The role of Dam methylation in phase variation of *Haemophilus influenzae* genes involved in defence against phage infection

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Haemophilus influenzae uses phase variation (PV) to modulate the activity of its defence systems against phage infection. The PV of the restriction–modification (R–M) system HindI, the main defence system against phage infection and incoming chromosomal and phage DNA in *H. influenzae* Rd, is driven by changes of the pentanucleotide repeat tract within the coding sequence of the *hsdM* gene and is influenced by lack of Dam methylation. Phase-variable resistance/sensitivity to phage infection correlates with changes in lipooligosaccharide (LOS) structure and occurs by slippage of tetranucleotide repeats within the gene *lic2A*, coding for a step in the biosynthesis of LOS. The lack of Dam activity destabilizes the tetranucleotide (5′-CAAT) repeat tract and increases the frequency of switching from sensitivity to resistance to phage infection more than in the opposite direction. The PV of the *lgtC* gene does not influence resistance or sensitivity to phage infection. Insertional inactivation of *lic2A*, but not *lgtC* or *lgtF*, leads to resistance to phage infection and to the same structure of the LOS as observed among phase-variable phage-resistant variants. This indicates that in the *H. influenzae* Rd LOS only the first two sugars (Glc–Gal) extending from the third heptose are part of bacterial phage receptors.

INTRODUCTION

Successful infection by bacteriophages requires attachment to a susceptible host cell, injection of the phage nucleic acid, transcription, translation, replication of genetic information and, finally, release of infectious progeny. Resistance to phage infection is due to the lack of a bacterial receptor, which blocks phage adsorption on the bacterial surface and results in complete loss of ability to generate phage progeny; the presence of an active restriction–modification (R–M) system can decrease the chance of productive infection but rarely stops infection completely. *Haemophilus influenzae* Rd encodes several active R–M systems (Roberts & Macelis, 2001) of which only HindI (a type I R–M system) is active against HP1c1 phage infection (Glover & Piekarowicz, 1972) and plays an important role in protection against foreign DNA uptake during transformation (Piekarowicz *et al.*, 1974).

A characteristic feature of *H. influenzae* is the ability to switch between genetic variants of alternative phenotypes with high frequency (Hood *et al.*, 1996b; Bayliss *et al.*, 2002, 2004). The phase variation (PV) of the R–M system and resistance/sensitivity to phage infection could modulate the

defence against phage infection without loss of the ability to acquire foreign DNA through horizontal transfer mediated by transformation or transfection. Previously it was shown (Glover & Piekarowicz, 1972) that the type I R–M phenotype of *H. influenzae* and the gene *mod*, a part of the type III R–M system of *H. influenzae*, are subject to reversible, high-frequency ON/OFF PV (De Bolle *et al.*, 2000). However, nothing is known about the PV of resistance/sensitivity to phage infection.

We do not know the cell structures of *H. influenzae* responsible for adsorption of HP1c1 or other *H. influenzae* phages. Bacterial phage receptors are present in the outer-membrane proteins, or the lipopolysaccharide (LPS) (Nesper *et al.*, 2000; Kutter *et al.*, 2005). In *H. influenzae* the particular surface-exposed epitopes of LOS are subject to high-frequency PV (Hood *et al.*, 1996a; Patrick *et al.*, 1987; Weiser *et al.*, 1989; Bayliss *et al.*, 2002; De Bolle *et al.*, 2000), as are the outer-membrane proteins (Bayliss *et al.*, 2004). PV is an adaptive mechanism that is advantageous for survival of bacteria confronted by differing microenvironments and immune responses of the host (Hood *et al.*, 2004).

The process of switching surface antigens (PV) is driven by highly mutable loci, called contingency loci, which generate repertoires of phenotypic variants. There are many mechanisms of hypermutation in contingency loci, but one of the commonest is mediated by changes in the number of

Abbreviations: Hep, heptose; LOS, lipooligosaccharide(s); MMR, mismatch repair; MTase, methyltransferase; PV, phase variation; R–M, restriction–modification.

tetranucleotide repeats within the coding sequence of a gene (Hood *et al.*, 1996b; Moxon *et al.*, 1994; Bayliss *et al.*, 2001, 2002). PV also involves alterations in the number of 5'-TA repeats located between the -10 and -35 promoter elements of the overlapping, divergently orientated promoters of *hifA* and *hifBCDE*, whose products mediate the biosynthesis and assembly of *H. influenzae* pili (Bayliss *et al.*, 2004). The rate of PV of genes containing tetranucleotide repeats is influenced by mutations in *polI* but not genes responsible for mismatch repair, MMR (i.e. *mutS*, *mutL*, *mutH*, *dam*), or other DNA repair pathways (i.e. *mfd* and *recA*) (Bayliss *et al.*, 2002). On the other hand, it was shown that the MMR system is active on dinucleotide repeat tracts on artificially constructed repeat regions connected to reporter genes, but does not affect 5'-TA-mediated pilin PV *in vivo* (Bayliss *et al.*, 2004).

We have shown previously (Zaleski & Piekarowicz, 2004) that MMR is necessary to repair DNA damage caused by oxidative compounds and that Dam methylation plays an important role in the infectivity of phage HP1c1. Thus, the major role of Dam methylation in different aspects of DNA stability and phage/host relations prompted us to investigate its role in other phage/host relations such as the activity of the R-M system and resistance and sensitivity to phage infection. In this paper, we present results indicating that Dam methylation changes the PV frequency of resistance/sensitivity to phage infection, which is correlated with the PV frequency of one of the genes involved in LOS expression.

This allowed us to determine the role of LOS as a receptor for HP1c1 phage.

METHODS

Bacterial strains, phages, plasmids and growth conditions.

All strains used in this study are described in Table 1. *H. influenzae* strains were grown in BHI (Difco) supplemented with 2 µg NAD ml⁻¹ and 10 µg haemin ml⁻¹ at 37 °C (Barcak *et al.*, 1991). *Escherichia coli* strains 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.

Test for R-M and resistance or sensitivity to phage HP1c1 infection.

Quantitative and qualitative tests (cross-streaking test) for R-M of phage HP1c1 were in general carried out as described previously (Glover & Piekarowicz, 1972). Restriction-proficient derivatives of *H. influenzae* strains restrict unmodified HP1c1 phage (grown on *H. influenzae* Rd30 strain) with an efficiency of about 10⁻² to 10⁻³. To carry out the spot test for restriction, a phage suspension (1 × 10⁴ to 1 × 10⁵ p.f.u. ml⁻¹) was streaked across supplemented BHI agar plates and allowed to dry. Bacterial cultures (1–5 × 10⁸ bacteria ml⁻¹) were cross-streaked over the phage and the plate incubated at 37 °C for between 18 and 24 h. In the absence of restriction, the phage produced confluent lysis of the bacteria. In the presence of restriction, only a few isolated plaques were visible. When necessary, we checked the restriction level quantitatively as described previously (Glover & Piekarowicz, 1972). To test for the resistance of bacteria to infection, a phage suspension containing 1 × 10⁹ p.f.u. ml⁻¹ was used. The strain resistant to phage HP1c1 gave no plaques, while the sensitive strain produced confluent lysis of bacteria. For the determination of the R-M or HP1^{R/M} phenotype the liquid culture of a particular clone was always tested with two phage

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>H. influenzae</i>		
Rd30	Str ^R HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	Glover & Piekarowicz (1972)
Rd30Nov	Str ^R Nov ^R HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	Zaleski & Piekarowicz (2004)
Rd30dam4	Str ^R <i>dam</i> ::Cm ^R HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	Zaleski & Piekarowicz (2004)
Rd30dam4mutH	Str ^R <i>dam</i> ::Cm ^R <i>mutH</i> :: <i>ter</i> ^R HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	Zaleski & Piekarowicz (2004)
Rd30lic2A	Str ^R HP1 ^S <i>lic2A</i> ::Cm ^R HP1 ^R r _{HinDI} ⁻ m _{HinDI} ⁻	This study
Rd30lgtC	Str ^R HP1 ^S <i>lgtC</i> ::Cm ^R HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	This study
Rd30lgtF	Str ^R HP1 ^S <i>lgtF</i> ::Cm ^R HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	This study
RM118	HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	Derivative of KW20 (Bayliss <i>et al.</i> , 2002)
RM118Δdam	Δ <i>dam</i> HP1 ^S r _{HinDI} ⁻ m _{HinDI} ⁻	Bayliss <i>et al.</i> (2002)
<i>E. coli</i>		
K-12 ER1944	Δ(<i>mcr</i> - <i>mmr</i>)102:: <i>zjj202</i> ::Tn10 <i>e14</i> ⁻	New England Biolabs
Plasmids		
pUC19	Cloning vector, replicates in <i>E. coli</i> ER1944	BRL
pK19	Cloning vector, replicates in <i>E. coli</i>	D. C. Stein*, unpublished
pMPMT4Ω	Cloning vector, replicates in <i>E. coli</i> ER1944	Mayer (1995)
pMPMK4Ω	Cloning vector, replicates in <i>E. coli</i> ER1944	Mayer (1995)
Phage		
HP1c1	Infects <i>H. influenzae</i>	Originally obtained from R. M. Herriot*

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suspensions, containing 1×10^9 p.f.u. ml⁻¹ and 1×10^4 p.f.u. ml⁻¹. A bacterial culture that did not give plaques using the phage suspension with high phage titre was considered phage resistant (HP1^R) (without the possibility to determine its R-M phenotype). A bacterial culture that gave only single plaques when the low phage titre was used was considered r_{HindI}⁺ m_{HindI}⁺, while a culture that gave a confluent lysis under the same conditions was considered r_{HindI}⁻ m_{HindI}⁻ and obviously HP1^S. Such tests allowed us to distinguish the R-M from the HP1^{R/S} phenotype. The probability that change of these two phenotypes will occur at the same time is very low.

PV frequencies of the R-M properties and resistance or sensitivity were determined as follows. The frozen stock culture of the strain to be tested was spread on BHI plates to obtain single colonies. After 18 h growth at 37 °C, single colonies were resuspended in 2 ml BHI. These cultures were tested for restriction proficiency and/or resistance to HP1c1 phage infection to establish parental phenotype and for preparation of serial dilutions, subsequently spread on BHI plates to obtain single colonies. Material from each colony served as an inoculum for liquid cultures grown in BHI at 37 °C for 18 h and then tested for restriction and phage-resistance phenotype. For each experiment several hundred colonies were screened by these methods. At least four colonies of each variant or construct were tested.

PV frequencies of the *lic1* gene (Hood *et al.*, 2001) were tested in a similar way, except that after obtaining colonies from serial dilutions, they were transferred to nitrocellulose filters, and probed with mAb TEPC 15 to detect the presence of a phosphocholine residue on the LOS of bacterial cells. The mutation rates were determined by the fluctuation method (Lea & Coulson, 1949) as described by De Bolle *et al.* (2000). Alterations in repeat tracts were determined by PCR amplification of the DNA sequence that contained the repeat tract from the parental and variant bacterial cells, and by direct sequencing of the products.

DNA manipulations and cloning. All general techniques were used according to 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 of *H. influenzae* were prepared by the anaerobic-aerobic method and were transformed with 0.1 µg chromosomal DNA as described by Barcak *et al.* (1991). For transfection, competent cells were exposed to 0.1 µg HP1c1 phage DNA according to Beattie & Setlow (1971). The efficiency of transfection is expressed as number of p.f.u. per concentration of DNA.

Chemicals, reagents and enzymes. Restriction enzymes and T4 DNA ligase were from Fermentas. All chemicals used for this study were reagent-grade or from Sigma unless otherwise specified. Tris-Tricine gels (15%) were prepared according to Schagger & von Jagow (1987). mAb TEPC 15 was obtained from Sigma.

LOS purification. Quick preparations of *Haemophilus* LOS were made from plated cultures as described by Jones *et al.* (1992). LOS were diluted 1:25 in lysing buffer (Hitchcock & Brown, 1983). The suspension was boiled for 10 min before loading. Approximately 0.1 µg LOS was subjected to SDS-PAGE on 15% Tris-Tricine gel in Tris-Tricine running buffer, following the protocol suggested by Bio-Rad. The gel was fixed overnight in 40% ethanol/5% acetic acid, and the LOS was visualized by silver staining (Tsai & Frasch, 1982).

Immunological methods. Purified LOS was transferred onto nitrocellulose membrane (Schleicher & Schuell). After air drying for 1 h, the membrane was processed in buffer [20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2% casein (fat free)] to block all nonspecific binding sites, and then screened for reactivity with mAb TEPC 15. Bound mAb was detected by covering the nitrocellulose filter with alkaline-phosphatase-labelled goat anti-mouse IgG, and tested for the presence of bound alkaline phosphatase using BCIP/NBT solution (Sigma). When colony blotting was performed, overnight colonies were transferred to a nitrocellulose membrane (Schleicher & Schuell) and processed as described above.

PCR. Primers used in this study are listed in Table 2. PCRs were performed using Pfu polymerase (Fermentas) and carried out according to the manufacturer's specifications. Primers were purchased from IBB Warsaw, Poland. PCR reactions were resolved on 1% agarose gels in Tris/borate/EDTA running buffer (Sambrook *et al.*, 1989). Sequencing of the DNA was performed at IBB Warsaw, Poland.

Strain construction. DNA fragments carrying the *lgtC*, *lic2A* and *lgtF* genes of *H. influenzae* Rd30 were amplified using primers LgtC_F and LgtC_R, Lic2A_F; and Lic2A_R and LgtF_F, LgtF_R respectively. The first two resulting amplicons (1100 bp and 950 bp) were cloned into pPMT4Q DNA, into the EcoRI and SmaI and the EcoRI and HindIII sites respectively, resulting in the formation of plasmids pPMT4*lgtC* and pPMT4*lic2A*. The third amplicon (980 bp) was cloned into the EcoRI and BamHI sites of pUC19 DNA, resulting in the formation of plasmid pUC19*lgtF*. In order to construct the *lgtC* mutant of *H. influenzae* Rd30, the chloramphenicol-resistance cassette, derived from pK19, was introduced into the HindIII restriction site found within the coding sequence of the *lgtC* gene cloned in pPMT4*lgtC*, generating pPMT4*lgtC*::Cam^R. Similarly, the chloramphenicol-resistance cassette was introduced into the XbaI

Table 2. Primers

Primer	Sequence (5'–3')	Description
Lic2A_F	GCCCATATGTTTGTATAGTAGAATGCGAAAAATTTAG	Amplifies the 5' end of <i>lic2A</i> with an NdeI site
Lic2A_R	GCGCAAGCTTCTACATAAAACGAACAATTTCTTTACC	Amplifies the 3' end of <i>lic2A</i> with a HindIII site
LgtC_F	GCCGAATTCAAATAACCCAAAAAATAATAATTCAACG	Amplifies the 5' end of <i>lgtC</i> with an EcoRI site
LgtC_R	CGGCCCGGGCATATATTAATTATCTTTTATTCTCTTTCTTAATC	Amplifies the 3' end of <i>lgtC</i> with a SmaI site
LgtF_F	GGCGGATCCGTGGGCGTAAAAAGTCGTAAAAAGTA	Amplifies the 5' end of the <i>lgtF</i> with a BamHI site
LgtF_R	CGGGCGAATTTCGAGCCAAAGTTAGAAGCCATTGTGA	Amplifies the 3' end of <i>lgtF</i> with an EcoRI site
Lic2A_S	ACTGGCTGTAAAAAGTTTCTAAACG	Primer for PCR sequencing of <i>lic2A</i>
LgtC_S	GTTCGTATATCTATATTCCAAAGTTC	Primer for PCR sequencing of <i>lgtC</i>

restriction site found within the coding sequence of the *lic2A* gene present in pMPMT4 *lic2A*, generating pMPMT4*lic2A*::Cam^R, and into the MluI restriction site found within the coding sequence of the *lgtF* gene in pUC19*lgtF*, generating pUC19*lgtF*::Cam^R. These constructs were introduced into *H. influenzae* Rd30 by separately transforming competent Rd cells with pMPMT4*lgtC*::Cam^R and pMPMT4*lic2A*::Cam^R linearized with NheI and pUC19*lgtF*::Cam^R DNA linearized with ScaI. The recombinants were selected on supplemented BHI medium containing 2 µg chloramphenicol ml⁻¹. The insertion of the chloramphenicol-resistance cassette into the all three tested genes was confirmed by PCR amplification of this region and restriction digestion analysis of the PCR amplicon (data not shown).

RESULTS

PV of the HindI type I R-M system is driven by pentanucleotide repeats and its frequency depends on Dam methylation

The HindI type I R-M system undergoes PV in *H. influenzae* Rd (Glover & Piekarowicz, 1972). Comparison of the genetic organization of this system with other known type I R-M systems (Murray, 2000; Roberts & Macelis, 2001) suggests that the expression of the *hsd* genes starts from the two promoters located 5' upstream of the *hsdM* gene and 5' upstream of the *hsdR* gene. Wild-type strains of *H. influenzae* Rd segregate into two phenotypic classes of clones, which differ in their abilities to restrict and modify phage HP1c1. The genetic organization of the HindI R-M system, and the fact that the phase variants either express both the modification and restriction activity or do not express either of them ($r_{\text{HindI}}^+ m_{\text{HindI}}^+$ or $r_{\text{HindI}}^- m_{\text{HindI}}^-$), suggest that PV could be driven by sequence repeats 5' upstream of *hsdM*.

According to Roberts & Macelis (2001), the HindI *hsdM* gene starts at position 1367826 and spans up to 1369157 bp of the genome sequence, encoding a protein of 443 amino acids. However, analysis of the DNA sequence of this region as well as the gene annotations (accession no. NC_000907), indicate that *hsdM* may begin 400 bp upstream and that the protein consists of 559 amino acids (Fig. 1).

This assumption is substantiated by the fact that the sequence of these additional amino acids shows very high homology to different HsdM proteins belonging to type I R-M systems (data not presented). If this is true, then the observed PV could be driven by the repeat sequence of four pentanucleotides (GACGA or CGAGA or GAGAC) present right at the beginning of the gene: 5'-ATGCAAG CGA GACGA GACGA GACGA GACGA CCA-3'. In the wild-type *H. influenzae* Rd 118 strain, the PV rate was higher for the phenotypic change from $r_{\text{HindI}}^- m_{\text{HindI}}^-$ to $r_{\text{HindI}}^+ m_{\text{HindI}}^+$ than from $r_{\text{HindI}}^+ m_{\text{HindI}}^+$ to $r_{\text{HindI}}^- m_{\text{HindI}}^-$ (Glover & Piekarowicz, 1972) (Table 3). The lack of Dam methylation decreases the mutation rate of the HindI R-M system of *H. influenzae* Rd 118 two- to fourfold compared to the wild-type strain. The frequency of PV from $r_{\text{HindI}}^- m_{\text{HindI}}^-$ to $r_{\text{HindI}}^+ m_{\text{HindI}}^+$ (OFF to ON) and from $r_{\text{HindI}}^+ m_{\text{HindI}}^+$ to $r_{\text{HindI}}^- m_{\text{HindI}}^-$

Fig. 1. Schematic representation of the chromosome region of *H. influenzae* Rd containing the ORFs encoding the HindI, HsdM and HsdS proteins (arrows). The 'long' form of HindI MTase is shown as a white arrow. The 'short' form of HindI MTase (M.HindI*) is shown as a black arrow. The numbers below the lines show the start and stop codons of the two forms of HindI MTase on the chromosomal map of *H. influenzae* (Fleischmann *et al.*, 1995). The position and the repeat sequence present at the beginning of the 'long' form of MTase gene are shown at the top of the figure.

(ON to OFF) was two to three times higher in the wild-type compared to the *dam* mutant (Table 3). The analysis of the DNA sequence of this region in the $r_{\text{HindI}}^+ m_{\text{HindI}}^+$ variant always showed the presence of four pentanucleotide repeat blocks compared to three or five repeat blocks in the $r_{\text{HindI}}^- m_{\text{HindI}}^-$ variant (Table 4). This confirms the assumption that this tract is responsible for PV of genes encoding the HindI R-M system and that change from the ON to OFF or OFF to ON state is accompanied by a change in the number of pentanucleotide repeats.

Inactivation of Dam influences the PV rates of the genes responsible for resistance/sensitivity to phage infection

H. influenzae Rd30 is a natural $r_{\text{HindI}}^- m_{\text{HindI}}^-$ variant of *H. influenzae* Rd that shows PV frequency of the $r_{\text{HindI}}^- m_{\text{HindI}}^-$ phenotype less than 10^{-3} (Glover & Piekarowicz, 1972).

The *H. influenzae* Rd30 *dam* mutant is sensitive to HP1c1 infection although the phage burst in this strain is decreased 10-fold compared to the wild-type strain (Zaleski & Piekarowicz, 2004). Wild-type *H. influenzae* Rd shows PV of resistance/sensitivity to phage infection (Table 3) that is 10-fold higher in the *dam* strain. The variation frequency from the HP1c1^S (ON) to HP1c1^R (OFF) phenotype in the *Dam* strain was 8–10-fold higher than that in the opposite direction.

The phage-resistant variants of *H. influenzae* Rd wild-type and *dam* strains showed about 7–10-fold less ability to adsorb phage particles. Phage DNA introduced by transfection into resistant and sensitive wild-type variants gave the same number of progeny phage particles, indicating that

Table 3. Influence of Dam methylation on PV in *H. influenzae*

Strain	Type of PV	$f (\times 10^{-2})^*$	Frequency range ($\times 10^{-2}$)/ $n^\dagger$	Mutation rate ($\times 10^{-3}$) ‡
RM118	$r_{\text{HindI}}^- m_{\text{HindI}}^- \rightarrow r_{\text{HindI}}^+ m_{\text{HindI}}^+$ (OFF-to-ON)	9.30	6.05–12.40/4	8.77 (9.20–7.80)
RM118	$r_{\text{HindI}}^+ m_{\text{HindI}}^+ \rightarrow r_{\text{HindI}}^- m_{\text{HindI}}^-$ (ON-to-OFF)	3.20	2.22–4.30/4	3.02 (3.89–2.70)
RM118 Δ dam	$r_{\text{HindI}}^- m_{\text{HindI}}^- \rightarrow r_{\text{HindI}}^+ m_{\text{HindI}}^+$ (OFF-to-ON)	3.20	2.40–4.10/4	3.20 (4.12–1.90)
RM118 Δ dam	$r_{\text{HindI}}^+ m_{\text{HindI}}^+ \rightarrow r_{\text{HindI}}^- m_{\text{HindI}}^-$ (ON-to-OFF)	1.10	0.85–1.65/4	1.03 (1.55–0.85)
RM118 Δ dam Δ mutH	$r_{\text{HindI}}^- m_{\text{HindI}}^- \rightarrow r_{\text{HindI}}^+ m_{\text{HindI}}^+$ (OFF-to-ON)	2.25	1.10–3.35/4	2.12 (3.41–1.87)
RM118 Δ dam Δ mutH	$r_{\text{HindI}}^+ m_{\text{HindI}}^+ \rightarrow r_{\text{HindI}}^- m_{\text{HindI}}^-$ (ON-to-OFF)	1.15	0.90–1.55	1.08 (2.20–1.75)
Rd30	$\text{HP1}^S \rightarrow \text{HP1}^R$ (ON-to-OFF)	1.10	0.75–1.75/4	1.24 (2.31–1.04)
Rd30	$\text{HP1}^R \rightarrow \text{HP1}^S$ (OFF-to-ON)	0.65	0.36–0.92/4	0.61 (1.34–0.98)
Rd30dam4	$\text{HP1}^S \rightarrow \text{HP1}^R$ (ON-to-OFF)	9.35	6.15–12.40/4	8.82 (9.32–6.34)
Rd30dam4	$\text{HP1}^R \rightarrow \text{HP1}^S$ (OFF-to-ON)	1.10	0.80–1.60/4	1.03 (2.20–0.97)
Rd30	TEPC 15 reactivity OFF-to-ON	0.55	0.42–0.61/4	0.51 (1.21–0.88)
Rd30dam4	TEPC 15 reactivity OFF-to-ON	0.52	0.39–0.62/4	0.50 (1.23–0.87)

* f is the median value for the frequency of variants per colony.

$^\dagger n$ is the number of colonies examined.

‡ Calculated using the method of the median according to Lea & Coulson (1949). Numbers in parentheses are 95 % confidence intervals calculated according to Kokoska *et al.* (1998).

resistance to phage infection was due to changes in the cells' receptors (data not shown).

Changes in the LOS of *H. influenzae* are responsible for the PV of resistance and sensitivity to phage infection

For a number of phages the LPS (LOS) structure or its components (Nesper *et al.*, 2000; Kutter *et al.*, 2005), are the

phage receptor(s). It was therefore reasonable to check whether there is a difference between the LOS structure of the resistant and sensitive variant derivatives from both wild-type and *dam* cells. *H. influenzae* LOS is a complex glycolipid composed of a membrane-anchoring lipidA portion linked by a single 2-keto-3-deoxyoctulosonic acid (Kdo) molecule to a heterogeneous oligosaccharide composed of neutral heptose and hexose sugars (Zamze & Moxon, 1987; Hood *et al.*, 1996a). Detailed analysis of LOS from *H. influenzae* Rd strain RM118 showed that this strain produces LOS with a globotetraose [β -D-GalpNAc-(1 $\rightarrow$ 3)- α -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)- β -D-Glcp] extension from the third heptose (Hep_{III}) and a single glucose as an

Table 4. Analysis of the repeat sequences localized in the genes responsible for PV of the HindI R-M system and resistance/sensitivity to phage infection

Strain*	Repeat sequences in:		
	<i>lic2A</i>	<i>lgtC</i>	<i>hsdM</i>
Rd30 HP1c1 ^S	22 $\times$ CAAT	22 $\times$ GACA	NT
Rd30dam4 HP1c1 ^S	22 $\times$ CAAT	22 $\times$ GACA	NT
Rd30 HP1c1 ^R	21 $\times$ CAAT	22 $\times$ GACA	NT
Rd30dam4 HP1c1 ^R	21 $\times$ CAAT	22 $\times$ GACA	NT
Rd30dam4 HP1c1 ^S	22 $\times$ CAAT	22 $\times$ GACA	NT
Rd30dam4#1 HP1c1 ^S	22 $\times$ CAAT	23 $\times$ GACA	NT
Rd30dam4#2 HP1c1 ^S	22 $\times$ CAAT	23 $\times$ GACA	NT
Rd30dam4#16 HP1c1 ^R	21 $\times$ CAAT	22 $\times$ GACA	NT
RM118 $r_{\text{HindI}}^- m_{\text{HindI}}^-$	NT	NT	3/5 $\times$ GACGA
RM118 $r_{\text{HindI}}^+ m_{\text{HindI}}^+$	NT	NT	4 $\times$ GACGA
RM118 Δ dam r_{HindI}^-	NT	NT	3/5 $\times$ GACGA
RM118 Δ dam r_{HindI}^+	NT	NT	4 $\times$ GACGA

*DNA from four colonies of each variant was used for determination of the repeat numbers.

Fig. 2. Schematic representation of the LOS structure of *H. influenzae* Rd strain RM118. The structure shown represents the known potential LOS molecule synthesized by *H. influenzae* Rd (modified from Hood *et al.*, 2001). Genes involved in the various steps of LOS biosynthesis are shown. The phase-variable loci are *lic1*, *lic2A* and *lgtC*. Abbreviations: Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-manno-heptose; Glc, D-glucose; Gal, D-galactose; GalNAc, N-acetylglactosamine; PCho, phosphocholine.

Fig. 3. Migration patterns of LOS from *H. influenzae* Rd30 and its phase-variable mutants after T-SDS-PAGE and staining with silver. (a) Lanes: 1, Rd30 HP1c1^S; 2, Rd30 *dam* HP1c1^S; 3, Rd30 *dam mutH* HP1c1^S. (b) Lanes: 1, Rd30 HP1c1^R; 2, Rd30 HP1c1^R; 3, Rd30 *dam* HP1c1^R; 4, Rd30 *dam mutH* HP1c1^R; 5, Rd30 HP1c1^S variant of Rd30 HP1c1^R. The lower intense-staining bands in each profile represent the major glycoform(s). Other bands represent alternative minor glycoforms produced mainly through phase-variable expression of LOS biosynthesis genes.

extension from the first heptose (Hep_I) (Fig. 2). This single glucose is decorated by the addition of a phosphocholine residue (Hood *et al.*, 1996b; Risberg *et al.*, 1999). Several of the surface-exposed epitopes of *H. influenzae* are subject to high-frequency PV (Patrick *et al.*, 1987; Weiser *et al.*, 1989). The addition of the globotetraose extension to Hep_{III} is dependent on three phase-variable genes, *lgtC*, *lic2A* and *lex2A* (*lpsA*) (Hood & Moxon, 1999; Bayliss *et al.*, 2002; Hood *et al.*, 2004). The PV of these genes is related to the differences in the number of the tetranucleotide repeats, which is not influenced by the MMR system except for the *polI* gene (Bayliss *et al.*, 2002, 2004).

LOS from the original wild-type strains (phage-sensitive) and its mutants defective in the MMR system (*dam* and *dam mutH*) showed the same migration (Fig. 3a), indicating that the presence of the mutations in themselves does not influence the structure of the LOS. However, the LOS from the phage-HP1c1-resistant variants (Fig. 3b) obtained from *H. influenzae* Rd30 and its *dam* or *dam mutH* mutants showed a different pattern of migration. The LOS from the phage-sensitive variants derived from the phage-resistant clone showed the same migration (Fig. 3b) as the original wild-type phage-sensitive strain, confirming that the phage resistance/sensitivity variation parallels the variation in LOS migration (structure).

The phase variants with a different LOS structure described above were detected by screening for phage resistance or sensitivity. In order to know whether the same variants can be seen without this type of detection, we isolated the LOS from 50 independent colonies of *H. influenzae* Rd30 and Rd *dam*, and analysed their LOS structure. We observed two types of variants among these clones. The LOS isolated from variants such as Rd30dam4#1 and Rd30dam4#2 (phage-sensitive) migrated differently than the LOS from the wild-type cells (Fig. 4a, lanes 2 and 3) and HP1c1-phage-resistant clones. LOS isolated from the variants represented by Rd30dam#16 (phage-resistant) showed the same migration pattern of LOS as the variant selected directly for the resistance to phage HP1c1 (Fig. 4b, lanes 2 and 3).

The original *H. influenzae* Rd and its *dam* mutant HP1c1-sensitive strains showed reactivity with TEPC 15 antibodies, indicating the presence of a phosphocholine moiety on the glucose extension from the first heptose (Hep_I) in the LOS structure (see Fig. 2) (data not shown). Among the phage-resistant or -sensitive variants derivatives of the wild-type and *dam* strains, the frequency of PV, as measured by reactivity with TEPC 15 antibodies, was the same (Table 3), indicating that *dam* does not influence the frequency of the PV of the gene responsible for the addition of this molecule.

Fig. 4. Migration patterns of LOS from *H. influenzae* Rd30 phase variants and derivatives with mutations in glycosyltransferase genes after T-SDS-PAGE and staining with silver. (a) Lanes: 1, Rd30 HP1c1^S; 2 and 3, variants exhibiting the first type of LOS migration and HP1c1^S phenotype; 4 and 5, *lgtC* insertional mutants sensitive to phage HP1c1. (b) Lanes: 1, Rd30 HP1c1^S; 2 and 3, HP1c1^R variants exhibiting the second type of LOS migration; 4 and 5, *lic2A* insertional mutants resistant to phage HP1c1.

The *H. influenzae* Rd HP1c1 phage receptor is localized in the Galp-(1→4)-β-D-Glcp part of the globotetraose extension from the Hep_{III} of the LOS molecule

In the LOS structure of *H. influenzae* Rd the second heptose (Hep_{II}) does not contain any extension. This leads us to assume that the main role of the HP1c1 phage receptor is played either by the globotetraose [β -D-GalpNAc-(1→3)- α -D-Galp-(1→4)- β -D-Galp-(1→4)- β -D-Glcp], extending from Hep_{III}, or by the glucose attached to Hep_I of the LOS molecule, or by both these extensions. The fact that the majority of the phage-resistant/sensitive variants showed reactivity to TEPC 15 antibodies could suggest that the glucose residue extending from Hep_I (Fig. 2) is probably not a part of the phage receptor. To confirm this assumption we constructed an *lgtF* mutant of the phage-sensitive *H. influenzae* Rd30 strain (see Methods) and tested for phage resistance and sensitivity. All 50 colonies of this mutant tested were still phage-sensitive, which indicates that lack of this glucose residue does not play a role in phage adsorption.

The addition of sugars to Hep_{III} is dependent on four genes, *lgtD*, *lgtC*, *lic2A* and *lpsA* (Hood *et al.*, 2001, 2004; Bayliss *et al.*, 2002) (see Fig. 2). In order to test which sugars are necessary to retain the sensitivity to phage infection, *lgtC* and *lic2A* mutants of phage-sensitive *H. influenzae* Rd30 were constructed (see Methods) and 20 single colonies of each mutant were tested for phage infectivity and LOS migration. The *lic2A* mutants were always resistant to phage infection and their LOS migrated in the same way as that of HP1c1-resistant variants of *H. influenzae* Rd30 (Fig. 4b, lanes 4 and 5). The *lgtC* mutants were HP1c1-sensitive and their LOS migrated faster than LOS of the wild-type cells but slower than LOS from *lic2A* mutant (Fig. 4a, lanes 4 and 5). This would be in accordance with the loss of the digalactoside residue in the globotetraose extension from Hep_{III} of the LOS molecule.

As an additional test to see whether *lic2A* mutation is responsible for the resistance to phage HP1c1 infection, chromosomal DNA isolated from *H. influenzae* Rd30 *lic2A* was used to transform wild-type *H. influenzae* Rd30. All the chloramphenicol-resistant transformants were resistant to HP1c1 phage infection and showed changes in LOS migration (data not shown).

The PV of resistance/sensitivity to phage infection is related to the changes in the number of tetranucleotide repeats

The above results indicate that the LOS molecule of *H. influenzae* Rd plays the role of the phage receptor and that while the digalactoside [β -D-GalpNAc-(1→3)- α -D-Galp] residue (see Fig. 2) is not needed for phage adsorption, the loss of the third galactose residue results in the loss of ability to adsorb the phage. According to Hood *et al.* (2001), the addition of all these sugar residues depends on the *lgtC*

and *lic2A* genes, which in strain *H. influenzae* Rd and strain KW-20 contain 22 5'-GACA and 22 5'-CAAT repeats respectively (Fleischmann *et al.*, 1995). To check whether the PV of the *lgtC* and *lic2A* genes observed in this paper is related to the changes in the number of the tetranucleotide repeats we tested these repeats in the original *H. influenzae* Rd30 strain and its spontaneous resistant/sensitive and LOS variants. Table 4 shows that in all tested cases a change from phage sensitivity to resistance was accompanied by a change in the number of 5'-CAAT repeats from 22 to 21 in the *lic2A* gene (i.e. a switch from ON to OFF). The phage-resistant *lic2A* variant (strain Rd30dam4#16) also showed a change in the number of 5'-CAAT repeats from 22 to 21. A change in the number of repeats (5'-GACA) was also observed in the *lgtC* gene present in strains Rd30dam4#1 and Rd30dam4#2, which were HP1c1 phage sensitive and showed a change in the LOS migration, confirming the assumption that this gene and its PV does not play a role in phage sensitivity.

DISCUSSION

The main line of defence against phage infection is the presence of R-M systems and mutations that lead to the inability to adsorb phage particles. Previously it was shown that HindI, the main R-M system that acts against phage infection and acquiring free DNA from the environment (Glover & Piekawicz, 1972; Piekawicz *et al.*, 1974) undergoes PV (Glover & Piekawicz, 1972). Similarly, the *mod* genes with homology to type III DNA methyltransferases (MTases) undergo PV with a high frequency (De Bolle *et al.*, 2000; Bayliss *et al.*, 2002). In *H. influenzae* Rd, the *mod* gene has two potential start codons that code for proteins of either 72 or 86 kDa, depending on the number of repeats that are present (De Bolle *et al.*, 2000). The distal start codon gave maximum expression while the proximal start gave only about 2.5% of the maximal expression (Srikhanta *et al.*, 2005).

The discrepancy between the HindI *hsdM* structures presented in the literature (Roberts & Macelis, 2001; Fleischmann *et al.*, 1995) makes it difficult to interpret the mechanism of PV of the HindI R-M system. It seems that the 'long' HsdM represents an active form of the enzyme. The N-terminal 123 amino acid end of this protein contains domains that are required for the normal activity of the DNA MTases (data not presented). The lack of these domains seems to preclude the possibility that the 'short' HsdM would be an active enzyme. If the 'long' HsdM represents an active form of the enzyme then it can be synthesized only in the presence of the four pentanucleotide repeats. In the presence of three or five repeats it would be synthesized out of frame as an inactive protein. If the synthesis in the presence of three or five repeats starts from another start codon, the 'short' inactive form of the enzyme will be formed.

In this report we also show that the resistance/sensitivity to phage infection is changed by PV. The PV of this phenotype

correlates with the PV of one of the genes engaged in LOS biosynthesis, *lic2A*. Analysis of the resistant/sensitive PV variants and insertion mutants made in *lic2A*, *lgtC* and *lgtF* clearly indicated that in the LOS structure of *H. influenzae* Rd, phage adsorption (receptor) is mediated by the Glc-Gal extending from the third heptose residue. The loss of the terminal digalactoside, as an extension of the Glu-Gal at the third heptose, or the Glc extending from the first heptose and/or phosphocholine residue decorating this Glc, does not influence the ability to adsorb HP1c1 phage. In *H. influenzae*, LOS is a principal component of the outer membrane, mediating interactions between the bacterium and the host's immune system, and contributing to its pathogenicity. The use of the LOS as a receptor for phage HP1c1 increases the probability that it will always be present on the surface of bacterial cells and increases the chance of the bacteriophage surviving in the environment.

In *H. influenzae*, PV is mediated mainly by long tetranucleotide or dinucleotide repeat tracts (Bayliss *et al.*, 2002, 2004; Hood *et al.*, 2004). The frequency of PV of the gene containing tetranucleotide and dinucleotide repeats strongly depends on the number of such repeats. This becomes visible when this number is greater than 10 (De Bolle *et al.*, 2000). Although the presence of penta- or heptanucleotide repeats was reported (van Belkum *et al.*, 1997), their influence on PV in *H. influenzae* was not known. Here we show for the first time that pentanucleotide repeats are responsible for PV of the HindI R-M system. However, the striking difference between PV mediated by tetranucleotide and dinucleotide tracts, compared to pentanucleotide tracts, is that the same frequency of PV is observed for the genes containing 3–5 pentanucleotide repeats as for genes containing more than 20 tetranucleotide or dinucleotide repeats (De Bolle *et al.*, 2000; Bayliss *et al.*, 2002).

All the phage-resistant variants that were tested demonstrate the lack of one of the 5'-CAAT repeats in the *lic2A* gene, while reversion to phage sensitivity restores the original number of repeats. Similarly, the difference between the variants that are able or unable to restrict the HP1c1 phage is related to the change of one or two pentanucleotide repeats. The same results observed by De Bolle *et al.* (2000) showed that 90 % of ON-to-OFF phase variants in the *mod::lacZ* reporter *H. influenzae* Rd strain exhibited a single repeated unit change.

A striking observation in this paper is that the lack of Dam methylation in *H. influenzae* increases the rate of PV of sensitivity/resistance to phage infection, which correlates with the PV of *lic2A* activity. Since the resistance to phage infection is a consequence of an inactive *lic2A* gene, this indicates that the lack of Dam methylation increases the frequency of PV of this gene. Although not so rigorously tested, our data also indicate that the lack of Dam methylation increases the PV frequency of the *lgtC* gene. Our results are in contrast with the observation of Bayliss *et al.* (2002), who showed that among seven MMR genes of *H. influenzae*, none, except for *polI*, influenced PV mediated

by tetranucleotide repeats. The increase of PV of phage resistance/sensitivity phenotype due to switching ON or OFF of expression of *lic2A* in a *dam* mutant is a spontaneous process not influenced by any selection. The test for phage resistance/sensitivity was a tool that allowed the detection of such events. The production of HP1c1 phage in the *dam* mutant is only 10-fold lower than in the wild-type cells (Zaleski & Piekarowicz, 2004) and cannot be responsible for the observed resistance to phage infection (since the resistance in such a case means a complete loss of the ability to adsorb phage and not its production inside the cell). However, Bayliss *et al.* (2002) tested the influence of mutations in the MMR system using artificial constructs introduced into the chromosome of *H. influenzae*, while in our experiments tests for PV were carried out in *in vivo* conditions.

Our experiments have shown that the lack of Dam methylation does not change the frequency of PV of the *lic1* gene, responsible for the addition of the phosphocholine residue to the glucose extended from the first heptose. The PV of this property is governed by the instability of the 5'-CAAT within the *licA* gene (Weiser *et al.*, 1989). Although it was suggested that Dam methylation may play a role in the regulation of PV in *Neisseria meningitidis* (Bucci *et al.*, 1999), further studies have shown that it does not change the frequency of PV of several tested loci in which PV was due to the presence of poly(G) tracts (Alexander *et al.*, 2004). All these results suggest that the effect of Dam on PV may depend on the particular gene tested and whether it was tested *in vivo* or using artificial constructs.

In conclusion, our results, together with those of other groups, suggest that the *H. influenzae* MMR pathway can destabilize *in vivo* the PV mediated by tetranucleotide and pentanucleotide repeats. Dam methylation, as a part of the MMR system, is responsible for the correction of single- and double-stranded DNA breakage in *H. influenzae* (Zaleski & Piekarowicz, 2004) and could be responsible for correction of slippage mutations generated in particular tetranucleotide and pentanucleotide repeat tracts. This paper shows that *dam* mutation does not change the PV rates of *lic1A* (i.e. TEPEC 15 reactivity), and Bayliss *et al.* (2002) showed that this mutation does not affect a reporter in the *mod* locus. Therefore, the *dam* effect must be acting in a locus-specific manner.

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