INTRODUCTION

DNA adenine methylation (Dam) plays a number of key roles in bacterial processes, including mismatch repair, the timing of DNA replication and the transcription of certain genes (Noyer-Weidner & Trautner, 1993). Interruption of the dam gene has been implicated in an increase in the rate of spontaneous mutation, with the repair system unable to discriminate between the parental strand and the daughter strand of DNA (Barras & Marinus, 1989). It has been shown that altering the levels of Dam methylase in the bacterial cells adversely affects the timing of replication (Boye & Lobner-Olesen, 1990) and that strict levels of Dam methylase are required to coordinate chromosome replication. Dam has also been implicated as a global regulator of gene expression, and in Salmonella it represses the expression of at least 20 in vivo-induced genes (Heithoff et al., 1999). Dam has also been shown to influence the expression of a number of genes in Escherichia coli (Barras & Marinus, 1989; Oshima et al., 2002). It has been suggested that using methylation to control gene expression may result in the coupling of expression to replication.

In Salmonella enterica serovar Typhimurium, Serratia marcescens or E. coli, inactivation of dam resulted in an increased rate of mutation and an inability to grow in the presence of 2-aminopurine (2-AP), a purine base analogue that becomes aberrantly incorporated into DNA (Glickman et al., 1978; Torreblanca & Casadesus, 1996; Ostendorf et al., 1999). The S. enterica var. Typhimurium dam mutant showed increased sensitivity to detergents such as bile salts and sodium deoxycholate, which may be due to changes in the cell wall structure, and resulted in the shedding of membrane vesicles and the release of periplasmic proteins into the surrounding medium (Pucciarelli et al., 2002). The mutant also showed reduced virulence by the oral or the intraperitoneal routes of challenge (Heithoff et al., 1999). In the murine model, immunization with the dam mutant provided protection against challenge with wild-type S. enterica serovar Typhimurium (Heithoff et al., 1999, 2001). This immunization regime also provided protection against S. enterica serovars Dublin and Enteriditis (Heithoff et al., 1999, 2001). These promising results suggested that a dam mutant of S. enterica serovar Typhimurium might be exploited as a live attenuated vaccine. In addition, because homologues of dam appeared to be present in a range of other bacterial species, it was suggested that dam might serve as a target for the construction of other rationally attenuated mutants, and also that the gene product might be a target for novel antimicrobial drugs (Heithoff et al., 1999).

 Interruption of the dam gene in the Yersinia pseudotuberculosis strain YPIII resulted in a lethal mutation (Julio et al., 2001), although overexpression of dam resulted in attenuation. The reasons for the non-viability of the dam mutant are not known, though inactivation of the dam gene has also been shown to be lethal in Vibrio cholerae. Y. pseudotuberculosis is a divergent species (Achtman et al., 1999) that has a wide distribution among domestic animals, wild
animals, soil and fresh water (Nagano et al., 1997) and can survive in a broad range of environments. The species has been separated into 21 serotypes; these different serotypes are associated with different diseases in humans and in animals. In humans Y. pseudotuberculosis is the occasional aetiological agent of gastroenteritis, which results in severe abdominal pain with fever and headaches (Putzker et al., 2001). Other serotypes preferentially infect animals, and disease caused by Y. pseudotuberculosis is especially problematic in farmed deer (Sanford, 1995). The bacterium is closely related to Yersinia pestis, the aetiological agent of plague, as shown by DNA–DNA hybridization, and similarity of 16S rRNA sequences (Achtman et al., 1999). Indeed, on the basis of this genetic similarity Y. pestis has been proposed by some to be a subspecies of Y. pseudotuberculosis.

Against this background we set out to construct a dam mutant in a strain of Y. pseudotuberculosis that had not been extensively passaged in the laboratory and which may be more representative of the species. This work describes the construction of an isogenic dam knockout mutant in Y. pseudotuberculosis strain IP32953, isolated from a human patient presenting with diarrhoea in France in 1990 (de Almeida et al., 1993), its phenotypic characterization by in vitro assays, virulence studies in the mouse model, and its ability to induce immune responses when used as a live vaccine.

### METHODS

**Bacterial strains, plasmids, growth conditions and chemicals.**

Bacterial strains and plasmids used in this study are listed in Table 1. E. coli was cultured at 37 °C, on Luria–Bertani (LB) agar or broth. Y. pseudotuberculosis was routinely cultured at 30 °C on LB agar or broth, Yersinia selective agar (YSA; Oxoid) or Magox agar (per litre: 10 g Tryptone peptone (Difco), 5 g Bacto yeast extract (Difco), 5 g NaCl, 20 g Bacto agar, 0-05 g Congo red, 2-68 g sodium oxalate and 1-0 g magnesium chloride). Y. pestis was routinely cultured at 28 °C in Blood Agar Base (BAB) broth or on BAB agar (Oyston et al., 1996). All chemicals were purchased from Sigma–Aldrich. Medium was supplemented with antibiotics as required, to final concentrations of 50 μg kanamycin ml⁻¹ and 55 μg ampicillin ml⁻¹. Medium was supplemented with the base analogue 2-AP to final concentrations ranging from 200 to 400 μg ml⁻¹. Medium was supplemented with bile salts and sodium deoxycholate to a final concentration of 1 % (w/v). Plasmids were introduced into E. coli by electroporation (Dower et al., 1988) and into Y. pseudotuberculosis by conjugation (Oyston et al., 1996), with E. coli BW 19851 as donor strain. Unless otherwise stated, plasmid and genomic extractions, restriction enzyme digestions, DNA ligations and transformations into E. coli were performed by standard procedures (Sambrook et al., 1989) using enzymes provided by Promega, Roche or Amersham Pharmacia.

**Analysis of the Y. pseudotuberculosis IP32953 genome.**

Although the genome sequence of Y. pseudotuberculosis IP32953 has not yet been published, BLAST analysis of the genome can be carried out at http://bbrp.llnl.gov/bbrp/bin/y.pseudo.complete.blast.

**PCR and cloning procedures.** The dam gene was identified in the Y. pestis genome (http://www.sanger.ac.uk/Projects/Y_pestis),

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y. pseudotuberculosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP 32953</td>
<td>Isolated from a human patient in 1990</td>
<td>E. Carniel, Institut Pasteur, Paris, France This study</td>
</tr>
<tr>
<td>C18</td>
<td>Y. pseudotuberculosis IP32953 dam mutant</td>
<td></td>
</tr>
<tr>
<td><strong>Y. pestis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GB</td>
<td>Orientalis (virulent)</td>
<td>Human – UK</td>
</tr>
<tr>
<td>CO92</td>
<td>Orientalis (pneumonic)</td>
<td>Human – USA</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Cloning strain</td>
<td>Clontech</td>
</tr>
<tr>
<td>CC118/pir</td>
<td>Cloning strain</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>IP19851pir (BW 19851)</td>
<td>Stably integrated pir</td>
<td>Metcalf et al. (1994)</td>
</tr>
<tr>
<td>S17/pir/pNJ5000</td>
<td>Triple mating strain with Tet⁺ helper plasmid</td>
<td>Grinter (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T-Easy</td>
<td>T-cloning vector, Amp⁺</td>
<td>Promega</td>
</tr>
<tr>
<td>pCVD442</td>
<td>Suicide vector, Suc⁺ Amp⁺</td>
<td>Donnenburg &amp; Kaper (1991)</td>
</tr>
<tr>
<td>pUC18</td>
<td>Amp⁺</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>pK2</td>
<td>pGEM-T-Easy containing kanamycin cassette</td>
<td>P. Oyston</td>
</tr>
<tr>
<td>pdamF</td>
<td>pGEM with beginning of dam gene</td>
<td>This study</td>
</tr>
<tr>
<td>pdamR</td>
<td>pGEM with end of dam gene</td>
<td>This study</td>
</tr>
<tr>
<td>pdamFRK</td>
<td>pUC18 with both incomplete parts of dam gene</td>
<td>This study</td>
</tr>
<tr>
<td>pdamFRK</td>
<td>pdamFRK in pCVD442, Kan⁺</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Amp⁺, ampicillin resistant; Tet⁺, tetracycline resistant; Kan⁺, kanamycin resistant; Suc⁺, sucrose sensitive.*
using the sequence for the S. enterica var Typhimurium dam gene in a BLAST search. Fragments of the Y. pestis dam gene were amplified from Y. pestis CO92 DNA using the PCR with the primer pair VT1-Sacl (5'-AGCTCTACACCCATATGATTG-3') and VT2-BglII (5'-AGATCTCCAGCAGGTATATTCCCCACCG-3') (restriction sites are underlined) and VT3-BglII (5'-AGATCTGGATATCATCAGGCCGGGTTCGC-3') and VT6-SalI (5'-GTCGACTCTACACCGTTAACCCGGGCTTC-3') (restriction sites are underlined). The PCR was performed with 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. The fragments of the dam gene were separately ligated into the expression vector using the restriction endonucleases SacI and BglII, and the 3' fragment was excised using SalI and BglII. The two segments were ligated into the plasmid pUC18, yielding pdamFR. Plasmid pdamFR was linearized using the unique BglII site and a kanamycin cassette was introduced into the linearized plasmid, yielding kanamycin-resistant pdamFRK.

**Construction of a Y. pseudotuberculosis dam mutant.** Plasmid pdamFR was digested with SacI and SalI, and the excised fragment containing the mutated dam gene was ligated with similarly digested pCVD442, yielding pCdamFRK. The plasmid pCdamFRK was electroporated into E. coli BW 19851 cells and subsequently introduced into Y. pseudotuberculosis strain IP32953 by conjugation in a three-way mating (Li et al., 1996), using a Y. pseudotuberculosis IP32953 wild-type recipient, E. coli BW 19851/pCdamFRK, and E. coli S17-1/pRK1000 (Guo et al., 1997). Equal volumes (50 µl) of the three strains were mixed, and 100 µl was spotted onto an LB agar plate. After incubation at 30°C for 5 h the bacteria were recovered, resuspended in 1 ml LB broth and cultured on YSA supplemented with ampicillin and kanamycin. The plates were incubated for 48 h at 30°C. Individual colonies were inoculated into LB broth, grown overnight at 30°C, and plated onto LB agar supplemented with kanamycin and 10% (w/v) sucrose. Resultant colonies were screened using the PCR with the primer pair CdamF (5'-TCAGACACCCTGAAT-3') and CdamR (5'-GCTTTATCACAAGTGGA-3'). A kanamycin-resistant Y. pseudotuberculosis dam double-crossover mutant, termed C18, was identified and used for further studies.

**Determination of the methylation state of Y. pseudotuberculosis dam gene DNA.** Genomic DNA (2 µg) isolated using the Puregene DNA isolation kit (Genta Systems) from Y. pseudotuberculosis IP32953 and C18, the dam knockout, was digested by one of Mbol (10 U), SsaAI (8 U) or DpnI (10 U) for 4 h at 37°C. Buffer mixtures were those recommended by the manufacturer (Promega). Restricted DNA was analysed by electrophoresis on 0.7% (w/v) agarose gels, together with DNA subjected to the same conditions in the absence of enzyme. After electrophoresis the gels were visualized under UV illumination.

**Determination of sensitivities.** Y. pseudotuberculosis IP32953 or C18 was grown for 18–22 h at 30°C in LB broth. The broth was then serially diluted in PBS and plated out in duplicate on LB plates containing bile salts, sodium deoxycholate, 2-AP or no supplements. The plates were then incubated at 30 or 37°C for 48 h and the colonies were enumerated.

**Determination of virulence in mice.** The median lethal doses (MLDs) of the Y. pseudotuberculosis wild-type or dam mutant were determined after oral gavage or intravenous (i.v.) inoculation of groups of five female 6-week-old BALB/c mice (Charles River Laboratories). For the oral challenge, bacteria were grown in 100 ml LB broth for 18–22 h at 30°C and centrifuged at 6000 r.p.m. for 20 min. The pellet was resuspended in 500 µl PBS and serially diluted to the required concentrations. For the i.v. challenge, bacteria were grown in 100 ml LB broth for 18–22 h at 30°C, then harvested and serially diluted in PBS. Mice were challenged with 100 µl in both the oral and i.v. challenges. Humane end-points were strictly observed in both experiments, and animals deemed incapable of survival were humanely killed by cervical dislocation. The MLD of the dam mutant was determined by the method of Reed & Muench (1938).

For immunization studies, groups of five female 6-week-old BALB/c mice (Charles River Laboratories) were dosed orally or i.v. with Y. pseudotuberculosis dam mutant C18. Six weeks after immunization, the mice were challenged subcutaneously (s.c.) with 100 c.f.u. Y. pestis GB or i.v. with 300 c.f.u. Y. pseudotuberculosis IP32953. Mouse survival was recorded for 5 weeks. In organ colonization studies, spleens were removed from mice 4 weeks after the original challenge with Y. pseudotuberculosis dam mutant and 2 weeks after the challenge with Y. pestis GB or Y. pseudotuberculosis IP32953. Spleens were homogenized in 3 ml LB broth and plated onto LB agar to count the numbers of viable bacteria per organ.

**Hybridization of RNA.** Y. pseudotuberculosis IP32953 RNA was used as the control and labelled with Cy3-dCTP. The dam knockout strain was treated as the test sample and was labelled with Cy5-dCTP (Amersham Biosciences). Labelling of 2–3 µg denatured RNA was performed using SuperScript II (Invitrogen) in the presence of 5 mM dATP, 5 mM dGTP, 5 mM dTTP, 2 mM dCTP and 750 pM Cy5-dCTP or Cy3-dCTP. After incubation at 25°C in the dark for 10 min, followed by incubation at 42°C for 90 min, control and test strain labelled RNA were mixed and purified together using a single Qiagen mini-elite column. Microarray slides were pre-hybridized in 3×5× saline sodium citrate buffer (SSC), 0-1% (w/v) SDS, 10 µg BSA ml⁻¹ at 65°C. The denatured RNA was applied to the microarray slide in hybridization solution (4× SSC, 0.3%, w/v, SDS) and hybridized for 18 h at 65°C. The slides were then washed once in 1× SSC, 0.05% (w/v) SDS at 65°C, and twice in 0.06× SSC for 2 min. All hybridizations were performed in duplicate.

**Microarray analysis.** All the Y. pestis CO92 predicted coding sequences are represented on the DNA microarray. Gene numbers refer to the published CO92 genome sequence (Parkhill et al., 2001). Microarray slides were scanned using an Affymetrix 428 scanner (MWG Biotech), the scanned images were loaded into Imagegene 5.0 (MWG Biotech). All the microarray data were analyzed using LOWESS normalization with 40% of the data used for smoothing and a cut-off value of 0-01. Data were interpreted using the ‘log of ratio’ with the cross-gene error model switched off. A 1.5-fold difference in expression level between the wild-type and the mutant was set as the cut-off. Genes that demonstrated at least a 1.5-fold difference in expression level were tested by Student’s t-test to determine that the difference was statistically significant. The Benjamini and Hochberg false discovery rate was used to correct for multiple testing. The results are a compilation of the gene expression profiles of three biological replicates grown on different days and two arrays of each replicate also performed on different days, resulting in a total of six arrays.

**RESULTS**

**Construction of the Y. pseudotuberculosis dam mutant, strain C18**

Analysis of preliminary genome sequence data available at the Lawrence Livermore National Laboratory website
 revealed that the genome of *Y. pseudotuberculosis* strain IP32953 possessed a gene that would encode a Dam enzyme identical to that previously identified in *Y. pseudotuberculosis* strain YPIII. The *Y. pseudotuberculosis dam* gene showed 99% similarity to the *Y. pestis dam* gene, and we therefore considered that a single plasmid could be designed to interrupt either the *Y. pseudotuberculosis* or the *Y. pestis dam* gene. A DNA fragment corresponding to an internal fragment of the *Y. pestis dam* gene was amplified, the central region removed and replaced with a kanamycin cassette, and the resulting fragment cloned into the suicide vector pCVD442. An isogenic mutant was constructed by allelic replacement using the inactivated *dam* gene. Several potential *dam* mutants were identified after growth on agar containing kanamycin and in the presence of 10% sucrose. Two colonies were selected for further analysis. A confirmatory PCR was used to verify the mutation; the *dam* allele was amplified from selected clones, yielding an 1154 bp product versus an 816 bp product of the wild-type *dam* (data not shown). After the deletion in the *dam* gene and the insertion of the kanamycin cassette had been confirmed, one *Y. pseudotuberculosis* clone (C18) was selected for further study.

**Dam-deficient *Y. pseudotuberculosis* no longer methylates DNA at GATC**

To demonstrate that the *Y. pseudotuberculosis dam* mutant no longer had a functional *dam* gene, DNA isolated from *Y. pseudotuberculosis* IP32953 or *Y. pseudotuberculosis* C18 was incubated with *MboI*, *Sau3AI* or *DpnI*. All of these enzymes recognize the sequence GATC and have previously been used to confirm the presence or absence of Dam methylation (Boye & Lobner-Olesen, 1990). *Sau3AI* cleaves at the adenine regardless of the state of methylation of the DNA, *MboI* cleaves at the adenine only if the sequence is non-methylated, whereas *DpnI* can only cleave at the adenine residue if the DNA is adenine methylated. The DNA of *Y. pseudotuberculosis* IP32953 was cleaved by *Sau3AI* and *DpnI* but was resistant to digestion with *MboI*. The DNA from *Y. pseudotuberculosis* C18 was cleaved by *MboI* and *Sau3AI* but not by *DpnI* (Fig. 1). These results indicate that although *Y. pseudotuberculosis* IP32953 possesses a methylation system that modifies DNA at the expected site, this system is not functional in *Y. pseudotuberculosis* C18.

**Dam-deficient *Y. pseudotuberculosis* is susceptible to bile salts, but not to 2-AP**

Dam-deficient mutants of *Salmonella* show an increased sensitivity to detergents, such as bile salts or sodium deoxycholate. This sensitivity has been suggested as a reason for reduced virulence (Pucciarelli et al., 2002; Heithoff et al., 2001). To establish if the *Y. pseudotuberculosis dam* mutant was similarly sensitive, we grew the wild-type and the *dam* mutant in the presence of 1% sodium deoxycholate and found that the strains grew equally well (Fig. 2). However, when incubated in medium in the presence of 1% bile salts, the *dam* mutant showed reduced growth compared to the wild-type (Fig. 2).

Dam-deficient mutants of *E. coli* are inhibited in their growth by the presence of 2-AP and thus 2-AP is frequently used to confirm the identity of *dam* mutants (Glickman et al., 1978; Ostendorf et al., 1999). Wild-type *Y. pseudotuberculosis* and the *dam* mutant were grown in the presence of various concentrations of 2-AP up to 400 μg ml⁻¹ in solid medium. Neither strain demonstrated sensitivity to the presence of 2-AP at 400 μg ml⁻¹ (Fig. 2). Dam-deficient strains of *E. coli* and *Serratia marcescens* showed sensitivity to 2-AP at concentrations of 100 μg ml⁻¹ (Glickman et al., 1978; Ostendorf et al., 1999).

**Microarray analysis**

Gene expression analysis indicated that of the 4042 predicted coding sequences represented on the microarray, 167 genes showed altered expression in C18 compared to *Y. pseudotuberculosis* IP32953. With the exception of *uvrB*, none of the 91 up-regulated genes were involved in the SOS response; this is unlike the situation in other *dam* mutants, in which a number of the up-regulated genes encode portions of the SOS response machinery (Peterson et al., 1985; Jonczyk et al., 1989; Oh & Kim 1999; Oshima et al., 2002). However, genes encoding proteins involved in the heat-shock response are up-regulated in the Dam-deficient *Y. pseudotuberculosis* mutant; these include *dnaK*, *ihpB*, *clpB*, *lon* and *rpoH* (Yura et al., 2000), indicating that the mutants are experiencing stressful conditions. The
Dam-deficient mutant also demonstrates up-regulation of a number of transposases. Of the 76 genes that were down-regulated, 52 were located on the pYV plasmid. Other genes that demonstrated reduced expression encode ribosomal proteins, formate dehydrogenase subunits and \( \text{tig} \), which encodes a molecular chaperone called trigger factor.

**The pYV plasmid is unstable in Dam-deficient *Y. pseudotuberculosis***

The down-regulation of the genes on the virulence plasmid, pYV, led to the possibility that Dam regulates the pYV plasmid or that the plasmid was not present in *Y. pseudotuberculosis* C18. To investigate whether the bacteria possessed the pYV plasmid they were cultured on Magox medium, where bacteria containing pYV give rise to red colonies while those that have lost the plasmid produce pink colonies. The wild-type strain retained pYV in 99% of cells after overnight culture in liquid medium, but only 4% of C18 cells retained pYV under the same in vitro conditions.

**Dam-deficient *Y. pseudotuberculosis* is attenuated in orally or i.v. infected mice**

Due to the low maintenance of the pYV plasmid in Dam-deficient *Y. pseudotuberculosis* the animal experiments to determine attenuation were carried out using plasmid-cured *Y. pseudotuberculosis dam* (pYV\(^{-}\)).

Groups of five BALB/c mice were challenged with increasing doses of *Y. pseudotuberculosis* IP32953 or the plasmid-cured dam mutant by either the oral or the i.v. route. The MLD of the wild-type strain was approximately 1000 c.f.u. by the oral route of challenge, while pYV\(^{-}\) *Y. pseudotuberculosis dam* did not cause deaths when given at 1-31 \( \times 10^{9} \) c.f.u. By the i.v. route the MLD of strain IP32953 is 1 c.f.u.; the highest dose of pYV\(^{-}\) dam mutant delivered intravenously was 2-13 \( \times 10^{7} \) c.f.u. At this dose no mice died, indicating that the plasmid-cured strain is avirulent by this route.

These results indicate that the plasmid-cured *Y. pseudotuberculosis* dam is attenuated by at least \( 10^{6} \) c.f.u. by both routes.

When the virulence studies were repeated using *Y. pseudotuberculosis* C18, which maintains pYV at 4%, the levels of attenuation were similar to those seen with the plasmid-cured strain: MLD 1-78 \( \times 10^{9} \) c.f.u. by the oral route and \( \sim 1-65 \times 10^{7} \) c.f.u. by the i.v. route.

**Dam-immunized mice are protected from a *Y. pseudotuberculosis* challenge**

Groups of five mice were immunized by the oral or i.v. route with a single dose of *Y. pseudotuberculosis dam* pYV\(^{-}\), and challenged 6 weeks later with 300 c.f.u. *Y. pseudotuberculosis* IP32953 given i.v. Naïve mice challenged with 300 c.f.u. *Y. pseudotuberculosis* IP32953 by the i.v. route died within 17 days. Mice that had been immunized with *Y. pseudotuberculosis dam* were protected against a subsequent challenge with *Y. pseudotuberculosis* IP32953. Complete protection was seen when the immunizing dose by the i.v. route was \( 10^{5} \) c.f.u., or when the immunizing dose by the oral route was \( 10^{8} \) c.f.u. (Table 2). On day 22 post-challenge, all surviving mice were humanely culled and the spleens were removed, homogenized and plated onto LB agar for isolation of *Y. pseudotuberculosis*. No bacteria were detected in the spleen homogenates.

**Dam-immunized mice are protected from a *Y. pestis* challenge**

We next investigated whether immunization with the dam mutant of *Y. pseudotuberculosis* would provide protection against *Y. pestis*. Groups of five mice were immunized by the
Table 2. Numbers of survivors following i.v. challenge with Y. pseudotuberculosis IP32953 or s.c. challenge with Y. pestis GB

<table>
<thead>
<tr>
<th>Dose (c.f.u.)</th>
<th>Y. pseudotuberculosis challenge</th>
<th>Y. pestis challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^5$</td>
<td>–</td>
<td>5/5</td>
</tr>
<tr>
<td>$10^6$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$10^7$</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$10^8$</td>
<td>5/5</td>
<td>–</td>
</tr>
<tr>
<td>$10^9$</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Mice, immunized with Y. pseudotuberculosis dam by oral gavage or by the i.v. route, were challenged 6 weeks after immunization with either 300 c.f.u. Y. pseudotuberculosis IP32953 by the i.v. route or 100 c.f.u. Y. pestis GB by the s.c. route. –, Not determined.

oral or i.v. routes with a single dose of Y. pseudotuberculosis dam pYV−, and challenged 6 weeks later with 100 MLD of Y. pestis GB by the s.c. route. Naïve mice challenged s.c. with 100 c.f.u. Y. pestis GB died within 10 days. Although mice immunized by either route showed protection against Y. pestis challenge, immunization with Dam-deficient Y. pseudotuberculosis by the i.v. route offered the highest levels of protection, with complete protection seen if immunizing doses of $10^9$ c.f.u. or greater were used (Table 2).

On day 22 post-challenge, all surviving mice were humanely culled and the spleens were removed, homogenized and plated onto Congo red agar for isolation of Y. pestis. No bacteria were detected in the spleen homogenates.

**DISCUSSION**

Previously it has been reported that the inactivation of the dam gene in Y. pseudotuberculosis strain YPIII was lethal, but the overexpression of dam in this strain resulted in attenuation (Julio et al., 2001). We have been able to construct an isogenic dam mutant in Y. pseudotuberculosis strain IP32953. Gene sequence comparisons of the dam genes from strains IP32953 and YPIII show that the gene targeted in both studies is the same one. The gene used, therefore, does not explain the different outcomes. This leaves the possibility that the reason for the successful inactivation in this study was the result of using a different strain of Y. pseudotuberculosis.

In Salmonella enterica serovar Typhimurium and E. coli the growth of dam mutants is limited in the presence of 2-AP because the mismatch repair function of the bacteria is disrupted, allowing lethal mutations to accumulate (Ritchie et al., 1986; Glickman et al., 1978; Ostendorf et al., 1999). In this study, the dam mutant of Y. pseudotuberculosis did not show increased susceptibility to 2-AP, but the reason for this is not clear. However, it is possible that there is some redundancy in the function of Dam. The dam gene of group B Neisseria meningitidis is not expressed due to a mutation in the coding sequence and this has led to an inactive methyl-directed mismatch repair mechanism (Bucci et al., 1999). One result of this loss is an increased frequency of phase variation in the neuraminic acid capsule controlled by the sapD gene. However, despite the lack of mismatch repair the bacteria are still virulent. In the Y. pseudotuberculosis dam mutant, it is possible that the methyl-directed mismatch repair mechanism is still intact but the lack of Dam methylation has led to an attenuated phenotype—possibly due to the loss of Dam-controlled gene expression in a number of virulence genes.

It has been proposed that an indication of a defect in the cell envelope may be observed by looking at the sensitivity of strains to bile salts. Y. pseudotuberculosis IP32953 shows a slight reduction in growth in the presence of bile salts compared to nutrient agar when incubated at either 30°C or 37°C, whereas strain C18 shows a large reduction in growth in the presence of bile salts when incubated at 30°C and hardly grows at all when incubated at 37°C. It is possible that this increased sensitivity to bile salts could account in part for the reduction in oral virulence of Y. pseudotuberculosis dam. Y. pseudotuberculosis dam shows only a small reduction in growth in the presence of sodium deoxycholate, which is a component of bile salts. It is therefore possible that the Dam-deficient strain does have a defect in its cell envelope. However, the level of sensitivity is far lower than that seen in S. enterica var. Typhimurium (Pucciarelli et al., 2002). These results suggest that Y. pseudotuberculosis dam has sensitivity to another component found in bile salts.

The attenuation and protection studies using the plasmid-cured Y. pseudotuberculosis dam and the Y. pseudotuberculosis dam containing a low percentage of pYV gave very similar results. This may indicate that the pYV plasmid is not maintained under in vivo conditions or that 4% maintenance of pYV does not result in enough plasmid to have an effect. The presence of the pYV plasmid is crucial for Y. pseudotuberculosis to cause infection, as the ability to resist phagocytosis is a requirement for its extracellular survival in the Peyer’s patches and the loss of the plasmid could account for the reduction in virulence seen in the Dam-deficient Y. pseudotuberculosis.

The induction of a protective immune response to Y. pestis after exposure to Y. pseudotuberculosis has previously been reported (Simonet et al., 1985). However, the protection demonstrated was lower than shown here and was not induced by a defined mutant. The identity of the antigens that are responsible for the induction of this cross-protective immunity has not been determined.

To date, two protective subunits from Y. pestis have been reported (Titball & Williamson 2004): the capsular F1 antigen and the V-antigen component of the type III system LcrV), which is encoded on the pYV plasmid (Brubaker
1991; Roggenkamp et al., 1997; Schmidt et al., 1999). The F1 antigen is not produced by any strains of Y. pseudotuberculosis and so is not responsible for the protection seen here. In this study we have demonstrated protection against Y. pestis after immunization with a pYV-cured derivative of the dam mutant of Y. pseudotuberculosis. This indicates that there are protective antigens additional to LcrV, and these must be chromosomally encoded in Y. pseudotuberculosis. We are able to exclude lipopolysaccharide (LPS) as the protective antigen because LPSs from Y. pseudotuberculosis and Y. pestis are not identical (Skurnik et al., 2000; Prior et al., 2001) and because LPS does not appear to play a significant role in protective immunity against plague (Prior et al., 2001).

Microarray analysis revealed that a number of molecular chaperones are differentially expressed in the dam mutant compared to the wild-type; this could result in altered protein folding. The analysis also indicated that a number of surface-related proteins are up-regulated in the absence of dam. The result of this may be an increase in the presentation of potential protective antigens to the immune system. The identification of these cross-protective antigens is potentially the most important aspect of any future work.

Alternative approaches to the development of a live attenuated mutant of Y. pestis, by inactivating the araA, phoP or htrA genes, have resulted in only modest levels of attenuation, which are insufficient for the development of these mutants as live attenuated vaccines (Oyston et al., 1996, 2000; Williams et al., 2000). The safety in humans of any dam mutant as a live attenuated vaccine has not yet been tested. However, our findings suggest that the dam mutant of Y. pseudotuberculosis we have constructed could form the basis of an orally delivered single-dose vaccine against plague.

The significance of the cross-protective immunity we have observed for the epidemiology of plague is not clear. Infection of humans with Y. pseudotuberculosis is relatively rare (Ljunberg et al., 1995), and it seems unlikely that a significant proportion of the population would be immune to Y. pestis. However, Y. pseudotuberculosis is frequently isolated from livestock such as piglets (Niskanen et al., 2002), and the exposure of humans to the bacterium in rural environments might be more frequent. Prior exposure to Y. enterocolitica might also provide protection against plague. It has been suggested that the different incidences of human disease in plague foci areas of China reflect the degree of exposure of the population to Y. enterocolitica (Fukushima et al., 2001). It is generally accepted that during the great pandemics of plague some individuals did not succumb to the disease even though they were almost certainly exposed to the pathogen. In the light of our findings it is possible that prior exposure to Y. pseudotuberculosis (and possibly Y. enterocolitica) would have provided protection against plague.

ACKNOWLEDGEMENTS

The authors wish to thank the Bacterial Microarray Group, based at St Georges Hospital Medical School, and Dr Karen Isherwood for their help with the microarrays. This work was funded by the Ministry of Defence, UK.

REFERENCES


