A DNA adenine methylase mutant of *Shigella flexneri* shows no significant attenuation of virulence

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Mutants of *Salmonella* defective in DNA adenine methylase (*dam*) have been reported to be attenuated for virulence and to provide protective immunity when used as vaccine strains. To determine whether these observations could be extended to *Shigella*, a *dam* mutant of *Shigella flexneri* 2a was characterized and examined for the role of *dam* in pathogenesis. The *Shigella* *dam* mutant showed some unique characteristics; however, it retained virulence *in vivo* as well as *in vitro*. The mutant invaded cultured L2 monolayer cells as efficiently as the wild-type parent, but its intracellular growth was suppressed up to 7 h post-invasion. Furthermore, the invading *dam* mutant formed smaller plaques in cell monolayers compared to the parent strain. However, the mutant produced keratoconjunctivitis in the Sereny test in guinea pigs only slightly more slowly than the wild-type. While the effect of the *dam* mutation on virulence was modest, the rate of spontaneous mutation in the *dam* mutant was 1000-fold greater compared with the wild-type. The virulence and high mutability displayed by the *dam* mutant of *Sh. flexneri* suggest that a general anti-bacterial pathogen vaccine strategy based on mutations in *dam* needs to be re-evaluated.

INTRODUCTION

*Shigella flexneri*, one of the causative agents of bacillary dysentery, invades and spreads in epithelial cells of the colon and causes extensive destruction of the mucosal surface. Virulence of *Shigella* spp. is multigenic, involving genes both on the chromosome and on a large virulence-associated plasmid (Parsot & Sansonetti, 1996; Sansonetti et al., 1982).

DNA methylation is a mechanism by which bacteria regulate gene expression and control cellular functions such as DNA replication, mismatch repair, and segregation of chromosomal DNA (Marinus, 1996). Enzymes known as DNA-methyltransferases mediate methylation, which occurs at the C-5 or N-4 positions of cytosine and at the N-6 position of adenine. DNA-methyltransferases are found in many genera of enteric bacteria, but DNA methylation is not essential for viability of *Escherichia coli* or *Salmonella* (García-del Portillo et al., 1999; Low et al., 2001).

Recently, DNA adenine methylase (*Dam*), which methylates the N-6 of adenine in GATC sequences, has been reported to play a key role in regulation of virulence of several bacterial pathogens. *Dam−* *Salmonella typhimurium* display reduced M-cell cytotoxicity and reduced invasion in tissue culture (García-del Portillo et al., 1999). In an animal model, these mutants colonize Peyer’s patches but fail to invade enterocytes and fail to cause disease (Heithoff et al., 1999). It has also been reported that Dam is required for expression of virulence genes in *Yersinia pseudotuberculosis* and *Vibrio cholerae*. In these organisms, Dam activity is essential for viability. However, overexpression of *dam* inhibits colonization of *V. cholerae* in the suckling mouse model and strongly attenuates virulence of *Y. pseudotuberculosis* in a murine model (Julio et al., 2001). Heithoff et al. (1999, 2001) showed that *dam* mutants of *Salmonella* provide effective immunity when used as live attenuated vaccines in the mouse model. These authors also proposed that because of the high conservation of DNA adenine methylases among pathogenic bacteria, these proteins are potential targets for the development of vaccines and antimicrobial agents.

In light of these reports, it has been suggested that Dam is likely to be important for pathogenesis of other bacterial pathogens as well as those just described (Low et al., 2001). In this study, we tested this hypothesis by constructing a *dam* mutant of *Shigella flexneri* 2a and examining the virulence properties of the mutant. We report that, contrary to what has been observed in *Salmonella*, a *dam* mutant of *Sh. flexneri* retains the ability to invade, grow intracellularly and spread intercellularly, and is only minimally altered in its virulence phenotypes.
METHODS

Bacterial strains, growth conditions and mutant construction. 2457T, a wild-type strain of *Sh. flexneri* 2a (Formal et al., 1958), and BS103, a virulence plasmid-cured derivative of 2457T (Maurelli et al., 1984), were used. *dam* mutants were obtained by P1L4 transduction of the *dam-13*::Tn9 mutant allele from *E. coli* K-12 (Marinus et al., 1983) into 2457T and BS103. Chloramphenicol-resistant transductants were selected and designated 2457T/*dam* and BS103/*dam*, respectively. The *dam* null phenotype was confirmed by digestion of plasmid DNA isolated from the *dam* mutants with *MboI*, which cleaves only unmethylated GATC sequences. Bacteria were grown in tryptic soy broth (TSB), Luria broth (LB) or on Congo red agar (TSB, 0.02% Congo red, 1:5% Bacto agar) with or without chloramphenicol (10 µg ml⁻¹).

Secretion of invasion plasmid antigens (Ipas). Secretion of IpaB and IpaC from 2457T and 2457T/*dam* was examined as previously described (Bahrani et al., 1997).

Virulence assays. Invasion assays with 2457T and 2457T/*dam* were performed using a gentamicin protection assay with semiconfluent monolayers of L2 mouse fibroblasts, as described before (Hromocký & Maurelli, 1989). Plaque assays were performed using confluent L2 monolayers, as described by Oaks et al., (1985). The Sereny test was used as an *in vivo* virulence assay (Hartman et al., 1991).

Bacterial growth in liquid culture. Strains 2457T, 2457T/*dam*, BS103 and BS103/*dam* were cultured overnight with shaking at 30 °C, subcultured 1:100 into fresh TSB, and then incubated at 30 °C or 37 °C (for 2457T and 2457T/*dam*), or at 37 °C (for BS103 and BS103/*dam*). Samples were taken every hour and plated to determine viable cell numbers.

Measurements of intracellular bacterial growth. Assays for growth of intracellular bacteria were carried out as previously described, with some modifications (Schuch et al., 1999). Briefly, strain 2457T and 2457T/*dam* were grown to early–mid log phase in TSB. Bacteria were washed with PBS and suspended in Dulbecco’s minimal essential medium (DMEM) to an OD600 of 0.2. For measurement of intracellular bacteria, monolayers were subcultured 1:100 into fresh TSB, and then incubated at 30 °C or 37 °C, with or without 100 µg rifampicin ml⁻¹. After incubation overnight at 37 °C, the plates were scored for rifampicin-resistant colonies and the frequency of spontaneous mutation calculated.

Statistical analysis. Values are reported as mean ± standard error of the mean (SEM) in tables. All results were analysed using Student’s *t*-test.

RESULTS

Transduction of *dam-13*::Tn9 into 2457T and BS103

*dam* mutants were obtained by transducing the *dam-13*::Tn9 mutant allele from *E. coli* K-12 into *Sh. flexneri* 2457T and BS103. Chloramphenicol-resistant transductants of 2457T (2457T/*dam*) and BS103 (BS103/*dam*) were chosen and analysed further. The *dam* null phenotype was confirmed by digestion of plasmid DNA isolated from the mutants with restriction endonuclease *MboI*, which cleaves only unmethylated DNA sequences. Plasmid DNA isolated from *dam* mutants was sensitive to cleavage by *MboI*, while plasmid DNA isolated from the isogenic parent strains was completely resistant (data not shown).

Secretion of Ipa proteins

The mutant strain 2457T/*dam* was first tested for the ability to secrete invasion effectors via the *Shigella* type III secretion system. This phenotype is tightly regulated by growth temperature and is essential for invasion and intracellular growth. 2457T/*dam* synthesized as much IpaBC as the parent 2457T, after centrifugation. At the indicated intervals, cell monolayers (in triplicate) were washed to remove extracellular bacteria and gentamicin, lysed with 0.5% Triton X-100 in H₂O to release intracellular bacteria, and the recovered bacteria were plated on Congo red agar for counting.

Spontaneous mutation rate. To determine the mutation rate in *dam* mutants of *Shigella*, spontaneous mutation to rifampicin resistance was examined, as described previously (Bale et al., 1979). Briefly, a few hundred cells from each strain were inoculated into 5 ml LB and the cells were cultured overnight to saturation at 37 °C with shaking. Bacteria from each culture were plated on LB agar, with or without 100 µg rifampicin ml⁻¹. After incubation overnight at 37 °C, the plates were scored for rifampicin-resistant colonies and the frequency of spontaneous mutation calculated.

Table 1. Virulence assays of strain *Sh. flexneri* 2a 2457T and its *dam* mutant derivative

Values are reported as mean ± SEM.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Invasion ratio*</th>
<th>Plaque-forming ratio†</th>
<th>Plaque size after 3 days (mm)</th>
<th>Plaque size after 5 days (mm)</th>
<th>Sereny test (no. positive/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2457T</td>
<td>0.0022 ± 0.0004</td>
<td>0.0612 ± 0.0088</td>
<td>2.0–2.5</td>
<td>3.6–4.0</td>
<td>2/211</td>
</tr>
<tr>
<td>2457T/*dam</td>
<td>0.0017 ± 0.0003§</td>
<td>0.0309 ± 0.0103§</td>
<td>1.0–2.1</td>
<td>1.7–3.5</td>
<td>3/3§</td>
</tr>
</tbody>
</table>

*Expressed as number of gentamicin-protected c.f.u. per input c.f.u.
†Expressed as number of plaques per input c.f.u.
§P value in *t*-test was 0.01 compared to the wild-type strain.
||Positive reaction 48 h after inoculation.
§§Positive reaction 48–96 h after inoculation.
and secreted similar amounts of these effector proteins into the growth medium, as detected by Western blotting (data not shown).

Virulence assays
Next, 2457T/dam was tested for the ability to invade tissue culture cells and to spread from cell to cell (as measured in the plaque assay). No significant difference in the efficiency of invasion was detected between 2457T and 2457T/dam (Table 1, \( P = 0.1 \)) and only a modest difference in plaque formation ratio was found (Table 1, \( P = 0.04 \)). Plaques formed by 2457T/dam tended to be smaller in size compared to 2457T (Fig. 1). In the Sereny test, 2457T/dam produced keratoconjunctivitis in all three of the animals tested, although fully developed keratoconjunctivitis was delayed in two of the animals (Table 1).

Bacterial growth in liquid culture and in invaded cells
Because of the observed difference in plaque size, we measured in vitro and in vivo growth of the dam mutants. The strains were cultured in TSB at 30 °C or 37 °C (for 2457T and 2457T/dam), or at 37 °C (for BS103 and BS103/dam). The generation time in exponential phase at 37 °C was about 30 min for 2457T and 45 min for 2457T/dam (Fig. 2). The generation time at 30 °C of 2457T/dam was essentially unchanged from 2457T: 41 min and 39 min, respectively (Fig. 2). The generation times of both BS103 and BS103/dam at 37 °C were identical, at about 30 min (Fig. 2).

Assays for growth of intracellular bacteria were carried out, and the slower growth rate of 2457T/dam was again observed (Fig. 3). At 2 h post-invasion, the number of recoverable c.f.u. of 2457T began to increase, and bacterial numbers continued to increase until 4 h post invasion. At this point, c.f.u. recovered began to decrease as infected L2 cells died and detached from the plates. 2457T/dam displayed no

![Fig. 1. Plaque formation in an L2 monolayer by a dam mutant of _Sh. flexneri_ 2a. A, wild-type parent 2457T; B, isogenic dam mutant 2457T/dam. Plaque formation is shown 3 days post-invasion. Culture dish diameter, 35 mm.](image1)

![Fig. 2. Growth curves of _Sh. flexneri_ 2a 2457T, BS103, and their isogenic dam derivatives. Strains were grown in TSB at 30 °C or 37 °C. Samples were taken every hour and plated to determine viable bacteria. Each data point represents the mean ± SD of three independent experiments.](image2)

![Fig. 3. Intracellular growth of _Sh. flexneri_ 2a 2457T and 2457T/dam in L2 cells. Beginning of the incubation was defined as _t_ = 0. Experiments were repeated three times and one representative experiment is shown. Each data point represents the mean ± SD of triplicate data in the experiment.](image3)
intracellular growth up to 7 h, and only then started to show an increase in bacterial numbers (Fig. 3). Cells infected by 2457T/dam were still attached to the culture plates at 8 h post-invasion (data not shown). Therefore, it is unlikely that the decrease in viable counts of the dam mutant was due to detachment of dam-infected cells from the monolayer.

**Morphology of intracellular bacteria**

Giemsa staining of infected monolayers was performed to visualize intracellular bacteria. The wild-type parent 2457T multiplied rapidly within the infected cells, filling the cytoplasm within 4 h (Fig. 4). The infected cells also displayed numerous cytoplasmic extensions that contained bacteria (data not shown), a phenotype that is associated with intracellular motility. After 7 h, most cells had detached from the culture plate, but the cells that remained attached contained many bacteria. In contrast, 2457T/dam formed long, snaking filaments in the L2 cells after invasion, in addition to normal-length bacteria (Fig. 4). About 20% of the infected cells contained long filamentous bacteria at each time point observed post-infection. There were no cells that were filled only with long filamentous bacteria.

**Spontaneous mutability**

The effect of the dam mutation on the rate of spontaneous mutation was measured by determining the frequency of spontaneous resistance to rifampicin. 2457T/dam showed a mutation frequency of $2.2 \pm 0.7 \times 10^{-6}$, which was about 1000-fold higher than that of the parent, 2457T ($2.3 \pm 0.7 \times 10^{-9}$). For comparison, the spontaneous mutation rate in E. coli dam mutants is only 20- to 80-fold higher than the wild-type (Bale et al., 1979; Glickman, 1979).

**DISCUSSION**

Many different genetic approaches have been used to attenuate pathogenic strains of bacteria in order to produce live vaccine strains capable of generating protective immunity without causing disease. It has recently been reported that DNA adenine methylase is required for expression of virulence genes in Salmonella enterica (Heithoff et al., 1999), Yersinia pseudotuberculosis and Vibrio cholerae (Julio et al., 2001). A dam mutant of Salmonella typhimurium shows reduced invasion of enterocytes, and is avirulent when given orally and interperitoneally (Garcia-del Portillo et al., 1999). Moreover, Dam Salmonella, administered orally or intraperitoneally, provides significant protection against challenge with wild-type Salmonella (Heithoff et al., 1999). Accordingly, although the mechanism of the dam requirement for Salmonella virulence remains unknown, it has been proposed that dam mutants could serve as live attenuated vaccines and that Dam itself may provide a potential target for broad antimicrobial activity (Heithoff et al., 1999).

In this study, we constructed a dam mutant of Sh. flexneri 2a strain 2457T. The mutant 2457T/dam was slightly attenuated in virulence in tissue culture invasion assays, plaque assays and, in vivo, in the guinea pig Sereny test. However, 2457T/dam formed smaller plaques in the plaque assay, and gave a delayed positive reaction in some of the animals in the Sereny test, compared to 2457T. Growth in TSB was also slower for the dam mutant, and in invaded cells some 2457T/dam bacteria were filamentous. Genes involved in stress and the SOS response are expressed at higher levels in dam mutants of E. coli (Marinus, 1996; Oshima et al., 2002), and the filamentous shape of some dam mutant Sh. flexneri in invaded cells suggested an increased SOS response in the Sh. flexneri dam mutant.
We were rather surprised that the efficiency of invasion of the Sh. flexneri dam mutant was indistinguishable from that of the wild-type parent, in spite of its high mutability and slower growth. In contrast, a Sal. typhimurium dam mutant is non-invasive in tissue culture (García-del Portillo et al., 1999). The Sh. flexneri dam mutant was also virulent in an animal model, while a Sal. typhimurium dam mutant is completely attenuated in an animal model (Heithoff et al., 2001). It has been proposed that since motility is decreased in a dam mutant of E. coli (Oshima et al., 2002), this phenotype might be responsible for the lack of invasiveness by the mutant of Sal. typhimurium. Motility is important for invasiveness of Salmonella typhi (Liu et al., 1988), and increases the rate of invasion of Sal. typhimurium (Khoramian-Falsafi et al., 1990; Moens & Vanderleyden 1996), but it is not important for invasion of Shigella, which is non-motile. The different behaviour of the dam mutants underscores the fact that the fundamental mechanism of pathogenesis of Shigella differs from that of Salmonella.

DNA adenine methylase in bacteria regulates a variety of functions, including chromosome replication, transcription, and DNA repair (Marinus, 1996). Spontaneous mismatch mutations that occur in rapidly growing bacteria are normally repaired by the dam-directed mismatch repair system. Thus, one phenotype of dam mutants is that they display an increased rate of mutation. We found that 2457T/dam had a mutation rate about 1000-fold higher compared to the wild-type parent. This result is in stark contrast with the spontaneous mutation rate of a dam mutant of E. coli, which was reported to be only 20- to 80-fold higher than wild-type (Bale et al., 1979). The molecular basis for this increased mutation rate remains to be elucidated.

These results suggest that a general anti-bacterial pathogen vaccine strategy based on dam mutants needs to be re-evaluated. Although inactivation or overexpression of dam has a strong attenuating effect on pathogens such as Salmonella, Yersinia and V. cholerae (Heithoff et al., 2001; Julio et al., 2001), a mutation in dam does not significantly alter virulence in Sh. flexneri. Although the Sh. flexneri dam mutant produced a positive reaction in guinea pigs in the Sereny test, we cannot exclude the possibility that the mutant might be attenuated in a human host. However, the increased rate of spontaneous mutation we observed in the Sh. flexneri dam mutant would not be a desirable trait for a live vaccine strain.

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