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Effects of DNA Methylation on Expression of Virulence Genes in *Streptococcus mutans*▽†

Jeffrey A. Banas,[1]* Saswati Biswas,[2] and Min Zhu[1]

Dows Institute for Research, University of Iowa College of Dentistry, Iowa City, Iowa 52242,[1] and Department of Microbiology, Molecular Genetics and Immunology, University of Kansas Medical Center, Kansas City, Kansas 66160[2]

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Bacteria produce a variety of enzymes capable of methylating DNA. In many species, the majority of adenine methylation is accomplished by the DNA adenine methylase Dam. In *Escherichia coli* the Dam methylase plays roles in the initiation of replication, mismatch repair, and gene regulation. In a number of other bacterial species, mutation or overexpression of Dam leads to attenuation of virulence. Homologues of the *dam* gene exist in some members of the *Firmicutes*, including *Streptococcus mutans*, a dental pathogen. An *S. mutans* strain inactivated in the *dam* gene (SMU.504; here designated *damA*) was engineered, and phenotypes linked to cariogenicity were examined. A prominent observation was that the *damA* mutant produced greater amounts of glucan than the parental strain. Real-time PCR confirmed upregulation of *gtfB*. To determine whether other loci were affected by the *damA* mutation, a microarray analysis was carried out. Seventy genes were upregulated at least 2-fold in the *damA* mutant, and 33 genes were downregulated at least 2-fold. In addition to *gtfB* (upregulated 2.6-fold; 1.7-fold when measured by real-time PCR), other upregulated virulence factors included *gbpC* (upregulated 2.1-fold) and loci predicted to encode bacteriocins (upregulated 2- to 7-fold). Various sugar transport operons were also upregulated, the most extreme being the cellobiose operon (upregulated nearly 40-fold). Expression of *sacB*, encoding fructosyltransferase, was downregulated 2.4-fold. The sequence 5′-GATC-3′ appeared to constitute the recognition sequence for methylation. These results provide evidence that DNA methylation in *S. mutans* has a global effect on gene expression, including that of genes associated with cariogenic potential.

DNA methylation plays a role in providing epigenetic information that affects gene expression and other cellular events. The annotated *Streptococcus mutans* genome (1) contains several loci encoding potential DNA methylases. SMU.504 has been annotated as *dam*, encoding an α-group adenine methyltransferase. Homologues of Dam are found in numerous species, particularly among Gram-negative enterics (15). In *Escherichia coli*, Dam is responsible for regulating an assortment of cellular activities that include mismatch repair, initiation of chromosomal replication, DNA segregation, transposition, and regulation of gene expression (16, 18). In some species, *dam* is an essential gene (18). In other species, mutation or overexpression of Dam has been linked to alterations in virulence (12, 16, 18). The potential mechanisms behind these effects have been postulated to be indirect, secondary to effects on basic cellular fitness, or direct, affecting transcriptional regulation (12).

In *S. mutans*, SMU.504 appears to be part of a type II restriction-modification system that includes three structural genes (SMU.504 to SMU.506) similar to those found in other streptococcal or related species (Fig. 1). We propose the designations *damA*, *damB*, and *damC* for SMU.504, SMU.505, and SMU.506, respectively. The DpnM-DpnA-DpnII (*Streptococcus pneumoniae*), *Ssu*DAT1IA-*Ssu*DAT1IB-*Ssu*DAT1IC (*Streptococcus suis*), and the *Lla*DCHIA-*Lla*DCHIB-*Lla*DCHIC (*Lactococcus lactis*) systems include a Dam-like methylase that recognizes the nucleotide sequence 5′-GATC-3′, which is followed by a second methyltransferase that methylates single-stranded DNA, and finally a restriction endonuclease (5, 19, 23). The *Streptococcus sanguinis Ssan*MA-*Ssan*MB-*Ssan*RB system is predicted based on its annotated genome sequence (28). Much of the focus on restriction-modification systems in these species has centered on their roles in interacting with foreign DNA introduced via horizontal transfer, either in a protective capacity (e.g., defense against bacteriophage) (19) or in facilitating conjugation, as the methylase for single-strand DNA is postulated to do (23). In this report, we describe a role for *damA* in gene regulation, including genes that affect the cariogenic properties of *S. mutans*.

Understanding the regulation of gene expression in bacteria, particularly the expression of genes associated with virulence properties, has been a high priority. Knowledge of the conditions and pathways that influence gene expression can reveal potential targets that would allow manipulation of expression levels that could reduce or eliminate the ability of an organism to cause disease. There are multiple layers of control for many of the *S. mutans* genes linked to cariogenicity. Here we report the novel finding that DNA methylation also plays a role.

MATERIALS AND METHODS

Strains and growth conditions. *Streptococcus mutans* UA159 was used as the parental strain for all genetically engineered *S. mutans* strains. *Escherichia coli* JM109 was used for intermediate cloning steps in mutant generation. *Escherichia*

* Corresponding author. Mailing address: Dows Institute for Research, University of Iowa College of Dentistry, N436 Dental Science Building, Iowa City, IA 52242. Phone: (319) 335-9911. Fax: (319) 335-8895. E-mail: Jeffrey-banas@uiowa.edu.

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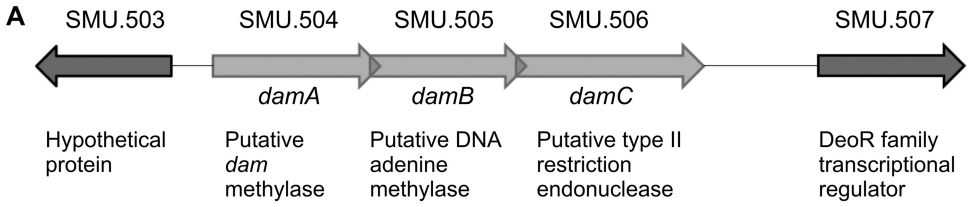


B

Comparable Proteins In Other Species	SMU.504		SMU.505		SMU.506	
	Percent Identity	Percent Similarity	Percent Identity	Percent Similarity	Percent Identity	Percent Similarity
S. sanguis	73	81	82	86	35	47
L. lactis	69	77	84	88	35	46
S. pneumoniae	66	74	86	89	27	36
S. suis	67	74	86	90	36	47

FIG. 1. (A) The genetic map of the putative *dam* methylase operon in *Streptococcus mutans* UA159. (B) Percent identities and similarities for the derived amino acid sequences of the three products encoded by the putative *dam* methylase operon with similar operons in closely related species, based on CLUSTAL-W sequence alignments; the Dam methylase (SMU.504), a single-strand DNA-specific adenine methylase (SMU.505), and a type II restriction endonuclease (SMU.506).

coli INV110 was used to propagate a cloned copy of *damA*. *E. coli* strains were cultured in Luria broth (Fisher Scientific, Pittsburgh, PA) at 37°C with shaking. *S. mutans* strains were cultured on brain heart infusion (BHI) plates, with or without 1% sucrose, BHI broth (Becton, Dickinson and Co., Sparks, MD), or Todd-Hewitt broth (Neogen Corp., Lansing, MI) and were grown at 37°C in a 5% CO_2 incubator. When tested for bacteriocin activity, *S. mutans* strains were grown on THY (Todd-Hewitt–yeast extract) plates and incubated in a microaerophilic atmosphere at 37°C.

Strains mutated in *damA*, *damB*, or *damC* were engineered by insertional inactivation. Approximately 500 bp, just downstream of the translational start sites of *damA*, *damB*, or *damC*, was amplified by PCR, cloned into the suicide vector pVA8912 (17), and propagated in *E. coli*. Each construct was recovered from *E. coli* and transformed into *S. mutans*, wherein a single homologous recombination event generated two incomplete copies of *damA*, *damB*, or *damC*. Erythromycin-resistant clones were screened by PCR, and quantitative real-time PCR was used to confirm the lack of *damA*, *damB*, or *damC* transcription. The *damA* mutant was complemented by amplifying *damA* by PCR and cloning it into the shuttle vector pDL276 (9). Table 1 lists the oligonucleotide primers used in strain construction.

Real-time PCR. Total RNA from each bacterial strain was isolated using the RNeasy minikit (Qiagen, Inc., Valencia, CA). Relative expression levels of *damA*, *damB*, or *damC* and *gtfBC* were measured by quantitative real-time PCR, using equivalent amounts of RNA. Results were normalized to *gyrA* transcription. Gene expression for the parental strain was set at 1. Reactions were set up using the iScript one-step reverse transcription-PCR kit with SYBR green (Bio-Rad, Hercules, CA) and tested in an iQ5 real-time PCR detection system. Each sample was tested in duplicate during a trial, and three independent trials were performed.

Microarrays. Total RNA from the parental strain and *damA* and *damC* mutants was isolated using the RNeasy minikit (Qiagen, Inc., Valencia, CA) and further purified using RNAeasy spin columns (Qiagen, Inc., Valencia, CA). The RNA samples were sent to the Roy J. Carver Center for Genomics, Department

TABLE 1. Primers used in this study

Strain	Primer[a]
damA mutant	F: 5′-cg(ggatcc)CAGATAAAATTAGCAAATACTACT-3′ (BamHI)
	R: 5′-cg(gaattc)TTGATAGATTAATTCACGATCAAT-3′ (EcoRI)
damB mutant	F: 5′-cg(ggatcc)ATGATGAATAACGAATATAAGTAT-3′ (BamHI)
	R: 5′-cg(gaattc)ATCATTTTTACGTGCCCACAGAAT-3′ (EcoRI)
damC mutant	F: 5′-cg(ggatcc)ATGGTCAATACTGTTGATGACAAC-3′ (BamHI)
	R: 5′-cg(gaattc)CACTCCAACAACATAATCATGAAC-3′ (EcoRI)
damA complemented strain	F: 5′-cg(ggatcc)TGAAATGATTATGTAACATAAAGG-3′ (BamHI)
	R: 5′-cg(gaattc)TTATTCATCATAATTAGTTACTAT-3′ (EcoRI)

[a] F, forward; R, reverse. Lowercase type indicates sequence that was not complementary to the DNA to which the primer was directed but which included a restriction enzyme recognition site to facilitate cloning. The recognition site and enzyme name for each primer are in parentheses.

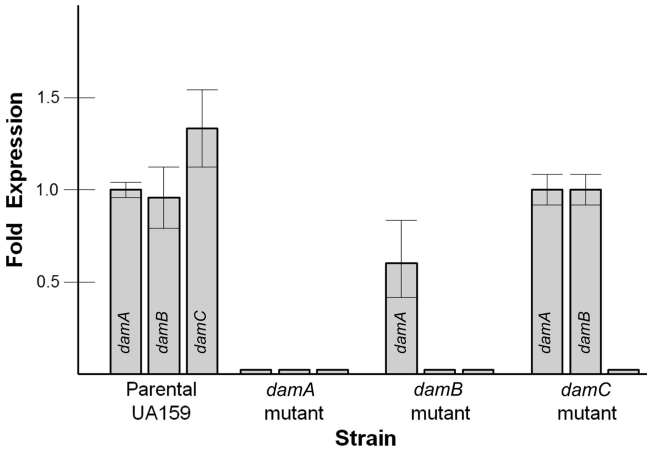


FIG. 2. Quantitative real-time PCR showing expression of the putative *dam* methylase operon genes in the parental strain (wild type) and *damA*, *damB*, and *damC* mutants. The results represent the means of results from three independent experiments.

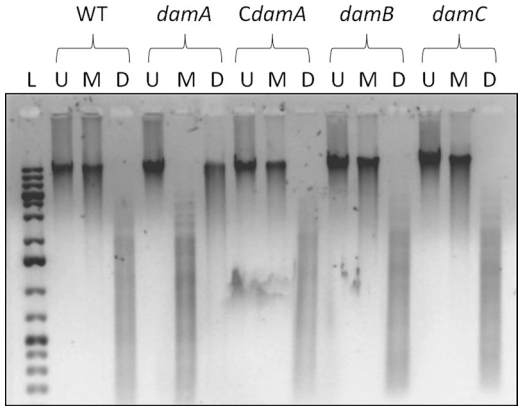


FIG. 3. Determination of methylation status by restriction digestion. Chromosomal DNA obtained from the parental UA159 strain (wild type [WT]), *damA*, *damB*, and *damC* mutants, or the complemented *damA* strain (C*damA*) was restricted with either MboI (M) or DpnI (D) or left uncut (U). L, GeneRuler DNA marker (Fermentas).

of Biology, at the University of Iowa, where the RNA was confirmed to be of high quality and then used for cDNA synthesis, labeling, and hybridization. The Roche NimbleGen catalogue array (T1210007) for *S. mutans* UA159 was used in a 12-subarray format with 135,000 60-mer probes per array. Therefore, there were four technical replicates per sample/strain. Results were normalized and analyzed using robust multichip analysis (RMA) and significance analysis of microarrays (SAM). A threshold of 2-fold change in expression relative to the parental strain was chosen for tabulating the data in this report.

Phenotypic assays. (i) DNA purification for analyzing methylase activity. Chromosomal DNA isolated from the *damA*, *damB*, or *damC* mutant strain as well as the complemented *damA* strain was digested with DpnI or MboI. Both DpnI and MboI recognize the sequence 5′-GATC-3′, but DpnI will cut the DNA only if the adenine within the recognition sequence is methylated, while MboI will digest only unmethylated DNA.

(ii) Glucosyltransferase activity gel. Overnight cultures, grown in Todd-Hewitt broth, were adjusted to an optical density at 600 nm (OD_{600}) of 0.8. Fifteen-milliliter cultures were centrifuged, and bacterial pellets resuspended in 50 μl H_2O and 50 μl 4× loading buffer (4× concentrations of 0.075 M Tris, 2% SDS, 5% beta-mercaptothanol, 30% glycerol, and bromphenol blue), incubated at room temperature for 2 h, and centrifuged. Twenty-five microliters of the supernatant was subjected to electrophoresis using a 12% SDS-PAGE precast gel (Invitrogen, Carlsbad, CA). After electrophoresis, the gel was washed in 0.05 M Tris (pH 7.5) for 1.5 h to remove SDS, then placed in 0.05 M phosphate buffer (pH 6.5) with 1% sucrose and 1% Triton X-100, and incubated overnight at 37°C.

(iii) Dextran-dependent aggregation. Overnight cultures, grown in BHI, were pelleted by centrifugation, washed, and resuspended in phosphate-buffered saline (PBS; pH 7.4) to an OD_{600} of 1.0. Dextran T2000 (Sigma, St. Louis, MO) was added to a final concentration of 100 μg/ml. The suspensions were then incubated at 37°C for 1 h, with optical density measurements taken at 0, 5, 15, 30, and 60 min.

(iv) Growth in different sugars. The growth levels of the *S. mutans* parental strain and *damA* and *damC* mutant strains were compared in a tryptone-vitamin base (TV) medium (3.5% tryptone with 0.04 μg of *p*-aminobenzoic acid/ml, 0.2 μg of thiamine-HCl/ml, 1 μg of nicotinamide/ml, and 0.2 μg of riboflavin/ml) supplemented with 0.5% of a selected carbohydrate (fructose, glucose, raffinose, sorbitol, mannose, or cellobiose) as described by Burne et al. (4). Growth of the parental strain in TV medium supplemented with glucose was used as the positive control. Optical densities were measured after overnight growth in the various carbohydrates.

(v) Mutacin activity. *S. mutans* parental and mutant strains were stabbed on a THY plate and incubated overnight at 37°C in a microaerophilic atmosphere. The indicator strains *Streptococcus gordonii* (ATCC 10558) or *Lactococcus lactis* (MG1363) were also grown overnight in THY broth. The plate was then overlaid with either indicator strain mixed with soft agar and incubated overnight under the same conditions. The diameters of clear zones in the lawn of the indicator strain, indicative of bacteriocin activity (11, 22), were measured.

RESULTS

Gene organization and sequence homology. The *damA*, *damB*, and *damC* genes appear to form an operon putatively consisting of a *dam*-like methylase, a second methylase, and a type II restriction endonuclease. Extensive amino acid sequence identity is evident with similar operons in related bacterial species (Fig. 1B; see also Fig. S1 in the supplemental material), though the methylase sequences are more similar to one another than are the restriction endonucleases. Insertional inactivation of *damA* or *damB* had a polar effect as measured by real-time PCR (Fig. 2).

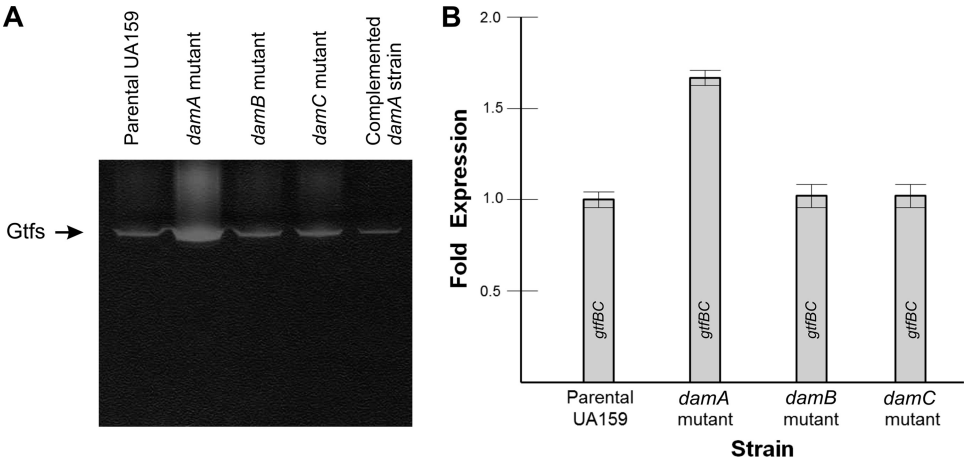


FIG. 4. (A) An activity gel for glucosyltransferase activity. Glucosyltransferases associated with *S. mutans* were removed by incubation with SDS and then separated by SDS-PAGE. After washing away the SDS, the gels were incubated in the presence of sucrose, which resulted in the *in situ* synthesis of glucan, appearing as a white precipitate within the gel. (B) Transcription of the *gtfB* and *gtfC* genes was measured in the parental and mutant strains via quantitative real-time PCR. The probes did not discriminate between *gtfB* and *gtfC*, so the values represent the cumulative expression of these two contiguous genes. The expression in the *damA* mutant strain was significantly higher than that in each of the other strains by a Student *t* test ($P < 0.001$).

TABLE 2. Microarray results of significantly upregulated or downregulated genes

Locus	Gene description[b]	Approx fold change	
		damA mutant vs parental strain	*damC* mutant vs parental strain[a]
SMU.27	Acyl carrier protein	2.3	NC
SMU.137	Malate dehydrogenase	0.35	NC
SMU.138	Putative malate permease	0.27	NC
SMU.139	Hypothetical; *B. subtilis yvrK*	0.25	NC
SMU.140	Glutathione reductase	0.23	NC
SMU.141	Hypothetical	0.29	NC
SMU.150	Hypothetical; bovicin peptide precursor	5.5	2.0
SMU.151	Hypothetical; lactacin F precursor	3.6	NC
SMU.152	Hypothetical	6.2	NC
SMU.179	Hypothetical	2.2	NC
SMU.180	Putative oxidoreductase; fumarate reductase	3.8	NC
SMU.279	Hypothetical	0.46	NC
SMU.309	Regulator of sorbitol operon	2.6	NC
SMU.310	Sorbitol operon activator	2.5	NC
SMU.311	Sorbitol PTS enzyme	2.3	NC
SMU.312	Sorbitol PTS enzyme	2.3	NC
SMU.313	Sorbitol PTS enzyme IIA	2.6	NC
SMU.314	Hypothetical	2.5	NC
SMU.423	Hypothetical bovicin peptide precursor	5.1	NC
SMU.438c	Hydroxyglutaryl-CoA dehydratase	3.6	NC
SMU.505	Adenine methylase	0.1	NC
SMU.506	Type II restriction enzyme	0.1	0.1
SMU.574c	Hypothetical; *B. subtilis ysbB*	4.5	NC
SMU.575c	Hypothetical; *B. subtilis ysbA*	7.0	NC
SMU.576	Putative response regulator LytR	2.2	NC
SMU.577	Putative histidine kinase LytS	2.1	NC
SMU.595	Dihydroorotate dehydrogenase	2.1	NC
SMU.700c	Putative phosphoglycerate mutase-like	2.5	NC
SMU.701c	Hypothetical; integral membrane protein	2.3	NC
SMU.702c	Putative transcriptional regulator	2.3	NC
SMU.703c	Hypothetical	2.2	NC
SMU.873	Homocysteine *S*-methyltransferase	3.0	NC
SMU.874	Homocysteine *S*-methyltransferase	2.9	NC
SMU.877	Alpha-galactosidase	2.5	NC
SMU.878	Sugar-binding; ABC transport; MsmE	2.5	NC
SMU.879	Permease; ABC transport; MsmF	2.6	NC
SMU.880	Permease; ABC transport; MsmG	2.5	NC
SMU.881	*gtfA*	2.7	NC
SMU.882	ATP-binding; ABC transport; MsmK	2.2	NC
SMU.883	*dexB*	2.5	NC
SMU.925	Hypothetical; *S. pneumoniae* BlpL-like	2.1	NC
SMU.932	Putative methyltransferase isozyme A	0.46	NC
SMU.976	Spermidine/putrescine-binding; ABC transp.	2.3	NC
SMU.998	Ferrichrome-binding; ABC transporter	2.7	NC
SMU.1001	Putative DNA processing *smf* protein	0.45	0.50
SMU.1004	*gtfB*	2.6	NC
SMU.1007	Permease; ABC transporter	2.1	NC
SMU.1032	Transposase	2.1	NC
SMU.1035	ATP-binding; ABC transporter	2.0	NC
SMU.1327c	*B. subtilis yhbA*-like	2.0	NC
SMU.1334	Putative phosphopantetheinyl transferase	0.28	NC
SMU.1335c	Putative enoyl reductase	0.37	NC
SMU.1336	Hypothetical; *B. subtilis pksD*	0.30	NC
SMU.1337c	Alpha-beta superfamily hydrolase	0.21	NC
SMU.1338c	Multidrug ABC transporter permease	0.25	NC
SMU.1339	Putative bacitracin synthetase	0.26	NC
SMU.1340	Putative surfactin synthetase	0.25	NC
SMU.1341c	Putative gramicidin S synthetase	0.21	NC
SMU.1342	Putative bacitracin synthetase BacA	0.20	NC
SMU.1343c	Putative polyketide synthetase	0.22	NC
SMU.1344c	Putative malonyl-CoA transacylase	0.21	NC
SMU.1345c	Putative peptide synthetase MycA	0.20	NC
SMU.1346	Putative thioesterase BacT	0.29	NC
SMU.1347c	Hypothetical; permease	0.33	NC
SMU.1348c	ATP-binding protein; ABC transporter	0.41	NC
SMU.1396	*gbpC*	2.1	NC
SMU.1410	Putative reductase	2.2	NC
SMU.1421	Acetyl-CoA synthesis	2.0	NC
SMU.1423	Putative pyruvate dehydrogenase	2.0	NC
SMU.1424	Putative dihydrolipoamide dehydrogenase	2.0	NC
SMU.1425	ClpB; ATP-binding subunit	2.2	NC
SMU.1495	Galactose-6-P isomerase LacB	0.49	NC
SMU.1496	Galactose-6-P isomerase LacA	0.47	NC
SMU.1596	Cellobiose PTS IIC	10.0	NC
SMU.1597c	Hypothetical	31.2	2.1
SMU.1598	Cellobiose PTS IIA	39.5	2.1
SMU.1599	Putative antiterminator; transcript. reg.	33.6	2.2
SMU.1600	Cellobiose PTS IIB	23.1	NC
SMU.1601	6-Phospho-beta-glucosidase	5.9	NC
SMU.1674	Aminotransferase	2.0	NC
SMU.1882c	Hypothetical	0.45	NC
SMU.1903c	Hypothetical	7.8	2.7
SMU.1904c	Hypothetical	5.4	2.6
SMU.1905c	Putative bacteriocin secretion protein	3.8	2.0
SMU.1906c	Hypothetical; BlpU	4.4	2.3
SMU.1907	Hypothetical	7.4	2.8
SMU.1908c	Hypothetical	4.6	2.9
SMU.1909c	Hypothetical	4.5	2.3
SMU.1910c	Hypothetical; calcium channel alpha subunit	4.5	2.1
SMU.1912c	Hypothetical	5.1	2.3
SMU.1913c	Hypothetical; BlpL	3.3	NC
SMU.1914c	Hypothetical; BlpO	2.8	NC
SMU.1933c	Cobalt permease	2.2	NC
SMU.1956c	Hypothetical	3.5	NC
SMU.1957	Mannose PTS IID	3.1	NC
SMU.1958c	Mannose PTS IIC	2.6	NC
SMU.1960c	Mannose PTS IIB	3.1	NC
SMU.1961c	Fructose PTS IIA	3.0	NC
SMU.1980c	Hypothetical	0.45	0.50
SMU.1981c	Hypothetical; *S. gordonii* ComYD	0.46	NC
SMU.1982c	Hypothetical	0.46	NC
SMU.1987	ATP-binding; ABC transporter; ComYA	0.49	NC
SMU.2028	*sacB*; beta-D-fructosyltransferase	0.41	NC

[a] NC, signifies no change in expression or a change that was less than 2-fold.
[b] CoA, coenzyme A; transcript. reg., transcriptional regulator.

Functional homology. Chromosomal DNA isolated from the *damA*, *damB*, and *damC* mutant strains, as well as the complemented *damA* strain, was digested with DpnI or MboI. Both DpnI and MboI recognize the sequence 5′-GATC-3′, but DpnI will cut the DNA only if the adenine within the recognition sequence is methylated, while MboI will digest only unmethylated DNA. As expected, MboI digested DNA from the *damA* mutant strain, whereas DpnI did not (Fig. 3). DNA isolated from the *damB* mutant, *damC* mutant, and complemented *damA* strain was digested by DpnI but undigested by MboI, indicating that these strains had a functional methylase.

Gene expression. When propagating the *damA* mutant, it was noted that its growth phenotype on agar containing sucrose differed from that of the parental strain. Colonies of the

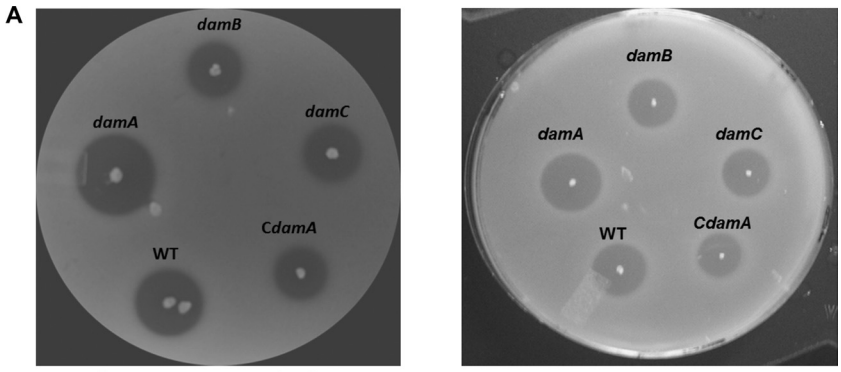
TABLE 3. Decrease in optical density associated with dextran-dependent aggregation[a]

Strain	OD_{600} at indicated min after addition of high-mol-wt dextran				
	0**	5	10	30	60
Parental UA159	1.0	0.996	0.985	0.977	0.913
damA mutant	1.0	0.784	0.714	0.682	0.620
damC mutant	1.0	0.994	0.976	0.960	0.911
damA complemented strain	1.0	0.996	0.985	0.969	0.910

[a] The OD values presented are the averages for three independent trials. In each instance the standard deviation was ≤0.01. The OD values for the *damA* mutant at 5, 10, 30, and 60 min were significantly different from those of each of the other strains when tested by analysis of variance (ANOVA) ($P \leq 0.01$). **, initial OD values at 0 min were 1.000 ± 0.030.

damA mutant appeared to have a larger slime layer and pitted the agar to an even greater extent than the parental strain. Cell-associated proteins were isolated from the parental and *damA* strains, separated by gel electrophoresis, renatured by washing, and incubated in sucrose for detection of glucosyltransferase activity. The *damA* mutant showed greater activity than the parental strain and *damB* and *damC* mutant strains (Fig. 4A). Complementation of the *damA* mutant with a plasmid-based copy of *damA* decreased glucosyltransferase activity to the parental level. Expression of the tandem *gtfB* and *gtfC* genes was measured by real-time PCR and found to be approximately 1.7-fold higher in the *damA* mutant than in the parental strain (Fig. 4B).

In order to measure the effect of *damA* on genome-wide gene expression, microarray analyses were carried out using RNA from parental and mutant organisms growing in mid-log phase. Table 2 lists numerous loci that were either up- or downregulated at least 2-fold. Since the mutation of *damA* was polar, a mutant in the downstream *damC* was also compared to the parental strain. Mutating *damC* affected only a small subset of the genes affected by mutation in *damA*, and in those instances in which similar loci were affected, the magnitude of the change was much smaller. Therefore, the changes associated with the inactivation of *damA* are likely attributable to loss of the methylase rather than a polar effect. Of particular note was that the *damA* mutation resulted in a 2-fold increase in the expression of cell surface glucan receptor GbpC, a 2-fold increase in the ATP-binding subunit for the Clp protease, 2- to 7-fold increases at four loci putatively encoding bacteriocins, and a 20-to-40-fold increase in expression of the cellobiose phosphotransferase (PTS) system, as well as increases in other sugar transport systems. The *sacB* gene encoding fructosyltransferase was downregulated over 2-fold. Phenotypic assays were then undertaken to verify selected results. Since GbpC is linked to dextran-dependent aggregation (DDAG), the DDAG properties of the *damA* mutant were compared to those of the parental strain, the *damC* mutant, and a complemented *damA* strain. The *damA* mutant was the only strain that had visible clumping of the bacteria after addition of high-molecular-weight dextran, and this was supported by reductions in optical density (Table 3). The *damA* strain was also the only strain to display enhanced bacteriocin activity against *Streptococcus gordonii* and *Lactococcus lactis* (Fig. 5). Growing the parental and mutant strains in various sugars did not reveal differences in growth patterns, with the exception of the observation that the parental UA159 strain did not grow in TV



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Indicator strain	Diameter of Zone of Clearing (mm)* *S. mutans* strains				
	Wild type	*damA*	*damB*	*damC*	*CdamA*
***S. gordonii* (ATCC 10558)**	11.6±0.6	15.3±1.0	9.0±0.0	10.3±1.0	9.6±1.0
***L. lactis* (MG1363)**	12.3±0.6	14.7±0.6	11.3±0.6	12.0±0.0	10.6±0.6

*Clearing-zone diameter is expressed as the mean ± standard deviation obtained from two independent experiments performed in triplicate.

FIG. 5. Assay for mutacin production. (A) Parental UA159 (WT) and *damA*, *damB*, and *damC* mutants, along with the complemented *damA* strain (C*damA*), were compared for bacteriocin activity against *S. gordonii* or *L. lactis*. Clear zones produced around the stabbed colonies indicate lysis of the indicator strain by mutacin. (B) Quantification of the zones of inhibition.

medium with fructose, whereas the *damA* strain grew to about half the optical density of growth in TV medium with glucose.

DISCUSSION

Homologues of the Dam methylase have an assortment of cellular functions in a variety of bacterial species (12, 16, 18). Often, but not always, they are part of a restriction-modification system. Among streptococci, homologues are found on the chromosomes of a subset of strains of *S. pneumoniae* (5), *S. suis* (23), *S. sanguinis* (28), and *S. mutans* (1). In the closely related species *Lactococcus lactis*, the presence of a *dam* homologue is associated with the presence of a particular plasmid (19). Each of the *dam* homologues has been found to methylate the adenine within the sequence 5′-GATC-3′. A similar recognition site was demonstrated for DamA, as chromosomal DNA from strains with an intact *damA* gene was digested with DpnI, which did not cut DNA isolated from a *damA* mutant strain. MboI, which cuts at unmethylated 5′-GATC-3′ sites, gave results opposite of those for DpnI, as expected.

A novel finding was that mutation of *damA* had a wide-ranging effect on gene expression, including genes important for the cariogenicity of *S. mutans*. A 2-fold change in expression was chosen as the threshold for organizing the microarray data, and each was calculated to be statistically significant based on the analyses of four technical replicates. It is acknowledged that changes in expression of lesser magnitude can nonetheless be of profound biological significance, and conversely, statistically significant changes that met the 2-fold threshold may not represent biological change of great consequence. In this study, the microarray data were used primarily as a screening tool to authenticate the potential impact of methylation by DamA. Changes in the expression of genes related to cariogenic properties were confirmed by real-time PCR and/or by phenotypic assays.

A majority of the changes resulted in upregulation of particular genes, implying that DNA methylation most often has a moderating effect on gene expression. A notable exception was a 2.4-fold reduction in the expression of *sacB*. Since the *sacB* gene product, fructosyltransferase, competes with the glucosyltransferases for the substrate sucrose and *gtfB* was upregulated, it appears that the absence of DamA shifts synthesis toward enhanced amounts of glucan at the expense of fructan. The upregulation of the glucan-binding protein GbpC in conjunction with the increase in glucan may be the basis for enhanced DDAG. Similar autoaggregating phenotypes have been associated with the promotion of adhesion and biofilm formation (8) or alternatively may be associated with stress whereby the organisms attempt to protect themselves from adverse environmental conditions (10, 29).

Enhanced bacteriocin (mutacin) activity produced by the *damA* knockout strain was detected using *Streptococcus gordonii* or *Lactococcus lactis* as indicator strains. The increased activity cannot be ascribed to a particular mutacin, as eight different genes at four distinct loci associated with mutacin production (20) were upregulated and one gene was downregulated. There are layers of regulation influencing the expression of various mutacins that respond to quorum sensing and environmental conditions. The identification of DNA methylation as another contributing factor is novel.

The repertoire of sugars fermented by *S. mutans* is linked to its ability to produce acid in sufficient quantities to promote demineralization of enamel. The loss of DamA was associated with upregulation of loci dedicated to carbohydrate transport. Ajdić and Pham (2) report constitutive high expression of PTSs for glucose, fructose, maltose, and sucrose and speculate that *S. mutans* is primed for quick utilization of these dietary sugars. Mutation of *damA* increased the expression of a mannose/fructose PTS that is fructose-inducible (2). Other sugar transport operons upregulated in the *damA* mutant were secondary carbohydrate sources such as mannitol/sorbitol, cellobiose, and the multiple-sugar metabolism (MSM) operon, each potentially employing multiple mechanisms of regulating expression.

Analysis of the operator/promoter regions upstream of the genes affected by mutation of *damA* did not reveal a consistent presence of 5′-GATC-3′ sites. Only about one-quarter of loci affected by a factor of two or more had a 5′-GATC-3′ site in the untranslated region upstream of the affected gene or operon. In the absence of a 5′-GATC-3′ site, the influence of methylation may be indirect. One possibility is that methylation affects DNA topology (16). Another possibility is that expression of one or more global regulators is affected. Some of the genes affected by the loss of the methylase, notably *gtfB*, *gbpC*, and genes for mutacin production, are regulated by the competence regulon and other two-component signal transduction systems (3, 7, 13, 14, 21, 24, 26, 27). However, microarray data from the *damA* mutant indicated a 2-fold increase in the expression of a single global regulator, the LytRS two-component signal transduction system which has previously been linked to autolytic activity and sucrose-independent adhesion (6, 25). Therefore, DamA appears to function independently of other global regulators that affect the repertoire of genes noted in this investigation. The possibility remains that expression of *damA* is coordinated with that of other regulatory factors, perhaps by recognizing a similar signal, such as cell density. Still, this can only be speculated upon since as yet there is no evidence that the expression of *damA* is regulated. Further investigation will be necessary to determine the mechanism(s) by which DamA-catalyzed methylation contributes to the regulation of gene expression.

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