Structures of *Escherichia coli* DNA adenine methyltransferase (Dam) in complex with a non-GATC sequence: potential implications for methylation-independent transcriptional repression

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ABSTRACT

DNA adenine methyltransferase (Dam) is widespread and conserved among the γ-proteobacteria. Methylation of the Ade in GATC sequences regulates diverse bacterial cell functions, including gene expression, mismatch repair and chromosome replication. Dam also controls virulence in many pathogenic Gram-negative bacteria. An unexplained and perplexing observation about *Escherichia coli* Dam (EcoDam) is that there is no obvious relationship between the genes that are transcriptionally responsive to Dam and the promoter-proximal presence of GATC sequences. Here, we demonstrate that EcoDam interacts with a 5-base pair non-cognate sequence distinct from GATC. The crystal structure of a non-cognate complex allowed us to identify a DNA binding element, GTYTA/TARAC (where Y = C/T and R = A/G). This element immediately flanks GATC sites in some Dam-regulated promoters, including the Pap operon which specifies pyelonephritis-associated pili. In addition, Dam interacts with near-cognate GATC sequences (i.e. 3/4-site ATC and GAT). Taken together, these results imply that Dam, in addition to being responsible for GATC methylation, could also function as a methylation-independent transcriptional repressor.

INTRODUCTION

*Escherichia coli* DNA adenine methyltransferase (EcoDam) methylates the exocyclic amino nitrogen (N6) of the Ade in GATC sequences (1,2). Orthologs of the *dam* gene are widespread among γ-proteobacteria (3), and among a number of their bacteriophages (4). DNA-adenine methylation at specific GATC sites plays a pivotal role in methylation-dependent bacterial gene silencing. DNA replication and DNA mismatch repair (5,6). For example, there is a cluster of GATC sites near the origins of replication of *E. coli* and *Salmonella typhimurium*, each of which is conserved between the two species. This is not limited to the order Enterobacteriales, as there is evidence that Dam methylation controls replication of *Vibrio cholerae* chromosome II (7) and possibly chromosome I (8).

The Dam-dependent control of the various affected cell functions is in response to hemimethylated GATC sites, produced immediately following DNA replication (9). Specifically, Dam activity is relatively low (10), so there is a delay between chromosome replication and methylation of the new daughter strand. This delay is essential to the post-replicative mismatch repair system, where the methylation directs repairs to the new daughter strand (11).

Dam methylation also regulates the expression of specific genes in *E. coli* (12,13). For example, the expression of pyelonephritis-associated pili (Pap) in uropathogenic *E. coli* is epigenetically controlled by the methylation state of the two GATC sites in the Pap regulon (14). As another example, in *Salmonella enterica serovar* Typhimurium, Dam methylation modulates expression and translocation of the secreted *Salmonella* effector protein SopB (15). The interaction of EcoDam with its GATC target sequences has been studied structurally (16,17) and functionally (see below). For example, GATC sites preceded by an A4 tract are poorly methylated, while the most-efficiently methylated GATC sites have no obvious flanking sequence pattern (18). However, GATC sites may not be associated with all EcoDam effects.

Puzzlingly, studies of global gene expression changes in *dam* mutant *E. coli* suggest that EcoDam can regulate gene expression in a methylation- and GATC-independent manner. Some of this is very likely due to indirect effects, but not necessarily all cases. There have been at least three stud-
Schematic summary of EcoDam-DNA base contacts in the specific complex. The flipped target adenine base of the top strand is shaded. The R124–Gua4 interaction is conserved in cognate (GATC) and non-cognate (GTYTA/TARAC) complexes. Y119 intercalates between the inner AT base pairs.

Figure 1.

MATERIALS AND METHODS

His-tagged EcoDam was expressed in HMS174(DE3) cells using autoinduction procedures (28), and purified on Ni2+-affinity, UnoS and S75 Sepharose sizing columns (GE Healthcare) as previously described (16,17). A 0.5 liter autoinduced culture yielded ~7 mg purified enzyme. In the last purification step and during concentration, AdoMet, AdoHcy or sinefungin was added to the protein at ~2:1 molar ratio. Concentrated binary complexes were mixed with oligonucleotide duplex (synthesized by New England Biolabs, Inc.) at a protein to DNA ratio of ~2:1 and allowed to stand on ice for at least 2 h before crystallization. Final protein concentration for crystallization trials was ~15–30 mg/ml.

Initially, in sitting drop crystallization screens, showers of small needles appeared in 5–10% of 2-methyl-2,4-pentanediol (MPD) and polyethylene glycol (PEG) 4000–8000 as precipitants, but it appeared that larger, more single crystals could be grown with low molecular weight PEGs. Ternary complexed crystals utilized for data collection were best grown in hanging drops, with well solution containing 100 mM KCl, 10 mM MgCl2 and with variation of PEG 200 from 5 to 30% and 100 mM MES or HEPES (pH 6.4-7.0). The PEG 200 concentration was increased to ~40% in the crystallization drop before picking single crystals in cryoloops and froze and stored in liquid nitrogen until data collection. In addition, a few larger crystals appeared in PEG 4000 conditions and 25% ethylene glycol was added to mother liquor before freezing.
Figure 2. Structure of EcoDAM in complex with a non-canonical site. (a) DNA sequence used in the crystallization containing one GATC in the middle region of the duplex. The end sequence of the duplex was chosen such that the sequence at the joint of two molecules (green and blue) mimics a GATC site if the DNA duplexes are stacked head-to-tail. (b) EcoDam binds at the joint of two DNA duplexes. The hairpin recognition loop is colored in red. (c) Schematic summary of the protein-DNA contacts. Two DNA duplexes are stacked head-to-tail with one T:T mispair in the joint of two duplexes. Backbone-mediated interactions are indicated with main chain amine nitrogen (N) or carbonyl oxygen (O). (d) EcoDam-DNA interactions involve a 5-bp non-canonical site (5'-GTCTA-3'). (e) R124 interacts with the first G:C pair. (f) L122 and P134 interact with the T:T mispair. (g) Y119 interacts with the third C:G pair. (h) N120 interacts with the fourth T:A pair. (i) R249 interacts with the fifth A:T pair. (j) K9 and Y138 are involved in phosphate interactions.
Structures were determined by molecular replacement with the program REPLACE (29), using an EcoDam protomer from the previously determined cognate complex structure (PDB: 2G1P) (16) as a search model and DNA was manually built into its obvious electron density. Most refinement was completed using the program CNS (30) and manual manipulation by the program O (31). The last rounds of refinement utilized the PHENIX package (32) and final maps and models were visualized and manual manipulation was completed with COOT (33,34). Molecular graphics were generated using PyMol (DeLano Scientific LLC).

DNA binding was measured by two assays. For electrophoretic mobility shift assays, EcoDam (20 μM) was pre-incubated with the AdoMet analog sinefungin (Sigma) at a 1:1.5 molar ratio in 20 mM HEPES–HCl (pH 7.5), 100 mM NaCl, 5% glycerol, 1 mM dithiothreitol (DTT). The binary complex (at the indicated amount) was mixed with a 32-bp 6-carboxy-fluorescein (FAM)-labeled double-stranded (ds)DNA (5 nM) for 30 min at room temperature (∼25°C) in a 20 μl reaction containing 0.1 mg/ml bovine serum albumin (New England Biolabs), with or without 10 μg/ml salmon sperm genomic DNA (Rockland Inc.). Samples were loaded onto a 10 cm × 10 cm 6% native acrylamide gel in 0.5× Tris/Borate/EDTA (TBE) buffer and run 45 min at 80 V. FAM labeled DNA was visualized by Typhoon Trio+ (GE Healthcare).

Fluorescence polarization measurements were carried out at room temperature on a Synergy 4 microplate reader (BioTek). The FAM-labeled dsDNA (5 nM) was incubated for 30 min with increasing amounts of EcoDam–sinefungin complex in 20 mM HEPES–HCl (pH 7.5), 200 mM NaCl, 10% glycerol, 1 mM DTT. Curves were fit individually using GraphPad Prism 5.0 software (GraphPad Software, Inc.). Binding constants (K_D) were calculated as [mP] = [maximum mP] × [C]/(K_D + [C]) + [baseline mP], and saturated [mP] was calculated as saturation = ([mP] − [baseline mP])/[maximum mP] − [baseline mP], where mP is millipolarization and [C] is protein concentration. Averaged K_D and its standard error are reported.

RESULTS

EcoDam interaction with a non-canoncal site

We previously crystallized a complex containing EcoDam and a 12-bp DNA duplex containing a single centrally-located GATC target site (16). The end sequence of the duplex was chosen such that the sequence at the joint of two molecules mimics a GATC target site, if the DNA duplexes are stacked head-to-tail (Figure 2a). Indeed, two Dam molecules were bound to each duplex, one at the central GATC and one at the joint. However, a second crystal form (space group: P2_12_1_2) was also produced using the same 12-bp blunt-end DNA duplex (Figure 2a; Table 1).

There were three unexpected observations in the second crystal form. First, while an EcoDam molecule was again bound to the joint between neighboring DNA duplexes, no EcoDam molecule was bound to the unbroken GATC site in the middle of the duplex (Figure 2b). Second, only 11 of the 12 base pairs in each DNA duplex are stacked head-to-tail along the crystal a-axis with the length of ∼36 Å (average helical rise per base pair of ∼3.3 Å). The electron density maps indicate that the two 3’ Ade bases at the ends of each DNA duplex were flipped out, with one being disordered and the other stabilized by Dam (Figure 2, panels b and c). This is surprising because, in the cognate complex, only the target (methylatable) Ade is flipped out and into the active site (16), whereas in this non-cognate complex both Ade are flipped and neither one is in the active site. Third, the two 5’ Thy bases formed a T:T mismatch at the joint of the two DNA molecules (Figure 2c).

EcoDam is thus in contact with five base pairs spanning the joint, which constitutes a non-canonical site. Three contacted bases are from the green/left duplex, one is from the T:T mispair, and one is from the blue/right duplex (using the colors and orientation of Figure 2, panels c and d). In this particular complex, the 5’ Gua (blue DNA) interacts with R124 (Figure 2c), and the interactions of its four 5’ phosphates are identical to those of EcoDam interacting with cognate DNA (16). One thymine of the T:T mispair makes a van der Waals contact with P134 and a C−H...O hydrogen bond with L122 (Figure 2f) (35). Other residues, previously identified in cognate complexes as being involved in intercalation (Y119), base-amino acid hydrogen bonding (N120), and first base pair recognition (K9 and Y138), are in the case of this non-cognate complex located in the major groove of the green DNA. It is as if they were positioned for invasion into the DNA at a GATC sequence, but then switched their roles to making phosphate contacts (Y119 and K9), base contacts (Y119 and N120), or water-mediated DNA interactions (Y138 (Figure 2g–j)). An additional base contact is formed in the minor groove of the green DNA by R249 (Figure 2i). Taken together, these interactions suggest EcoDam recognizes and binds to a non-canonical 5-bp sequence of 5’-GTCTA-3’ / 5’-TAGTC-3’. This non-canonical binding might be to a more degenerate sequence, but the structure provides direct evidence for recognition of this particular sequence.

Comparing the cognate complex (16) and the non-canonical complex, the protein components are structurally similar (root mean squared deviation = 0.4 Å across 245 Ca atoms; Figure 3a), as are the analogous cofactors (AdoHcy in the cognate complex and sinefungin in the non-canonical complex). The associated active-site residues are in equivalent positions (Figure 3b). In addition, there is one particularly well-conserved protein–DNA base interaction: R124–Gua (Figure 3c). The interactions with GTCTA/HTAGC (Figure 2, panels d–j) suggest that the Dam binding is likely to be specific for the 5’ G bp, but could be degenerate at the other positions. This partially resembles a 1/4-site recognition of GATC, in which EcoDam traps the sequence that mimics part (1/4) of the GATC sequence.

Interactions with pap promoter sequences

As mentioned in the introduction, the Pap operon contains two GATC sites (Figure 4a). In contrast to most GATC sites in the E. coli genome, the pap-associated sites are not always completely re-methylated after DNA replication, and their methylation state determines in part the phase variation of pilus formation, which is under epigenetic control (14). The failure to methylate these sites is due in part to the bind-
Table 1. Statistics of X-ray diffraction and refinement

<table>
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<th>Character</th>
<th>Crystal</th>
<th>Non-cognate</th>
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<th>Pap (prox)</th>
<th>GTTA (prox)</th>
<th>GTCTA (dist)</th>
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<td></td>
<td>4RTL</td>
<td>4TN</td>
<td>4RTO</td>
<td>4RTS</td>
</tr>
</tbody>
</table>

Note: Wavelength=1 \(\AA\); Space group P2_12_1; One ternary complex per asymmetric unit; Synchrotron beamline APS 22-BM or 22-ID

Values in parentheses correspond to highest resolution shell;

\(\sigma = \sqrt{\sum (I - \langle I \rangle)^2}/\langle I \rangle\), where \(I\) is the observed intensity and \(\langle I \rangle\) is the averaged intensity from multiple observations;

\(\text{R}_{\text{int}} = \sum |I_{\text{obs}} - I_{\text{calc}}|/\sum |I_{\text{obs}}|\), where \(I_{\text{obs}}\) and \(I_{\text{calc}}\) are the observed and calculated structure factors, respectively;

\(\text{R}_{\text{merge}} = \text{R}_{\text{merge}}\) was calculated using a randomly chosen subset (5%) of the reflections not used in refinement.

\(\text{Estimated error}\) is maximum-likelihood based.
Figure 3. Structural comparison of cognate and non-canonical complexes. (a) Superimposition of EcoDam–cognate DNA–AdoHcy ternary complex (PDB: 2G1P in cyan) and the non-canonical complex (in gray). (b) The active sites containing Sinefungin (yellow) and AdoHcy (orange) and conserved sequence motifs (defined in (57)). (c) Conserved R124–Gua interaction.

pairs, via a water-mediated interaction (base pair 4) or a weak interaction with the methyl group of thymine of base pair 5 (Figure 4d, bottom).

For the Dist site, we suspected that the same R124 could interact with the 5′ Gua of GTCTA on the bottom strand (see Figure 5). Surprisingly, we also obtained crystals in which R124 was interacting with the 5′ Gua of GACGA of the top strand in the joint between two neighboring DNA duplexes (Figure 4b). This complex resembles a 1/2-site recognition, in which the 5′ Gua forms hydrogen bonds with R124 while the second-position Ade interactions with P134 and L122 (Figure 4e, top left and middle) are identical to those of EcoDam interacting with cognate GATC sequence (16). It seems that the interactions for the first 2-bp of the GACGA are specific, while the interactions for the last 3-bp are less so and those bp could be replaced with alternatives (comparing Figure 4d and e). In this orientation, the Dam molecule occupying the Dist site is in the same direction as the one in Prox site (i.e. both R124–Gua interactions occur for the top strand (Figure 4b and c).

EcoDam alters DNA conformation in non-cognate complexes

We next designed oligos containing the two repeated 5-bp GTYTA elements, from either Dist or Prox site (Figure 5a and b), with one repeat within the DNA duplex and the other at the joint of two neighboring DNA molecules. Again, we observed that a Dam molecule occupies the 5-bp element formed in the joint between two neighboring DNA duplexes (Figure 5). Interestingly, we observed two distinct Y119 interactions with DNA at the Prox and Dist sequences. In the Prox site, Y119 intercalates into the DNA between the two thymine bases of GT^TTA (Figure 5c), similarly to the cognate complex where Y119 inserts into the DNA between Ade and Thy of GA^TC (16). In the Dist site, Y119 stacks with the thymine base at position 2, GTCTA, but pushes the rest of DNA away, resulting in two DNA molecules shifted relative to one another perpendicularly to the DNA axis (Figure 5d). This second phenomenon has been observed previously with the phage T4 Dam (23).

We have observed previously that a mammalian SRA domain protein could bind the junction between the two DNA duplexes (40). In addition, protein–DNA complex crystals of some DNA repair glycosylases were obtained in which the enzyme did not bind to the middle region of the duplex containing the lesion, but bound at the joint (41–43). The examples mentioned here, Dam, SRA, and DNA glycosylases, all use a base-flipping mechanism (44) to access their target base, whether for the purpose of mismatch excision, damage repair or generating/recognizing modification. It is possible that the junction between the two DNA duplexes mimics the altered B-DNA conformation generated during or after base flipping. In fact, HhaI methyltransferase binds more tightly to DNA sequences containing a mismatch at the target base (45).
Figure 4. Structure of EcoDam in complex with pap promoter sequences. (a) Organization of pap regulatory sequence: numbers (1–6) indicate six leucine-responsive-regulatory-protein (Lrp) binding sites (14). Among the six Lrp binding sites, sites 2 (Prox) and 5 (Dist) contain a GATC sequence. The Pap GATC flanking sequences, shown underneath, share sequence similarity with the non-canonical site (inset box). (b and c) The 11-mer DNA duplexes are stacked head-to-tail with the 5’ overhangs A and T forming a base pair, resulting in a sequence representing the Dist site or Prox site (underlined). The Dam molecule is trapped at the 5’ Gua of GACGA (Dist) or GTTTA (Prox). (d and e) EcoDam-DNA interactions involves 5-bp at the Prox site (GTGTA) or Dist site (GACGA).
Figure 5. Structure of EcoDam in complex with the 5-bp GT(C/T)TA element. (a and b) The repeated 5-bp elements used in the crystallization. The organization of the DNA sequences mimics what appears in the pap regulatory sequence (see Figure 4). (c) Y119 intercalates into DNA resembling the Prox site, and stacks between two thymine bases. (d) Y119 pushes the green DNA, resulting in the two DNA molecules (blue and green, resembling the Dist site) being shifted relative to one another perpendicularly to the DNA axis. (e) A hypothetical model of EcoDam molecules sliding along DNA and binding at adjacent noncognate and cognate sites. The model was generated based on T4Dam structure (PDB: 1YFJ) (23).
EcoDam–DNA interactions in solution

To explore the effect of DNA sequence variation on EcoDam binding, we first measured the binding activity of EcoDam using a 32-bp DNA oligonucleotide containing two GAT/ATC sites, but no GATC (Figure 6). In electrophoretic mobility-shift assays, specific band shifts were observed at \(\sim 40\), 160 and 630 nM of EcoDam, respectively (Figure 6a). EcoDam maintains the specific band shifts in the presence of salmon sperm DNA, though the shifts occur at higher EcoDam concentrations at approximately 0.63, 1.25 and 2.5 \(\mu\)M, respectively (Figure 6b). This observation of non-GATC specific interactions was in agreement with a previous study using a long 150-bp DNA fragment containing a single GATC site and six 3/4 sites (ATC or GAT) (27), where the appearance of a ladder of bands indicated multiple DNA molecules binding to non-GATC sites. In contrast, when the same long DNA molecule, which contained a single EcoRV binding site (GATATC), was incubated with EcoRV methyltransferase (a restriction-modification enzyme which is related to EcoDam by protein sequence homology and overlapping substrate specificities), only a single specifically-shifted band resulted (46).

We next measured the dissociation constants \(K_D\) between EcoDam and a 12-bp oligonucleotide containing either a GATC, GTTTA or no G:C base pair using fluorescence polarization analysis (Figure 6c). Surprisingly, the binding affinity for GTTTA is slightly stronger than that of GATC under the assay conditions (Figure 6c). While the difference is only \(\sim 40\)%, it is clear that the Dam affinity for GTTTA is no lower than that for GATC. Changing the single G:C base pair of GTTTA to A:T reduced binding affinity by a factor of >2 (Figure 6c), indicating nonspecific DNA binding, which might account for the smear observed in the electrophoretic mobility-shift assays. The 32-bp oligonucleotide exhibited the strongest binding at \(K_D\) of \(\sim 1.1\) \(\mu\)M, probably because it allowed binding of multiple EcoDam molecules at the same time.

\[\begin{align*}
5' - \text{TACACTATCATGCGCTGACCCACAACATC} & - 3' \\
3' - \text{ATGTGATAGTACCGACTGGGTGTGTTGTAAGC} & - 5' - (\text{FAM})
\end{align*}\]
Table 2. Examples of GTYTA/TARAC elements in the GATC-regulated promoters

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<th>Fragment</th>
<th>Sequence</th>
<th>Notes</th>
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<td>ATCTGctagAAAT.....ATTTTctagCAAAT</td>
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<tr>
<td>TAGACgatcTTTTA.....TAAAGagacGTATA (foo (49))</td>
<td>ATCTGctagAAAT.....ATTTTctagCAAAT</td>
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<tr>
<td>TAGACgatcTTTTA.....GTAAAagacGTAAA (clp (50))</td>
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Note: GATC sites are in lower case and boxed. Variations of GATC sequence (GAT or GA or their complements) are in red. Motif GTYTA/TARAC is in blue and its variation GTYAA/TTRAC in green.

DISCUSSION

A new EcoDam binding site on DNA

We have demonstrated, by means of X-ray crystallography, that EcoDam binds to a 5-bp sequence distinct from the cognate GATC. This is different from the previously described preferences for certain sequences flanking GATC sites (18), or from the sometimes substantial binding preference for particular repeat symmetries in non-cognate DNA sequences (47). However, the new type of EcoDam binding site may affect binding to an adjacent GATC. The new sequence, GTYTA/TARAC (Y = C/T, R = A/G), immediately flanks the proximal and distal GATC sites in the Pap operon. These sites control the expression of pyelonephritis-associated pili. In the Dist site, we also found that EcoDam binds GACGA, the sequence overlapping with the 5-bp element and the cognate GATC (Figure 4a), which share two base pairs. The two elements near the Dist GATC, GTCTA (top strand) and GACGA (bottom strand), are oriented in opposite directions, suggesting EcoDam linear diffusion along DNA in either direction will result in the enzyme passing over a non-cognate site and thus affecting processivity of GATC methylation. The same could be true for the Prox site, where the top strand GTTTA and the bottom strand contains a 3/4-site (GAT) (Figure 4a). These non-cognate sites contain at least one G:C base pair (recognized by R124) and may also affect Dam intrasite hopping (48). A previous study also suggested that a GATC site with immediate 5′ polyA-tract (as occurred in the Pap operon) was methylated at a lower rate (18). The GATC site itself is palindrome, but the neighboring sequences at the Dist and the Prox sites are inverted (Figure 4a) as well as differing in a C:G versus A:T base pair, contributing to and possibly explaining the different methylation rates of the two target Ade on the same strand. T4Dam, the Dam ortholog of phage T4, has a similar ability to bind DNA sequences containing part (1/4-, 1/2- or 3/4-site) of GATC sequences (23). In T4Dam, we previously observed five different modes of T4Dam–DNA interaction ranging from nonspecific, through noncognate to specific (23). We suggest that EcoDam might be able to bind neighboring cognate and noncanonical sites in pap (Figure 5e), providing a potential Dam–Dam contact mechanism (either recruiting, or clashing with the second Dam molecule if the two sites are too close) that would work at adjacent cognate–noncognate DNA sites.
Table 3 was assembled because multiple earlier analyses of gene expression changes in \textit{dam} vs. \textit{WT} \textit{E.coli} indicated a surprising lack of correlation between gene expression level and the presence of GATC sites upstream of the \textit{Dam}-responsive transcriptional start sites (19,22). To further assess the role of the 5-bp element as a regulatory motif at the transcriptional level, we analyzed the upstream sequences of 35 transcription units that have been experimentally verified as being differentially expressed in a \textit{dam} mutant. Of these, 18 (51%) have TANAC or GTNTA (Table 3). In addition, 28 (80%) have ATC or GAT (i.e., the 3/4-site of cognate GATC sequence). Combining these two together, either the 5-bp element or GAT or both, it accounts for 33 (94%). The shorter or more degenerate sequences will, of course, occur more often in random sequences so, while they may nonetheless show significant non-cognate EcoDam binding (particularly where such binding is cooperative), it is necessary to be more cautious in interpreting those subsites. In particular, the promoter regions for \texttt{hns} and \texttt{yfeXY} have only 2 bp (1/2-site) sequences, while the single 3/4 site associated with \texttt{treBC} is downstream of the transcript start, and it may be that these three promoters are Dam-responsive only via indirect effects.

Regulatory implications of non-GATC EcoDam binding sites

To assess the possible role of EcoDam as a methylation-independent regulator, we searched the literature for Dam-regulated promoters. Table 2 shows promoters confirmed to be Dam responsive where GATC methylation plays a role, while Table 3 shows Dam-responsive promoters (whether due to direct or indirect effects) where methylation is not known to play a role—in fact only 2/35 of these Table 3 promoter regions have a GATC at all, and in those two cases the GATC is downstream of the transcription start. Focusing first on Table 2, and the methylation-dependent promoters, we found that two copies of the motif GTYTA are present in the regulatory region for another pilus type, \textit{foo} (49), where the GTYTA and GATC sites are equivalent to the way they appear in the Pap operon. One copy of GTCTA is upstream of the protease gene \texttt{clp} (50), while a second copy contains a variation at fourth position of the element, GTTAA. Two copies of GTYAA are present in \texttt{sfa} (51) and \texttt{fot} (49). In addition, antigen 43 (52) and \texttt{fae} (53) contain one copy of GTTTA or GTTAA. In addition, variations of cognate GATC sequence (i.e. 3/4-site GATC or 1/2-site GA) can be found in \texttt{agn43} (the gene for antigen 43) and \texttt{fae}. For the division inhibitor \texttt{sulA}, the GATC overlaps the putative –35 hexamer, with an overlapping upstream GTTGA.
binding of EcoDam occurs at near-cognate sites (Figure 6). A recent study of genome-wide mapping of methylated ad
nine residues in a pathogenic E. coli strain detected adenine-specific methylation of GATC sites as well as ATC/GAT sites, when the dam homologs were expressed in a plasmid system (54).

Based on our evidence for specific Dam binding to non-GATC sequences, and the occurrence of such sequences in Dam-responsive promoters, we suggest that Dam may function as a transcriptional repressor, in a methylation- and GATC-independent manner. It would be informative to know if a non-catalytic mutant of Dam still binds the non-cognate sites and has regulatory effects.

Finally, we note one technical implication of these findings. EcoDam has been used as a tool to assess mammalian chromatin structure by determining relative accessibility of GATC sites for methylation (55), or preferential methylation of specific GATC sites when EcoDam is fused to a mammalian regulatory protein (DamID) (56). The binding of Dam to noncanonical sites might subtly bias these uses of EcoDam.

ACCESSION NUMBERS
Protein Data Bank: the coordinates and structure factors of EcoDam–DNA complexes have been deposited (see Table 1 for accession numbers).

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