A role for the bacterial GATC methylome in antibiotic stress survival

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Antibiotic resistance is an increasingly serious public health threat1. Understanding pathways allowing bacteria to survive antibiotic stress may unveil new therapeutic targets2–8. We explore the role of the bacterial epigenome in antibiotic stress survival using classical genetic tools and single-molecule real-time sequencing to characterize genomic methylation kinetics. We find that Escherichia coli survival under antibiotic pressure is severely compromised without adenine methylation at GATC sites. Although the adenine methylome remains stable during drug stress, without GATC methylation, methyl-dependent mismatch repair (MMR) is deleterious and, fueled by the drug-induced error-prone polymerase Pol IV, overwhelms cells with toxic DNA breaks. In multiple E. coli strains, including pathogenic and drug-resistant clinical isolates, DNA adenine methyltransferase deficiency potentiates antibiotics from the β-lactam and quinolone classes. This work indicates that the GATC methylome provides structural support for bacterial survival during antibiotic stress and suggests targeting bacterial DNA methylation as a viable approach to enhancing antibiotic activity.

Bacteria exposed to antibiotics mount complex stress responses that promote survival9–14, and accumulating evidence suggests that inhibiting such responses potentiates antimicrobial activity in organisms sensitive, tolerant and resistant to drugs2,3,5,8,15–18. In both prokaryotes and eukaryotes, genetic pathways underlying responses to environmental insults have been widely studied and involve some of the most phylogenetically conserved proteins known19. In eukaryotes, stress can also elicit epigenetic modification of histones and DNA that support long-lasting downstream responses20–23. The role of prokaryotic epigenomes in stress, however, is much less clear.

Bacteria lack histones but harbor a diverse group of enzymes able to insert epigenetic modifications in the form of sequence-specific methylation of DNA bases24. Prokaryotic DNA methyltransferases (MTases) function either alone or as part of restriction modification systems, participating in various cellular processes including antiviral defense, cell cycle regulation, DNA replication and repair, and transcriptional modulation24–26. Although several methylation-dependent epigenetic switches have been described27–32, genome-wide methylation patterns and kinetics have, until recently, been difficult or impossible to study in a high-throughput manner33–36. In this study, we use genetic and genomic tools to explore the function and behavior of the bacterial methylome during antibiotic stress.

To assess the role of DNA methylation in antibiotic stress survival, we first tested the ability of E. coli lacking different MTases to withstand sublethal doses of β-lactam antibiotics. Laboratory E. coli K12 possesses four functional MTases that methylate adenines or cytosines in distinct target sequences24,36–40 (Fig. 1a). Survival of subinhibitory ampicillin exposure by log-phase E. coli was unaffected in mutants lacking the HsdM, YhdJ or Dcm MTase. However, bacteria deficient in DNA adenine methyltransferase (Dam) were highly susceptible to this low drug dose (Fig. 1b and Supplementary Fig. 1a,b). Increased ampicillin susceptibility in dam-deficient E. coli was also reflected in a reduced minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (Supplementary Fig. 1c). Complementation with a plasmid expressing dam but not gfp restored wild-type survival levels in Δdam E. coli (Fig. 1c and Supplementary Fig. 2a,b). Because Dam might also behave as a transcriptional repressor independently of its DNA methyltransferase function37, we tested the ability of plasmids expressing previously characterized methylation-incompetent Dam variants38 (Supplementary Fig. 2a) to rescue ampicillin hypersensitivity in Δdam E. coli. Consistent with a role for GATC methylation, mutant Dam expression minimally altered the ampicillin hypersensitivity of Δdam E. coli, if at all (Supplementary Fig. 2b,c). Finally, we sought to determine whether Δdam E. coli hypersensitivity extended to drugs other than ampicillin.

Subinhibitory treatment with aztreonam, meropenem and cephalexin, other β-lactams commonly used in the clinic, was also significantly potentiated in the absence of dam (Fig. 1d). Together, these results...
suggest that Dam-dependent methylation is important for bacterial survival during β-lactam stress.

Dam methylates GATC sites throughout the genome of organisms belonging to multiple orders of γ-proteobacteria, including the clinically relevant genera *Escherichia*, *Salmonella*, *Yersinia* and *Vibrio*. To explore the behavior of the Dam methylome in the context of antibiotic pressure, we extracted genomic DNA from *E. coli* growing in the presence or absence of ampicillin stress and analyzed genome-wide GATC methylation over time using single-molecule real-time (SMRT) sequencing. With SMRT technology, epigenetic modifications on template DNA strands are inferred through the unique kinetic signature they engender during sequencing and the fraction of DNA molecules methylated at each GATC site is estimated. In all samples, consistent with the previous genetics of Dam, the majority of GATC sites were detected as methylated in a high fraction of the DNA molecules sequenced (0.97 ± 0.05 on average). The dynamic methylation patterns, remained unchanged by treatment (Fig. 2d, Supplementary Table 2 and Supplementary Data Set 1). This event's biological consequences are unclear, however, as expression of the surrounding gene (gdhA) was unperturbed by ampicillin treatment (data not shown). Thus, ampicillin stress does not majorly alter the *E. coli* Dam methylome.

Given the remarkable stability of adenine methylation during antibiotic exposure and the contrasting drug-sensitive phenotype of *dam* deletion mutants, we reasoned that the GATC methylome must provide structural rather than regulatory support for bacterial survival during antibiotic stress. Widespread genomic Dam methylation enables cellular processes requiring discrimination between the fully methylated parental DNA strand and the newly synthesized DNA strand whose GATC sites are not yet modified. Specifically, transient hemimethylation at replication forks orients the MMR system, guiding replacement of mismatched bases to nascent DNA strands only. Notably, without GATC methylation, the methyl-dependent endonuclease MutH can introduce double-strand breaks (DSBs) near mismatches targeted for repair. Mismatches are rare in log-phase DNA replication (<1 per replication cycle), but, under conditions of stress, their frequency can increase, in part, through induction of the error-prone polymerase Pol IV (encoded by *dinB*)53–55. We thus hypothesized that potentiation of β-lactam killing in the absence of Dam was a result of drug-induced mutagenesis fueling a genotoxic MMR pathway.

To test this hypothesis, we assessed the effect of deleting *dinB*, *mutH* or the mismatch-binding component of the MMR complex, *mutS*, on antibiotic hypersensitivity in Δ*dam* bacteria. Strikingly, without the mutagenic polymerase Pol IV, Δ*dam* *E. coli* survival of ampicillin stress returned to wild-type levels (Fig. 3a and Supplementary Fig. 4a). Similarly, removal of *mutS* or *mutH* on the Δ*dam* background also abrogated ampicillin hypersensitivity (Fig. 3b and Supplementary Fig. 4b). In Δ*mutH* Δ*dam* bacteria, optical density (OD) was soon diminished in ampicillin (Supplementary Fig. 4b), but this did not reflect decreased viability during treatment (Fig. 3b).

The finding that genomic GATC methylation supports survival of β-lactam stress in *E. coli* evokes the possibility of targeting Dam
to therapeutically potentiate antibiotic drug activity. Dam is an attractive target, as it lacks mammalian homologs but is conserved in several enteric pathogens. Furthermore, because multiple drugs can induce mutagenic responses in bacteria, treatment with antibiotics other than β-lactams should also be potentiated in the absence of GATC methylation. Indeed, survival of dam-deficient E. coli in the presence of subinhibitory doses of the quinolones norfloxacin, ofloxacin and ciprofloxacin was severely compromised in comparison to wild-type bacteria (Fig. 4a). As seen with ampicillin, hypersensitivity to ofloxacin could be abrogated by deleting dinB, mutH or mutS in Dam E. coli (Supplementary Fig. 5a,b). Consequently, drug potention in the absence of GATC methylation occurs via a similar mechanism across different antibiotic classes and may be broadly exploitable.

Next, we sought to determine whether virulent clinical isolates could also be sensitized to treatment by the removal of Dam. As in E. coli K12, dam deletion in uropathogenic E. coli (UPEC) strain UTI89 (ref. 63) substantially increased sensitivity to ciprofloxacin (Fig. 4b). Ciprofloxacin is a valuable drug for UPEC treatment, but its use is increasingly restricted by the spread of quinolone resistance. To assess whether targeting Dam might allow reconstitution of resistant strains, we deleted dam in a highly ciprofloxacin-resistant (CiproR) clinical UPEC isolate bearing multiple common quinolone resistance–confering mutations (Supplementary Table 3). Remarkably, although dam deletion did not restore full sensitivity to this isolate, the ciprofloxacin MIC for the CiproR UPEC was reduced by over half and its MBC90 value was reduced by 4.6-fold (Fig. 4c). Thus, removing GATC methylation can potentiate antibiotic lethality in both drug-sensitive and drug-resistant pathogenic organisms.

Together, our results define an important structural role for the bacterial epigenome in antibiotic stress survival. Characterization of the adenine methylome demonstrated highly stable global GATC methylation levels during log-to-stationary phase transition and subinhibitory β-lactam stress, and, although we identified several previously uncharacterized GATC sites with variable methylation over time, antibiotic stress did not significantly alter these patterns.

Despite the remarkable stability of the GATC methylome, E. coli lacking Dam are hypersensitive to antibiotic stress. Deletion of E. coli dcm or Neisseria meningitidis Mod11A (an adenine Mtase) was also reported to alter bacterial sensitivity to toxic compounds, but increased resistance rather than hypersensitivity was observed and attributed to altered gene expression. Although we cannot exclude additional involvement of transcriptional dysregulation, our data suggest that the GATC methylome represents an important backbone structure enabling DNA repair processes to function in the context of β-lactam and quinolone stress. Specifically, GATC methylation likely supports antibiotic-elicited mutagenesis dependent on Pol IV.
an error-prone polymerase induced transcriptionally or posttranslationally in the presence of several antibiotics. In the absence of GATC methylation, MMR machinery can convert postreplicative mismatches to DSBs, which accumulate to toxic levels in the absence of GATC methylation. Thus, a feedback loop may establish itself (Fig. 3e).

Our findings raise the possibility of targeting Dam to enhance the therapeutic activity of existing drugs. Several classes of antibiotics induce mutagenesis at subinhibitory concentrations and may thus be subject to potentiation by this mechanism. Enhancement of drug activity could be harnessed to lower effective therapeutic doses in drug-sensitive infections and may also allow resensitization of resistant organisms. Indeed, our data suggest that targeting Dam methylation can partially reverse ciprofloxacin resistance in UPEC.

Figure 3 Pol IV-dependent mutagenesis fuels MMR-mediated DNA damage in β-lactam-stressed Δdam E. coli. (a) Wild-type, Δdam, ΔdinB and ΔdinB Δdam (a) and wild-type, Δdam, ΔmutH, ΔmutH Δdam, ΔmutS and ΔmutS Δdam (b) strains were grown in LB to an OD of 0.3 and then treated with ampicillin (2.5 µg/ml) or left untreated. CFUs in bacterial cultures were monitored hourly to assess survival. Data are shown as the mean percent survival ± s.e.m. of n = 2 independent experiments. (c) Log-phase E. coli grown in LB alone or in LB with hydrogen peroxide (H2O2; 100 mM) or ampicillin (2.5 µg/ml) for the indicated time were assayed for DNA breaks by TUNEL analysis. The fluorescence distribution of each sample incubated with fluorescent label in the presence (solid line) or absence (shaded histogram) of TdT is displayed. A representative experiment is shown. MFI, mean fluorescence intensity. (d) DNA damage assessed as in e at 1 h. Data are shown as the mean percent of cells positive for DNA damage ± s.e.m. of n = 3 independent experiments. Statistical comparisons between each mutant strain and wild-type bacteria were not significant unless otherwise indicated; **** P < 0.0001. (e) Model of antibiotic potentiation in the absence of Dam methylation.

Figure 4 Quinolone toxicity is potentiated in laboratory and pathogenic Δdam E. coli. (a) Wild-type and dam-deficient E. coli BW25113 grown to an OD of 0.3 were treated for 3 h with or without the indicated drugs. (b) Log-phase UT819 UPEC and dam-deficient UT819 were treated with 15 ng/ml ciprofloxacin or left untreated. (c) Determination of ciprofloxacin MIC (left) and MBC90 (right) values for CiproR UPEC by broth microdilution in LB. Wild-type and Δdam MIC values were 133 µg/ml and 59 µg/ml, respectively, and wild-type and Δdam MBC90 values were 316 µg/ml and 68 µg/ml. Dotted lines indicate cutoff values for MIC (OD < 0.1) and MBC90 (10% survival). MBC90 values were interpolated using a sigmoidal curve fit model as shown. In a–c, data are shown as means ± s.e.m. of n = 2–3 independent experiments: NS, not significant; **** P < 0.0001.
More broadly, this observation suggests that mutagenic stress responses can occur and be therapeutically exploited in highly drug-resistant pathogenic organisms. In addition to drug potentiation, inhibiting Dam has been proposed as a strategy to weaken bacterial pathogenicity in vivo25–27,74, as GATC methylation controls virulence gene expression in some organisms. Although elevated rates of mutagenesis and induction of certain prophages25 in the absence of Dam could complicate a Dam inhibitor–based monotherapy, these drawbacks may be mitigated in the context of combination treatment. In summary, our results suggest that targeting bacterial epigenomic structures that support mutagenic stress responses may be a viable strategy for enhancing antibiotic activity.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

N.R.C. conceived of the project, designed and performed experiments, and wrote the manuscript. C.A.R. performed bioinformatics analyses. S.J. performed experiments. R.S.S. performed experiments and bioinformatics analyses. A.G. contributed intellectually to the project and helped design experiments. P.B. contributed intellectually to the project. H.L. performed bioinformatics analyses and provided mentorship. J.J.C. oversaw the project and provided mentorship.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Bacterial strains and plasmids. The laboratory bacterial strains used are derived from *E. coli* K12 (BW25113 obtained from the Coli Genetic Stock Center or MG1655 obtained from the American Type Culture Collection). UPEC strain UT89 was kindly provided by M. Conover and S. Hultgren. The ciprofloxacin-resistant UPEC isolate (UPEC CipR) was collected from the Brigham and Women’s Hospital specimen bank (Supplementary Table 3). Deletion mutants on the BW25113 background were derived from the Keio collection following Kan^R cassette removal. Deletion mutants on the MG1655 background were constructed by allelic transduction from Keio collection strains using classical P1 phage transduction followed by Kan^R cassette excision. The *dam*-null phenotype was confirmed by PCR alone or with electrophoresis of genomic DNA digested with DpnII, which cleaves only unmethylated GATC sites. For construction of the *Adam* UT89 and *Adam* UPEC CipR strains, the parent strain bearing a KM208 plasmid-based Red-recombinase system was electroporated with a PCR amplicon encoding the *Adam*:Kan^R allele. Recovered cells were selected for kanamycin-resistant homologous recombinants. The plasmid was cured, and the Kan^R cassette was removed. The genotype of each deletion strain was verified by colony PCR. The plasmid used in the *dam* complementation studies, namely pZS151 (Fig. 1c and Supplementary Fig. 1), was obtained from Expressys and belongs to the pZ vector family. pZS*31 has a plasmid was cured, and the Kan^R cassette was removed. The genotype of each deletion strain was verified by colony PCR. The plasmid used in the *dam* complementation studies, namely pZS151 (Fig. 1c and Supplementary Fig. 1), was obtained from Expressys and belongs to the pZ vector family. pZS*31 has a pSC101 origin of replication (which yields a low copy number of 3–5 plasmids per cell) and a chloramphenicol resistance marker. Sequence encoding either Dam (with the 500-bp upstream region flanking the gene) or GFP was inserted into the multiple-cloning site. For complementation experiments using mutated versions of *dam*, the plasmid containing the *dam* insert was engineered using either Gibson cloning or site-directed mutagenesis (New England Biolabs, Q5 Site-Directed Mutagenesis kit). Quinolone resistance–confering mutations in the CipR UPEC clinical isolate were identified through whole-genome Illumina sequencing of genomic DNA (PureLink Pro-96 Genomic Purification kit, Life Technologies). Libraries were prepared as previously described38. Raw sequencing reads were processed by trimming adaptor sequences and discarding reads shorter than 28 bp. Processed reads were aligned to the *E. coli* MG1655 genome using breseq^7. The genome alignments were searched for known quinolone resistance–confering mutations using the `acr,` `acr,` `besS,` `cpxA,` `cpxB,` `envZ,` `gyrA,` `gyrB,` `marA,` `marR,` `mdtA,` `mdtB,` `mdtC,` `ompC,` `ompF,` `ompR,` `parC,` `parE,` `sxxR,` `sxxS` and tolC genes and their regulatory regions.

Bacterial kill curves and determination of MBC and MIC. For time course kill curves and MBC assays, stationary-phase bacterial cultures were diluted by 1:1,000 in 25 ml of LB medium in 250-ml baffled flasks. Cultures were grown at 37 °C and 200 r.p.m. until they reached an OD of ~0.3. Cultures were transferred to 24-well plates at a final volume of 500 µl per well or to 96-well plates at a final volume of 150 µl per well and were either left untreated or treated with the indicated drugs at the specified doses. Plates were sealed using breathable membranes (BreatheEasy, BEM-1) and incubated at 37 °C and 900 r.p.m. for the remainder of the experiment. CFUs were enumerated at desired time points (4 h for MBC determination) by spot plating 5 µl of tenfold serially diluted culture onto LB agar and counting colonies after overnight growth at 37 °C. Percent survival at each time point was calculated in relation to the CFUs immediately before treatment (0 h). For MIC determination, antibiotics were serially diluted in a 96-well plate and mixed with stationary-phase bacterial cultures diluted by 1:10,000 in a final volume of 150 µl of LB per well. OD was measured from plates after 24 h of growth at 37 °C and 900 r.p.m.

Genomic DNA extraction and PacBio sequencing. Genomic DNA was extracted from *E. coli* K12 MG1655 LB cultures grown in the presence or absence of ampicillin using the GenElute Bacterial Genomic DNA Extraction kit (Sigma). To assess genomic methylation status, genomic DNA extracted from stationary-phase cultures was quantified, digested using DpnII (New England Biolabs) and run on a 0.8% agarose gel containing ethidium bromide. For methylome analyses, samples were sent to the University of Massachusetts Medical School Deep Sequencing Core, where methylome data were obtained by PacBio Core Enterprise instrument SMRT. SMRTbell DNA templates for SMRT sequencing were prepared according to the instructions in the Procedure & Checklist for 10-kb Template Preparation and Sequencing (Pacific Biosciences). Briefly, genomic DNA samples were first sheared to a target size of 10 kb using g-Tube devices ( Covaris), treated with DNA damage repair mix, end- repaired and ligated to hairpin adapters. SMRTbell libraries were prepared using the DNA Template Prep kit 2.0 (3–10 kb) from Pacific Biosciences. Incompletely formed SMRTbell templates were digested using exonuclease III (New England Biolabs) and exonuclease VII (Affymetrix). The prepared SMRTbell libraries were sequenced using a 120-min movie acquisition time and P4 polymerase-C2 DNA sequencing reagent kits following standard instructions for a PacBio RS II instrument (Pacific Biosciences). Each *E. coli* sample was sequenced on four or more SMRT cells, yielding a total of approximately 200-fold double-stranded coverage of the bacterial genome, and two or three biological replicates were sequenced for each antibiotic treatment condition (Supplementary Data Set 2). Sequencing coverage was comparable for methylated and unmethylated sites (Supplementary Table 1 and Supplementary Data Set 2), ruling out coverage loss as an explanation for the absence of methylation.

Bioinformatics analyses of SMRT sequencing data. Genome-wide detection of base modification and affected motifs was performed using the standard (default) settings in the RS Modification and Motif Analysis.1 protocol included in SMRT Analysis version 2.3.0 Patch 5. The FASTA reference genome sequence (*E. coli* K12 MG1655, NC_000913.2) used for base modification detection analyses was obtained from Pacific Biosciences. For motif identification, the base modification quality value (QV) threshold setting was left at the default value of 30. Interpulse distance (IPD) values were measured for all nucleotide positions in the genome and compared with expected distances in an in silico kinetic model of the polymerase for significant associations. Frac values were calculated in SMRT Analysis using a standard mixture-model analysis of the pooled kinetic data for a given sample. The frac output value provides information about the fraction of individual molecules displaying a methylation signal at each identified motif site within the genome (Supplementary Data Set 1). Methylation frac values were derived from IPD data within the SMRT pipeline using the single-site mixture model.39 A value of 0 was substituted for frac values that were below detection limits. The values from two or three experimental replicates were compared by Student’s t test, and false discovery rate (FDR)-adjusted P values were obtained by the method of Benjamini and Hochberg (Supplementary Data Set 1). Circular graphs were generated using the Circos software package.

Flow cytometry assessment of DNA damage. *E. coli* log-phase cultures were transferred to a 96-well plate (200 µl/well) and treated with ampicillin (2.5 µg/ml) or hydrogen peroxide (100 mM) for 30 min to 2 h at 37 °C and 900 r.p.m. Bacteria were pelleted by centrifugation at 3,000g for 5 min. The supernatant was discarded. Cell pellets were resuspended vigorously in 1 µl of cold 4% paraformaldehyde in PBS and incubated at room temperature for 30 min to allow fixation. Bacteria were centrifuged again and then resuspended in 200 µl of cold permeabilization buffer (0.1% Triton X-100 in 0.1% sodium citrate). After 2 min at room temperature, bacteria were centrifuged and washed in PBS. After pelleting the cells and discarding the supernatant, cells were resuspended in 50 µl of TUNEL labeling mix (dUTP–FITC and TdT enzyme) or 50 µl of TUNEL labeling reagent (dUTP–FITC) according to the manufacturer’s instructions (Roche, In Situ Cell Death Detection Kit, Fluorescein). Bacteria were stained for 1 h at 37 °C. Cells were then washed twice with PBS, resuspended in 1 µg/ml propidium iodide in PBS and analyzed by flow cytometry (BD LSORT Fortessa). Propidium iodide–negative cells, which lack genomic material, were excluded from the analysis. Gating was determined using single-color and unstained controls as references. For Figure 3d and statistical analysis, background staining with labeling reagent only was subtracted for each sample to account for treatment–dependent shifts in autofluorescence or stain retention.

Statistical analyses. Statistical analysis was performed on log_{10}-transformed data (for survival experiments) or on untransformed data (for TUNEL assays) using two-way ANOVA followed by a post hoc test using Sidak’s multiple-comparison test correction. In all cases, the P values indicated are multiplicity adjusted. 76. Baym, M. et al. Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS One 10, e0128036 (2015). 77. Barrick, J.E. et al. Identifying structure I variation in haploid microbial genomes from short-read resequencing data using bresq. BMC Genomics 15, 1039 (2014).