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Characterization of a *dam* mutant of *Haemophilus influenzae* Rd

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The gene encoding Dam methyltransferase of *Haemophilus influenzae* was mutagenized by the insertion of a chloramphenicol-resistance cassette into the middle of the Dam coding sequence. This mutant construct was introduced into the *H. influenzae* chromosome by transformation and selection for Cam^R transformants. The authors have shown that several phenotypic properties, resistance to antibiotics, dyes and detergent as well as efficiency of transformation, depend on the Dam methylation state of the DNA. Although the major role of the methyl-directed mismatch repair (MMR) system is to repair postreplicative errors, it seems that in *H. influenzae* its effect is more apparent in repairing DNA damage caused by oxidative compounds. In the *dam* mutant treated with hydrogen peroxide, MMR is not targeted to newly replicated DNA strands and therefore mismatches are converted into single- and double-strand DNA breaks. This is shown by the increased peroxide sensitivity of the *dam* mutant and the finding that the sensitivity can be suppressed by a *mutH* mutation inactivating MMR. In the *dam* mutant treated with nitrofurazone the resulting damage is not converted into DNA breaks but the high sensitivity is also suppressed by a *mutH* mutation.

INTRODUCTION

DNA methylation carried out by DNA methyltransferases (MTases) is widespread in prokaryotes and eukaryotes (Bestor, 2000; Cheng & Roberts, 2001). In eukaryotes, this process is essential for controlling transcription, genomic imprinting, developmental regulation, mutagenesis, DNA repair, and chromatin organization. In bacteria, methylation is primarily associated with cognate restriction endonucleases (Ahmad & Rao, 1996), serving to protect the cell's own DNA from degradation. Some MTases, called solitary MTases, modify DNA for purposes distinct from restriction. The best-known enzymes in this group are Dam of *Escherichia coli* (Herman & Modrich, 1981) and M.CcrMI (Reisenauer *et al.*, 1999; Kossykh & Lloyd, 2004). The Dam MTase methylates adenosine residues in 5′-GATC-3′ sequences using *S*-adenosylmethionine as the methyl donor (Lacks & Greenberg, 1977); it is found in diverse groups of *Proteobacteria* (Marinus, 1996; Torreblanca & Casadesus, 1996; Reisenauer *et al.*, 1999). Dam MTase plays a role in several main cell functions: (i) regulation of gene expression through changes in the efficiency of transcription, (ii) DNA repair processes, and (iii) integrity of the bacterial genome.

Abbreviations: 2-AP, 2-aminopurine; EMS, ethyl methanesulfonate; MMR, methyl-directed mismatch repair; MTase, methyltransferase; 8-oxoG, 7,8-dihydro-8-oxoguanine.

Recent studies using a high-density DNA microarray of 4097 *E. coli* genes showed that the expression, as measured by the level of transcription, of the majority of genes was affected by *dam* deficiency (Oshima *et al.*, 2002). Genes involved in aerobic respiration, stress and SOS responses, and amino acid and nucleic acid metabolism were expressed at higher levels in the mutant cells. In contrast, transcription of genes participating in anaerobic respiration, flagellar biosynthesis, chemotaxis and motility was decreased in the *dam* mutant strain under both aerobic and microaerobic conditions. It was proposed that Dam-mediated methylation plays an important role in the global regulation of genes, particularly those for fumarate nitrate reduction (Fnr) and catabolite activator protein binding site (CRP). These observations could explain previous observations that the Dam DNA MTase regulates such cell functions as chromosome replication (Messer *et al.*, 1985; Russell & Zinder, 1987), phase variation (Bucci *et al.*, 1999; Cantalupo *et al.*, 2001) or synthesis of fimbriae (Blyn *et al.*, 1990).

Methyl-directed mismatch repair in *E. coli* (MMR, MutHLS system) appears to remove mispaired and unpaired bases from newly synthesized DNA strands, thus correcting replication errors (Modrich, 1987; Hsieh, 2001). The discrimination between the parental and newly synthesized strand is based on the transient undermethylation of GATC sequences in nascent strands (Low *et al.*, 2001, Friedberg, 2003). In the absence of the Dam methylation an increased rate of spontaneous and induced mutation

is observed (Modrich, 1987). MMR requires the presence of MutH, MutL, MutS and MutU (helicase DNA II) proteins. MutS recognizes the replication error in the DNA and binds to it. MutL helps to make the complex between MutH and MutS, and tracks this complex to the nearest 5′-GATC-3′ sequence. MutH makes a scision in the newly replicated unmethylated DNA strand within the 5′-GATC-3′ sequence, helicase separates the two strands while one of the four possible exonucleases removes nucleotides from the point of scission beyond the mismatch. Finally, the polymerase synthesizes the missing nucloetides and the ligase joins the strand (Modrich, 1987; Yang, 2000; Hsieh, 2001; Friedberg, 2003). Recently it was also shown that the MMR system of *E. coli* can prevent oxidative mutagenesis either by removing 7,8-dihydro-8-oxoguanine (8-oxoG) directly or by removing adenine misincorporated opposite 8-oxoG, or both (Wyrzykowski & Volkert, 2003). It was also shown that Dam methylation plays a role in the integrity of the bacterial chromosome, regulating transposition of insertion elements (Roberts *et al.*, 1985), conjugation (Camacho & Casadesus, 2002) and recombination (Stambuk & Radman, 1998).

Haemophilus influenzae is an obligate commensal of the upper respiratory tract that has the potential to cause diseases such as otitis and meningitis. The *H. influenzae* strain Rd genome sequence contains homologues (67–84 % similarity) of all of the known *E. coli* MMR genes (see The Institute for Genomic Research microbial database at http://www.tigr.org), indicating that *H. influenzae* has a fully functional MMR system. Although a genome-scale analysis for identification of genes required for *H. influenzae* Rd growth and survival suggested that the *dam* gene could be essential for cell viability (Akerley *et al.*, 2002), a viable *dam* mutant was obtained (Bayliss *et al.*, 2002, 2004). It was shown that this mutant, depending on marker tested, either does not exhibit higher spontaneous mutation rates, or exhibits only a three- to fourfold increase compared to the wild-type. These authors have also shown that although lack of Dam methylation destabilizes the 5′-AT tracts (Bayliss *et al.*, 2004), it does not influence the phase variation of the pilin. In *E. coli*, Dam methylation alone and as a part of the MMR system plays a much broader role than guarding against the introduction of mutations into the genome and in stabilizing dinucleotide tracts. To learn more about the role of Dam methylation in *H. influenzae* we first tested its effect on gene expression and its role in the maintenance of chromosome integrity. Assuming that changes of the phenotypic properties of *H. influenzae dam* mutant should reflect changes in the expression of the genes, we then tested some of the phenotypic properties that are governed by a wide spectrum of genes, such as sensitivity to different antibacterial agents. We were also interested to know whether, as in *E. coli*, the MMR system plays a role in defence against DNA damage by oxidative agents. Our results extend our understanding of the influence exerted by Dam methylation on gene expression and as a defence system against such damage.

METHODS

Bacterial strains, plasmids, and growth conditions. *H. influenzae* Rd30 (Glover & Piekarowicz, 1972), Rd30dam4 (this study), Rd30Nov (this study), Rd30 *mutH*::Tet^R (Bayliss *et al.*, 2002) and Rd30dam4 *mutH*::Tet^R (this study) were grown at 37 °C in BHI (Difco) supplemented with 2 μg NAD ml^{-1} and 10 μg haemin ml^{-1} (sBHI) as described by Barcak *et al.* (1991). *E. coli* strains ER1944, GM2163 *dam dcm* Cm^R and ER1470 (New England Biolabs) were grown in Luria–Bertani broth (LB) at 37 °C. If required, the media contained antibiotics or other supplements as detailed later in the text. Plasmid pMPMT4INT is a derivative of pMPMT4Ω (Mayer, 1995); pUC19 and pK19 were obtained from Dr D. C. Stein (Department of Cell Biology and Molecular Genetics, University of Maryland). The construction of the pMPMT4INT::Dam was described previously (Bujnicki *et al.*, 2001).

Inactivation of DNA metabolism genes. Cloning of the gene encoding the Dam MTase into pMPMT4INT was described previously (Bujnicki *et al.*, 2001). The R.NdeI-R.SmaI fragment encoding this gene was introduced into pUC19 plasmid DNA on the same restriction sites. This plasmid was linearized with R.EcoRV and ligated to an R.SmaI fragment carrying a chloramphenicol-resistance cassette (derived from plasmid pK19). The R.EcoRV site within the cloned *dam* gene is located 220 bp from the start codon. The plasmid construct was checked by restriction digestion and sequencing the ends of the inserts, using primers that generate sequences across the multiple cloning site. This plasmid was linearized by digestion with R.Cfr10I and used to transform competent *H. influenzae* Rd30 cells. Transformants were selected on sBHI plates containing 2 μg chloramphenicol ml^{-1}. One *dam*::Cam^R strain, designated *H. influenzae* Rd30dam4, was chosen for further studies. The *dam mutH* double mutant of *H. influenzae* Rd30 was constructed by transforming *H. influenzae* Rd30dam4 with chromosomal DNA prepared from *H. influenzae* Rd *mutH*::Tet^R (Bayliss *et al.*, 2002), and selecting transformants on sBHI plates containing 3 μg tetracycline ml^{-1}. One of the Tet^R Cam^R transformants, designated *H. influenzae* Rd30dam4mutH, was used for further studies.

DNA manipulations and cloning. All general techniques followed protocols described for the two host organisms – *E. coli* (Sambrook *et al.*, 1989) and *H. influenzae* (Barcak *et al.*, 1991). Isolation of plasmids and chromosomal DNA, restriction analysis of DNA, cloning of DNA fragments and PCR were done by standard procedures (Sambrook *et al.*, 1989).

Transformation and transfection assays. Competent cells were prepared by the anaerobic–aerobic method and transformed with 1 μg chromosomal DNA as described by Barcak *et al.* (1991). Transformation frequency was calculated as the number of novobiocin-resistant colonies divided by the total number of colonies. For transfection, competent cells were exposed to 0·1 μg HP1 phage DNA according to Jablonska & Piekarowicz (1976). The efficiency of transfection is expressed as number of plaque-forming units per 0·1 μg HP1 DNA.

Treatment of cells with antibiotics, dyes, detergents, 2-aminopurine (2-AP), ethyl methanesulfonate (EMS), hydrogen peroxide and nitrofurazone

The following experiments were done at least in triplicate with cultures that each started from a single colony.

(i) Determination of sensitivity to antibiotics, dyes and detergents. MICs were determined by serial twofold dilution in BHI medium, using 5×10^4 late-exponential-phase cells, grown in sBHI, as the inoculum. The concentration at which there was no visually detectable bacterial growth was taken as the MIC.

(ii) Treatment of cells with EMS and hydrogen peroxide. Frozen stocks were streaked onto sBHI plates containing, when necessary, 2 μg chloramphenicol ml^{-1}, and were grown overnight at 37 °C. Single colonies were then inoculated into sBHI medium. Cultures were grown with gentle aeration overnight and then diluted 1 : 50 in fresh sBHI containing, when necessary, 2 μg chloramphenicol ml^{-1}, and grown with aeration to mid-exponential phase. Samples (5 ml) were withdrawn and EMS or hydrogen peroxide was added to an appropriate final concentration. After 60 or 15 min of incubation, respectively, at 37 °C in a shaking incubator, the cells were centrifuged, washed with sBHI and suspended in the 5 ml sBHI. The samples were withdrawn and plated after appropriate dilution on sBHI agar containing, when necessary, 2 μg chloramphenicol ml^{-1}, and incubated for 24 h at 37 °C to determine survival. Of the remaining suspension treated with EMS, 2 ml was added to 5 ml pre-warmed sBHI and incubated for 4 h at 37 °C. The cells were then centrifuged, suspended in 1 ml BHI and appropriately diluted samples were plated on selective media (sBHI with antibiotics) and nonselective (viable count) media to determine the frequencies of resistant mutants.

(iii) Treatment of cells with 2-AP. Frozen stocks were streaked onto sBHI plates containing, when necessary, 2 μg chloramphenicol ml^{-1} and were grown overnight at 37 °C. Single colonies were then inoculated into sBHI medium. Cultures were grown with gentle aeration overnight, when necessary in the presence of 2 μg chloramphenicol ml^{-1}. Samples of 0·1 ml were withdrawn and placed in 5 ml sBHI in fresh culture vessels, and 2-AP from an aqueous stock solution (5 mg ml^{-1}) was added to a final concentration of 0, 10, 50 and 100 μg ml^{-1}. The cultures were aerated at 37 °C until the sample without 2-AP reached a titre of 2×10^9 (about 4 h). The cells of all samples were then harvested by centrifugation, washed twice in sBHI, and an appropriate dilution was plated on selective and nonselective media to determine the survival and frequencies of resistant mutants. Mutation frequencies were determined for at least five colonies of mutants. Mutation rates were estimated from these frequencies using the median value by the method of Drake (1991).

(iv) Treatment with nitrofurazone. Frozen stocks were streaked onto sBHI plates containing, when necessary, 2 μg chloramphenicol ml^{-1} and grown overnight at 37 °C. Single colonies were then inoculated into 5 ml sBHI medium. Cultures were grown with gentle aeration overnight, when necessary in the presence of 2 μg chloramphenicol ml^{-1}. Samples of 0·1 ml were withdrawn and placed in 5 ml sBHI in fresh culture vessels, and nitrofurazone from an aqueous stock solution (1 mg ml^{-1}) was added to a final concentration of 0, 0·25, 0·5 and 0·75 μg ml^{-1}. The cultures were aerated at 37 °C for 6 h. The cells of all samples were then harvested by centrifugation, washed twice in BHI, and suspended in 1 ml BHI. Appropriate dilutions were then plated on sBHI plates to determine survival.

Determination of nitroreductase activity. Frozen stocks were streaked onto sBHI plates containing, when necessary, 2 μg chloramphenicol ml^{-1} and were grown overnight at 37 °C. Single colonies were then inoculated into sBHI medium. Cultures were grown with gentle aeration overnight and then centrifuged (Sorvall SS-34, 6000 r.p.m., 15 min), washed and suspended in 1× PBS buffer prepared according to Sambrook *et al.* (1989). Cultures were centrifuged (6000 r.p.m., 30 min, 4 °C), then resuspended in 5 ml 100 mM Tris/HCl pH 7·5 and kept on ice. Cells were disrupted by sonication and the cell debris was removed by centrifugation at 10 000 r.p.m. for 30 min at 4 °C. The protein concentration was determined by the Lowry method (Smith *et al.*, 1987). The enzyme activity was determined spectrophotometrically at 365 nm. The sample for reaction assay contained 800 μl protein suspension at the same concentration, 100 μl 1 mM nitrofurazone and 100 μl 2 mM NADPH. The reaction was measured as absorbance decrease every 5 s for 1 min.

Determination of DNA degradation after treatment of cells with hydrogen peroxide and nitrofurazone. This was done according to Sisson *et al.* (2000). Overnight cultures of *H. influenzae* strains were diluted 1 : 50 in 5 ml fresh sBHI and grown to mid-exponential phase. The cultures were then treated with hydrogen peroxide (0·1 %, v/v) for different times or with 5·0 μg nitrofurazone ml^{-1} for 6 h. After treatment the cells were harvested by centrifugation, washed with fresh sBHI and resuspended in 200 μl TE buffer to give an OD_{600} value of 7·0 for all samples. Lysis of bacterial cells in low-melting-point agarose, DNA agarose gel electrophoresis under alkaline conditions, staining and visualization of the gel were done according to Sisson *et al.* (2000), using the same volume of prepared bacterial suspension.

One-step growth of phage HP1. The procedure for a single cycle of growth of HP1 in *H. influenzae* Rd30 was described previously (Jablonska & Piekarowicz, 1976).

RESULTS

Isolation of *dam* and *dam mutH* mutants of *H. influenzae*

Previously we cloned the *H. influenzae dam* gene and showed that it encodes an active Dam MTase (Bujnicki *et al.*, 2001). In order to construct the *dam* mutant of *H. influenzae* a chloramphenicol-resistance cartridge was introduced into the R.EcoRV restriction site found within the coding sequence of the *dam* gene, generating the plasmid pUC19 *dam*::Cm^R. This plasmid was then used to construct the *H. influenzae dam* strain (see Methods). The insertion of the chloramphenicol-resistance cassette into the *dam* gene was confirmed by PCR amplification of the *dam* region and restriction digestion analysis of the PCR amplicon (data not shown). Lack of a functional Dam MTase in the *H. influenzae* Rd30dam4 mutant was verified by restriction analysis of the chromosomal DNA isolated from the *H. influenzae* mutant. The data presented in Fig. 1 show that the wild-type chromosomal DNA is not cleaved by R.MboI but is cleaved by R.DpnI and R.Sau3AI, while the mutant DNA is cleaved by R.MboI and R.Sau3AI but not cleaved by R.DpnI (Fig. 1). The R.MboI enzyme will cleave the DNA only when the adenine residue within the sequence 5′-GATC-3′ is not methylated at the N-6 position, while R.DpnI will cleave only when the adenine residue within the sequence 5′-GATC-3′ is methylated. R.Sau3AI will cleave DNA within this sequence independently of the presence or absence of a methylated adenine residue within the sequence (Roberts & Macelis, 2000). The data presented in Fig. 1 clearly indicate that the isolated *H. influenzae* Rd30dam4 mutant had lost the ability to methylate the chromosomal DNA within the sequence 5′-GATC-3′.

Analysis of the *dam* mutant of *H. influenzae*

Bayliss *et al.* (2002, 2004) and we have shown that it is possible to construct viable *H. influenzae dam* mutants. The

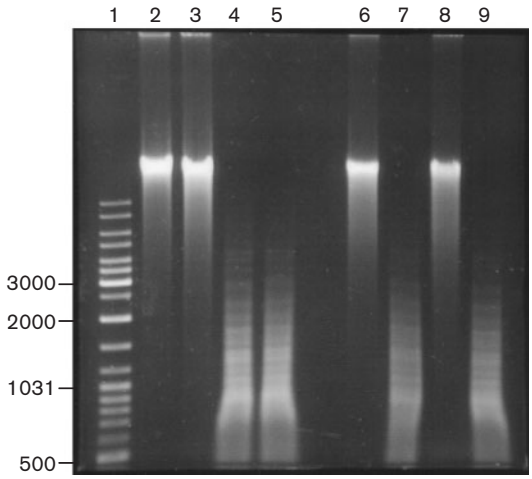

Fig. 1. Chromosomal DNA treatment by restriction endonucleases indicating lack of Dam DNA methylation. Lane 1, size standard (GeneRuler DNA Ladder Mix from MBI Fermentas). Lanes 2–5, chromosomal DNA from Rd30: lane 2, with no restriction endonuclase; lane 3, cleaved by R.MboI; lane 4, cleaved by R.DpnI; lane 5, cleaved by R.Sau3AI. Lanes 6–9, chromosomal DNA from Rd30dam4: lane 6, with no restriction endonuclease; lane 7, cleaved by R.MboI; lane 8, cleaved by R.DpnI; lane 9, cleaved by R.Sau3AI.

H. influenzae dam mutant isolated by us showed under aerobic growth conditions a decrease of the doubling time of about 30 % compared to the wild-type strain Rd, and under low-oxygen conditions up twofold longer doubling (data not presented). Microscopic examination of the morphology of cells grown under aerobic and low-oxygen conditions showed only a small increase in the numbers of filamentous cells of the *dam* mutant in comparison with the wild-type.

In *E. coli* the expression of most of the genes is changed in *dam* mutant cells (Oshima *et al.*, 2002), which can influence their phenotypic properties. Thus, the observed changes of phenotypic properties of a *dam* mutant should reflect the changes in transcription level of particular genes. To test the influence of the lack of Dam methylation in *H. influenzae* we examined changes in some of the phenotypic properties regulated by a broad spectrum of gene products.

Effect of *dam* mutation on the drug-efflux transporter system

Genomic sequencing of *H. influenzae* has identified a three-gene complex that is homologous to the *acrRAB* multidrug-resistance efflux transporters of *E. coli* (Fleischmann *et al.*, 1995). The disruption of these multidrug-efflux genes causes hypersusceptibility of *H. influenzae* to several antibiotic and dyes (Sanchez *et al.*, 1997). The data presented in Table 1 indicate that the presence of a *dam* mutation made *H. influenzae* Rd30dam4 more susceptible to several compounds tested by Sanchez *et al.* (1997): antibiotics (erythromycin, tetracycline, kanamycin, spectinomycin), dyes (crystal violet, Congo red) and a detergent (SDS). The *dam* mutant showed in most cases not only a lower MIC, but also increased susceptibility to higher concentrations of the compounds. For example, the efficiency of plating (EOP) of the wild-type strain on medium with 2 μg kanamycin ml^{-1} was 10^{-4} while that of the mutant strain was 10^{-8}.

Table 1. MICs of antibiotics, dyes and detergents estimated for *H. influenzae* Rd30 and Rd30dam4

Substance	MIC (μg ml^{-1})	
	Rd30 (WT)	Rd30dam4
Erythromycin	2·5	0·2
Rifampicin	0·6	0·4
Tetracycline	0·5	<0·2
Vancomycin	100	25
Kanamycin	1·0	<0·1
Crystal violet	3·0	0·2
Congo red	100	2·0
SDS	20·0	6·25

Influence of Dam activity on transformation and transfection

H. influenzae is capable of taking DNA from its environment and integrating it via recombination into the bacterial chromosome. When linear bacteriophage DNA is transfected into wild-type cells, viable phage particles are produced (Notani & Setlow, 1974). Whereas several genes have been shown to be required for transformation and transfection in *H. influenzae* (Beattie & Setlow, 1971; Williams *et al.*, 1994; Dougherty & Smith, 1999), the *dam* gene has not been implicated in this process. Most of these mutants show a defect in DNA binding and uptake, reflecting probable changes in the surface of the bacteria. If the lack of Dam MTase activity affected the expression one of these genes, the *H. influenzae dam* mutant should show decreased frequency of transformation. The results presented in Table 2 show that the frequency of transformation of a chromosomal marker is decreased only about 10-fold in comparison to the wild-type, while the transfection efficiency is decreased more than 200-fold. Deschavanne & Radman (1991) showed that the transfection efficiency of phage ϕX174 DNA containing GATC sites is much lower in an *E. coli dam* mutant than in wild-type cells and that the wild-type level was restored in *dam mutH* mutants. It was assumed that the Dam methylation protects the DNA containing the GATC sites from degradation by MutH endonuclease activity. However, the efficiency of transfection of HP1 DNA is the same in both *dam* and *dam mutH H. influenzae* mutants (Table 2). The difference between the efficiency of transfection of the HP1 phage DNA and the efficiency of transformation may be due to the fact

Table 2. Comparison of transformation and transfection values for *H. influenzae* Rd30, Rd30dam4 and Rd30dam4mutH

Strain	Transformation*†	Transfection*‡
Rd30 (WT)	$1{\cdot}1 \times 10^{-3}$	$3{\cdot}4 \times 10^{3}$
Rd30dam4	$1{\cdot}4 \times 10^{-4}$	$1{\cdot}3 \times 10^{1}$
Rd30dam4mutH	$1{\cdot}2 \times 10^{-4}$	$1{\cdot}5 \times 10^{1}$

*Each value is the mean of three determinations.
†Values are presented as transformation frequencies (antibiotic-resistant c.f.u./total c.f.u.).
‡Values are presented as transfection frequencies (no. of p.f.u. per 0·3 μg phage HP1 DNA).

Table 3. Single-cycle growth of HP1 phage in *H. influenzae* Rd30 and Rd30dam4

	Rd30 (WT)	Rd30dam4
No. of bacteria ml^{-1}	$4{\cdot}5 \times 10^{8}$	$4{\cdot}8 \times 10^{8}$
No. of phage ml^{-1}	$5{\cdot}0 \times 10^{7}$	$5{\cdot}0 \times 10^{7}$
M.o.i.	0·1	0·1
Adsorption	70 %	70 %
No. of infective centres	$2{\cdot}0 \times 10^{7}$	$2{\cdot}0 \times 10^{7}$
Progeny phage	$1{\cdot}5 \times 10^{9}$	$1{\cdot}2 \times 10^{8}$
Mean burst size	70	7

that the propagation of the wild-type HP1 phage is about 10-fold lower in the *dam* mutant than in the wild-type of *H. influenzae* (Table 3).

Effect of *dam* mutation on mutation frequency

A major role of the methyl-directed mismatch repair (MMR) in *E. coli* and other bacteria is to initiate the repair of postreplicative errors (Hsieh, 2001). *H. influenzae* is not mutable to any significant degree by UV or X-radiation, methyl methanesulfonate, or by nitrogen mustard, presumably owing to the absence of an error-prone repair system (Kimball *et al.*, 1977). However, ethyl methanesulfonate (EMS), which does not induce the SOS system and error-prone repair system, is an effective mutagen (Barcak *et al.*, 1991). The similar MIC for rifampicin and novobiocin of the *H. influenzae* wild-type and *dam* mutant cells allowed us to use the resistance to them as markers to determine the frequency of mutation. Compared to the wild-type, the frequency of mutation to novobiocin and rifampicin resistance was increased only four- to sixfold (Table 4). The *dam* mutant was only slightly more sensitive to EMS than the wild-type; in the presence of 0·5 % EMS, the survival of the *dam* mutant was 40 % (wild-type 50 %), while in the presence of 1 % EMS, its survival was 25 % (wild-type 35 %). However, the frequency of induced mutations was increased only three- to fivefold over the spontaneous frequency. For example, in the presence of 0·5 % EMS the mean numbers of mutants was about 1×10^{2} per 10^{9} wild-type cells compared to $2{\cdot}5 \times 10^{2}$ per 10^{9} *dam* mutant cells.

2-AP is a mutagen causing A:T to G:C transitions in prokaryotic systems. The *dam* mutant showed very high sensitivity to 2-AP at the concentration of 10 μg ml^{-1}: after 4 h, the survival rate was less than 10^{-5} compared to the 100 % survival of the wild-type at the same concentration (Fig. 2a). However, as in the case of EMS, the frequency of 2-AP induced mutations was increased only threefold over the spontaneous frequency (data not shown). The *dam* mutant was also slightly more sensitive to mitomycin C than the wild-type (data not shown).

Sensitivity of the *H. influenzae dam* mutant to nitrofurazone and hydrogen peroxide

In *E. coli*, resistance to multiple environmental hazards such as antibiotics, disinfectants and oxidative stress is regulated by the MarA, SoxS and Rob global regulator systems, which modulate the expression of a large number of genes (Barbosa & Levy, 2002). The biological activity of nitro-substituted compounds is derived from reductive metabolism of the parent compound's nitro moiety, a process catalysed by a variety of nitroreductase activities (Bryant & DeLuca, 1991). The end products of metabolism are biologically inactive; the biological activities of these chemicals are due to the reactivities of short-lived intermediates with proteins and DNA (Whiteway *et al.*, 1998). In *Helicobacter pylori* and *E. coli*, nitrofurans (nitrofurazone, nitrofurantoin and furazolidone) exhibit antimicrobial activity but are only weakly mutagenic, and none of them cause DNA breakage (Sisson *et al.*, 2002). The data presented in Fig. 2(b) show that the *H. influenzae dam* mutant

Table 4. Spontaneous mutability of *H. influenzae* Rd30 and Rd30dam4

Antibiotic (μg ml^{-1})	Mutation frequency/10^{9} c.f.u.*		Mutant/wild-type ratio
	Rd30 (WT)	Rd30dam4	
Novobiocin (0·25)	3·4 ± 0·5	18·7 ± 4·2	5·5
Rifampicin (5·0)	32·1 ± 0·4	146·8 ± 3·1	4·5

*Mean frequency of rifampicin- and novobiocin-resistant mutants in *H. influenzae* populations after 48 h growth. Each value is the mean of four replicate experiments.

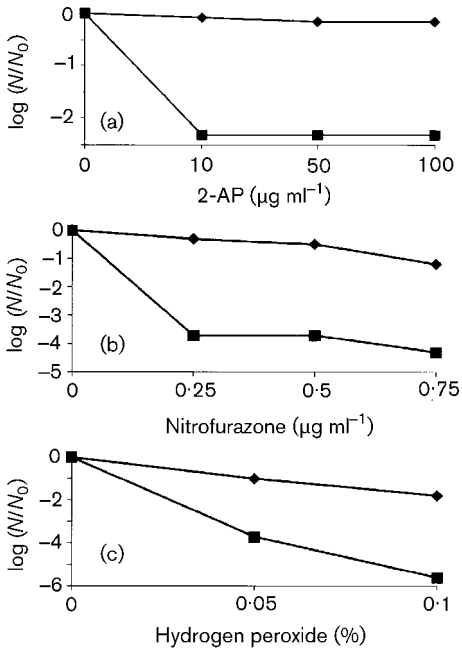

Fig. 2. Effect of 2-AP treatment (a), nitrofurazone (b) and hydrogen peroxide (c) on survival of the wild-type (◆) and the *dam* mutant (■) of *H. influenzae*. N_0 is the viable count and N is the number of c.f.u. ml^{-1} after each treatment.

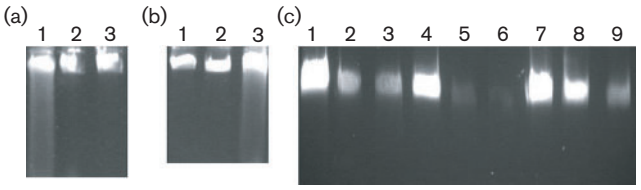

Fig. 3. Effect of *in vivo* action of hydrogen peroxide and nitrofurazone on the degradation of chromosomal DNA. Experiments were carried out as described in Methods. (a) *H. influenzae* Rd30: lane 1, cells treated with 0·1 % hydrogen peroxide for 15 min; lane 2, cells treated with 2 μg nitrofurazone ml^{-1} for 6 h; lane 3, cells treated with 5 μg nitrofurazone ml^{-1} for 6 h. (b) *H. influenzae* Rd30dam4: lane 1, cells treated with 2 μg nitrofurazone ml^{-1} for 6 h; lane 2, cells treated with 5 μg nitrofurazone ml^{-1} for 6 h; lane 3, cells treated with 0·1 % hydrogen peroxide for 15 min. (c) Lane 1, *H. influenzae* Rd30 cells not treated; lane 2, Rd30 cells treated with 0·05 % hydrogen peroxide for 30 min; lane 3, Rd30 cells treated with 0·1 % hydrogen peroxide for 30 min; lane 4, *H. influenzae* Rd30dam4 cells not treated; lane 5, Rd30dam4 cells treated with 0·05 % hydrogen peroxide for 30 min; lane 6, Rd30dam4 cells treated with 0·1 % hydrogen peroxide for 30 min; lane 7, *H. influenzae* Rd30dam4mutH cells not treated; lane 8, Rd30dam4mutH cells treated with 0·05 % hydrogen peroxide for 30 min; lane 9, *H. influenzae* Rd30dam4mutH cells treated with 0·1 % hydrogen peroxide for 30 min.

is much more sensitive to nitrofurazone (by several orders of magnitude) than the wild-type cells. Treatment with nitrofurazone is not mutagenic (data not presented) and does not cause the fragmentation of DNA (Fig. 3). The nitroreductase activity in Rd30 and Rd30dam4 was tested and both strains showed a comparable level of nitroreductase activity (data not shown). Strong sensitivity of the *dam* mutant strain to nitro-substituted compounds suggested that it may be sensitive to other reactive oxygen species like hydrogen peroxide. Hydrogen peroxide is relatively stable but reacts with Fe^{2+} to produce highly reactive –OH radicals (Wyrzykowski & Volkert, 2003; Imlay *et al.*, 1988). These radicals can react with DNA to produce a variety of DNA lesions and cause the degradation of DNA (Sisson *et al.*, 2002). We found that *H. influenzae dam* mutant cells are more sensitive to hydrogen peroxide treatment than are the wild-type cells (Fig. 2c), and such treatment causes the fragmentation of DNA (Fig. 3). These differences between the wild-type and mutant in the degradation of DNA are especially apparent after a longer time of incubation with hydrogen peroxide, when degradation probably leads to low-molecular-mass DNA fragments, manifested as a disappearance of the DNA (Fig. 3).

Sensitivity of the *dam* mutant to peroxide and nitrofurazone sensitivity is mediated by the MMR system

In *E. coli*, reactions with the DNA of the free radicals generated after treatment of the cells with hydrogen peroxide lead to production of 8-oxoG. This predominant oxidative lesion is readily bypassed by DNA polymerase and has little effect on cell viability (Henderson *et al.*, 2002). It was recently shown that this lesion is repaired not only by MutM or MutY but also by the MMR system of *E. coli* (Wyrzykowski & Volkert, 2003). It was postulated that in *E. coli* the DNA containing oxidized bases will acquire single-strand breaks if they are repaired by MMR. In a *dam* mutant strain, DNA containing 8-oxoG acquires not only single- but also double-stranded breaks, which would result in increased sensitivity to hydrogen peroxide treatment (Wyrzykowski & Volkert, 2003). Since *H. influenzae* also encodes homologous of MutM and MutY (Fleischmann *et al.*, 1995), the same system of repair as in *E. coli* may operate in these cells. To check whether in *H. influenzae* the oxidative lesions generated by hydrogen peroxide and nitrofurazone are repaired by MMR we tested whether the sensitivity to these compounds can be suppressed by a mutation inactivating MMR. The results (Fig. 4) demonstrate that a *dam mutH* strain is much more resistant to hydrogen peroxide than the *dam* single mutant and even slightly more resistant to it than the wild-type. The restoration of the wild-type level of sensitivity to hydrogen peroxide is also manifested by the degradation of DNA (Fig. 3c). The double mutant *dam mutH* is much more resistant to nitrofurazone than the *dam* mutant alone but still more sensitive than the wild-type cells (Fig. 4). These results suggest then that in *H. influenzae* MMR can participate in the repair of the oxidative DNA damage.

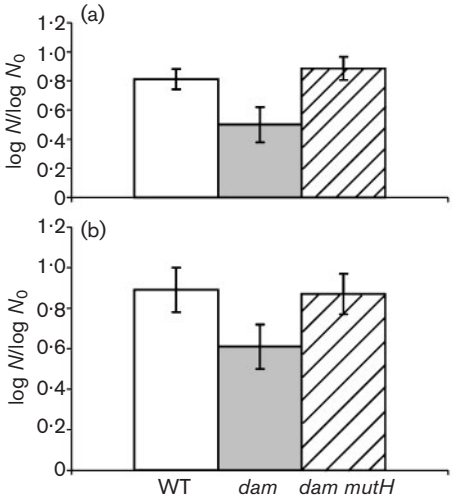

Fig. 4. MutH supression of Dam-mediated sensitivity to hydrogen peroxide (a) and nitrofurazone (b). The cells were treated with hydrogen peroxide (0·1 %) for 15 min or with nitrofurazone (0·75 μg ml^{-1}) for 6 h. The survival of the cells was measured as log (no. of resistant cells)/log (total no. of cells) (log *N*/log N_0). White bars, wild-type Rd30; grey bars, Rd30dam4; hatched bars, Rd30dam4mutH.

DISCUSSION

Recent experimental data (Oshima *et al.*, 2002; Lobner-Olesen *et al.*, 2003) indicate that the lack of Dam methylation in *E. coli* influences the expression of a variety of genes. The impact of the expression on such a broad diversity of genes could be the reason for the loss of pathogenic properties in *dam* mutants of *Salmonella typhimurium* (Torreblanca & Casadesus, 1996) or lethality of such mutations in *Vibrio cholerae* and *Yersinia pseudotuberculosis* (Julio *et al.*, 2001). As shown by Bayliss *et al.* (2002, 2004) and this paper it is possible to obtain viable *dam* mutants of *H. influenzae*. We have shown that in *H. influenzae* the lack of Dam methylation influences the expression of a variety of genes as indicated by hypersensitivity to different antibiotics and dyes, or decrease in the efficiency of transformation and transfection of a *dam* mutant and the decrease of HP1 phage growth.

H. influenzae has three different genes homologous to *E. coli acrRAB*, as well as homologues for *emrAB* and *bcr*, disruption of which leads to changes in the structure of the multidrug efflux pumps resulting in hypersusceptibility to different antibiotics and dyes (Sanchez *et al.*, 1997; Ma *et al.*, 1993). In an *E. coli dam* mutant strain the *acrRAB* genes showed a decreased level of transcription (Oshima *et al.*, 2002). The change in the expression of these genes in the *H. influenzae dam* mutant could lead to different levels of the particular gene products building the drug efflux complex. Different levels of these proteins could result in changes of the structure of the drug efflux complex and the sensitivity to the same group of antibiotics and dyes tested previously (Sanchez *et al.*, 1997). The observed decrease in efficiency of transformation and transfection could also be explained by changes in the expression of many genes involved in DNA binding and uptake in *H. influenzae* (Dougherty & Smith, 1999). It was observed that the deletion of one gene from among *mtrR*, *acrA* and *acrB* results in a relatively less drastic effect on transformation (Dougherty & Smith, 1999) than changes in the other genes responsible for DNA binding and uptake and recombination. The much higher effect of the *dam* mutation on transfection than on transformation cannot be explained by the higher susceptibility of the transfecting phage DNA to the action of MutH nuclease since the efficiency of transfection is the same in *dam* and *dam mutH* mutants. It can be explained, however, by the fact that phage HP1 gives about 10 times fewer progeny phages in the *dam* mutant than in the wild-type. Why the phage growth in the *dam* mutant is affected by the lack of Dam methylation is not known at present. All these results suggest then that in *H. influenzae*, as in *E. coli*, the Dam methylation alters the expression of a great variety of genes.

The principal effect of Dam MTase activity in the γ subdivision of *Proteobacteria* is to prevent spontaneous and induced mutation. This implies that the Dam enzyme plays a role in differential strand tagging at the replication fork for methyl-instructed mismatch repair of newly synthesized DNA (Hsieh, 2001). The mutation frequency in our mutant is similar to the frequency in the *H. influenzae dam* mutant isolated by Bayliss *et al.* (2004). The *dam* mutant of *H. influenzae* described here shows only a moderate increase of spontaneous mutability and mutability after treatment with EMS. This phenotype is very similar to that of *dam* mutants of *E. coli* (Palmer & Marinus, 1994), *S. typhimurium* (Torreblanca & Casadesus, 1996) or *Serratia marcescens* (Ostendorf *et al.*, 1999), suggesting a similar role.

Our mutant is also very sensitive to nitrosubstituted compounds, whose mutagenic and antimicrobial activity is caused by short-lived intermediates formed during their reduction by nitroreductases (Whiteway *et al.*, 1998), and to hydrogen peroxide, which produces free radicals. It was observed (Wyrzykowski & Volkert, 2003) that in an *E. coli dam* mutant sensitivity to hydrogen peroxide is due to conversion of oxidative lesions to strand breaks by MMR since mutation in the *mutH* or *mutS* genes restores peroxide resistance. Similarly, the MMR system of *H. influenzae* seems also to be responsible for the action on DNA containing oxidized bases. This conclusion is based on the fact that hydrogen peroxide induces breaks in a *dam* mutant and that mutants carrying *dam* and *mutH* mutations restore the wild-type level of sensitivity to this compound.

H. influenzae encodes only one oxygen-insensitive nitroreductase, a homologue of *E. coli nfnB* gene. Upregulation of the activity of this nitroreductase in the *dam* mutant could be responsible for its extreme sensitivity to nitrofurazone. However, the fact that the level of the enzyme in the *dam* mutant is the same as in the wild-type cells argues

against this explanation. Moreover, the disruption of the *nfnB* (also called *nfsB*) gene in *E. coli* results in only a slight increase (6–19 %) in susceptibility to nitrofurantoin or nitrofurazone, while the *dam* mutant of *H. influenzae* is several orders of magnitude more sensitive. Thus, it seems that the extreme sensitivity of the *dam* mutant of *H. influenzae* to nitrofurazone is not mediated through changes in the regulation of *nfnB*.

Similar to the situation in *E. coli* and *Campylobacter jejuni* (Sisson *et al.*, 2002), nitrofurazone does not produce DNA single- or double-stranded breaks in the chromosome of *H. influenzae* and is not mutagenic. We do not know what type of lesions are induced in the DNA by nitrofurazone or what type of nucleotide is affected by its action. Independently of the type of lesions produced by nitrofurazone, sensitivity of the *dam* mutant is at least partially due to the MMR system. This conclusion is based on the observation that the double mutant *dam mutH*, which has an inactive MMR system, is much more resistant to nitrofurazone than the *dam* single mutant. Since the action of MMR on DNA damaged by the oxidative product derived from nitrofurazone is influenced by the *dam* mutation, and the hemimethylated state required for MMR (Palmer & Marinus, 1994), it is possible that the role of MMR is to immediately repair the products of misreplication past oxidative lesions. Recently, it has been shown that incorporated 8-oxo-dGMP derived from the dNTP pool is effectively removed by the yeast MMR system (Colussi *et al.*, 2002) from the newly synthesized DNA strand. These authors suggested that the role of MMR is to immediately repair the products of misreplication past oxidative lesions and to remove 8-oxoG incorporated by the replication machinery. In this process the yeast MMR initiates correction of the A-containing daughter strand at 8-oxoG : A pairs formed during replication. A similar system may operate in *H. influenzae*. We propose that in wild-type *H. influenzae* the MMR recognizes the mispaired bases caused by the treatment of cells with nitrofurazone. MutH makes scissions in the non-methylated newly replicated strand containing, for example, 8-oxoG, incorporated into the daughter strand opposite template A. After the scission is completed, the MMR removes 8-oxoG incorporated during replication, which involves a relatively short patch within the replication fork. Due to such a mechanism we cannot observe the DNA degradation products. In the absence of Dam methylation MutH makes scissions not precisely in proximity to mispaired bases but more randomly, which in consequence blocks the replication of DNA and causes the cell's death.

In conclusion, the presence of a *dam* mutation in the *H. influenzae* genome is not lethal under laboratory conditions. However, the resulting sensitivity to hazardous compounds, and increased sensitivity to antibiotics and dyes, may make the effect of this mutation lethal under natural conditions, as this pathogen colonizes the upper respiratory tract and invades the respiratory mucous membranes.

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