INTRODUCTION

The association of Pasteurella multocida with bovine respiratory disease (BRD) has been well known since the early 1950s (Carter, 1954). In most survey studies of feedlot BRD, Mannheimia haemolytica has been the most commonly isolated species, followed closely by Pasteurella multocida, with fewer cases of Haemophilus somnus (Collier, 1969; Purdy et al., 1997; Scheifer et al., 1978). However, in some studies, P. multocida is the most common isolate (Allen et al., 1991; Singer et al., 1998), and many BRD investigators have become convinced that P. multocida is an important primary pathogen in BRD (Gourlay et al., 1989; Purdy et al., 1997). In young dairy calves, P. multocida is the most frequently isolated species from cases of pneumonia (Bryson et al., 1978; Virtala et al., 1996). P. multocida is also an important pathogen of poultry, swine, rabbits, and occasionally humans.

P. multocida is a commensal of the bovine upper respiratory tract (Allen et al., 1991). Induction of disease is often associated with stress, especially from transportation. Proposed virulence factors include polysaccharide capsule (Boyce & Adler, 2000), lipopolysaccharide (Brogden et al., 1986; Rimler & Rhoades, 1989), iron-regulated outer-membrane proteins (Confer et al., 2001; Geschwend et al., 1997), proteases (Negrete-Abascal et al., 1999; Straus et al., 1998), neuraminidase (Straus et al., 1998; White et al., 1995), and porins (Galdiero et al., 1998; White et al., 1995). Using signature-tagged mutagenesis, 25 genes were identified that, when inactivated, reduce virulence using a mouse intraperitoneal model (Fuller et al., 2000). These genes were classified into four general categories: regulatory, biosynthetic, known virulence factors, and unknown/novel. The whole genome sequence of an avian P. multocida isolate was published in 2001 (May et al., 2001), which allowed identification of 104 potential virulence-related genes by homology searches. However, the mechanisms that control
expression of these potential virulence factors have not been determined.

DNA adenine methylase (Dam) is an important virulence gene regulator in the Enterobacteriaceae. In Escherichia coli, Dam regulates transcription of several pili operons, including the pap (pyelonephritis-associated pili) (Blyn et al., 1990; Braaten et al., 1994), sfa (S pili), fae (K88 pili) and dda (F1845 pili) operons (van der Woude & Low, 1994), and it regulates expression of a major outer-membrane protein (Ag43) (Henderson & Owen, 1999). A Salmonella entereica serovar Typhimurium dam mutant strain had altered expression of more than 20 in vivo-induced (ivi) genes (elevated by 2- to 18-fold in a Dam-inactivated mutant compared to a wild-type Dam-positive strain) (Heithoff et al., 1999).

In several bacterial species that possess a dam gene, alteration of dam expression causes substantial attenuation, as well as enhanced protective immunogenicity (Heithoff et al., 1999). A S. enterica serovar Typhimurium dam mutant had an LD$_{50}$ that was $>10^4$ higher than the wild-type parent strain, and it was effective as a live attenuated vaccine after a single oral dose (Heithoff et al., 1999). A dam-overexpressing strain was also highly attenuated in mice (Heithoff et al., 1999). In Yersinia pseudotuberculosis and Vibrio cholerae, inactivation of the dam gene was shown to be a lethal mutation (Julio et al., 2001). However, plasmid-mediated overexpression of the dam gene in Y. pseudotuberculosis resulted in a >6000-fold increase in LD$_{50}$ in mice compared to wild-type and a fivefold defect in colonization of V. cholerae in a suckling mouse model compared to wild-type (Julio et al., 2001).

Although dam genes have been identified in the Pasteurellaceae as a result of genome sequencing projects (Fleischmann et al., 1995; May et al., 2001), there have been no reports of functional characterization of Dam in any of these species. In this study, we demonstrate the presence of Dam function in P. multocida, and show that alteration of Dam function in a serotype A1 fowl cholera strain causes increased spontaneous mutation rate and attenuation in a mouse model.

**METHODS**

**Bacterial strains and plasmids.** A list of the bacterial strains and plasmids used in this study is shown in Table 1. Pasteurella multocida ATCC 11039, which is a fowl cholera strain, was selected for this study because it has a well-characterized mouse model that can be used to assess virulence (Chung et al., 2001; Homchampa et al., 1992). Escherichia coli strains were grown at 37°C on Luria–Bertani (LB) agar or broth. P. multocida strains were grown at 37°C on brain-heart infusion (BHI) agar or broth. For plasmid maintenance, antibiotics were used at the following final concentrations: ampicillin, 200 μg ml$^{-1}$; kanamycin, 50 μg ml$^{-1}$; and streptomycin, 80 μg ml$^{-1}$. IPTG and X-Gal were used at final concentrations of 80 μM and 70 μg ml$^{-1}$, respectively, for blue/white screening on LB agar.

**Detection of Dam function in P. multocida.** To determine whether P. multocida contains a functional dam gene, we used differential digestion with restriction endonucleases SauI-AI, MboI and DpnI (New England BioLabs) (Palmer & Marinus, 1994). First, genomic DNA was isolated from a 100 ml culture of P. multocida 11039 by phenol/chloroform extraction followed by precipitation with 2-propanol (Ausubel et al., 1994). Genomic DNA (1 μg) was then digested for 1 h with 2 units of SauI-AI, which cleaves GATC sites regardless of methylation state, 10 units of DpnI, which only

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td>P. multocida ATCC 11039</td>
<td>Wild-type serotype A1 fowl cholera strain</td>
<td>ATCC</td>
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<tr>
<td>E. coli XL-1 Blue MRF’</td>
<td>Cloning strain; contains an F’ episome with lac$^{+}$Z ΔM15 for blue/white screening</td>
<td>Stratagene</td>
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<tr>
<td>E. coli DG98</td>
<td>Parent strain for DG105</td>
<td>ATCC</td>
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<tr>
<td>E. coli DG105</td>
<td>dam mutant (dam-13, lacI$^{+}$)</td>
<td>ATCC</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pT7Blue</td>
<td>Commercial cloning vector</td>
<td>Novagen</td>
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<tr>
<td>pBluescript SK$^{-}$</td>
<td>Commercial cloning vector</td>
<td>Stratagene</td>
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<tr>
<td>pCLPm2</td>
<td>pT7Blue with 3.0 kb insert containing P. multocida dam in opposite orientation to lacZ promoter</td>
<td>This study</td>
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<td>pCLPm3</td>
<td>pBluescript with same insert as pCLPm2; dam gene oriented so it is expressed from lacZ promoter</td>
<td>This study</td>
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<td>pLS88</td>
<td>E. coli shuttle vector that replicates in Pasteurellaceae</td>
<td>Willson et al., 1989</td>
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<td>pLS88/pol</td>
<td>pLS88 modified by insertion of the Sept–HindIII portion of the pUC19 polylinker in the HindIII site of pLS88</td>
<td>J. Sanders, Univ. of Michigan Flint</td>
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<td>pLSdam</td>
<td>pLS88 with pCLPm3 insert; lacZ promoter removed</td>
<td>This study</td>
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<tr>
<td>pLSdam2</td>
<td>pLS88 with pCLPm3 insert; lacZ promoter intact</td>
<td>This study</td>
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cleaves GATC sites whose adenine residue has been methylated, and 2-5 units of MboI, which only cleaves unmethylated GATC sites. Digested DNA was visualized by agarose gel electrophoresis.

Cloning and sequencing the P. multocida A1 dam gene. The P. multocida A3 chromosomal sequence (May et al., 2001) flanking the dam gene was analysed for PCR primer suitability, and two oligonucleotide primers, Pm1219DamM (TGAAGCAACGGTGTTCTCTGCTGTCC) and Pm1223DamP (GCTTGAAATTGCGTCTCGTCTGTC) were selected. Using P. multocida ATCC 11039 genomic DNA as the template, a 3 kb ampiclon containing the P. multocida dam gene and flanking sequences was produced under the following cycling conditions: 95°C, 2 min; 35 × (95°C, 30 s; 64°C, 1 min; 72°C, 2 min); 72°C, 10 min.

The 3 kb P. multocida PCR fragment containing the dam gene and flanking sequences was ligated into pT7Blue (Novagen) by blunt-end ligation using the Perfectly Blunt Cloning Kit (Novagen) according to the manufacturer’s protocol, followed by transformation into NovaBlue Singles Competent Cells (Novagen). Plasmid DNA was isolated from white colonies using the QIAPrep spin miniprep kit (Qiagen), and one clone with the predicted insert size was selected and designated pCLPm2 (Fig. 1a).

To determine the complete sequence of the P. multocida A1 dam gene, a portion of the pCLPm2 insert was sequenced on both strands using the Applied Biosystems Dye Terminator Cycle Sequencing Ready Reaction Kit protocol for sequencing double-stranded plasmid DNA. Sequencing reactions were resolved on an Applied Biosystems Prism Reaction Kit protocol for sequencing double-stranded plasmid DNA. using the Applied Biosystems Dye Terminator Cycle Sequencing Ready Reaction Kit protocol for sequencing double-stranded plasmid DNA. Sequencing reactions were resolved on an Applied Biosystems Prism 310 Genetic Analyser. Percentage identities with previously published dam sequences were determined using the CLUSTAL W method (Thompson et al., 1994) with MegAlign v5.0 (DNAStar). Prokaryotic promoters were predicted with the assistance of Neural Network Promoter Prediction (Reese et al., 1996). The final sequence of the P. multocida A1 dam gene was deposited in GenBank (accession AF411317).

Expression of the P. multocida A1 dam gene from a lacZ promoter. To orient the P. multocida dam gene downstream of a lacZ promoter, the insert from pCLPm2 was subcloned into pBluescript. Using pCLPm2 DNA as template, a 3 kb fragment containing the P. multocida dam gene was amplified by PCR with primers T7 and U19 using cycle conditions 95°C, 2 min; 35 × (95°C, 30 s; 57°C, 1 min; 72°C, 2 min); 72°C, 10 min. The 3 kb band was excised from a 0.7% agarose gel, and DNA was eluted using the Qiaquick gel extraction kit. The ends of the 3 kb fragment were digested with BamHI and SalI and ligated into pBluescript. One clone with the predicted insert size was selected and designated pCLPm3 (Fig. 1b). One end of the insert was sequenced to confirm correct orientation of the dam gene in relation to the lacZ promoter using the T3 primer.

Complementation of an E. coli dam mutant. Plasmids pCLPm2 and pCLPm3 were transferred into E. coli dam mutant strain DG105 by electroporation (Ausubel et al., 1994). To assess Dam function in the resulting strains, genomic DNA was isolated from DG105, DG105/pCLPm2, DG105/pCLPm3 and E. coli parent strain DG98; 0-2-0-3 μg genomic DNA was digested for 1 h with 2 units of SmaI, 10 units of DpnI and 2-5 units of MboI, and analysed by 0.5% agarose gel electrophoresis.

Effect of Dam alteration in P. multocida. To alter Dam production in P. multocida strain 11039, the insert from pCLPm3 was transferred into pLS88, a shuttle vector that replicates both in E. coli and in the Pasteurellaceae (Willson et al., 1989). Two pLS88 derivatives were constructed from pCLPm3: pLSdam, which has the P. multocida dam gene with no exogenous promoter, and pLSdam2, which has the P. multocida dam gene expressed from the lacZ promoter.

To construct pLSdam, a 1-7 kb fragment was amplified from pCLPm3 (Fig. 1b) by PCR using primers T7P1 (GGATCCTGCGTTCTCTGCTGTCC) and CLDamM20 (TCTAGATGTTGCCAATTGGCGTGTCC) using cycle conditions 95°C, 2 min; 35 × (95°C, 30 s; 67-5°C, 1 min; 72°C, 30 s); 72°C, 10 min. The 1-7 kb ampiclon was ligated with BamHI, which removed the lacZ promoter from pBluescript, and XbaI, which digested within the CLDamM20 primer, and ligated into BamHI- and XbaI-digested pLS88 that had been modified by insertion of a pUC19 polylinker (pLS88/poly; J. Sanders, personal communication).

To construct pLSdam2, pCLPm3 was digested with AflIII and HindIII (Fig. 1b), followed by treatment with the Klenow fragment of DNA polymerase I to create blunt ends. The resulting 1-6 kb fragment

![Fig. 1. (a) Map of pCLPm2. Locations of T7 and U19 primers used to amplify the pCLPm2 insert are indicated by black triangles. (b) Map of pCLPm3. Locations of T7P1 and CLDamM20 primers used to amplify a portion of pCLPm3 for construction of pLSdam are indicated by black triangles. AflIII and HindIII sites were used in construction of pLSdam2.](http://mic.sgmjournals.org)
from pCLPm3 retained the \textit{P. multocida} dam gene downstream of the \textit{lacZ} promoter. The 1-6 kb fragment was excised from a 0.7% agarose gel, eluted using the Qiaquick gel extraction kit, and ligated into EcoRV-digested pLS88.

Both pLSDam and pLSDam2 were transferred into \textit{P. multocida} 11039 by electroporation using described conditions (Jablonski \textit{et al.}, 1992) with a Bio-Rad Gene Pulser II.

**Effect of altered Dam production on \textit{P. multocida} spontaneous mutation frequency.** Overproduction of Dam in \textit{E. coli} causes an increased spontaneous mutation frequency (Herman \\& Modrich, 1981; Marinus \textit{et al.}, 1984). To determine whether altered Dam production in \textit{P. multocida} has the same effect, we compared the spontaneous mutation frequencies of wild-type strain 11039, 11039/pLSDam, and 11039/pLSDam2 by measuring the spontaneous development of rifampicin resistance (Ostendorf \textit{et al.}, 1999).

Briefly, 5 ml cultures of each strain were started from single colonies, incubated for 18 h, and aliquots (0.1 ml) of the diluted suspensions were spread in triplicate on BHI plates with 100 μg rifampicin ml$^{-1}$. The cultures were also serially diluted in PBS, and viable bacterial counts were determined by spreading diluted bacterial suspensions on BHI plates without antibiotics.

Mutation rates for each strain were determined by dividing the number of rifampicin-resistant mutants by the total viable bacterial counts. Five independent replicates of the experiment were run from separate bacterial cultures, and the mean mutation rates of the strains were compared by analysis of variance (ANOVA) for a randomized complete block design with run as the blocking factor. If significant differences among strains were found at the 5% level of significance, means were separated using the least significant difference test, and 95% confidence intervals were calculated to characterize the biological importance of those differences. The homogeneity of variance and normality assumptions necessary for valid application of ANOVA were examined by Levene’s test and by stem-and-leaf and normal probability plots, respectively. Statistical computations were performed using the SAS System for Windows, Version 8 (SAS Institute).

**Mouse virulence assay.** The virulence of 11039/pLSDam2 was compared to 11039 and 11039/pLSDam using a mouse model with five mice per treatment. Female 6-8-week-old BALB/c mice were obtained from the Jackson Laboratory, randomly divided into nine cages, and allowed to acclimate for 1 week. Bacterial broth cultures obtained from the Jackson Laboratory, randomly divided into nine cages, and allowed to acclimate for 1 week. Bacterial broth cultures obtained from the Jackson Laboratory were transferred into agarose gel, eluted using the Qiaquick gel extraction kit, and ligated into EcoRV-digested pLS88.

The cultures were also serially diluted in PBS, and viable bacterial counts were determined by spreading diluted bacterial suspensions on BHI plates without antibiotics.

Differential digests of genomic DNA with Sau3AI, DpnI and MboI can be used to detect Dam activity. Because MboI failed to cleave \textit{P. multocida} 11039 DNA and DpnI cleaved the same DNA (Fig. 2, lanes 3 and 4), our results indicated that \textit{P. multocida} has functional Dam activity.

**P. multocida A1 dam gene sequence results**

The \textit{P. multocida} A1 dam sequence was found to be 99.4% identical to the published \textit{P. multocida} A3 dam sequence. The deduced amino acid sequences of the \textit{P. multocida} A1 and A3 Dam proteins were 100% identical.

As expected, the \textit{P. multocida} dam gene had higher sequence identity with the \textit{Haemophilus influenzae} dam gene than with the other reported bacterial dam gene sequences. Among Gram-negative species, the dam gene is well conserved across four families, with identities ranging between 50 and 60%. The \textit{P. multocida} dam ORF was the largest of the currently identified dam genes. By CLUSTAL W alignment, the additional coding sequence for the \textit{P. multocida} dam ORF was located at the 5’ end of the gene, with an extra 39 bp at the 5′ end of the gene compared to \textit{H. influenzae} dam and an extra 72 bp at the 5′ end compared to dam sequences from other species.

\textit{aroB} in \textit{P. multocida} is located immediately upstream of dam with only a 4 bp gap between the genes, which is similar to the arrangement in \textit{H. influenzae}. Our promoter analysis detected a potential promoter upstream of \textit{P. multocida} dam within the \textit{aroB} coding sequence. The

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**RESULTS**

**Phenotypic detection of Dam function in \textit{P. multocida}**

Sau3AI, DpnI and MboI all recognize and cleave GATC sequences. However, DpnI only cleaves GATC sites whose adenine residue has been methylated, while MboI only cleaves unmethylated GATC sites. Sau3AI cleaves GATC sites regardless of methylation state. Therefore, differential
The 5’ end of this promoter was located 115 bp upstream of the *P. multocida dam* start codon and had the sequence TGGAAA–17 bp–TAGCGT–5 bp–G.

**Complementation of an *E. coli* dam mutant**

As described in Methods, pCLPm2 contains the *P. multocida dam* gene cloned in the opposite orientation to the lacZ promoter in pT7Blue, while pCLPm3 contains the *P. multocida dam* gene oriented downstream of the lacZ promoter in pBluescript. When these plasmids were transferred into *E. coli dam* mutant strain DG105, both restored Dam function (Fig. 3). *Mbo*I-digested genomic DNA from DG105/pCLPm3 was indistinguishable from undigested DNA (Fig. 3, lanes 6 and 7), similar to parent strain DG98 (Fig. 3, lanes 10 and 11). However, it appeared that *Mbo*I did cause some partial digestion of DG105/pCLPm2 (Fig. 3, lane 3), suggesting that GATC methylation may not have been as efficient in this strain as in DG105/pCLPm3. The pCLPm2 insert contained only 117 bp of native *P. multocida* chromosomal sequence upstream of the *dam* start codon, suggesting that the sequence we identified as a potential promoter within the 115 bp upstream of the *P. multocida dam* gene is functional.

**Effect of Dam production on spontaneous mutation rate in *P. multocida***

Plasmid pLSDam is similar to pCLPm2 in that the insert contains the *P. multocida dam* gene under the control of its native promoter, while pLSDam2 is similar to pCLPm3 in that *dam* is expressed from a *lac* promoter. Both plasmids were transferred into *P. multocida* 11039 to compare the abilities of the *lac* promoter and the putative native *dam* promoter to cause altered Dam phenotypes in *P. multocida*.

Transfer of pLSDam into strain 11039 caused no statistically significant effect on the spontaneous mutation rate compared to the wild-type strain (0.3 spontaneous mutants per 10^7 c.f.u. for wild-type 11039 compared to 0.14 mutants per 10^7 c.f.u. for 11039/pLSDam). However, the spontaneous mutation rate for 11039/pLSDam2 was 7.9 times higher (2.4 mutants per 10^7 c.f.u.) than the rate for 11039, which was a statistically significant increase (*P* < 0.05). This indicates that the *lac* promoter was effective in altering *dam* expression in *P. multocida* and that altered Dam activity affects the mutability of *P. multocida*.

**Mouse virulence assay**

Our virulence assay demonstrated that 11039/pLSDam2 was clearly attenuated in mice compared to 11039 and 11039/pLSDam. Mice injected with 11039 at all three doses (down to 3 x 10^4 c.f.u. per mouse) had 100% mortality, and mice injected with 11039/pLSDam (down to 2 x 10^4 c.f.u. per mouse) also had 100% mortality. In contrast, mice injected with 11039/pLSDam2 at all four doses (up to 3 x 10^9 c.f.u. per mouse) had 0% mortality. Sham control mice also had 0% mortality. *P. multocida* was recovered from the spleens of all mice that died during the trial.

![Fig. 3. Differential digests of genomic DNA from *E. coli* dam mutant DG105, *E. coli* parent strain DG98, and DG105 carrying *P. multocida dam* under the control of its native promoter (pCLPm2) or a lacZ promoter (pCLPm3). Lanes: 1, 1 Kb Plus DNA ladder; 2, undigested genomic DNA from DG105/pCLPm2; 3, DG105/pCLPm2 genomic DNA digested with *Mbo*I; 4, DG105/pCLPm2 digested with *Dpn*I; 5, DG105/pCLPm2 digested with *Sau*3AI; 6, undigested DG105/pCLPm3; 7, DG105/pCLPm3 digested with *Mbo*I; 8, DG105/pCLPm3 digested with *Dpn*I; 9, DG105/pCLPm3 digested with *Sau*3AI; 10, undigested DG98; 11, DG98 digested with *Mbo*I; 12, DG98 digested with *Dpn*I; 13, DG98 digested with *Sau*3AI; 14, undigested DG105; 15, DG105 digested with *Mbo*I; 16, DG105 digested with *Dpn*I; 17, DG98 digested with *Sau*3AI; 18, 1 Kb Plus DNA ladder.](http://mic.sgmjournals.org/2287)
In this study, we demonstrated the presence of Dam activity in *P. multocida*, which is an important aetiological agent of BRD in cattle, and we examined the function of Dam from *P. multocida*. In other bacterial species, Dam is important in regulating and coordinating several cell functions, including initiation of chromosome replication, DNA repair, and gene transcription. As a result of the role of Dam in DNA repair, bacteria with altered Dam activity have increased mutability. Its role in regulation of gene transcription, particularly of genes involved in pathogenicity, causes strains with altered Dam activity to be attenuated. Therefore, in the current study we measured the effects of altered *P. multocida* Dam activity on these two phenotypes: spontaneous mutation frequency and virulence in a mouse model.

The *E. coli* dam gene is part of a superoperon that includes several genes under complex regulatory control (Jonczyk *et al.*, 1989). Immediately upstream of *E. coli* dam is *urf*, which is preceded by *aroB* and *aroK*. A 92 bp gap is between *aroB* and *urf*, and a 107 bp gap is between *dam* and *urf*. The *aroK* and *aroB* products function in aromatic amino acid biosynthesis, while *urf* and *dam* are involved in cell cycle regulation. No promoter activity is located immediately upstream of *E. coli* dam, but a weak promoter is located upstream of *urf* within the *aroB* sequence (Jonczyk *et al.*, 1989). Another strong promoter is located upstream of *aroB*.

*P. multocida* Dam has 55% identity with *E. coli* Dam, and our results demonstrated that it complements an *E. coli* dam mutant. The putative native dam promoter sequence we identified on pCLPm2 (located within the *aroB* coding sequence) appeared relatively weak, having 5/12 mismatches compared to the *E. coli* consensus promoter. Similarly, in *E. coli*, the promoter upstream of *dam* and *urf* located within the *aroB* coding sequence is also relatively weak, having three to four times less activity than another promoter located upstream of *aroB* (Jonczyk *et al.*, 1989). Our result from digestion of DG105/pCLPm2 with *MboI* also suggested that the *P. multocida* native promoter contained within the *aroB* coding sequence on the pCLPm2 insert is relatively weak. Partial digestion of chromosomal DNA was evident in DG105/pCLPm2 (Fig. 2), suggesting that GATC methylation of chromosomal DNA was incomplete. Perhaps *P. multocida* is similar to *E. coli* in that a promoter upstream of *aroB* is more important in expressing the *P. multocida* dam gene than the promoter immediately upstream of *dam*.

*E. coli* dam mutants have an increased rate of spontaneous mutations, increased sensitivity to ultraviolet radiation and to base analogues such as 2-aminopurine, and unviability when combined with a *recA*, *recB* or *recC* mutation (Bale *et al.*, 1979; Glickman *et al.*, 1978; Marinus & Morris, 1974). Dam methylates DNA at the N⁶ position of adenine within GATC recognition sequences (Geier & Modrich, 1979), which allows the methyl-directed mismatch repair system to distinguish between the template and nascent strands to correct misincorporated bases during DNA replication (Glickman *et al.*, 1978; Modrich, 1989). Interestingly, overproduction of Dam also increases mutability because methylation of the daughter strand occurs too quickly, which also prevents the mismatch repair system from distinguishing between the template and daughter strands (Herman & Modrich, 1981; Marinus *et al.*, 1984).

When the *P. multocida* dam gene was expressed from its native promoter in strain 11039 using shuttle vector pLS88 (pLSdam), there was no significant increase in spontaneous mutation frequency. On the other hand, expression of *P. multocida* dam from a lacZ promoter in 11039/pLSdam2 did cause increased spontaneous mutation frequency, which is probably a result of unregulated Dam production. Because pLSdam and pLSdam2 are derived from the same parent plasmid, the increased mutation frequency caused by pLSdam2 is not likely to be the result of increased copy number. Rather, it is probably the result of either a stronger promoter sequence (the lacZ promoter more closely matches the consensus *E. coli* promoter than the native dam promoter does) or the absence of transcriptional regulation that may affect the native *P. multocida* dam promoter.

Dam-overproducing strains of *S. enterica*, *Y. pseudotuberculosis* and *V. cholerae* are all attenuated (Heithoff *et al.*, 1999; Julio *et al.*, 2001). Similarly, our Dam-overproducing *P. multocida* strain was attenuated using a mouse model. The attenuation of strains with altered dam expression is apparently due to ‘inappropriate’ expression of virulence factors that results from the failure of Dam to regulate their transcription (Heithoff *et al.*, 1999). In *Salmonella*, a similar attenuating effect occurs in dam deletion mutants (Heithoff *et al.*, 1999); mutation of the dam gene is lethal in *Y. pseudotuberculosis* and *V. cholerae* (Julio *et al.*, 2001).

Interestingly, the inappropriate expression of virulence factors in Dam-altered *S. enterica*, *Y. pseudotuberculosis* and *V. cholerae* not only causes attenuation, but it also renders them highly effective as live attenuated vaccines (Heithoff *et al.*, 1999, 2001; Julio *et al.*, 2001). Although *P. multocida* antigens have been identified that have potential as vaccines (Adler *et al.*, 1999; Confer *et al.*, 1996), an effective *P. multocida* BRD vaccine is still lacking. Killed bacterins have little effect in preventing BRD (Cardella *et al.*, 1987); however, live vaccines have improved efficacy (Cardella *et al.*, 1987; Chengappa *et al.*, 1989; Panciera *et al.*, 1984). Therefore, alteration of dam expression in *P. multocida* may be a viable strategy for development of a live attenuated vaccine.

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