

Evolution and function of the neisserial *dam*-replacing gene

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Abstract Phase variation through slippage-like mechanisms involving homopolymeric tracts depends in part on the absence of Dam-methylase in several pathogenic isolates of *Neisseria meningitidis*. In Dam-defective strains *drg* (*dam*-replacing gene), flanked by pseudo-transposable small repeated elements (SREs), replaced *dam*. We demonstrate that *drg* encodes a restriction endonuclease (*NmeBII*) that cleaves 5'-GmeATC-3'. *drg* is also present in 50% of *Neisseria lactamica* strains, but in most of them it is inactive because of the absence of an SRE-providing promoter. This is associated with the presence of GATmeC, suggesting an alternative restriction-modification system (RM) specific for 5'-GATC-3', similar to *Sau3AI*-RM of *Staphylococcus aureus* 3A, *Lactococcus lactis* KR2 and *Listeria monocytogenes*. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Type II restriction endonuclease; *NmeBII*; DNA repair; Neisserial small repeated element; Non-pathogenic *Neisseria*; *Neisseria meningitidis*; *Neisseria lactamica*

1. Introduction

Neisseria meningitidis, a narrow-host-range parasite, undergoes sophisticated genetic switches for adapting to different microenvironments within the human host during the course of a natural infection. Control of retrievable genetic programmes by phase variation of several surface-associated components including capsule and outer membrane proteins enables the microorganism to choose between alternative life styles, commensal or pathogenic, intracellular or extracellular, and to evade the host immune system. The genetic basis for this variation depends on the evolution of iterative DNA motifs, especially homopolymeric tracts, to effect reversible, high-frequency molecular switching through slippage-like mechanisms [1]. We have recently clarified several aspects of the molecular mechanism responsible for phase variation by slipped-strand mispairing involving homopolymeric repeats [2]. Although the mutation rate at homopolymeric repeats is high in all strains, several strains isolated from patients may be hypermutable at these loci due in part to genetic defects in

the DNA mismatch repair process. Molecular analysis showed that the mutator phenotype depends on the absence of activity of the Dam-methylase, a component of the methyl-directed mismatch repair system [3], evolutionarily conserved along phylogenesis of the γ subgroup of the Proteobacteria, including *Vibrio*, *Haemophilus*, and *Escherichia* [4–6], present in *Neisseria*, belonging to the strictly related β subgroup [2], and also found in the spirochete *Treponema pallidum* [7]. The analysis of the meningococcal *dam* gene region revealed that in all Dam-defective strains a gene, named *drg* (*dam*-replacing gene), bordered by pseudo-transposable neisserial small repetitive elements (SRE) [8] replaced the functional *dam* gene. The putative gene product shared significant homology (44.5% identity and 66.9% similarity) with the *Streptococcus pneumoniae* type II restriction endonuclease *DpnI*, which cleaves the GATC sequence methylated at the adenine residue. On the basis of this and other indirect evidence, it was inferred that the product of *drg* was a restriction endonuclease, and that the *dam* and *drg* genes were mutually exclusive. In this paper we have investigated the function of the *drg* gene product, and have analysed the presence of *drg* in non-pathogenic *Neisseria* in an attempt to gain more information about its origin and evolution during speciation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Neisseria strains (Table 1) were cultured on GC agar or broth supplemented with 1% (v/v) Polyvitox (Bio-Merieux) at 37°C in 5% CO₂. When required rifampicin (36 µg/ml) was added.

Escherichia coli strain SMR843 (*dam*13::Tn9) and congenic SMR506 (wild type) were a gift of Dr. S.M. Rosenberg (University of Alberta, Canada). *E. coli* strains were grown in Luria–Bertani broth supplemented with ampicillin (50 µg/ml), when required.

2.2. Plasmids and cloning procedures

Plasmid pUC-Drg was constructed by cloning a 960-bp *Hind*III–*Bam*HI fragment, spanning the coding region of *drg*, into pUC19. The 960-bp fragment was obtained by amplifying a genomic region from strain BL859 by polymerase chain reaction (PCR) using the oligonucleotides 5'-AATCCAAAGCTTGAATTTATTTTCGATACCAA-ATTGG-3' and 5'-AGCGGCGGATCCGGCAGCTTCTTCAGCC-GGTTTGTC-3'.

2.3. DNA procedures

Genomic DNA from *Neisseria* strains was prepared as described [9]. DNA fragments were isolated through polyacrylamide slab gels and recovered by electroelution [10].

DNA labelling and Southern blot experiments were performed according to Sambrook et al. [10]. A 1-kb DNA ladder (Life Technol-

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Table 1
Neisseria strains

Strain	Drg ^a	Dam phenotype ^b	<i>Sau</i> 3AI restriction ^c	Source ^d
<i>N. meningitidis</i>				
BL859	+	—	S	i
BL847	+	—	S	i
B1940	+	—	S	ii
BL911	+	—	S	iii
BF2	—	+	S	iv
BF9	—	+	S	iv
BF52	—	+	S	v
BF18	+	—	S	v
<i>N. lactamica</i>				
NL21	—	—	R	iii
NL56	—	+	S	iii
NL76	—	+	S	iii
NL104	+	—	R	iii
NL172	—	+	S	iii
NL411	+	—	R	iii
NL995	+	—	S	iii
NL4627	+	—	R	iii
<i>N. mucosa</i>				
NM404	—	—	S	iv
NM405	—	—	S	iii
<i>N. sicca</i>				
NS407	—	+	S	iv
NS408	—	+	S	iii
<i>N. flava</i>				
NF410	—	+	S	iii
NF3264	—	+	S	iii
<i>N. subflava</i>				
NS3260	—	+	S	iii
NS5291	—	+	S	iii
<i>N. cinerea</i>				
NC415	—	—	S	iii
NC5917	—	—	S	iii

^aThe presence of *drg* was investigated by Southern blot (Fig. 2).

^bThe Dam phenotype was determined by treating the chromosomal DNAs with isoschizomers *Mbo*I and *Dpn*I which cleave the GATC sequence depending on the adenine methylation status, *Mbo*I cutting only non-methylated sites, and *Dpn*I cutting only methylated sequences.

^cS, sensitive to restriction by *Sau*3AI; R, resistant to restriction.

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ogies) was used as a molecular weight marker. The ³²P-labelled 960-bp *Hind*III–*Bam*HI fragment was used as a *drg*-specific probe.

DNA sequencing reactions were carried out by the dideoxy chain termination procedure using a PCR-based methodology. The oligonucleotides used to amplify the *leuS-drg* region were 5'-AGTGA-TGGTTCAAGTCAACGGCAAACCTGCG-3' and 5'-CGTATTCG-GCAGCTTCTTCAGCCGTTTGTC-3'.

The amplification consisted of 30 cycles of 1 min at 94°C, 1 min at 55°C and 1–2 min at 72°C and was performed in a Perkin Elmer Thermal Cycler 480.

2.4. RNA Procedures

Total RNA was extracted from logarithmically growing cells by the guanidine hydrochloride procedure [2]. Electrophoretic analysis was done by fractionating the total RNA on 1% agarose gels containing formaldehyde [10]. RNA transfer to Hybond (Amersham) membranes and hybridisation were according to standard procedures [10].

2.5. Transformation of meningococci

Transformations were performed as described [11] with 500 ng of chromosomal DNA extracted from rifampicin-resistant derivatives of strains BL847 or BF52. The recipient strain was BF52. Transformants were selected on GC agar supplemented with rifampicin.

2.6. Extract preparation

Logarithmically growing cells (OD₅₅₀ = 0.5, 200 ml) in LB medium were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 45–90 min at 37°C. Cells were harvested by centrifugation at 5000×g, resuspended in 4 ml of 50 mM Tris–HCl (pH 8) contain-

ing 1 mM phenylmethylsulfonyl fluoride, and broken by a French press. Crude extracts were centrifuged at 30 000×g for 30 min and the supernatants were collected and frozen at –80°C. S30 extracts were analysed by SDS–PAGE as described [10]. Protein molecular weight ladders (Rainbow 800) were from Amersham.

2.7. Assays for *NmeBII* activity

S30 extracts derived from IPTG-induced *E. coli* cells harbouring pUC-Drg (expressing *NmeBII*) or pUC19 were added to 20 µl reaction mixture containing 10 mM Tris–HCl, pH 7.6, 50 mM NaCl, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 1 mg/ml of bovine serum albumin and 1 µg of Dam⁺ or Dam[–] DNAs derived from rifampicin-resistant strains. After incubation for 2 h at 37°C, transformation activity was assayed on the rifampicin-sensitive strains BF52. Transformants were scored by plating 10⁶ colony-forming units (CFU) on selective plates; in control cultures no rifampicin-resistant clones were detected by plating 10⁶ CFU. A relative transforming activity equal to 1 corresponds to a transformation efficiency of 3.5×10^{–4}. Endonuclease activity was tested using pUC19-purified DNA.

3. Results and discussion

3.1. Function of the *N. meningitidis* *drg* gene

The coding sequence of *drg* was amplified by PCR from strain BL859, and cloned into pUC19 translationally in frame with *lacZ*, under expression of the IPTG-inducible *lac* promoter. Cloning was viable in a *dam*-defective *E. coli* host

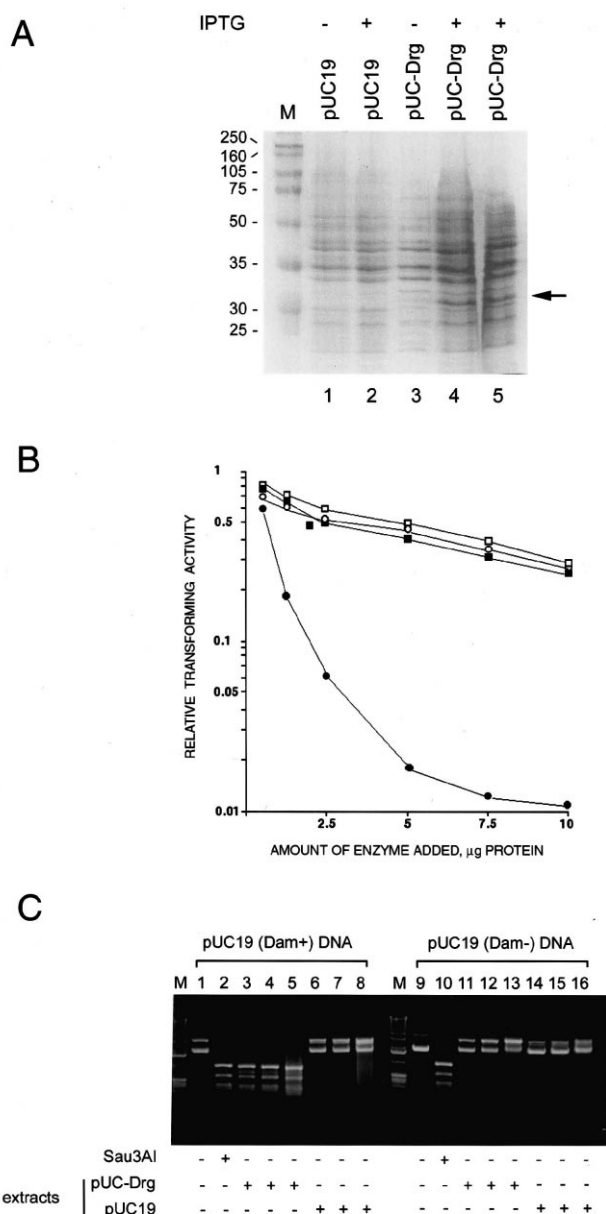


Fig. 1. A: Expression of recombinant *drg*. *E. coli* strain SMR843 harbouring pUC19 (lanes 1 and 2) or pUC-Drg (lanes 3–5) were induced with IPTG for 45 min (lanes 2 and 4), 90 min (lane 5), or not induced (lanes 1 and 3). S30 extracts were run on 12% SDS-PAGE. Bars, molecular weight ladders. Arrow, induced 31.5-kDa polypeptide. B: Action of endonuclease *NmeBII* on Dam+ and Dam- transforming DNA. Closed circles, Dam+ DNA pre-treated with *NmeBII* extract; open circles, Dam+ DNA pre-treated with control extract; closed squares, Dam- DNA pre-treated with *NmeBII* extract; open squares, Dam- DNA pre-treated with control extract. C: Site and DNA adenine methylation specificity of endonuclease *NmeBII*. pUC19 plasmid DNA, derived from either Dam-proficient (lanes 1–8) or Dam-defective (lanes 9–16) strains, was incubated in the presence of increasing amounts (2.5, 5, or 7.5 μ g) of *NmeBII* extract (lanes 3–5 and 11–13) or control extract (lanes 6–8 and 14–16). As a control, the pUC19 DNA (lanes 1 and 9) was digested with *Sau3AI* (lanes 2 and 10). Molecular weight ladders were run in parallel (M).

(SMR843) and led to generation of recombinant pUC-Drg. Expression of Drg protein in *E. coli* S30 extracts was checked by SDS-PAGE (Fig. 1A). This analysis evidenced a protein of about 31.5 kDa, very close to the expected size (31.482 kDa),

in IPTG-induced cells (Fig. 1A, lanes 4 and 5), absent in non-induced cells (Fig. 1A, lane 3) or in control cells harbouring the vector (Fig. 1A, lanes 1 and 2).

To have preliminary evidence of the activity of Drg, a transformation experiment was performed using a *N. meningitidis* Dam+ strain, and Dam-methylated or non-methylated DNA purified from rifampicin-resistant Dam+ or Dam- strains (Dam+ or Dam- Rif^R DNA). This experiment tested the ability of the extract containing the Drg protein to inhibit the transforming activity by Rif^R DNA (Fig. 1B). Dam+ and Dam- Rif^R DNAs were therefore pre-treated with amounts of extracts derived from IPTG-induced *E. coli* cells harbouring pUC-Drg or pUC19. This experiment demonstrated that the S30 extract expressing Drg was able to inhibit the transforming activity of the Dam+ Rif^R DNA. The level of inhibition was dependent on the amounts of extract added. In contrast, the extract was not effective on Dam- Rif^R DNA. pUC19 extracts did not inhibit transformation by either Dam+ or Dam- Rif^R DNA. These data indicated that recombinant Drg retained biological activity, and they are consistent with the hypothesis that Drg encodes a type II restriction endonuclease cleaving the Dam-methylated GmeATC sequence.

Direct evidence of the activity of the Drg protein was obtained by restriction analysis (Fig. 1C). pUC19 DNA derived from Dam+ or Dam- strains (Fig. 1C, lanes 1 and 9, respectively) was treated with various amounts of pUC-Drg or pUC19 extracts. As a control, pUC19 DNA was digested

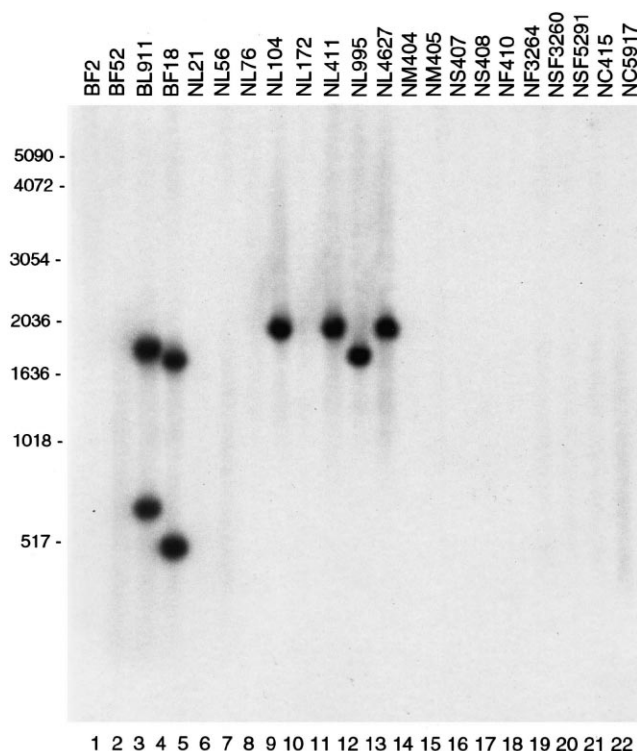


Fig. 2. Southern blot analysis of *drg* in pathogenic and non-pathogenic *Neisseriae*. *HinfI*-restricted chromosomal DNAs derived from two Drg- (lanes 1 and 2) and from two Drg+ (lanes 3 and 4) *N. meningitidis* strains, from eight *N. lactamica* strains (Fig. 3, lanes 5–12), and from pairs of *N. mucosa*, *N. sicca*, *N. flava*, *N. subflava* and *N. cinerea* strains (Fig. 3, lanes 13–22) were hybridised to a *drg*-specific probe. The name of each individual strain is indicated above the respective lane. Bars, molecular weight ladders.

with *Sau3AI*, which cleaves the GATC sequence irrespective of the adenine methylation status (Fig. 1C, lanes 2 and 10). Treatment of Dam⁺ pUC19 DNA with the extract containing Drg (Fig. 1C, lanes 3–5) resulted in the appearance of cleavage products corresponding in size to those obtained with *Sau3AI*. In contrast, Dam[−] pUC19 DNA was not digested by the extract (Fig. 1C, lanes 11–13). Digestion products were not observed with the control extract either on Dam⁺ or on Dam[−] plasmid DNA (Fig. 1C, lanes 6–8 and 14–16, respectively). This result confirmed the cutting specificity of the *drg* gene product, now named *NmeBII*.

3.2. Evolution of the *N. meningitidis* *drg* gene

The analysis of the structure of the *N. meningitidis* *leuS*–*dam* region evidenced that all strains belong to alternative biotypes: strains harbouring an intact *dam* gene, and strains

harbouring an identical rearrangement removing part of the *dam* gene, possibly promoted by pseudo-transposable SRE elements bordering the mutually exclusive *drg* gene [2]. This evidence suggested (i) that the *dam*-proficient biotype was the ancestral one, (ii) that *drg* came from outside, and (iii) that the rearrangement leading to introduction of *drg* into the *leuS*–*dam* region occurred during evolution of several meningococcal pathogenic lineages and/or during speciation of pathogenic *Neisseria*. In fact, there is evidence that the same rearrangement is also present in several strains of *N. gonorrhoeae* (strain FA1090, University of Oklahoma). To investigate in more detail the origin and evolution of *drg*, and its distribution among pathogenic and non-pathogenic *Neisseria* species, a Southern blot experiment was performed (Fig. 2). To this purpose, *HinfI*-restricted chromosomal DNAs derived from *Neisseria lactamica* (Fig. 2, lanes 5–12), from pairs of

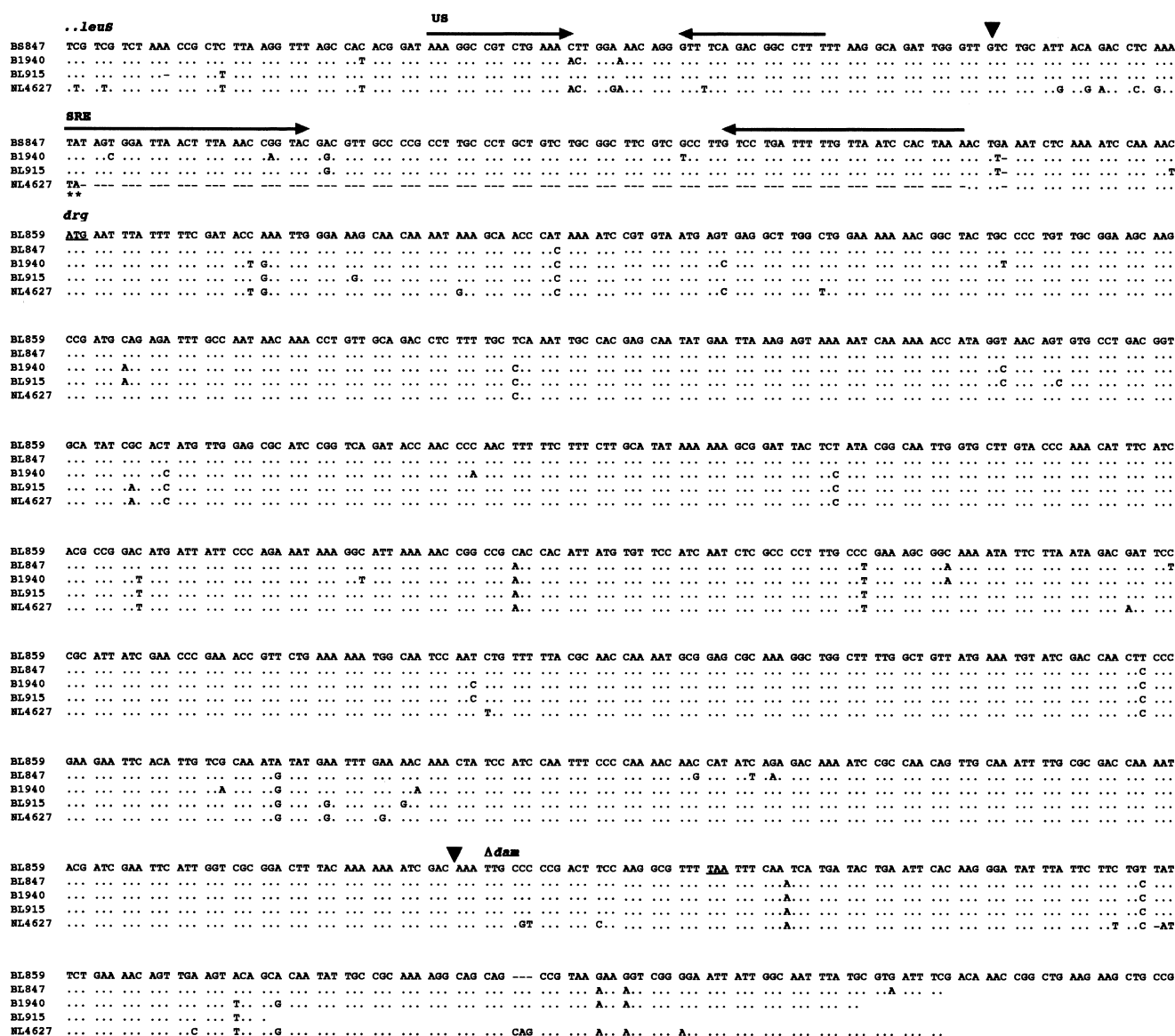


Fig. 3. Comparison of nucleotide sequences of the *leuS*–*drg* region from *N. meningitidis* and *N. lactamica* strains. The nucleotide sequences of the *leuS*–*drg* genetic region from *N. meningitidis* strains BS847, B1940, BL915, BL859 and from *N. lactamica* strain NL4627 are aligned. Dots, identical nucleotides; tracts, missing nucleotides. Start and stop codons of *drg* are underlined. Arrows, palindromic sequences that are part of the neisserial uptake sequence (US), and SRE. The nucleotide sequence between the two closed triangles is present in Dam[−] Drg⁺ strains and absent in Dam⁺ isolates.

Neisseria mucosa, *Neisseria sicca*, *Neisseria flava*, *Neisseria subflava* and *Neisseria cinerea* (Fig. 2, lanes 13–22), and from *N. meningitidis* Drg– (Fig. 2, lanes 1 and 2) or Drg+ (Fig. 2, lanes 3 and 4) strains were hybridised to a *drg*-specific probe. Specific DNA fragments were detected in four out of eight *N. lactamica* strains, but in none of the other non-pathogenic *Neisseriae* (Fig. 2, lanes 13–22). More in detail, strains NL104, NL411 and NL4627 (Fig. 2, lanes 8, 10 and 12, respectively) exhibited fragments of similar size (about 2150 bp). Strain NL995 (Fig. 2, lane 11) exhibited a fragment very close in size to one of the two fragments detectable in the *N. meningitidis* strain BF18 (Fig. 2, lane 4).

Consistently with previous observations in *N. meningitidis* [2], the presence of *drg* was generally associated with the absence of Dam methylation also in *N. lactamica*. However, *N. lactamica* NL21, *N. mucosa* NM404 and NM405, and *N. cinerea* NC415 and NC5917 strains were both Drg– and Dam– (Table 1). Significantly, the DNAs derived from Dam– strains NL21, NL104, NL411 and NL4627, but not that derived from NL995, from *N. meningitidis*, or from the other non-pathogenic *Neisseriae* were resistant to restriction by *Sau*3AI (Table 1). Cleavage by this enzyme is inhibited by methylation at the cytosine residue within the target GATC sequence [12]. We therefore speculate that several *N. lactamica* strains have acquired a type II restriction-modification system similar to that present in several Gram-positive bacteria including *Staphylococcus aureus* 3A [12], *Lactococcus lactis* KR2 [13], *Listeria monocytogenes* [14]. This system does not seem to be present in Dam+ *N. lactamica* strains.

More information about the evolution of *drg* was obtained by nucleotide sequence analysis (Fig. 3). Regions of DNA containing *drg* were sequenced from a *N. lactamica* strain and from four serogroup B *N. meningitidis* strains of distinct clonal types [2]. Comparison of the nucleotide sequences derived from the meningococcal strains evidenced that *drg* was highly variable. Indeed, we detected 37 polymorphic sites within the coding regions, giving rise to distinct alleles with diversity up to 2.65%. In contrast, *dam* was highly conserved (we only found 10 polymorphic sites in three different alleles and three different proteins, and a diversity up to 1.59%; data not shown). The different results with the *drg* and *dam* genes might reflect functional constraints of the respective gene products. Alternatively, variability of *drg* might result from hyper-mutation, inherent in the presence of *drg* (and loss of *dam*) within the mutator cell.

The diversity between *drg* genes from *N. meningitidis* and *N. lactamica* was about the same order of magnitude. However, the PCR and sequence analyses of the genomic DNA from different strains (Fig. 3 and data not shown) revealed that, in *N. lactamica*, *drg* was preceded by the SRE only in strain NL995. The absence of the SRE might be the consequence of excision of the element upon recombination involving the terminal TATA sequences of the SRE [8]. The TA dinucleotide in *N. lactamica* (Fig. 3) would represent a relic of the recombinational event. Alternatively, pseudo-transposition of SRE into the *leuS*–*drg* region was subsequent to the genomic rearrangement replacing *drg* for the *dam* gene, and it specifically occurred during speciation of meningococci. If the latter hypothesis is correct, the TA dinucleotide would represent the target site for pseudo-transposition. As a consequence, because the TA repeat borders SRE in most locations, the TA dinucleotide should not be considered a part of the inverted

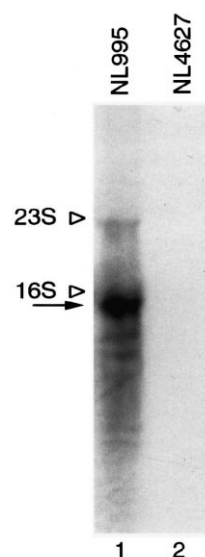


Fig. 4. Northern blot analysis of *drg*-specific transcripts. Total RNAs, extracted from *N. lactamica* strains NL995 (lane 1) and NL4627 (lane 2), were hybridised to the *drg*-specific probe. Arrow, *drg*-specific transcript. Bars, 23S and 16S rRNAs.

repeats (IRL and IRR) of the element [8], but a preferred target site, which undergoes duplication upon pseudo-transposition.

Transcription of *drg* was believed to be promoted by sequences lying within the SRE [2,15]. Due to lack of SRE, in most *N. lactamica* strains the *drg* coding region starts only 60 bp downstream of the putative transcription terminator of *leuS*. In the 60-bp region obvious consensus sequences for σ^{70} are not detectable. Indeed, the results of Northern blot experiments demonstrated that *drg* was transcriptionally active in NL995 (harbouring the SRE upstream of *drg*) (Fig. 4, lane 1), but not in NL4627 (lacking the SRE) (Fig. 4, lane 2). Altogether these findings will be useful to clarify the structure and the mechanisms responsible for pseudo-transposition of the neisserial SRE, and provide additional evidence of its nature of 'mobile' promoter.

In conclusion, our results demonstrate that *drg*, which is associated with most serogroup B meningococci isolated from patients, is present in about 50% of *N. lactamica* strains, and that the same strains are defective in Dam-methylase activity. These findings are consistent with the hypothesis that a continuous horizontal flow of genetic material affects the chromosomal composition not only of the pathogenic *Neisseria* species, but also of many non-pathogenic species. Genetic exchange between *N. meningitidis* and *N. lactamica* is documented by several studies attempting to provide phylogeny of these organisms. A recent study, based on sequence comparisons of housekeeping genes and the small subunit (16S) rRNA, demonstrates that *N. lactamica* and *N. meningitidis* are closely related species, and collocates them in one of five groups [16]. Indeed, *N. lactamica* and several serogroups of *N. meningitidis* share similar structures and genetic elements, including lipooligosaccharide epitopes [17], porins [18] and a cryptic plasmid [19], in addition to several metabolic properties [20]. Moreover, among the non-pathogenic *Neisseriae*, *N. lactamica* is the more frequently involved species in human diseases [21–28]. Further studies will address

the question whether the absence of Dam activity and/or the presence of an alternative restriction-modification system specific for the GATC sequence in *N. lactamica* has functional consequences for mutation rates and regulation of phase-variable genes, and plays a role in adaptation and, occasionally, in pathogenesis.

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