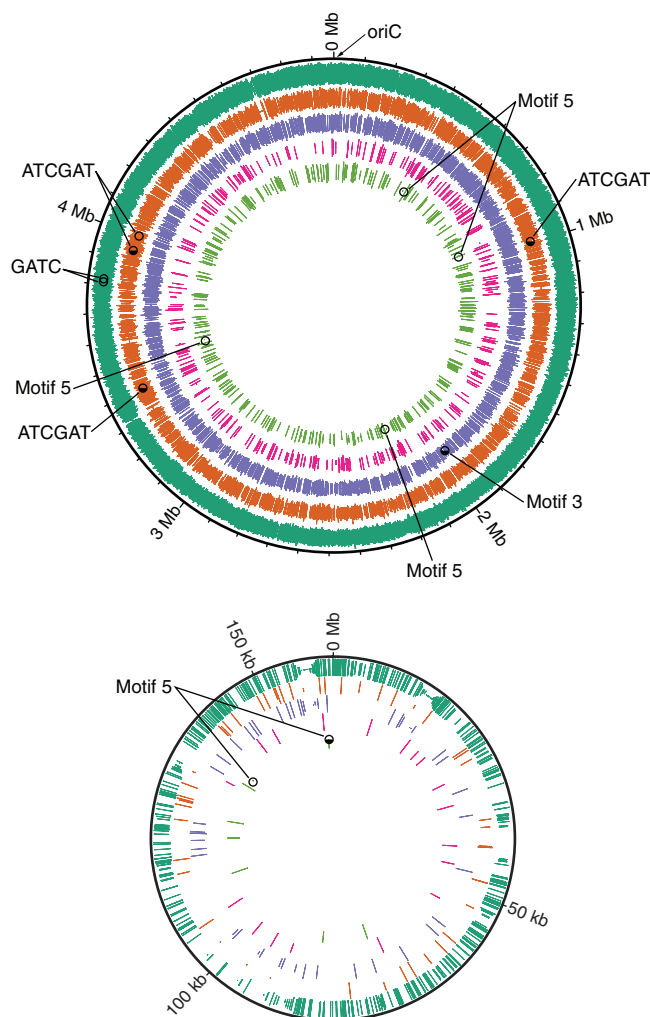


FIG 1 Locations of methylated sequence motifs in the chromosome and megaplasmid of *S. oneidensis* MR-1. Moving from the outer ring to the inner ring, the methylated motifs are as follows: GATC, ATCGAT, GCAN₄GTC/GACN₄TGC (motif 3), TACN₆GTNGT/ACN₆GTA (motif 4), and TGAYN₆TGAC/GTCAN₆RTCA (motif 5). The length of each methylation site marker is proportional to the confidence of methylation; all indicated sites have a *P* value of <0.0001. Hemimethylated (●) and nonmethylated (○) sites in cultures grown aerobically in minimal medium are indicated. OriC indicates the position of the origin of replication.

TABLE 2 Assigning target motifs to putative methyltransferases using SMRT sequencing of gene knockout mutants^a

Locus ID	R-M system	Predicted motif	Confirmed motif
SO_0383	Type I		<u>TGAYN₆TGAC/GTCAN₆RTCA</u>
SO_1457	Type I ^b		<u>GCAN₄GTC/GACN₄TGC</u>
SO_4265	Type I		<u>TACN₆GTNGT/ACN₆GTA</u>
SO_0289	Type II	GATC	ND
SO_0690	Type II		ND
SO_3004	Type II	GATC	ND
SO_A0004	Type II	ATCGAT	<u>ATCGAT</u>

^a Underlined bases were methylated. Target motifs were predicted for three putative methyltransferase in REBASE. Loss of a methylated motif in mutants confirmed the target of the knocked-out gene. Motifs for three predicted methyltransferases were not detected (ND). No mutant was available for SO_0289, whereas no methylated motifs were lost for SO_0690 and SO_3004.

^b Type II according to REBASE but annotated as type I elsewhere (e.g., GenBank and COG).

sequences we detected were novel R-M targets. The two methylated palindromes GATC and ATCGAT were also predicted in the restriction enzyme database REBASE (<http://rebase.neb.com/rebase/rebase.html>) based on sequence homology to methylases with known targets (35). However, *S. oneidensis* MR-1 has four putative type II methyltransferases, which is more than the number of methylated motifs detected once the three type-I-like bipartite motifs were excluded. All putative methyltransferases were expressed under these growth conditions, suggesting either redundancy among the methyltransferases in targeted motifs or possible misannotation of some genes.

To identify the specific target of each methyltransferase, we performed SMRT sequencing on methyltransferase transposon mutants. In these tests, a target motif was assigned to a predicted methyltransferase if the motif was not methylated in the transposon mutant. For example, the sequence motif ATCGAT was no longer methylated in a mutant lacking the putative methyltransferase SOA0004, thus indicating the target of this methyltransferase. The three bipartite motifs were also clearly assigned to the three predicted type I methyltransferases using this approach (Table 2). However, the protein(s) targeting GATC could not be identified unequivocally because this motif was always methylated in the individual transposon mutants. Interestingly, we were unable to generate a viable transposon mutant for predicted methyltransferase SO_0289, suggesting that this gene maybe required for viability. Two of the putative methyltransferase genes show homology to *dam* (SO_0289 and SO_0690) and were suspected to target

TABLE 1 Sequence motifs containing N6mA modifications (underlined bases) in *S. oneidensis* MR-1 grown aerobically on minimal medium^a

Motif	No. of motifs			No. (%) of motifs with methylation state			
	Total	CDS	Intergenic	Methylated	Hemi	Not	Conflict
<u>GATC</u>	16,376	14,328	2,048	16,281 (99.4)	0	2	93
<u>ATCGAT</u>	2,342	2,052	290	2,292 (97.9)	3	1	46
<u>GCAN₄GTC/GACN₄TGC</u>	1,803	1,543	260	1,793 (99.4)	1	0	9
<u>TACN₆GTNGT/ACN₆GTA</u>	306	246	60	301 (98.4)	0	0	5
<u>TGAYN₆TGAC/GTCAN₆RTCA</u>	291	243	48	282 (96.9)	1	5	3

^a The total number of motifs and their locations in either coding (CDS) or intergenic regions were determined. The methylation state of motifs with <25× coverage could not be confidently determined, and these were excluded from these counts. “Methylated” motifs were modified on both strands, while “Hemi” motifs were modified on only one strand. Motifs with disagreements among replicates were in “Conflict.” Methylated bases occurring simultaneously in two motifs were counted toward each motif, e.g., the 225 instances of the methylated sequence ATCGATC were included in the tallies of both GATC and ATCGAT motifs.

TABLE 3 Transcription factor binding sites containing type II motifs^a

Regulator	No. of sites		No. of operons regulated	No. of genes regulated	No. of differentially expressed genes		No. of differentially methylated sites	
	Total	With motif			Rich medium	Anaerobic	Rich medium	Anaerobic
ArgR	34	1	22	39	30 (4)	13 (11)	0	0
Crp	174	64	150	286	69 (48)	12 (6)	0	0
Fnr	30	21	26	73	34 (28)	8 (6)	0	0
Fur	32	1	32	58	12 (7)	7 (2)	1	0
ModE	2	2	2	4	0	0	0	0
SO1578	2	2	2	3	0	0	0	0
SO3385	1	1	1	3	0	0	0	0
SO3393	6	2	2	2	1 (1)	0	0	0

^a The numbers of genes and operons regulated by these transcription factors, as well as the number of genes differentially expressed when transferred to either rich medium or anaerobic minimal medium, are indicated. The numbers of upregulated genes are indicated in parentheses next to the total number of differentially expressed genes. Binding sites with different methylation states under different growth conditions are also displayed.

GATC (Table 2). The gene SO_3004 does not show strong homology to *dam* but was also predicted in REBASE to target GATC. Thus, it appears that *S. oneidensis* MR-1 may use multiple genes to methylate GATC. Similar redundancies have been observed in *Escherichia coli*, which has three separate enzymes that methylate GATC (3, 22). In addition, some strains of *Salmonella enterica* may have up to five copies of *dam* genes (see Table S3 in the supplemental material). The potential redundancy of GATC-targeting methyltransferases and the lack of corresponding restriction enzymes suggest that *S. oneidensis* MR-1 may use GATC methylation in some regulatory capacity.

To examine the efficiencies of methyltransferases in *S. oneidensis* MR-1, we determined the methylation state for each instance of the five sequence motifs throughout the genome. After excluding 418 motif locations with low sequence coverage, we found that 99.2% of the remaining 21,118 motif locations were methylated on both strands in all three replicates. The degree of methylation varied by motif, from as low as 96.9% to greater than 99.9% (Table 1). Interestingly, 5 sites were confidently hemimethylated, i.e., methylated on only one strand, in all three replicates, whereas 8 sites were not methylated on either strand (see Table S2 in the supplemental material). Five of the nonmethylated sites were in type I R-M motifs, two were located in GATC motifs, and one was found in an ATCGAT motif (Table 1; see also Table S2). Non-methylated and hemimethylated sites have been detected in other bacteria (8, 20, 22, 23, 51, 52), and these sites are often protected from methyltransferases by DNA binding proteins.

Changes in DNA methylation can impact gene expression levels by altering the binding affinities of regulatory proteins. For example, the binding of leucine-responsive protein (Lrp), which regulates expression levels of the *pap* operon in *E. coli*, is controlled by the methylation state of two GATC sites in the Lrp binding site (8, 53). In *S. oneidensis* MR-1, GATC motifs are commonly found in the binding sites for the transcription factors Crp and Fnr (32). Indeed, 64 of the 174 Crp binding sites and 21 of 30 Fnr binding sites have one or more type II motifs (Table 3). This enrichment of GATC motifs in transcription factor binding sites presents an opportunity for methylation to impact gene expression on a large scale. That is, if DNA binding activities of Crp and Fnr are sensitive to the DNA methylation, then changes in the methylation of GATC sites could potentially influence gene expression in *S. oneidensis* MR-1.

Dynamics of DNA methylation and gene expression. To explore changes in genome-wide methylation patterns and their possible impact on gene expression, we measured DNA methylation and mRNA levels in exponential-phase *S. oneidensis* MR-1 cultures grown under various conditions. More specifically, triplicate cultures grown aerobically in minimal medium were first analyzed by SMRT sequencing and RNAseq and then analyzed again after transfer to either aerobic rich medium or anaerobic minimal medium. Not surprisingly, gene expression varied from one condition to another, with 426 genes differentially expressed between minimal and rich media (201 upregulated and 225 downregulated) and 99 genes differentially expressed between aerobic and anaerobic conditions (51 upregulated and 48 downregulated) (Fig. 2; see also Tables S4 and S5 in the supplemental material).

We did not observe widespread changes in methylation in cultures growing under different conditions. However, reproducible differences in methylation state were observed at 8 sites (Table 4; see also Table S2 in the supplemental material). Of these sites, 5 were GATC motifs located within intergenic regions. One of these GATC sites (genome position 4,061,174) was located within the binding site of a ferric uptake regulator protein (Fur) transcription factor (Table 4), although neither gene regulated by this transcription factor was differentially expressed. The methylation state of two other GATC motifs flanking another Fur binding site (genome positions 3,823,765 and 3,823,792) also differed between minimal and rich media. One of the nine genes (SO_3667) regulated by this binding site was upregulated when cultures were transferred to rich medium (see Table S4). Finally, a differentially methylated GATC motif was detected 215 bp upstream of *argC* (SO_0275), a gene involved in arginine synthesis. The expression of *argC* and four other genes in the same operon was repressed when cultures were transferred from minimal medium lacking arginine into rich medium containing arginine (see Table S4). The latter two cases were suggestive of a possible connection between DNA methylation and gene expression, although the nature of the connection remains unclear. That is, did changes in the methylation state influence expression levels, or did binding of regulatory proteins inadvertently protect these sites from methylation? Establishing a direct causal link was not possible with the current data set and will require additional future investigations.

While these methylation dynamics are intriguing, it is clear that widespread changes in gene expression were not accompa-

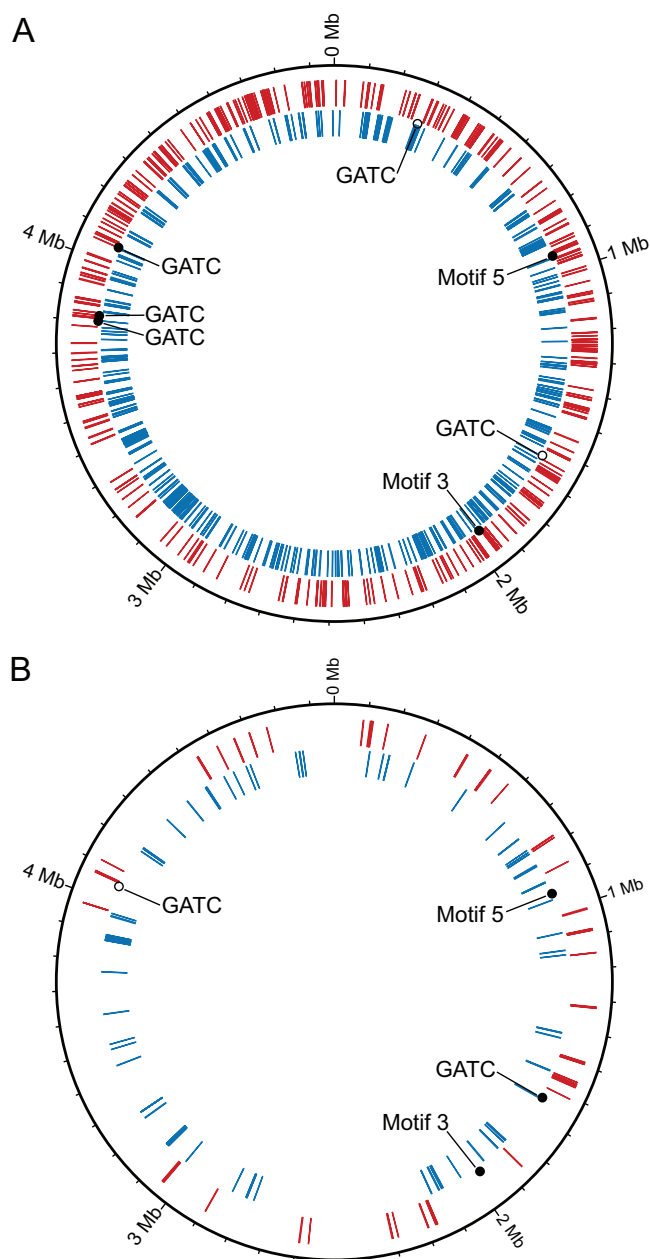


FIG 2 Locations of differentially expressed genes (red and blue rings) and differentially methylated motifs in *S. oneidensis* MR-1 cultures transferred from aerobic minimal medium to rich medium (A) or anaerobic minimal medium (B). Upregulated genes are red, and downregulated genes are blue. Motif locations that became methylated (●), hemimethylated (◐), or nonmethylated (○) after transfer to different culture conditions are indicated. The methylated motifs are as follows: GCAN₄GTC/GACN₄TGC (motif 3), TACN₆GTNGT/ACNACN₆GTA (motif 4), and TGAYN₆TGAC/GTCAN₆RTCA (motif 5).

nied by equally widespread changes in DNA methylation (Fig. 2). Nor was there an obvious relationship between methylation at known transcription factor binding sites and expression. For example, 69 Crp-regulated genes were differentially expressed when cultures were transferred from minimal medium to rich medium, but none of the Crp binding sites were differentially methylated (Table 3; see also Tables S4 and S5 in the supplemental material). The only differentially methylated transcription factor binding

site we observed did not exhibit a significant difference in expression for the corresponding genes. It is worth noting that *Shewanella* cultures were not synchronized, and it is conceivable that short-term changes in methylation state and gene expression within a small subset of the culture could have gone undetected. Still, while methylation may impact the expression of a few genes, our data suggest that methylation does not play a large and direct role in regulating gene expression in *S. oneidensis* MR-1, at least not under the conditions we tested.

To our knowledge, this is the first study to directly measure the genome-wide dynamics of methylation and gene expression in a wild-type bacterium. Three previous studies examined gene expression in *E. coli* mutants lacking *dam* (Dam^-), and in each case the expression patterns of the mutants differed from those of the wild-type strain (54–56). However, support for a direct regulatory role for methylation was inconsistent among studies. For example, Oshima et al. (54) found that a disproportionately large number of the differentially expressed genes in Dam^- mutants were regulated by transcription factors with GATC motifs within their binding sites. In contrast, Lobner-Olesen et al. (55) and Robbins-Menke et al. (56) found that the regulatory regions of differentially expressed genes were not enriched with GATC for both Dam^- and Dam -overproducing mutants. While there are clear cases where methylation directly regulates gene expression in *E. coli* (57), the last two studies suggest that most differential gene expression in Dam mutants results from indirect effects of variable Dam concentrations on cell physiology, not from direct regulation of expression via methylation. Similarly, >30% of genes in *E. coli* C227-11 were differentially expressed after deleting the RM.EcoGIII methyltransferase, yet there were no clear connections to RM.EcoGIII methylation sites for most of these genes (22). These studies highlight the challenges of interpreting a direct regulatory roles for DNA methylation by examining methyltransferase knockout mutants. Monitoring methylation dynamics in wild-type bacteria, which is now possible using SMRT sequencing, should provide a clearer picture of the regulatory roles of DNA methylation in bacteria.

Roles for DNA methylation in genome replication and DNA mismatch repair. If DNA methylation is not a major regulator of gene expression in *S. oneidensis* MR-1, then why does its genome encode orphan methyltransferases? One possibility is that DNA methylation plays a critical role in DNA mismatch repair and/or genome replication. For example, *S. oneidensis* MR-1 contains the DNA mismatch repair gene *mutH*, which in *E. coli* nicks the unmethylated strand near hemimethylated GATC sites when mismatches occur during genome replication. This enables removal of the unmethylated strand and resynthesis of the correct sequence from the methylated template (7). In addition, the non-uniform distribution of GATC sites throughout the genome suggests that *S. oneidensis* MR-1 may use DNA methylation to regulate genome replication in a manner similar to that of *E. coli*. Specifically, the origin of replication (*oriC*) is enriched with GATC sites in both organisms (Fig. 3; see also Fig. S1 in the supplemental material), and in *E. coli* the methylation state of these sites plays an important role in regulating genome replication (58–61). Briefly, genome replication in *E. coli* is initiated in part by the binding of DnaA to the origin of replication. Shortly after replication begins, SeqA binds to newly formed hemimethylated GATC sites in the origin, thus preventing additional binding of DnaA and the reinitiation of replication (58, 62). SeqA also binds to hemimethylated GATC sites in the promoter region of *dnaA* and reduces transcrip-

TABLE 4 Genomic contexts of motifs with differences in methylation state among conditions^a

Position (+/-)	Motif	Methylation state ^b			Genomic context		
		Min medium, aerobic	Rich medium	Min medium, anaerobic	Left flanking gene	Within gene	Right flanking gene
280309/280310	GATC	●	○	●	← SO_0274		SO_0275 →
1642124/1642125	GATC		○	●	← SO_1563		SO_1565 →
3823765/3823766	GATC	○	●	○	← SO_3669		SO_3670 →
3823792/3823793	GATC	○	●	○	← SO_3669		SO_3670 →
4061174/4061175*	GATC		●	○	← SO_3914		SO_3915 →
1965318/1965324	GCAN ₄ GTC/GACN ₄ TGC	◐	●	●	→ SO_1871		SO_1872 ←
938801/938809	TGAYN ₆ TGAC/GTCAN ₆ RTCA	○	●	●		SO_0912	
160490/160498 P	TGAYN ₆ TGAC/GTCAN ₆ RTCA	◐		●		SO_A0172	

^a Left and right flanking genes, as well as their orientation, are provided for motifs found within intergenic regions. Motifs were upstream of a left flanking gene if the arrow points to the left and upstream of right flanking genes if the arrow points to the right. "P" indicates a motif located in the plasmid, while "*" indicates a motif in a Fur transcription factor binding site.

^b Min, minimal; ●, methylated; ○, nonmethylated; ◐, hemimethylated.

tion of *dnaA* once the replication fork has passed (6, 63), thereby decreasing the chance of replication reinitiation. We hypothesize that *S. oneidensis* MR-1 uses similar mechanisms to control DNA replication based on the presence of *dam* and *seqA* in the genome as well as enrichment of GATC sites in the *oriC*.

To determine if DNA methylation regulates genome replication and mismatch repair in *S. oneidensis* MR-1, we attempted to construct *dam* and *seqA* mutants. DNA replication and cell division are not synchronized in Δdam and $\Delta seqA$ mutants of *E. coli* (63, 64), and we wished to observe if the same was true for *S. oneidensis* MR-1. However, both *dam* (locus tag SO_0289) and *seqA* appear to be essential for viability as determined by high-throughput transposon mutagenesis and sequencing (TnSEQ) (65) (K. M. Wetmore, M. Blow, and A. Deutschbauer, unpublished data), so mutants could not be recovered. *dam* is also essential for fellow *Gammaproteobacteria* members *Vibrio cholerae* and *Yersinia pseudotuberculosis*, where it plays a role in regulating genome replication (66, 67). Interestingly, mutants were viable for the two other *S. oneidensis* MR-1 genes that either showed some homology to *dam* (SO_0690) or were predicted to methylate GATC by REBASE (SO_3004). Both genes were expressed in wild-type *S. oneidensis* MR-1, indicating that they were functional but not completely redundant to SO_0289. The nonviability of both *dam* and *seqA* mutants suggests that methylation of GATC motifs plays a critical functional role in *S. oneidensis* MR-1, mostly likely in regulating genome replication.

Next, we asked if other *Shewanella* species might utilize methylation to regulate genome replication or mismatch repair by searching their genomes for *dam*, *seqA*, *mutH*, and GATC enrich-

ment around the origin of replication. In addition to *S. oneidensis* MR-1, there are 21 finished *Shewanella* genomes isolated from diverse environments, including marine and freshwater sediments, hydrothermal vents, and the nidamental gland of a squid. Nineteen isolates encoded the mismatch repair protein MutH, whereas all 21 isolates were positive for *dam*, *seqA*, and GATC enrichment around *oriC* (Fig. 4; see also Table S3 in the supplemental material), the same pattern observed in *S. oneidensis* MR-1 and *E. coli*. This suggests that regulation of genome replication through DNA methylation might be a universal feature of the *Shewanellaceae* family.

Looking beyond the *Shewanellaceae*, we searched all finished *Gammaproteobacteria* genomes for the presence of *dam*, *mutH*, *seqA*, and GATC enrichment in *oriC*. Of the 448 finished *Gammaproteobacteria* genomes in the Integrated Microbial Genomes database (42), 331 encoded one or more putative *dam* genes, while 266 encoded *seqA* and 278 encoded *mutH* (Fig. 4; see also Fig. S2 and Table S3 in the supplemental material). Interestingly, virtually all *Gammaproteobacteria* arising after a single evolutionary branch point were positive for *dam*, *mutH*, *seqA*, and GATC enrichment at *oriC* (Fig. 4; see also Fig. S2). There were 24 exceptions beyond this branch point that lacked *seqA* and GATC enrichment, and of these, 23 were endosymbionts with massively reduced genomes (see Table S3). Genome reduction, including loss of regulatory elements, is a common feature of endosymbionts (68), and it appears that both *dam* and *seqA* were lost during these reductions. *Glaciecola nitratireducens* FR1064 is not an endosymbiont, but its genome is >1Mbp smaller than those of the two other sequenced members of the genus *Glaciecola* (69), suggesting it too

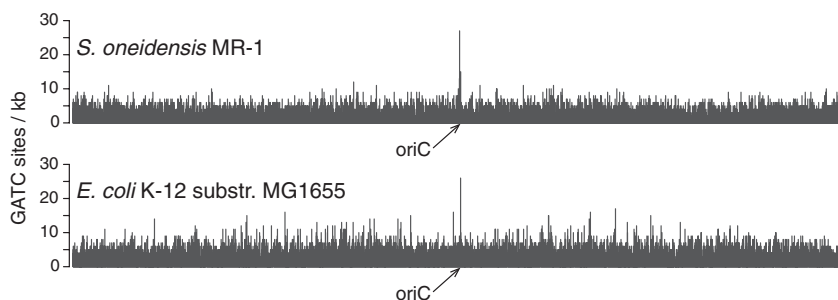


FIG 3 Distribution of GATC motifs throughout the genomes of *S. oneidensis* MR-1 and *E. coli*. The arrow indicates the origin of replication (*oriC*).

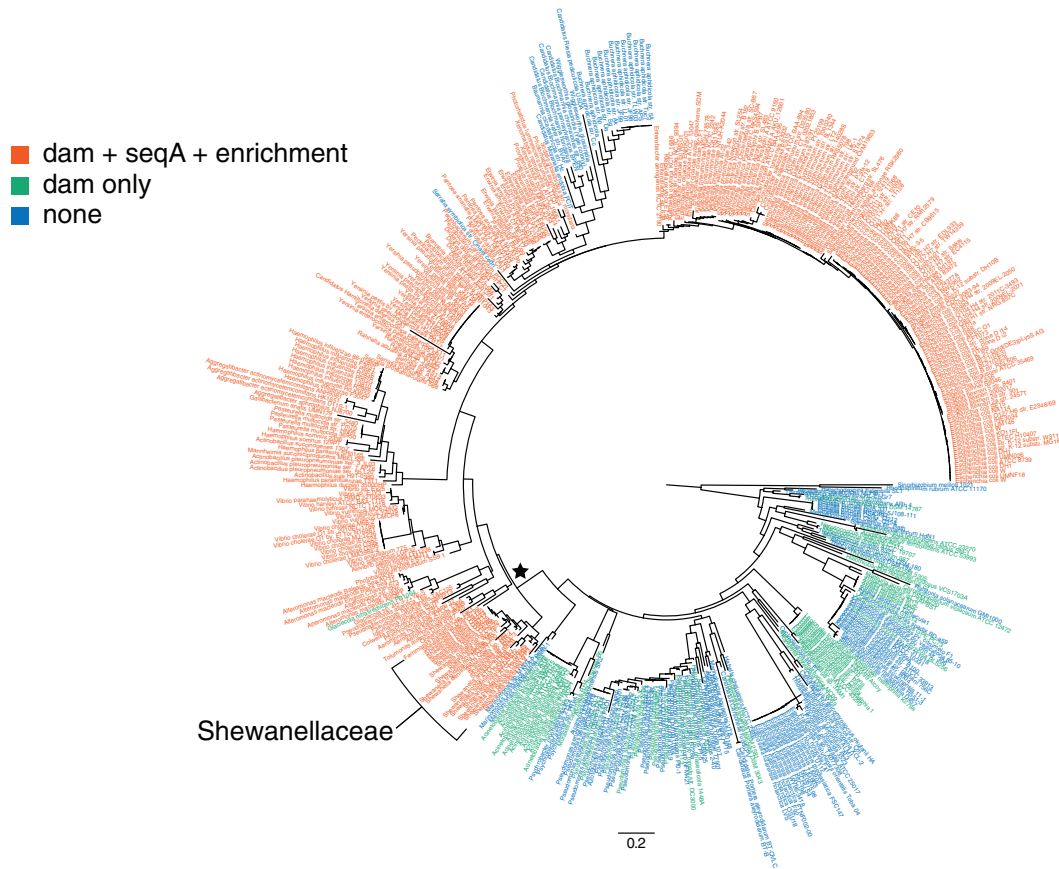


FIG 4 Phylogenetic patterns of DNA methylation usage in the *Gammaproteobacteria*. The presence of *dam*, *seqA*, and GATC enrichment at the origin of replication is illustrated on a phylogenetic tree constructed from the 16S sequences of 448 finished genomes. The scale bar represents the fraction of substitutions per site. The star indicates a node beyond which all but 24 genomes encoded *dam* and *seqA*, and were enriched for GATC motifs in the origin of replication. Although this node does not have strong bootstrap support based on 16S rRNA sequences, this node was supported by a rare indel in RpoB (70, 71).

may have lost *seqA* during genome reduction. While some basal lineages within the *Gammaproteobacteria* encoded *dam* and/or *mutH* genes, none encoded *seqA* or showed enrichment of GATC sites at the origin of replication (Fig. 4). Using the limited number of genomes available at the time, Lobner-Olesen et al. (3) identified a “DamMT clade” within the *Gammaproteobacteria*. Our more comprehensive analysis generally agrees with this earlier report, although it would be more appropriate to discuss a “*seqA*/GATC-*oriC* clade” since *dam* and *mutH* were not exclusive to one clade of the *Gammaproteobacteria* (Fig. 4).

The development of epigenetic regulation of genome replication appears to be a key evolutionary event within the *Gammaproteobacteria*. The phylogenetic pattern of *dam*, *seqA*, and GATC enrichment at the origin indicates that this mechanism for regulating chromosome replication via DNA methylation was acquired and maintained by more recent lineages, not lost by the more basal groups within the clade. Moreover, this mechanism has been maintained throughout the evolution of multiple families, even as these groups have diversified and expanded into a wide range of different aquatic, terrestrial, and host-associated environments. Dam has even become essential for viability of some *Gammaproteobacteria*, such as *S. oneidensis* MR-1 (this study), *Vibrio cholerae*, and *Yersinia pseudotuberculosis* (66). The maintenance of *dam*, *seqA*, and GATC enrichment at *oriC*

throughout their evolutionary history suggests that many members of the *Gammaproteobacteria*, regardless of their environment, experience strong selective pressure to synchronize genome replication with cell division and that more recently evolved lineages do so with DNA methylation. Presumably, the more basal lineages of *Gammaproteobacteria* lacking *seqA* and GATC enrichment at *oriC* use alternative strategies. Interestingly, many of these basal *gammaproteobacteria* lineages encode *dam*, but its functional role remains a mystery since they do not appear to use methylation for DNA mismatch repair or genome replication. Further analyses with SMRT sequencing will shed additional light on the role of DNA methylation in these bacteria.

Conclusions. DNA methylation appears to serve a variety of functions in *S. oneidensis* MR-1, including restriction/modification, DNA mismatch repair, and regulation of genome replication. Methylation may also regulate expression of a few genes, but it does not appear to be a major regulator of gene expression. This connection to gene expression is one of the more interesting, but poorly understood, facets of DNA methylation, and it remains to be seen if it is a major regulator of expression in any bacterium. However, with the exception of *E. coli* and *Caulobacter crescentus*, our understanding of DNA methylation is extremely limited for the vast majority of prokaryotes. The further application of SMRT sequencing will dramatically expand our understanding of DNA

methylation in these understudied clades. Large-scale surveys of diverse microbial groups using SMRT sequencing will help provide new insights into the scope and variety of DNA methylation in various phylogenetic groups, whereas analyses of wild-type and knockout mutants will help uncover and experimentally verify the functional roles of methylation.

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