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Dam Methyltransferase Is Required for Stable Lysogeny of the Shiga Toxin (Stx2)-Encoding Bacteriophage 933W of Enterohemorrhagic *Escherichia coli* O157:H7[▽]

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Shiga toxin 2 (Stx2), one of the principal virulence factors of enterohemorrhagic *Escherichia coli*, is encoded by 933W, a lambda-like prophage. 933W prophage induction contributes to Stx2 production, and here, we provide evidence that Dam methyltransferase is essential for maintenance of 933W lysogeny. Our findings are consistent with the idea that the 933W prophage has a relatively low threshold for induction, which may promote Stx2 production during infection.

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 bacteria are a major cause of food- and waterborne illness in the United States, Europe, and Japan (5, 7, 25). These bacteria are highly infectious and produce potent Shiga toxins that account for the most severe clinical manifestations of EHEC infection, including the hemolytic uremic syndrome. In the sequenced EHEC strain EDL933, the stx_1 and stx_2 genes are located within lambdoid prophages designated 933J (also referred to as 933V [21]) and 933W, respectively (21). The former prophage is thought to be defective (20). Induction of Stx-encoding prophages can markedly increase Stx production and trigger phage-mediated cell lysis, thereby providing a mechanism for Stx release (18, 27). Thus, understanding processes that regulate Stx prophage lysogeny can provide insight into EHEC pathogenicity. In *E. coli* K-12, *dam* is known to influence lambda lysogeny. Here, we investigated the influence of *dam* on the maintenance of Stx-encoding prophages.

The *dam* gene encodes a DNA methyltransferase that methylates the adenine in the sequence GATC in double-stranded DNA (9, 28). Dam methylation in nonpathogenic *E. coli* K-12 has been studied extensively (9, 28). Among other traits, *dam* mutants exhibit single-stranded interruptions in their DNA (12). These single-strand interruptions are a consequence of MutHLS DNA mismatch repair and are converted into double-strand breaks which require homologous recombination to restore genomic integrity (11, 19). The presence of these breaks induces the SOS response (14, 22), likely explaining the increased induction of prophage lambda observed in *E. coli* K-12 *dam* lysogens (13). Unlike *E. coli* K-12, EHEC strain EDL933 contains several putative Dam-like methyltransferases, but Campellone et al. (2) recently demonstrated that deletion of a chromosomal *dam* gene that is 99% identical to that in *E. coli* K-12 (21) was sufficient to abrogate DNA methylation in this strain.

We used lambda Red recombination to inactivate the *dam* gene of the prototype EHEC strain EDL933, as recently described for construction of *dam* mutants in TUV93-0, a stx_1- and stx_2-deficient derivative of EDL933 (2). Unexpectedly, the frequency of *dam* mutants in the EDL933 background was much lower than that which we observed in the TUV93-0 background. Most of the candidate EDL933 *dam* mutants proved to be false positives. For example, when fragments of plasmid pKM212 (Δ*dam*::*kan*) (2) were used to transform EDL933, only 1 out of the 10 Kan^r colonies tested by PCR showed deletion of the *dam* gene (Fig. 1). The other nine candidates were presumably plasmid transformants. We chose to further analyze this Δ*dam*::*kan* strain (designated GM7284) as well as KM69, an independently derived EDL933 *dam* deletion mutant which was made in two steps. First, a SacI-SphI digest of pKM213 (Δ*dam*::*cat-sacB*) was used to generate a deletion of the *dam* gene (2), and second, a SacI-SphI digest of pKM210 was used to generate an in-frame (markerless) deletion of the *dam* gene by selection of a sucrose-resistant Cam^s colony (indicative of loss of the *cat-sacB* cassette [17]). The deletion was confirmed by PCR (2). These mutants lacked methylation in GATC sequences, as determined by digestion with the restriction endonucleases DpnI and Sau3A, which do not cut unmethylated DNA (data not shown). As has been observed in *E. coli* K-12 *dam* mutants, each of these EDL933 *dam* mutants showed an increase in frequency of spontaneous mutation to rifampin resistance relative to EDL933 and a false-positive candidate. The fractions of overnight cultures showing spontaneous mutation to rifampin resistance (per 10^8 cells, ± standard errors) are as follows: for EDL933/pTP223, 0.125 ± 0.78; for KM68 (Δ*dam*::*cat sacB*), 2.67 ± 0.56; for KM69 (Δ*dam*), 2.83 ± 0.81; for GM7284 (Δ*dam*::Kn), 2.83 ± 0.81; and for false-positive candidate no. 1 (from Fig. 1), 0.121 ± 0.004. (Determinations were done in triplicate.) Also, as reported for *E. coli* K-12 *dam* mutants, the EDL933 *dam*

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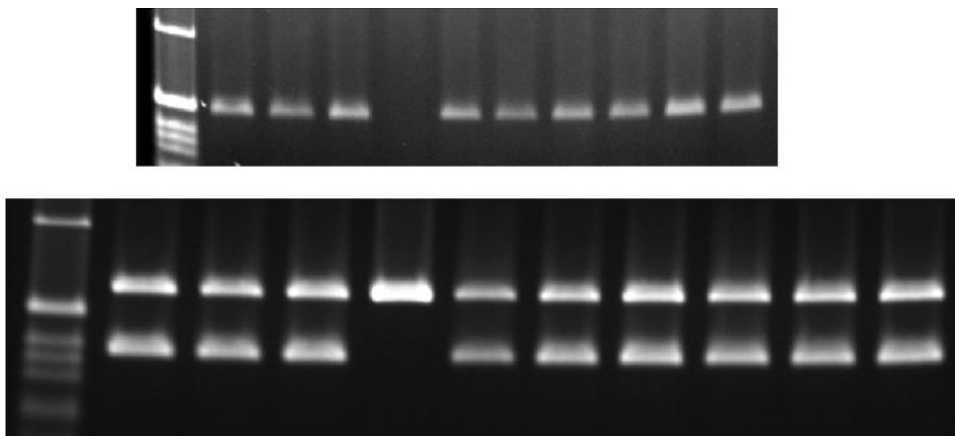

FIG. 1. PCR colony analysis of Kan^r transformants. Shown are results for PCR analysis (by colony PCR) of 10 Kan^r transformants of EDL933/pTP223 electroporated with a SacI-SphI digest of pKM212 (containing Δ*dam*::*kan*). (Top) PCR products (500 bp) used to detect the presence of the chromosomal *dam* gene. (Bottom) PCR products used to detect the presence of the Stx1 (top band, 624 bp) and the Stx2 (bottom band, 383 bp) phages.

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mutants exhibited heterogeneous morphology with many cells showing filamentation (2).

We initially suspected that *dam* might influence 933W lysogeny because we found that there was no detectable Stx2 in enzyme-linked immunosorbent assays (23, 24) of mitomycin C-treated cell lysates of the *dam* mutant strains (data not shown). In contrast, Stx1 was detectable in these lysates. Furthermore, PCR analyses revealed the loss of stx_2 but not stx_1 from both *dam* mutants; both stx_1 and stx_2 were present in the false-positive-candidate *dam* mutants mentioned above (Fig. 1). We performed microarray studies to investigate whether the 933W prophage transcriptome was lost from KM69. We plotted microarray signals from EDL933 and KM69 (Δ*dam*) against each other, which revealed a cluster of genes (bottom right of Fig. 2) that are expressed in EDL933 but not KM69 (Δ*dam*), and 55/56 of these are located in bacteriophage 933W (the other is *dam*). We confirmed our suspicion that the prophage was excised from KM69 by using a PCR assay that demonstrated that the *attB* site in this strain was unoccupied; furthermore, we were unable to amplify *intW-wrbA* from KM69 (data not shown). Thus, the data confirm the loss of bacteriophage 933W, and not just the stx_2 gene, in KM69 and suggest that *dam* may be required to maintain 933W lysogeny.

Our understanding of the relationship between Dam methylation, SOS induction, mismatch repair, and prophage induction is much more extensive for *E. coli* K-12 than for EDL933. *E. coli* K-12 was used, therefore, to test the hypothesis that *dam* mutants are nonviable due to enhanced 933W prophage sensitivity to induction by mismatch repair-induced SOS signaling. A 933W lysogen of *E. coli* K-12 strain MM294 (16) was isolated using a phage lysate from EDL933 and its identity confirmed by PCR using primers specific for the 933W immunity region (data not shown). An MM294(lambda$^+$) lysogen was also constructed for use as a control. The lambda phage used to prepare this lysogen produced turbid plaques at both 30°C and 42°C, and the MM294(lambda$^+$) lysogen was inducible with mitomycin C treatment, indicating that the lambda phage used did not harbor *c*I857 or *ind* mutations. The MM294(lambda$^+$) and MM294(933W) strains were transduced to Cam^r with a P1*vir* lysate propagated on a *dam-13*::Tn*9* donor. The number of transductants with MM294(933W) was more than 1,000-fold lower than that with MM294(lambda$^+$)

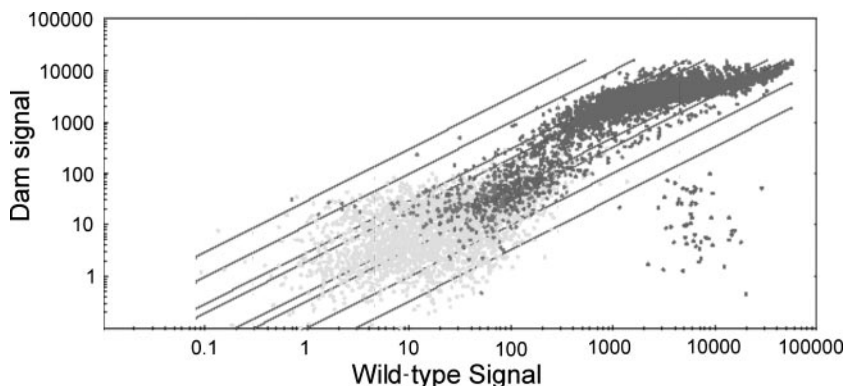

FIG. 2. Scatter graph comparing microarray signals from EDL933 and KM69 (Δ*dam*). Data points obtained from transcription microarrays are plotted to compare EDL933 and KM69 (Δ*dam*). The data points in the bottom right corner of the figure indicate that signal was obtained from EDL933 but not KM69. Almost all (55/56) of these are from phage 933W. Values shown are $\log_2$.

TABLE 1. Properties of wild-type and *dam* MM294(933W) transductants[a]

Strain	Cam phenotype	Presence of:		Plaque detection or amt (PFU/ml)[b]	2-AP phenotype
		Tn*9*	933W		
MM294(933W)	S	−	+	10^5–10^6	R
GM7258	R	+	+	±	S
GM7259	R	+	−	−	S
GM7260[c]	R	+	+	10^5–10^6	R
GM7261	R	+	−	−	S

[a] Strains GM7258 to GM7261 are independent transductants of MM294(933W) containing the *dam13*::Tn*9* mutation. Cam designates the phenotype of the strain (R, resistance; S, sensitivity) and Tn*9* the presence (+) or absence (−) of the *cat* (chloramphenicol acetyltransferase) gene as determined by PCR using flanking primers. The size of the *dam* gene was also shown to be increased when Tn*9* was present by using PCR and flanking primers (data not shown). The presence (+) or absence (−) of the 933W phage was determined by PCR using primers specific for the *stx2* genes. Numbers of PFU/ml were determined by plating supernatants of cultures grown overnight at 37°C on the *E. coli* K-12 indicator strain C600. 2-AP sensitivity (S) and resistance (R) were determined as described previously (15).

[b] ±, plaques that were pinpoint-sized at about 10^3 to 10^4 PFU/ml; −, not detected.

[c] Strain GM7260 has a strong mutator phenotype which is complemented by a plasmid-borne *mutS*$^+$ gene.

or with nonlysogenic MM294 (data not shown). The four Cam^r transductants obtained from MM294(933W) were shown to contain Tn*9* in the *dam* gene and characterized further (Table 1). Two of the *dam*::Tn*9* transductants (GM7259 and GM7261) did not produce bacteriophage plaques either spontaneously or after mitomycin C induction, did not contain the 933W prophage, as determined by PCR, and were sensitive to 2-aminopurine (2-AP), indicating an active mismatch repair system. Ordinarily, sensitivity to 2-AP is indicative of *dam* mutants, and these strains can become resistant when DNA mismatch repair is abrogated by mutation in the *mutH*, *mutL*, or *mutS* genes (6). Thus, these two transductants appear to be *dam* mutants that have lost the 933W prophage. The third transductant (GM7258) was 2-AP sensitive and contained the 933W prophage, but this phage produced pinpoint plaques, suggesting that it had acquired a mutation in either the host or the prophage, compromising phage development. The fourth transductant (GM7260) produced wild-type levels of spontaneously induced phage but was resistant to 2-AP, suggesting that mismatch repair was compromised in this strain. Strain GM7260 has a strong mutator phenotype which is complemented by a plasmid-borne *mutS*$^+$ gene. Inactivation of mismatch repair in *dam* mutants abolishes the number of detectable double-strand breaks (19), the processing of which signals SOS induction. Thus, none of the four Cam^r transductants isolated proved to have both a fully functional mismatch repair system and a wild-type 933W prophage, suggesting that *dam* is essential for maintenance of 933W lysogeny.

The data obtained with both EDL933 and *E. coli* K-12 (933W) are consistent with the hypothesis that the level of SOS induction in *dam* mutants generated by mismatch repair-mediated DNA breaks is sufficient to induce the 933W prophage, leading to phage-mediated cell lysis. In contrast, in *E. coli* lambda$^+$ lysogens, only a small fraction of the *dam* population shows full SOS induction (14) and undergoes prophage induction and cell lysis (13), consistent with our finding that the transduction frequencies were the same in the lambda lysogen and the nonlysogen. Thus, our observations suggest that 933W induces more easily than lambda at a given level of SOS induction. Indeed, Livny and Friedman (8) showed that at a given level of inducing signal, a greater fraction of lysogens with Stx-encoding prophages are induced than lysogens with non-Stx-encoding prophages. A possible explanation for the "hair-trigger" induction of prophage 933W is the observation that, unlike other lambdoid phages, which have three operators on the left side (O_L), prophage 933W has only two such operators (26). The lack of a third O_L in 933W precludes a lambda-like model in which interactions between *c*I repressors at O_R and O_L are critical for repression (4).

Dam has also been reported to influence the lysogeny of other prophages. Increased excision of the defective prophage ST64B from a *dam* mutant of *Salmonella enterica* is also due to enhanced SOS regulon expression (1). In this case, however, there was also a direct effect on the transcription of genes putatively involved in phage induction due to the presence of *dam* sites in the regulatory regions of these genes. Increased prophage excision in *dam* mutants may not be a general phenomenon, however, as Alonso et al. (1) found that of four prophages in *S. enterica* tested, only ST64B was affected.

The virulence of *S. enterica* and some other pathogenic bacteria is greatly reduced by a *dam* mutation and has led to the proposal that *dam* strains can be used as vaccines (3, 10). An alternative approach would be to inactivate the Dam methyltransferase in vivo by the use of a small molecule as a therapeutic. A similar strategy for EHEC would seem unwise, since inhibition of Dam in intestinal EHEC would likely lead to both increased induction of prophage 933W and Shiga toxin production.

Finally, our conclusion that loss of *dam* leads to inviability of EDL933 through prophage induction is a caution for studies where the ability to delete a particular gene is often used to determine if the gene is essential or not to the viability of the organism. In this case, Dam does not perform an essential function but the cells die due to an indirect cause.

Microarray data accession numbers. Complete microarray data for these strains are available at http://users.umassmed.edu/martin.marinus/arrays/index.html.

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REFERENCES

1. **Alonso, A., M. G. Pucciarelli, N. Figueroa-Bossi, and F. Garcia-del Portillo.** 2005. Increased excision of the *Salmonella* prophage ST64B caused by a deficiency in Dam methylase. J. Bacteriol. **187:**7901–7911.
2. **Campellone, K. G., A. J. Roe, A. Lobner-Olesen, K. C. Murphy, L. Magoun, M. J. Brady, A. Donohue-Rolfe, S. Tzipori, D. L. Gally, J. M. Leong, and M. G. Marinus.** 2007. Increased adherence and actin pedestal formation by *dam*-deficient enterohemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. **63:**1468–1481.
3. **Casadesús, J., and D. Low.** 2006. Epigenetic gene regulation in the bacterial world. Microbiol. Mol. Biol. Rev. **70:**830–856.
4. **Dodd, I. B., A. J. Perkins, D. Tsemitsidis, and J. B. Egan.** 2001. Octamerization of lambda CI repressor is needed for effective repression of P(RM) and efficient switching from lysogeny. Genes Dev. **15:**3013–3022.
5. **Donnenberg, M. S., and T. S. Whittam.** 2001. Pathogenesis and evolution of

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virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. J. Clin. Investig. **107:**539–548.
6. **Glickman, B. W., and M. Radman.** 1980. *Escherichia coli* mutator mutants deficient in methylation-instructed DNA mismatch correction. Proc. Natl. Acad. Sci. USA **77:**1063–1067.
7. **Kaper, J. B., J. P. Nataro, and H. L. Mobley.** 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. **2:**123–140.
8. **Livny, J., and D. I. Friedman.** 2004. Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system. Mol. Microbiol. **51:**1691–1704.
9. **Lobner-Olesen, A., O. Skovgaard, and M. G. Marinus.** 2005. Dam methylation: coordinating cellular processes. Curr. Opin. Microbiol. **8:**154–160.
10. **Low, D. A., N. J. Weyand, and M. J. Mahan.** 2001. Roles of DNA adenine methylation in regulating bacterial gene expression and virulence. Infect. Immun. **69:**7197–7204.
11. **Marinus, M. G.** 2000. Recombination is essential for viability of an *Escherichia coli dam* (DNA adenine methyltransferase) mutant. J. Bacteriol. **182:** 463–468.
12. **Marinus, M. G., and N. R. Morris.** 1974. Biological function for 6-methyladenine residues in the DNA of *Escherichia coli* K12. J. Mol. Biol. **85:**309–322.
13. **Marinus, M. G., and N. R. Morris.** 1975. Pleiotropic effects of a DNA adenine methylation mutation (*dam-3*) in *Escherichia coli* K12. Mutat. Res. **28:**15–26.
14. **McCool, J. D., E. Long, J. F. Petrosino, H. A. Sandler, S. M. Rosenberg, and S. J. Sandler.** 2004. Measurement of SOS expression in individual *Escherichia coli* K-12 cells using fluorescence microscopy. Mol. Microbiol. **53:** 1343–1357.
15. **McGraw, B. R., and M. G. Marinus.** 1980. Isolation and characterization of Dam^+ revertants and suppressor mutations that modify secondary phenotypes of *dam-3* strains of *Escherichia coli* K-12. Mol. Gen. Genet. **178:**309–315.
16. **Meselson, M., and R. Yuan.** 1968. DNA restriction enzyme from *E. coli*. Nature **217:**1110–1114.
17. **Murphy, K. C., K. G. Campellone, and A. R. Poteete.** 2000. PCR-mediated gene replacement in *Escherichia coli*. Gene **246:**321–330.
18. **Neely, M. N., and D. I. Friedman.** 2003. Analyzing transcription antitermination in lambdoid phages encoding toxin genes. Methods Enzymol. **371:** 418–438.
19. **Nowosielska, A., and M. G. Marinus.** 2005. Cisplatin induces DNA double-strand break formation in *Escherichia coli dam* mutants. DNA Repair **4:**773–781.
20. **O'Brien, A. D., L. R. Marques, C. F. Kerry, J. W. Newland, and R. K. Holmes.** 1989. Shiga-like toxin converting phage of enterohemorrhagic Escherichia coli strain 933. Microb. Pathog. **6:**381–390.
21. **Perna, N. T., G. Plunkett III, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grotbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamousis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner.** 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. Nature **409:**529–533.
22. **Peterson, K. R., K. F. Wertman, D. W. Mount, and M. G. Marinus.** 1985. Viability of *Escherichia coli* K-12 DNA adenine methylase (*dam*) mutants requires increased expression of specific genes in the SOS regulon. Mol. Gen. Genet. **201:**14–19.
23. **Ritchie, J. M., G. R. Campbell, J. Shepherd, Y. Beaton, D. Jones, K. Killham, and R. R. Artz.** 2003. A stable bioluminescent construct of *Escherichia coli* O157:H7 for hazard assessments of long-term survival in the environment. Appl. Environ. Microbiol. **69:**3359–3367.
24. **Ritchie, J. M., C. M. Thorpe, A. B. Rogers, and M. K. Waldor.** 2003. Critical roles for *stx*$_2$, *eae*, and *tir* in enterohemorrhagic *Escherichia coli*-induced diarrhea and intestinal inflammation in infant rabbits. Infect. Immun. **71:** 7129–7139.
25. **Spears, K. J., A. J. Roe, and D. L. Gally.** 2006. A comparison of enteropathogenic and enterohaemorrhagic *Escherichia coli* pathogenesis. FEMS Microbiol. Lett. **255:**187–202.
26. **Tyler, J. S., M. J. Mills, and D. I. Friedman.** 2004. The operator and early promoter region of the Shiga toxin type 2-encoding bacteriophage 933W and control of toxin expression. J. Bacteriol. **186:**7670–7679.
27. **Wagner, P. L., M. N. Neely, X. Zhang, D. W. Acheson, M. K. Waldor, and D. I. Friedman.** 2001. Role for a phage promoter in Shiga toxin 2 expression from a pathogenic *Escherichia coli* strain. J. Bacteriol. **183:**2081–2085.
28. **Wion, D., and J. Casadesus.** 2006. N6-methyl-adenine: an epigenetic signal for DNA-protein interactions. Nat. Rev. Microbiol. **4:**183–192.

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