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ABSTRACT

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 methyltranferases were determined via SMRT sequencing of gene knockout mutants. In addition, we found *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 in these conditions, suggesting 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 indicate 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.

INTRODUCTION

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 or 4-methylcytosine [17]. Methyl-sensitive restriction enzymes have been used to identify the methylation state of specific sequence motifs [18-20], but complete methylome analyses are not possible without 1) prior knowledge of the entire set of methyltransferases and their sequence targets within a genome, and 2) 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

70 deviations in the polymerase kinetics observed during sequencing. The specific
71 type of DNA methylation can often be determined from the polymerase kinetics,
72 e.g. N6-methyladenine or 4-methylcytosine. With SMRT sequencing it is now
73 possible to identify the complete set of methylated sequence motifs within a
74 microbial genome as well as the methylation state for each instance of a motif
75 [22-24]. This represents a powerful tool for characterizing the functional roles of
76 DNA methylation in a wide variety of bacteria.

77 *Shewanella oneidensis* MR-1 is a bacterial isolate belonging to the
78 *Shewanellacea*, a family distinguished by the wide variety of electron acceptors
79 they can utilize (e.g. iron, manganese, uranium, chromium, and plutonium) [25-
80 28]. Because of their flexible respiratory pathways, *Shewanella* sp. are
81 recognized as potential agents for bioremediation at sites contaminated with
82 heavy metals and radionuclides [29]. To better exploit its metabolic potential, *S.*
83 *oneidensis* MR-1 has been characterized extensively, including analysis of gene
84 expression [30, 31], identification of regulatory regions [32], and the
85 determination of fitness levels for thousands of gene knockout mutants
86 [33]. However, the developing systems-level view of *Shewanella* does not yet
87 incorporate DNA methylation and its potential regulatory roles. Genomic
88 analyses reveal multiple putative methyltransferases in *S. oneidensis* MR-1 [34,
89 35], including several apparent 'orphans' that lack corresponding restriction
90 enzymes. It remains unclear what role these orphan methyltransferases might
91 play.

92 Here we use SMRT sequencing to provide the first look at DNA
93 methylation in *S. oneidensis* MR-1. We identify methylated sites throughout the
94 genome as well as the sequence motifs targeted by predicted
95 methyltransferases. To determine if DNA methylation regulates gene
96 expression, we examine whether changes in expression level correspond with
97 changes in DNA methylation state when cultures are transferred from one set of
98 growth conditions to another. Finally, we examine the finished genomes of all
99 *Gammaproteobacteria*, including the *Shewanellacea*, to determine which groups
100 appear to use DNA methylation for regulating genome replication and DNA
101 mismatch repair.
102
103 **RESULTS AND DISCUSSION**
104 *Methylation profile of S. oneidensis MR-1*
105 To identify methylated sites within the genome of *S. oneidensis MR-1*, we
106 performed SMRT sequencing on DNA extracted from triplicate exponential-phase
107 cultures grown aerobically on minimal media. Our analysis revealed 42,965
108 nucleotides that exhibited significant variations in polymerase kinetics that were
109 diagnostic of DNA modification [21]. Of those modified nucleotides, 41,853 were
110 identified as N6-methyladenine (N6mA) based on their distinct kinetic fingerprint.
111 The remaining kinetic variants included 396 cytosine, 301 guanine, and 415
112 thymine bases (Supplementary Table 1). While some of these were likely
113 analytical artifacts, the agreement among all three biological replicates suggests
114 additional, unidentified mechanisms for DNA modification at work in *S.*

115 *oneidensis* MR-1, e.g. glucosylation, putrescinylation, and glutamylation [36,
116 37]. The nature of these putative modifications could not be determined in this
117 study, but represent an interesting avenue for future investigation.
118 Next we analyzed the local sequence context of these N6mA bases to
119 determine if they were located within specific sequence motifs. We identified two
120 palindromic motifs (G<u>A</u>TC and ATCG<u>A</u>T) and three bipartite motifs (GC<u>A</u>N_4GTC /
121 G<u>A</u>CN_4TGC, T<u>A</u>CN_6GTNGT / ACN<u>A</u>CN_6GTA, and TG<u>A</u>YN_6TGAC /
122 GTC<u>A</u>N_6RTCA) enriched with N6mA bases (methylated bases are underlined).
123 Over 99% of all N6mA residues could be assigned to one of these five sequence
124 motifs, suggesting that methylation was performed by DNA methyltransferases
125 targeting specific DNA sequences. Genome annotations of *S. oneidensis* MR-1
126 predicted three Type I RM systems with unknown target sequences [34]. The
127 discovery of three bipartite motifs is consistent with Type I R-M systems [38], but
128 the specific motif sequences we detected were novel R-M targets. The two
129 methylated palindromes GATC and ATCGAT were also predicted in restriction
130 enzyme database REBASE (http://rebase.neb.com/rebase/rebase.html) based
131 on sequence homology to methylases with known targets [35]. However, *S.*
132 *oneidensis* MR-1 has four putative Type II methyltransferases, which is more
133 than the number of methylated motifs detected once the three Type-I-like
134 bipartite motifs were excluded. All putative methyltransferases were expressed
135 under these growth conditions, suggesting either redundancy among the
136 methyltransferases in targeted motifs or possible misannotation of some genes.

137 To identify the specific target of each methyltransferase, we performed
138 SMRT sequencing on methyltransferase transposon mutants. In these tests, a
139 target motif was assigned to a predicted methyltransferase if the motif was not
140 methylated in the transposon mutant. For example, the sequence motif ATCGAT
141 was no longer methylated in a mutant lacking putative methyltransferase
142 SOA0004, thus indicating the target of this methyltransferase. The three bipartite
143 motifs were also clearly assigned to the three predicted Type I
144 methyltransferases using this approach (Table 2). However, the protein(s)
145 targeting GATC could not be identified unequivocally because this motif was
146 always methylated in the individual transposon mutants. Interestingly, we were
147 unable to generate a viable transposon mutant for predicted methyltransferase
148 SO_0289, suggesting this gene maybe required for viability. Two of the putative
149 methyltransferases show homology to *dam* (SO_0289 & SO_0690) and were
150 suspected to target GATC (Table 2). Gene SO_3004 does not show strong
151 homology to *dam*, but was also predicted in REBASE to target GATC. Thus, it
152 appears that *S. oneidensis* MR-1 may use multiple genes to methylate
153 GATC. Similar redundancies have been observed in *E. coli*, which has three
154 separate enzymes that methylate GATC [3, 22]. In addition, some strains of
155 *Salmonella enterica* may have up to five copies of *dam* genes (Supplementary
156 Table 3). The potential redundancy of GATC-targeting methyltransferases and
157 the lack of corresponding restriction enzymes suggest that *S. oneidensis* MR-1
158 may use GATC methylation in some regulatory capacity.

159 To examine the efficiency of methyltransferases in *S. oneidensis* MR-1,
160 we determined the methylation state for each instance of the five sequence
161 motifs throughout the genome. After excluding 418 motif locations with low
162 sequence coverage, we found that 99.2% of the remaining 21,118 motif locations
163 were methylated on both strands in all three replicates. The degree of
164 methylation varied by motif from as low as 96.9% to greater than 99.9% (Table
165 1). Interestingly, 5 sites were confidently hemi-methylated, i.e. methylated on
166 only one strand, in all three replicates, whereas 8 sites were not methylated on
167 either strand (Supplementary Table 2). Five of the non-methylated sites were in
168 Type I R-M motifs, two were located in GATC motifs, and one was found in an
169 ATCGAT motif (Table 1; Supplementary Table 2). Non-methylated and hemi-
170 methylated sites have been detected in other bacteria [8, 20, 22, 23, 39, 40], and
171 these sites are often protected from methyltransferases by DNA binding proteins.
172 Changes in DNA methylation can impact gene expression levels by
173 altering the binding affinity of regulatory proteins. For example, the binding of
174 leucine responsive protein (Lrp), which regulates expression levels of the *pap*
175 operon in *E. coli*, is controlled by methylation state of two GATC sites in the Lrp
176 binding site [8, 41]. In *S. oneidensis* MR-1, GATC motifs are commonly found in
177 the binding sites for transcription factors Crp and Fnr [32]. Indeed, 64 of the 174
178 Crp binding sites and 21 of 30 Fnr binding sites have one or more Type II motif
179 (Table 3). This enrichment of GATC motifs in transcription factor binding sites
180 presents an opportunity for methylation to impact gene expression on a large
181 scale. That is, if DNA binding activities of Crp and Fnr are sensitive to the DNA

182 methylation, then changes in the methylation of GATC sites could potentially
183 influence gene expression in *S. oneidensis* MR-1.
184
185 *Dynamics of DNA methylation and gene expression*
186 To explore changes in genome-wide methylation patterns and their
187 possible impact on gene expression, we measured DNA methylation and mRNA
188 levels in exponential-phase *S. oneidensis* MR-1 cultures grown under various
189 conditions. More specifically, triplicate cultures grown aerobically in minimal
190 media were first analyzed by SMRT sequencing and RNAseq, and then analyzed
191 again after transfer to either aerobic rich media or anaerobic minimal media. Not
192 surprisingly, gene expression varied from one condition to another, with 426
193 genes differentially expressed between minimal and rich media (201 up-
194 regulated, 225 down-regulated), and 99 genes differentially expressed between
195 aerobic and anaerobic conditions (51 up-regulated, 48 down-regulated) (Fig 2;
196 Supplementary Tables 4&5).
197 We did not observe widespread changes in methylation in cultures
198 growing under different conditions. However, reproducible differences in
199 methylation state were observed at 8 sites (Table 4, Supplementary Table 2). Of
200 these sites, 5 were GATC motifs located within intergenic regions. One of these
201 GATC sites (genome position 4,061,174) was located within the binding site of a
202 ferric uptake regulator protein (Fur) transcription factor (Table 4), although
203 neither gene regulated by this transcription factor was differentially expressed.
204 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 up regulated when cultures were transferred to rich media (Supplementary Table 4). 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 media lacking arginine into rich media containing arginine (Supplementary Table 4). 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 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 dataset and will require additional future investigations.

While these methylation dynamics are intriguing, it is clear that widespread changes in gene expression were not accompanied by equally widespread changes in DNA methylation (Figure 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 media to rich media, but none of the Crp binding sites were differentially methylated (Table 3, Supplementary Tables 4 and 5). The only differentially methylated transcription factor binding site we observed did not exhibit a significant difference in expression for the

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

235 To our knowledge this is the first study to directly measure the genome-
236 wide dynamics of methylation and gene expression in a wild-type
237 bacterium. Three previous studies examined gene expression in *E. coli* mutants
238 lacking *dam* (Dam-), and in each case the expression patterns of the mutants
239 differed from those of the wild-type strain [42-44]. However, support for a direct
240 regulatory role for methylation was inconsistent among studies. For example,
241 Oshima et al. (2002) found that a disproportionately large number of the
242 differentially expressed genes in Dam- mutants were regulated by transcription
243 factors with GATC motifs within their binding sites. In contrast, Lobner-Olesen et
244 al (2003) and Robbins-Menke et al (2005) found that the regulatory regions of
245 differentially expressed genes were not enriched with GATC for both Dam- and
246 Dam-overproducing mutants. While there are clear cases where methylation
247 directly regulates gene expression *E. coli* [45], the latter two studies suggest that
248 most differential gene expression in dam mutants results from indirect effects of
249 variable Dam concentrations on cell physiology, not from direct regulation of
250 expression via methylation. Similarly, >30% of genes in *E. coli* C227-11 were

differentially expressed after deleting 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 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 hemi-methylated 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 *S. oneidensis* MR-1 may use DNA methylation to regulate genome replication in manner similar to *E. coli*. Specifically, the origin of replication (*oriC*) is enriched with GATC sites in both organisms (Figure 3; Supplementary Figure 1), and in *E. coli* the methylation state of these sites plays an important role in regulating genome replication [46-49]. 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 hemi-methylated GATC sites in the origin, thus preventing additional binding of DnaA and the re-initiation of replication [46, 50]. SeqA also binds to hemi-methylated GATC sites in the promoter region of *dnaA* and reduces transcription of *dnaA* once the replication fork has passed [6, 51], thereby decreasing the chance of replication re-initiation. We hypothesize *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 *dam*- and *seqA*- mutants of *E. coli* [51, 52], and we wished to observe if the same was true for *S. oneidensis* MR-1. However, both *dam* (locus id SO_0289) and *seqA* appear to be essential for viability as determined by high-throughput transposon mutagenesis and sequencing (TnSEQ) [53] (unpublished data; KMW, MB, AD), so mutants could not be recovered. *Dam* is also essential for fellow *Gammaproteobacteria Vibrio cholerae* and *Yersinia pseudotuberculosis,* where it plays a role in regulating genome replication [54, 55]. 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

297 SO_0289. The non-viability of both *dam*- and *seqA*- mutants suggests that
298 methylation of GATC motifs plays a critical functional role in *S. oneidensis* MR-1,
299 mostly likely in regulating genome replication.

300 Next we asked if other *Shewanella* species might utilize methylation to
301 regulate genome replication or mismatch repair by searching their genomes for
302 *dam*, *seqA*, *mutH*, and GATC enrichment around at the origin or replication. In
303 addition to *S. oneidensis* MR-1, there are 21 finished *Shewanella* genomes
304 isolated from diverse environments including marine and freshwater sediments,
305 hydrothermal vents, and the nidamental gland of a squid. Nineteen isolates
306 encoded mismatch repair protein MutH, whereas all 21 isolates were positive for
307 *dam*, *seqA*, and GATC enrichment around *oriC* (Figure 4, Supplementary Table
308 3), the same pattern observed in *S. oneidensis* MR-1 and *E. coli*. This suggests
309 that regulation of genome replication through DNA methylation might be a
310 universal feature of the *Shewanellaceae* family.

311 Looking beyond the *Shewanellaceae*, we searched all finished
312 *Gammaproteobacteria* genomes for the presence of *dam*, *mutH*, *seqA*, and
313 GATC enrichment in *oriC*. Of the 448 finished *Gammaproteobacteria* genomes
314 in the Integrated Microbial Genomes database [56], 331 encoded one or more
315 putative *dam* genes, while 266 encoded *seqA* and 278 encoded *mutH* (Figure 4;
316 Supplementary Figure 2; Supplementary Table 3). Interestingly, virtually all
317 *Gammaproteobacteria* arising after a single evolutionary branch point were
318 positive for *dam*, *mutH*, *seqA*, and GATC enrichment at *oriC* (Figure 4;
319 Supplementary Figure 2). There were 24 exceptions beyond this branch point

320 that lacked *seqA* and GATC enrichment, and of these, 23 were endosymbionts
321 with massively reduced genomes (Supplementary Table 3). Genome reduction,
322 including loss of regulatory elements, is a common feature of endosymbionts
323 [57], and it appears both *dam* and *seqA* were lost during these reductions.
324 *Glaciecola nitratireducens* FR1064 is not an endosymbiont, but its genome is
325 >1Mbp smaller than the two other sequenced members of the genus *Glaciecola*
326 [58], suggesting it too may have lost *seqA* during genome reduction. While some
327 basal lineages within the *Gammaproteobacteria* encoded *dam* and/or *mutH*
328 genes, none encoded *seqA* or showed enrichment of GATC sites at the origin of
329 replication (Figure 4). Using the limited number of genomes available at the
330 time, Lobner-Olesen et al. (2005) identified a "DamMT clade" within the
331 *Gammaproteobacteria*. Our more comprehensive analysis generally agrees with
332 this earlier report, although it would be more appropriate to discuss a
333 "*seqA*/GATC-*oriC* clade" since *dam* and *mutH* were not exclusive to one clade of
334 *Gammaproteobacteria* (Figure 4).
335 The development of epigenetic regulation of genome replication appears
336 to be a key evolutionary event within the *Gammaproteobacteria*. The
337 phylogenetic pattern of *dam*, *seqA*, and GATC enrichment at the origin indicates
338 this mechanism for regulating chromosome replication via DNA methylation was
339 acquired and maintained by more recent lineages, not lost by the more basal
340 groups within the clade. Moreover, this mechanism has been maintained
341 throughout the evolution of multiple families, even as these groups have
342 diversified and expanded into a wide range of different aquatic, terrestrial, and

343 host-associated environments. Dam has even become essential for viability of
344 some *Gammaproteobacteria* such as *S. oneidensis* MR-1 (this study), *Vibrio*
345 *cholerae*, and *Yersina pseudotuberculosis* [54]. The maintenance of *dam*, *seqA*,
346 and GATC enrichment at *oriC* throughout their evolutionary history suggests
347 many *Gammaproteobacteria*, regardless of their environment, experience strong
348 selective pressure to synchronize genome replication with cell division, and that
349 more recently evolved lineages do so with DNA methylation. Presumably, the
350 more basal lineages of *Gammaproteobacteria* lacking *seqA* and GATC
351 enrichment at *oriC* use alternative strategies. Interestingly, many of these basal
352 Gammaproteobacteria lineages encode *dam*, but its functional role is remains a
353 mystery since they do not appear to use methylation for DNA mismatch repair or
354 genome replication. Further analyses with SMRT sequencing will shed additional
355 light on the role of DNA methylation in these bacteria.
356
357 *Conclusions*
358 DNA methylation appears to serve a variety of functions in *S. oneidensis*
359 MR-1 including restriction/modification, DNA mismatch repair, and regulation of
360 genome replication. Methylation may also regulate expression of a few genes,
361 but it does not appear to be a major regulator of gene expression. This
362 connection to gene expression is one of the more interesting, but poorly
363 understood, facets of DNA methylation, and it remains to be seen if it is a major
364 regulator of expression in any bacteria. However, with the exception of *E. coli*
365 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 methylation.

METHODS AND MATERIALS

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 media contained per liter: 1.5 g NH_4Cl, 0.1 g KCl, 1.75 g NaCl, 0.61 g $MgCl_2$-$6H_20$, 0.6 g NaH_2PO_4, 30 mM PIPES buffer, 20 mM DL-lactate, Wolfe's vitamins, and Wolfe's minerals (pH7). Fumarate was added as an electron acceptor to anaerobic minimal media (30mM final conc.). Anaerobic minimal media experiments were set up in an anaerobic chamber (Coy) with a gas mix of 5% H_2, 10% CO_2, and 85% N_2, and incubated in serum bottles closed with butyl rubber stopper at 200 rpm. Aerobic minimal media 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 media (OD600 = 0.32). DNA and RNA were isolated with the DNeasy Blood & Tissue kit (Qiagen) and RNeasy

389 mini kit (Qiagen), respectively. Cells were treated with RNAprotect (Qiagen)
390 according the manufacturers instructions prior to RNA extraction, and total RNA
391 was isolated after an on-column DNAse treatment. To examine changes in
392 methylation and gene expression in wild-type cells, we inoculated exponentially
393 growing cells from aerobic minimal media (OD600 = 0.32) into either rich media
394 (Luria-Bertani broth) or anaerobic minimal media. DNA and RNA were collected
395 after cultures reached exponential phase in the new media (OD600 of 0.45 for
396 rich media, ~5 population doublings post transfer; OD600 of 0.16 for anaerobic
397 media, ~3 population doublings post transfer).
398 DNA methylation patterns were also determined in mutant strains whose
399 putative methyltransferases were inactivated by transposon insertions [33]. DNA
400 from transposon mutants was collected from stationary phase cultures growing in
401 aerobic minimal media (OD600 of ~0.80). All mutants grew to the same density
402 and did not display any substantial growth differences from wild-type.
403
404 *DNA methylation detection*
405 Libraries of replicate of wild-type *S. oneidensis* MR-1 cultures were
406 prepared for SMRT sequencing using a library construction protocol described
407 previously [59]. These libraries were sequenced to a mean genome coverage
408 depth of 118-222X on the Pacific Biosciences RS instrument using C2 chemistry.
409 One replicate grown in anaerobic minimal media was excluded from further
410 analysis due to poor coverage (<70X). Methyltransferase transposon mutants
411 were sequenced to a depth 72-113X. Average read lengths ranged from 2,296-

412 2,889bp in all libraries. Reads were mapped to the reference genome (RefSeq
413 NC_004347.1) using BLASR [60]. Base modification and motif detection were
414 performed using the Modification and Motif Detection protocol in SMRTPipe
415 v.1.3.3. Positions with coverage >25X and kinetic scores ≥ QV 40 were
416 considered modified. The kinetic score is the -10*log (p-value) where p-
417 value was determined from a t-test between the sample and the in silico model
418 (http://pacb.com/applications/base_modification/index.html). Positions with
419 coverage <25X were excluded from analysis.
420
421 *Gene expression analyses*
422 Total RNA collected from biological replicates was sequenced on the
423 Illumina platform to determine gene expression levels of *S. oneidensis* MR-1
424 grown under the three experimental conditions described above. Ribosomal
425 RNA was removed using the Ribo-Zero Meta-Bacteria kit (Epicentre) prior to
426 creation of a strand-specific RNAseq library [61]. Libraries were sequenced on
427 the Illumina HiSeq 2000 platform. Ten million mRNA reads randomly selected
428 from each library were analyzed for differential expression using DEseq [62],
429 edgeR [63], and RankProd [64] tools. Differentially expressed genes were those
430 confidently identified by all three tests (Q<0.005; FDR<0.01) as having a fold
431 change >2.
432
433 *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/) [56] as of March 6, 2013. The number of genes assigned to COGs 0338, 3057, and 3066 was 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) [65, 66]. For the small number of genomes missing from DoriC, the origin was found using Ori-Finder (http://tubic.tju.edu.cn/Ori-Finder) [67]. 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: 1) the origin was predicted by in silico analysis, which considers both *dnaA* and *gidA* to be "indicator genes" of the origin; 2) the proposed origin was located upstream of *dnaA*, whereas the predicted origin in other closely related strains was located upstream of *gidA*; and 3) the region upstream of *gidA* was significantly enriched with GATC motifs. This suggested the apparent anomalies were due to misannotation of the origin or replication. The origin was re-defined around *gidA* in these cases.

457

458 *Phylogeny of Gammaproteobacteria*

459 16S rRNA sequences from finished *Gammaproteobacteria* genomes were
460 aligned using MAFFT v6.864b [68] and used to construct a maximum likelihood
461 phylogeny with RAxmL v7.2.6 [69] using the GTRGAMMA model and rapid
462 bootstrap algorithm (1,000 iterations).

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466

467 **FIGURE LEGENDS**

468 Figure 1: Locations of methylated sequence motifs in the chromosome and
469 megaplasmid of *S. oneidensis* MR-1. Moving from the outer ring to the inner ring,
470 the methylated motifs are: G<u>A</u>TC, ATCG<u>A</u>T, GC<u>A</u>N_4GTC/G<u>A</u>CN_4TGC (motif 3),
471 T<u>A</u>CN_6GTNGT/ACN<u>A</u>CN_6GTA (motif 4), and TG<u>A</u>YN_6TGAC/GTC<u>A</u>N_6RTCA
472 (motif 5). The length of each methylation site marker is proportional to the
473 confidence of methylation; all indicated sites have a p-value < 0.0001. Hemi-
474 methylated (◓) and non-methylated (○) sites in cultures grown aerobically in
475 minimal media are indicated. OriC indicates the position of the origin of
476 replication.

Figure 2: Locations of differentially expressed genes (red and blue rings) and differentially methylated motifs in *S. oneidensis* MR-1 cultures transferred from aerobic minimal media to rich media (A) or anaerobic minimal media (B). Up-regulated genes are red and down-regulated genes are blue. Motif locations that became methylated (●), hemi-methylated (◓), or non-methylated (○) after transfer to different culture conditions are indicated. No differentially methylated motifs were found in the megaplasmid.

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

Figure 4: Phylogenetic patterns of DNA methylation usage in *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*, *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].

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Table 1. Sequence motifs containing N6mA modifications (underlined bases) in *S. oneidensis* MR-1 grown aerobically on minimal media. The total number of motifs and their their location in either coding (CDS) or intergenic regions was determined. The methylation state of motifs with <25X coverage could not be confidently determined and 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'. The percentage of total motifs methylated is indicated in ()'s. Methylated bases occurring simultaneously in two motifs were counted towards each motif, e.g. the 225 instances of the methylated sequence ATCGATC were included in the tallies of both GATC and ATCGAT motifs.

				Methylation state				
Motif	**Total**	**CDS**	**Intergenic**	**Methylated**		**Hemi**	**Not**	**Conflict**
GATC	16,376	14,328	2,048	16,281	(99.4%)	0	2	93
ATCGAT	2,342	2,052	290	2,292	(97.9%)	3	1	46
GCAN$_4$GTC / GACN$_4$TGC	1,803	1,543	260	1,793	(99.4%)	1	0	9
TACN$_6$GTNGT / ACNACN$_6$GTA	306	246	60	301	(98.4%)	0	0	5
TGAYN$_6$TGAC / GTCAN$_6$RTCA	291	243	48	282	(96.9%)	1	5	3

Table 2. Assigning target motifs to putative methyltransferases using SMRT sequencing of gene knockout mutants. 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.

Locus ID	R-M System	Predicted motif	Confirmed motif
SO_0383	Type I		TG<u>A</u>YN$_6$TGAC / GTC<u>A</u>N$_6$RTCA
SO_1457	Type I*		GC<u>A</u>N$_4$GTC / G<u>A</u>CN$_4$TGC
SO_4265	Type I		T<u>A</u>CN$_6$GTNGT / ACN<u>A</u>CN$_6$GTA
SO_0289	Type II	GATC	ND
SO_0690	Type II		ND
SO_3004	Type II	GATC	ND
SO_A0004	Type II	ATCGAT	ATCG<u>A</u>T

* Type II according to REBASE, but annotated as Type I elsewhere (e.g. Genbank, COG)

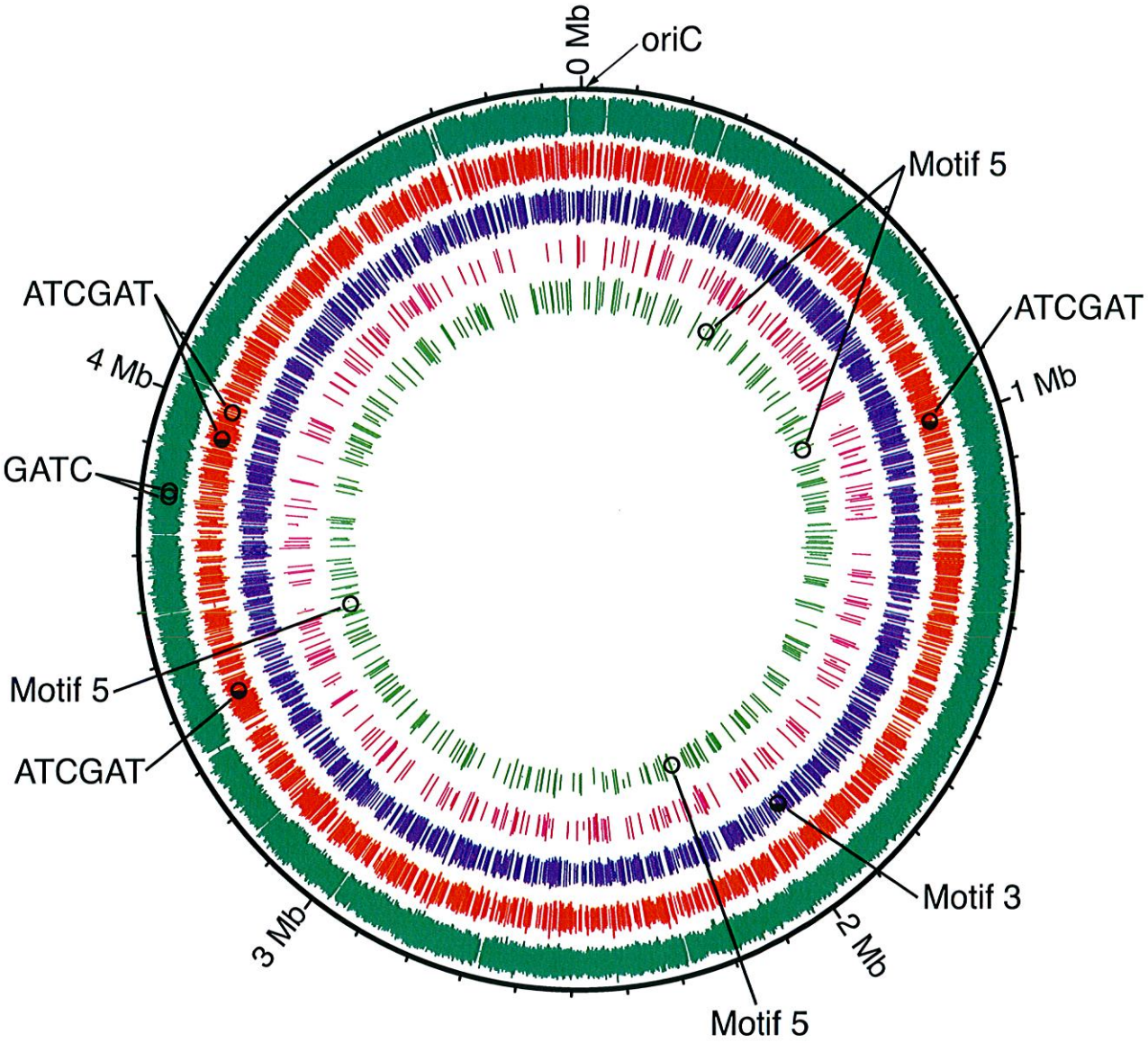
Table 3. Transcription factor binding sites containing Type II motifs. The number of genes and operons regulated by these transcription factors, as well as the number of genes differentially expressed when transferred to either Rich media or Anaerobic minimal media, is indicated. The number of up-regulated genes is indicated in ()'s next to the total number of differentially expressed genes. Bindings sites with different methylation states under different growth conditions are also displayed.

					Differentially expressed genes		Differentially methylated sites	
Regulator	Total Sites	Sites with motif	Operons regulated	Genes regulated	Rich media	Anaerobic	Rich media	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

Table 4. Genomic context of motifs with differences in methylation state among conditions. Left and right flanking genes, as well as their orientation, are provided for motifs found within intergenic regions. Motifs were down stream of a left flanking gene if the arrow points to the left, and down stream of right flanking genes of the arrow points to the right. "P" indicates motif located in the plasmid, while * indicated motif in a Fur transcription factor binding site.

		Methylation state			Genomic context		
Position (+/-)	Motif	Min Media Aerobic	Rich media	Min Media Anaerobic	Left flanking gene	Within gene	Right flanking gene
280309/280310	G<u>A</u>TC	●	○	●	← SO_0274	-	SO_0275 →
1642124/1642125	G<u>A</u>TC	-	○	●	← SO_1563	-	SO_1565 →
3823765/3823766	G<u>A</u>TC	○	●	○	← SO_3669	-	SO_3670 →
3823792/3823793	G<u>A</u>TC	○	●	○	← SO_3669	-	SO_3670 →
4061174/4061175*	G<u>A</u>TC	-	●	○	← SO_3914	-	SO_3915 →
1965318/1965324	GC<u>A</u>N$_4$GTC / G<u>A</u>CN$_4$TGC	◓	●	●	→ SO_1871	-	SO_1872 ←
938801/938809	TG<u>A</u>YN$_6$TGAC / GTC<u>A</u>N$_6$RTCA	○	●	●	-	SO_0912	-
160490/160498 P	TG<u>A</u>YN$_6$TGAC / GTC<u>A</u>N$_6$RTCA	◒	-	●	-	SO_A0172	-

● = methylated ○ = non-methylated ◓ = hemi-methylated


0 Mb
oriC
Motif 5
ATCGAT
ATCGAT
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GATC
Motif 5
ATCGAT
Motif 3
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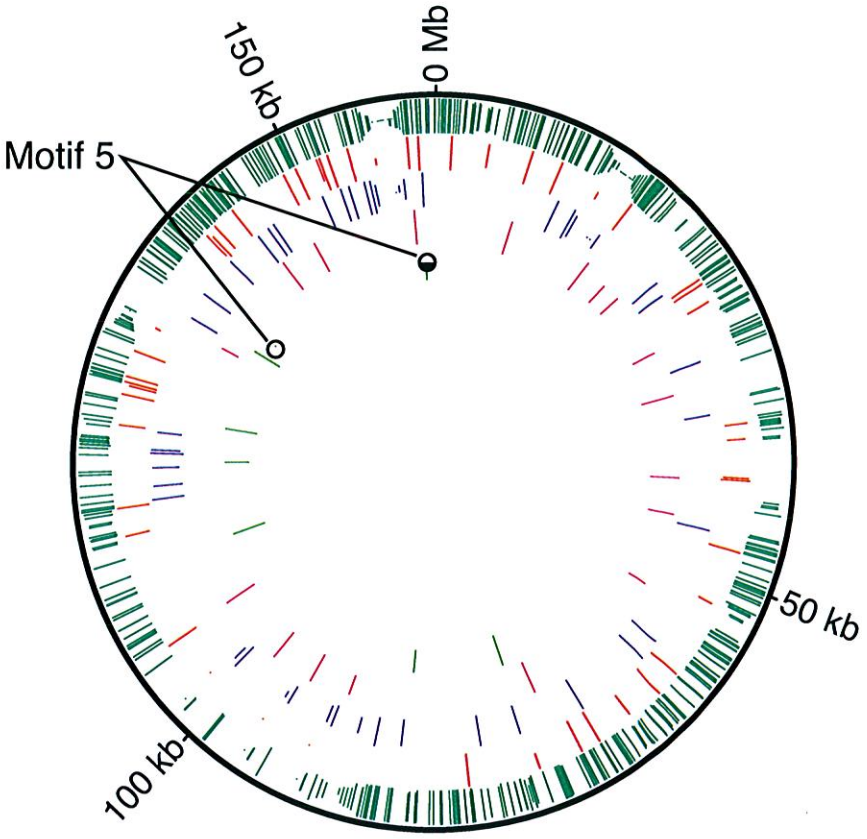
Motif 5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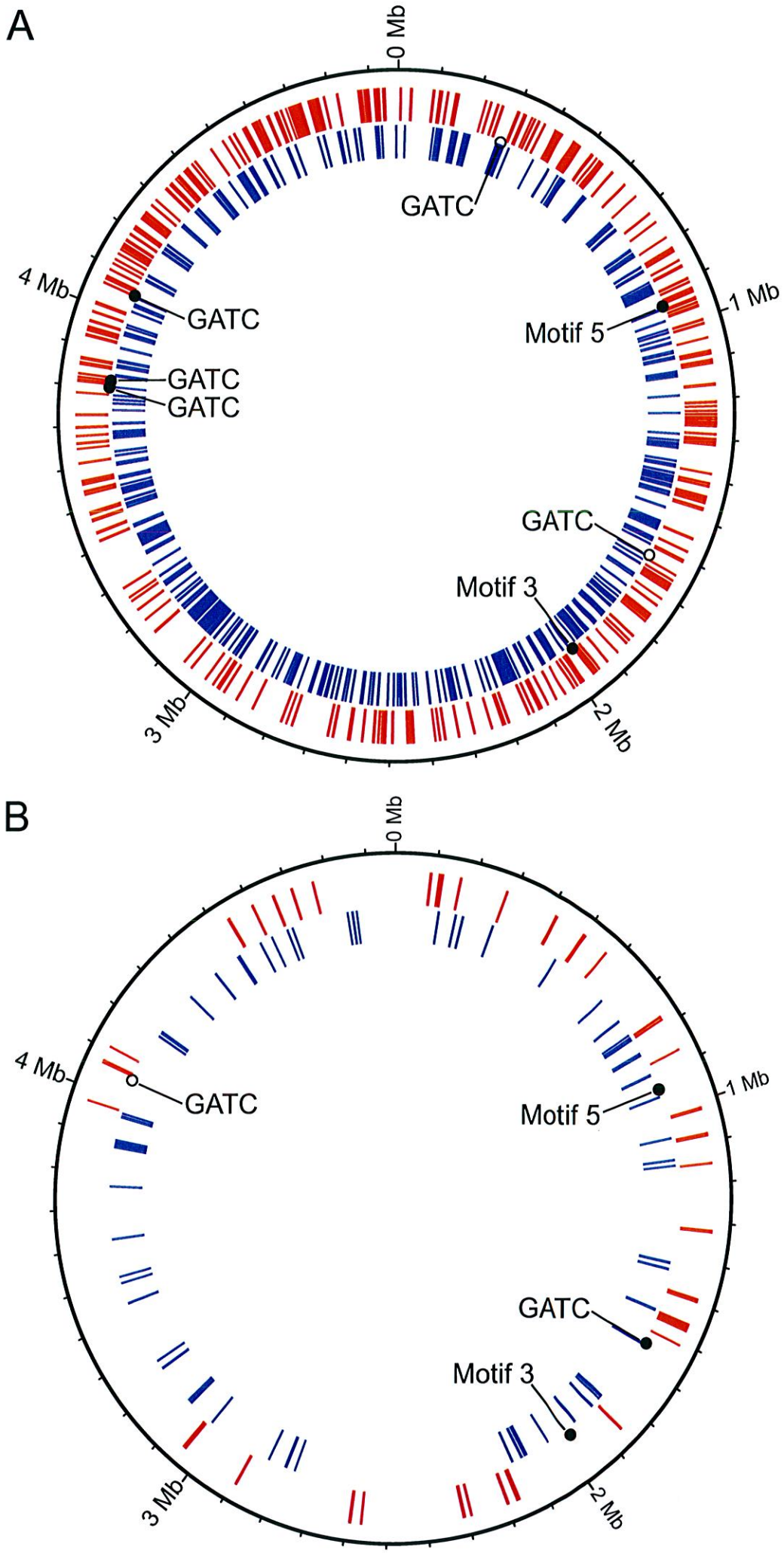
A
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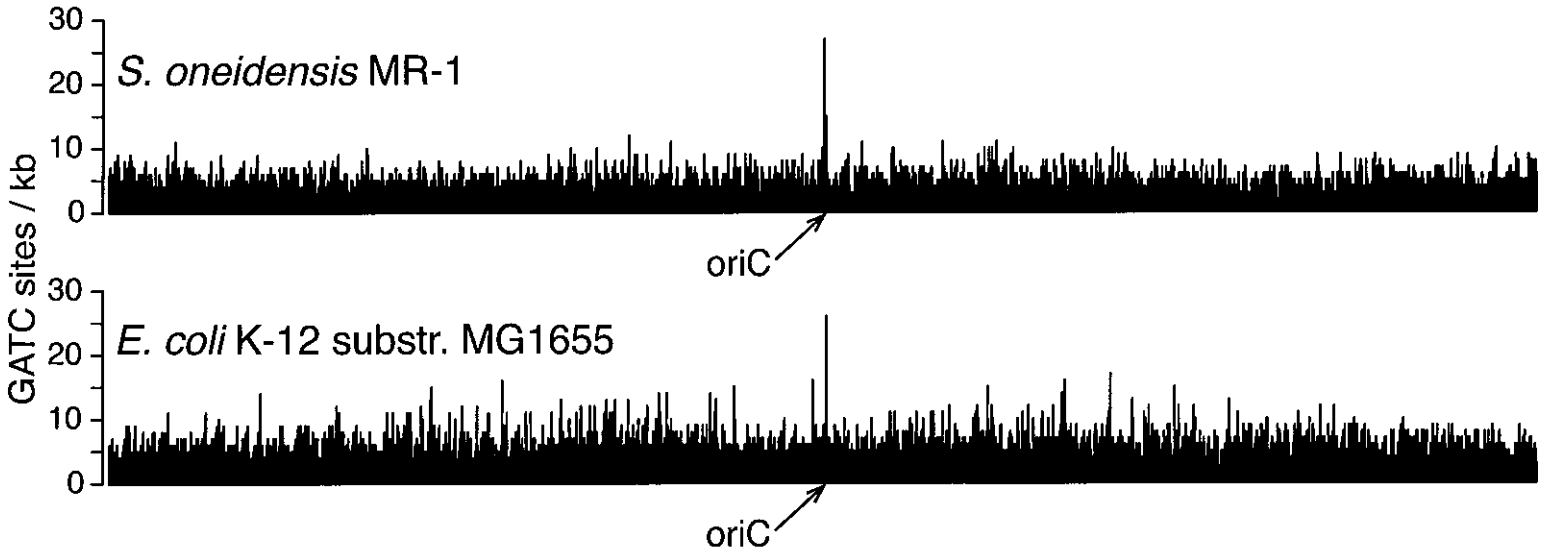
Motif 3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S. oneidensis MR-1
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E. coli K-12 substr. MG1655
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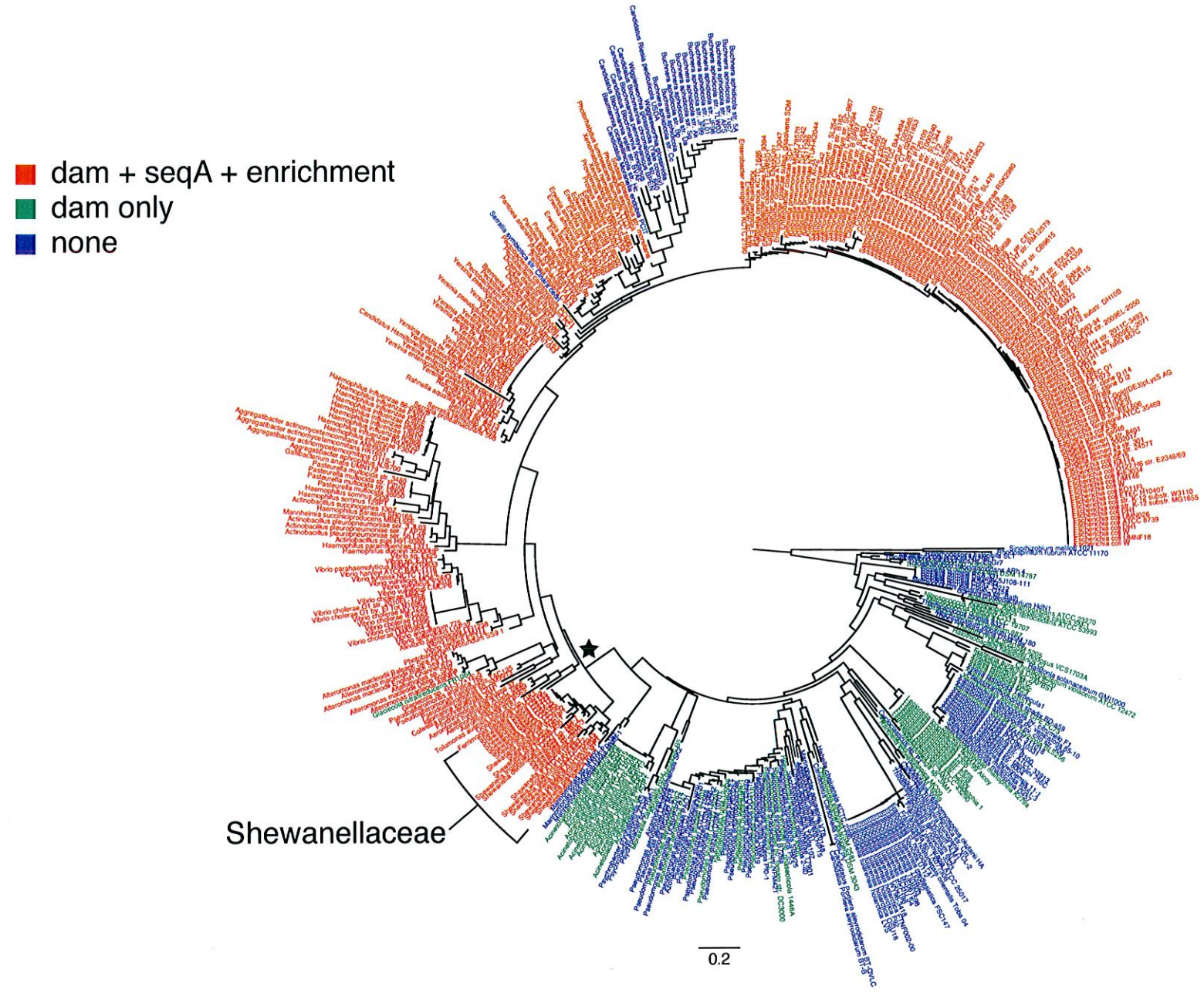
GATC sites / kb


dam + seqA + enrichment
dam only
none
Shewanellaceae
0.2