

HmbR, a Hemoglobin-Binding Outer Membrane Protein of *Neisseria meningitidis*, Undergoes Phase Variation

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Neisseria meningitidis uses hemoglobin (Hb) as an iron source via two TonB-dependent outer membrane receptors, HmbR and HpuB. Analysis of 25 epidemiologically unrelated clinical isolates from serogroups A, B, C, and Y revealed that 64% strains possessed both Hb receptor genes. Examination of the *hmbR* expression pattern in strains in which the *hpuB* gene was genetically inactivated revealed two distinct Hb utilization phenotypes. Five strains retained the ability to grow as a confluent lawn, while seven grew only as single colonies around Hb discs. The single-colony phenotype observed for some *hpuB* mutants is suggestive of phase variation of *hmbR*. The length of the poly(G) tract starting at position +1164 of *hmbR* absolutely correlated with the two Hb utilization phenotypes. All five strains that grew as confluent lawns around Hb discs possessed either 9 or 12 consecutive G residues. All seven strains that grew as single colonies around Hb discs had poly(G) tracts of a length other than 9 or 12. These single-colony variants that arose around the Hb discs had poly(G) tracts with either 9 or 12 consecutive G residues restoring the *hmbR* reading frame. Inactivation of *hmbR* in these strains resulted in a loss of Hb utilization, demonstrating that the change in the *hmbR* gene was responsible for the phenotypic switch. The switching rates from *hmbR* phase off to phase on were $\sim 5 \times 10^{-4}$ in four serogroup C strains, 2×10^{-2} in the serogroup A isolate, and 7×10^{-6} in the serogroup B isolate.

The gram-negative diplococcus *Neisseria meningitidis* is a major cause of bacterial meningitis and is a significant public health problem throughout the world (7, 38). The incidence of meningococcal disease in developing countries ranges between 10 and 25 per 100,000, with periodic epidemics having attack rates of up to 1,000 per 100,000 (6, 12, 45). Approximately 2,600 cases of meningococcal disease occur annually in the United States, predominantly in children less than 2 years old (7). Since the success of the current vaccine against *Haemophilus influenzae* type b, *N. meningitidis* is the leading cause of meningitis in children in the United States (7).

Despite its relatively low incidence, several facts about meningococcal disease make it a potential public health problem of major importance. Mortality from meningococcal meningitis remains, despite optimal antibiotic and supportive therapy, about 5% in children and 10 to 15% in adults (33). There have been recent reports from Europe, Canada, South Africa, and the United States of *N. meningitidis* isolates that show moderate resistance to penicillin (48, 51). The occurrence of a new, more virulent strain of *N. meningitidis* serogroup C associated with an increase in both incidence and mortality of meningococcal disease in Canada is a reminder that the evolution of *N. meningitidis* virulence is ongoing (49).

The best preventive measure against meningococcal disease is a polyvalent polysaccharide vaccine. The vaccine is not protective against serogroup B meningococcus, however, nor can it protect children under 2 years of age (7, 14). Among new vaccine targets against *N. meningitidis* being explored are iron-regulated outer membrane proteins (2, 24). These proteins are attractive vaccine candidates due to their important role in virulence and surface accessibility (2, 24, 39). *N. meningitidis*

expresses several outer membrane receptors that enable the bacterium to use human transferrin, lactoferrin, hemoglobin (Hb), heme, and haptoglobin-hemoglobin complexes (Hpt-Hb) as sources of iron (11, 13, 22, 23, 36, 42). Heme and Hb are the most abundant sources of iron in the body (29). Recently, two neisserial Hb-binding outer membrane receptors have been identified. HmbR is an iron-regulated, 89.5-kDa protein that binds Hb, extracts the heme from it, and transports the heme into the periplasm (42–44). The *hmbR* mutant of *N. meningitidis* was attenuated in an infant rat infection model, confirming the importance of Hb acquisition in meningococcal virulence (42). A second Hb-binding protein is the HpuAB bipartite receptor involved in use of Hb and Hpt-Hb complexes in *N. meningitidis* and *Neisseria gonorrhoeae* (8, 22, 23). In addition to Hb, the HpuAB receptor binds Hpt-Hb complexes and apo-Hpt (22). The HpuAB bipartite receptor is most likely the main Hb receptor of gonococci, since the *hmbR* gene contains a premature stop codon in all gonococcal strains tested (8, 43). Recently, the expression of the *hpuAB* receptor genes in *N. gonorrhoeae* was shown to undergo phase variation (9).

A limited analysis of meningococcal clinical isolates revealed functional redundancy in their Hb utilization systems (43). In this communication, we show that this functional redundancy is a common phenomenon in clinical isolates of *N. meningitidis*. Moreover, we show that the expression of *hmbR* undergoes phase variation due to the slipped-strand mispairing (SSM) of a poly(G) tract beginning at position +1164 in the *hmbR* coding sequence. The rate of switching between the off and on phases of *hmbR* differs more than 1,000-fold between different clinical isolates. These results establish a rational explanation for the existence of functional redundancy in Hb utilization systems and support a novel hypothesis correlating the rates of phase variation and virulence.

MATERIALS AND METHODS

Plasmids, bacteria, and media. Strains and plasmids used in this study are listed in Table 1. The meningococcal strains in this study were epidemiologically

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source(s)
Strains		
IR2781	<i>N. meningitidis</i> serogroup B clinical isolate	D. Stephens
IR2848 to IR2856	<i>N. meningitidis</i> serogroup A clinical isolates	M. Reeves
IR2857 to IR2863	<i>N. meningitidis</i> serogroup C clinical isolates	M. Reeves
IR2843 to IR2847	<i>N. meningitidis</i> serogroup Y clinical isolates	M. Reeves
IR1072 to IR1075	<i>N. meningitidis</i> strains	Lab collection
IR1113, IR1114, IR1311, IR2359, IR2864, and IR2865	<i>N. gonorrhoeae</i> strains	Lab collection
IR2363	<i>N. lactamica</i> O9238	Lab collection
IR2364	<i>N. lactamica</i> E6040	Lab collection
IR2365	<i>N. lactamica</i> O1748	Lab collection
IR2366	<i>N. kochii</i> 31291	Lab collection
IR2367	<i>N. kochii</i> 31292	Lab collection
IR2368	<i>N. flava</i> NS-6	Lab collection
IR2369	<i>N. ovis</i> 72-B	Lab collection
IR2370	<i>N. polysaccharea</i> N462	Lab collection
IR2371	<i>N. mucosa</i> A7895	Lab collection
IR2372	<i>N. cinerea</i> 34382	Lab collection
IR2373	<i>N. cinerea</i> 33683	Lab collection
IR2375	<i>N. subflava</i> B886	Lab collection
IR2376	<i>N. subflava</i> NS-6	Lab collection
IR3261 to IR3277	<i>hpuB::erm</i> derivatives of the following strains in order: IR2781, IR2844 to IR2846, IR2848, IR2850 to IR2853, IR2855 to IR2860, IR2862, and IR2863	This study
IR3287 to IR3292	<i>hmbR</i> phase-on revertants of the following <i>hmbR</i> phase-off strains in order: IR3261, IR3270, IR3272, IR3273, IR3275, and IR3277	This study
IR3297 to IR3318	<i>hmbR::kan</i> derivatives of the following strains in order: IR1074, IR2781, IR2849, IR2851, IR2855 to IR2860, IR2862, IR2863, IR3267, IR3271, IR3274, IR3276, and IR3287 to IR3292	This study
Plasmids		
pDS85	<i>DraI-StuI</i> fragment of <i>hpuB</i> cloned into pBluescript	D. Dyer and L. Lewis
pARR1500	Erm ^r from Tn1545 cloned into <i>ClaI</i> fragment of pDS85	This study
pIRS523	<i>EcoRI-SalI</i> fragment of <i>hmbR</i> cloned into pUC18	Lab collection
pIRS525	Kan ^r cassette cloned into <i>NotI</i> site of pIRS523	Lab collection
pIRS876	<i>PstI-SalI</i> fragment of <i>hmbR</i> cloned into pBluescript	Lab collection

unrelated clinical isolates obtained from Michael Reeves at the Centers for Disease Control and Prevention, Atlanta, Ga. (3, 10). The meningococci were grown on GCB (Difco) agar containing Kellogg's supplements and incubated at 37°C with 5% CO₂. *Escherichia coli* was grown in LB (Luria broth). When necessary, the following antibiotics were used in work with neisseriae: kanamycin at 100 mg/liter and erythromycin at 3 mg/liter (300 mg/liter in *E. coli*). Inactivation of *hpuB* and *hmbR* was performed by transforming meningococci with pARR1500 and pIRS525, respectively. pARR1500 was linearized by digestion with *XbaI*, and pIRS525 was linearized by digestion with *BamHI*. One microgram of linearized DNA was incubated with ~10⁷ bacteria in GCB broth supplemented with 5 mM MgCl₂ for 30 min. Reaction mixtures were transferred to 500 µl of GCB plus Kellogg's supplements and allowed to grow for 6 h. Transformants were selected on GCB plates with the appropriate antibiotic overnight. Southern blot analysis was performed to verify the inactivation of *hmbR* and *hpuB*.

Hb utilization assays. To test the ability of neisseriae to use Hb and/or hemin as the sole iron sources, a suspension of bacteria was plated onto GCB agar containing 50 µM deferoxamine mesylate (Desferal; Ciba Geigy). Filter discs (1/4-in. diameter; Schleicher & Schuell, Inc., Keene, N.H.) supplemented with 10 µl of either 5.0-mg/ml human Hb (Sigma) or 6.5-mg/ml bovine hemin (Sigma) stock solutions and placed onto plates inoculated with *N. meningitidis*. Zones of growth around the discs were recorded after overnight incubation at 37°C and 5% CO₂.

Chromosomal DNA preparations. Approximately one-eighth plate full of *N. meningitidis* or one quarter-plate full of other *Neisseria* species was resuspended in 500 µl of saline-EDTA (150 mM NaCl, 100 mM EDTA [pH 8.0]) and frozen at -80°C. The samples were thawed in the presence of 500 µl of Tris-sodium dodecyl sulfate (SDS) (100 mM Tris [pH 9.0], 100 mM NaCl, 1% SDS) at 50°C. DNA was extracted with saline-EDTA-equilibrated phenol and centrifuged at 14,000 × *g* for 20 min. The supernatant was removed, and DNA was precipitated with isopropanol and washed with 70% ethanol. DNA was resuspended in double-distilled H₂O (ddH₂O) and stored at 4°C.

Outer membrane preparations. Cells of *N. meningitidis* grown on one plate were resuspended in 20 ml of GCB medium containing Kellogg's supplements minus iron but with 50 µM deferoxamine mesylate and incubated for 4 h at 37°C with 5% CO₂. Cells were pelleted by centrifugation at 500 × *g* and placed on ice. The pellet was resuspended in 1.6 ml of 600 mM sucrose-6 mM EDTA-200 mM Tris (pH 8.0). Spheroplasts were generated by treating the cells with 200 µg of

lysozyme (2 mg/ml in 200 mM Tris [pH 8.0]) in 5 ml of ddH₂O. Outer membranes were extracted from spheroplasts with 2% Triton X-100 in 50 mM Tris (pH 8.0) in the presence of 10 mM MgCl₂ and 100 µg of DNase. Outer membranes were sedimented by centrifugation at ~40,000 × *g* for 60 min. Outer membranes were washed and resuspended in ddH₂O.

Southern blot hybridization, PCR, and DNA sequencing. Standard methods for plasmid DNA preparation, restriction endonuclease analysis, and ligation were performed by the methods of Sambrook et al. (33a). Southern blot analysis was done with a digoxigenin (DIG) nonradioactive DNA labeling and detection kit (Genius system; Boehringer, Mannheim, Germany) under medium-stringency conditions (two washes with 2× SSC-0.1% SDS [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at room temperature followed by two washes in 0.5× SSC-0.1% SDS at 60°C). The internal *hpuB*-specific probe was obtained via random priming of the *DraI-StuI* fragment of pDS85. The internal *hmbR*-specific probe was obtained as described above with the *PstI-SalI* fragment from pIRS876. PCRs were performed with *Taq* polymerase. The ~600-bp fragment containing the poly(G) tract of *hmbR* was amplified by using primers *hmbRpg1a* (5' GCTTATCAAATCAACGACAACCACC 3') and *hmbRpg1b* (5' GGTGTT TTGTACAAGCATGAC 3') and an annealing temperature of 60°C. This fragment was sequenced by dye terminator cycle sequencing on an ABI model 377 automated sequencer with primers *hmbRpg1a* and *hmbRpg1b* to obtain the nucleotide sequence of both DNA strands.

Determination of the rate of switching. Single colonies from *hpuB::erm* strains that were *hmbR* phase off were isolated on nonselective GCB plates and resuspended in 200 µl of GCB broth. Samples (50 µl) of 10⁻¹ to 10⁻³ serial dilutions were plated on GCB plates containing 50 µM deferoxamine mesylate and 100 mg of Hb per liter. Samples (50 µl) of 10⁻⁴ to 10⁻⁶ serial dilutions were plated on nonselective GCB plates. The *hmbR*-off to -on switching rates were determined by dividing the number of colonies on GCB-deferoxamine mesylate-Hb plates by the number of colonies on the nonselective GCB plates. Medians of eight independent measurements were used for comparisons between strains. Statistical significance of measurements was determined by nonparametric Wilcoxon signed-rank and rank sum tests. Validity of the assay was determined by plating *hmbR* phase-on strains and determining the ratio of colonies on restrictive versus nonrestrictive GCB plates.

Western blot analysis. Samples (2 µl) of outer membrane preparations were boiled in SDS buffer and loaded in a 10% polyacrylamide gel. Samples were

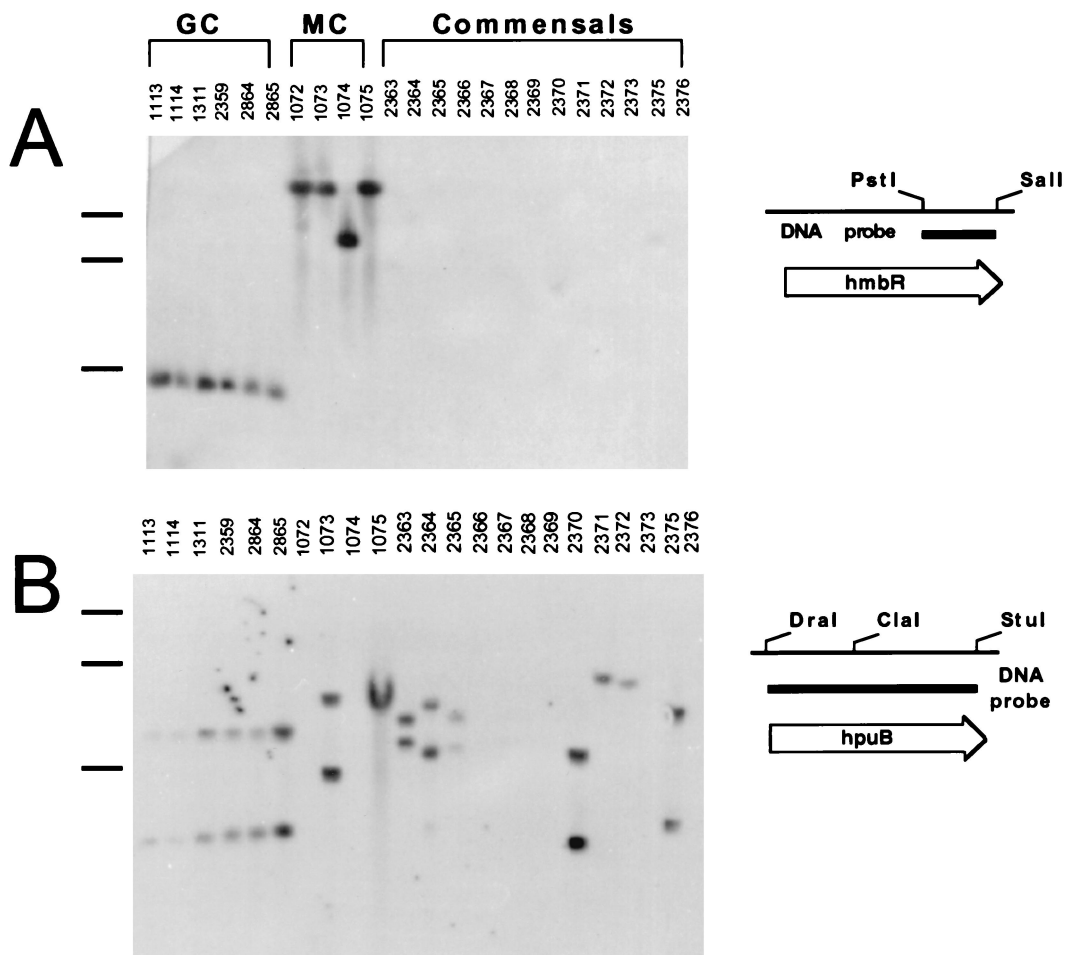


FIG. 1. Distribution of *hmbR* and *hpuB* genes in isolates of *N. gonorrhoeae* (GC), *N. meningitidis* (MC), and commensal *Neisseria* species. *N. meningitidis* 1072 and 1075 represent serotype C, strain 1074 represents serotype B, and strain 1073 represents serotype A. *ClaI*-digested chromosomal DNA from indicated strains was hybridized with *hmbR*-specific (A) or *hpuB*-specific (B) DIG-labeled DNA probes. The positions of 3-, 6-, and 12-kb marker bands are indicated by the black bars to the left of the gel (from the bottom to the top).

transferred to OPTITRAN BA-S 83 filter by using a Schleicher & Schuell Pronto Semi-Dry Electroblotter and discontinuous buffers. The membrane was probed with a 1:1,000 dilution of a polyclonal rabbit anti-glutathione *S*-transferase fused to HmbR' N terminus (anti-2532#2) and washed with phosphate-buffered saline containing 0.05% Tween 20. Samples were probed with a 1:10,000 dilution of goat anti-rabbit antibody conjugated to horseradish peroxidase. Signals were detected by fluorography, using equal volumes of Enhanced Luminol Reagent and Oxidizing Reagent (NEN Life Science Products Renaissance Western Blot Chemiluminescence Reagent).

RESULTS

Distribution of *hmbR* and *hpuB* among *Neisseria* species.

N. meningitidis can express two receptors, HmbR and HpuB, capable of utilizing free Hb as an iron source (23, 42). The distribution of these two receptors in different species and serotypes was investigated by DNA hybridization. Chromosomal DNA from six strains of *N. gonorrhoeae* hybridized with both *hmbR* and *hpuB* probes (Fig. 1A and B). Chromosomal DNA from only 2 of the 13 tested commensal *Neisseria* strains hybridized with the *hmbR* probe. These two commensal strains, 2370 (*Neisseria polysaccharea* N462) and 2375 (*Neisseria subflava* B886), yielded only very weak signals (Fig. 1A). In contrast, the *hpuB* probe hybridized strongly with chromosomal DNA from 7 of the 13 commensal strains: 2363 (*Neisseria lactamica* O9238), 2364 (*N. lactamica* E6040), 2365 (*N. lac-*

tamica O1748), 2370 (*N. polysaccharea* N462), 2371 (*Neisseria mucosa* A7895), 2372 (*Neisseria cinerea* 34382), and 2375 (*N. subflava* B886) (Fig. 1B). Chromosomal DNA from twenty-five *N. meningitidis* clinical isolates, representing four serotypes, was tested with *hmbR* and *hpuB* probes. The *hmbR*-specific probe hybridized with 18 of the 25 chromosomal DNAs, and the *hpuB* probe hybridized with 23 (Fig. 1 and 2). All nine serogroup C meningococcal strains tested possessed both *hmbR* and *hpuB* with the exception of strain 1072, which did not yield a signal with the *hpuB* probe. The single serogroup B isolate, strain 1074, possessed only *hmbR* (Fig. 1). The largest divergence in the distribution of *hmbR* and *hpuB* was found in serogroups A and Y. All of the 10 serogroup A isolates contained *hpuB*, but only 6 possessed *hmbR* (Fig. 1 and 2). Similarly, all five serogroup Y strains possessed *hpuB*, while only two contained *hmbR* (Fig. 2). The absence of *hmbR* in the serogroup A and Y isolates was confirmed by PCR (data not shown).

Characterization of different Hb utilization phenotypes in *N. meningitidis*. All wild-type *N. meningitidis* strains tested were able to use human Hb as an iron source with the exception of strain 2844. Furthermore, nearly all strains that used Hb grew as a uniform lawn around Hb-supplemented discs, strain 2781 being the only exception (Table 2). In order to study the function of HmbR, the *hpuB* gene was inactivated by insertion

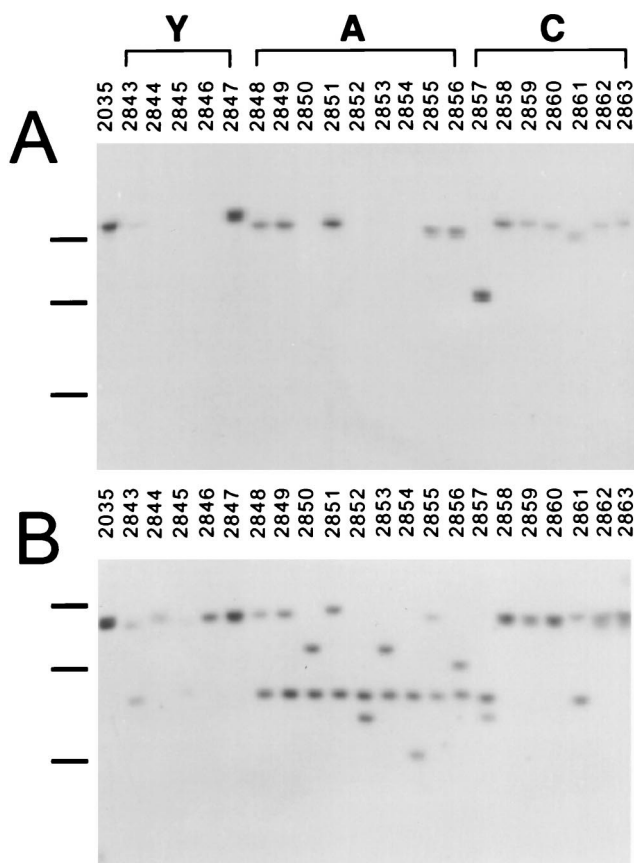


FIG. 2. Distribution of *hmbR* and *hpuB* in *N. meningitidis* clinical isolates representing serogroups Y, A, and C. *Cla*I-digested chromosomal DNA from indicated strains was hybridized with *hmbR*-specific (A) or *hpuB*-specific (B) DIG-labeled DNA probes. The positions of 3-, 6-, and 12-kb marker bands are indicated by the black bars to the left of the gel (from the bottom to the top). Due to the small amount of DNA loaded in lane 2845, the *hpuB*-hybridizable band is relatively weak.

of an erythromycin cassette. The *hpuB*-negative strains displayed two distinct Hb utilization phenotypes in the disc diffusion assay: (i) single-colony growth around a disc supplemented with Hb (3261, 3270, 3272, 3273, 3275, 3276, and 3277), and (ii) confluent growth around the Hb disc (1074, 3265, 3267, 3271, and 3274) (Table 2). The *hpuB::erm* strains that did not use Hb as a source of iron (3262, 3263, 3264, 3266, 3268, and 3269) were found not to possess *hmbR* (Fig. 2A and Table 2). Similar phenotypes with regard to growth around Hb discs were observed when the function of HpuB was tested in an *hmbR*-negative genetic background. Eleven strains were still able to grow as confluent lawns despite the loss of *hmbR*, whereas six grew only as single colonies (Table 2).

These data suggest that some meningococcal strains, although possessing both Hb receptor genes, were expressing only one receptor. The single-colony phenotype observed in some *hmbR::kan* and *hpuB::erm* genetic backgrounds may be due to the selection for rare switching-on events in *hmbR* and *hpuB* genes. Alternatively, Hb⁺ revertants may be the result of activation of some currently unknown Hb utilization mechanism in meningococci.

Correlation between *hmbR* genotypes and different Hb utilization phenotypes in *N. meningitidis*. Recently, the HpuAB receptor of *N. gonorrhoeae* was shown to undergo phase variation due to the SSM at the poly(G) tract in the *hpuA* gene (9).

Since the *N. meningitidis* *hpuA* and *hpuB* genes are highly homologous to *N. gonorrhoeae* *hpuAB* genes, it is very likely that the observed Hb⁺ revertants in the *hmbR::kan* background arose in *hpuAB* by a phase variation mechanism (9, 23).

The single-colony phenotype of the *hpuB::erm* strains could be also explained by the phase variation of *hmbR*. Indeed, the *hmbR* gene has a poly(G) tract located at position +1164 relative to the *hmbR* start codon that could be a substrate for SSM (42). Poly(G) tracts containing 9 or 12 consecutive G residues would allow *hmbR* to remain in frame with respect to the start codon. In order to determine the number of G residues in the poly(G) tract of *hmbR*, a 600-bp region surrounding the poly(G) tract of the wild-type isolates was PCR amplified and sequenced. The length of the poly(G) tract in the *hmbR* genes of 12 clinical isolates (Table 2) was compared to the Hb utilization phenotypes of their *hpuB::erm* derivatives (Table 2). All five strains (1074, 3265, 3267, 3271, and 3274) possessing either 9 or 12 G residues grew as a uniform lawn around Hb discs (putative *hmbR* phase on). All seven strains possessing poly(G) tracts of a length other than 9 or 12 (3261, 3270, 3272, 3273, 3275, 3276, and 3277) grew as single colonies around Hb discs (putative *hmbR* phase off). The length of poly(G) tracts in three clinical isolates, 2843, 2847, and 2849, were not correlated with the phenotypes of their *hpuB::erm* derivatives because these mutants could not be isolated (Table 2). These data, though merely a correlation, support the hypothesis that *hmbR* undergoes phase variation via SSM at a run of consecutive G residues within the coding region.

Demonstration of *hmbR* switching in *N. meningitidis*. When the Hb⁺ revertant colonies (3288, 3289, 3290, 3291, and 3292) of *hpuB::erm* mutants (3270, 3272, 3273, 3275, and 3277) were assayed for Hb utilization, all grew as confluent lawns (Fig. 3, right). The inactivation of *hmbR* in these *hpuB::erm* strains eliminated Hb utilization, indicating that the HmbR receptor is responsible for the Hb⁺ phenotype. Southern blot hybridization confirmed that the *hpuB* gene in each revertant was still inactive (data not shown). These data demonstrated that the single-colony phenotype originates from changes within *hmbR*, most likely due to the SSM in the poly(G) tract of *hmbR*. Indeed, when the poly(G) tracts of the putative phase-on *hmbR* revertants were analyzed, all possessed either 9 or 12 consecutive G residues, while the parent strains had 8, 10, or 13 (Fig. 3). The only exception was strain 2855. This strain 2855 grew as a confluent lawn, while both *hpuB::erm* (3270) and *hmbR::kan* (3301) derivatives grew as single colonies around Hb discs (Table 2). In addition, the *hmbR* poly(G) tract contained eight G residues, indicating that the gene is switched off. However, the single-colony phenotype of *hpuB* and *hmbR* derivatives of strain 2855 was different from single-colony phenotypes of other studied strains. While an average single-colony phenotype is characterized by a few dozen colonies growing around Hb discs (Fig. 3, middle), several hundred colonies were growing around Hb discs when *hpuB::erm* and *hmbR::kan* derivatives of 2855 strain were analyzed. Due to the large number of single colonies growing around Hb discs, the single-colony phenotype of strain 2855 appeared on the plates as a confluent lawn.

Confirmation of *hmbR* expression patterns in *N. meningitidis*. To confirm that the observed Hb utilization phenotypes corresponded with the actual expression of *hmbR*, a Western blot analysis was performed. HmbR was detected in outer membrane preparations from several *N. meningitidis* strains by using rabbit polyclonal anti-HmbR antibody (Fig. 4). DNA from strains 2845 and 2850 did not hybridize with the *hmbR* probe (Fig. 2), and these strains were used as negative controls. The strong signal from outer membranes of strain 2851 and its

TABLE 2. Genotypic and phenotypic characterization of *N. meningitidis* clinical isolates and isogenic *hpuB::erm* and *hmbR::kan* derivatives

Serotype	Clinical isolates			<i>hpuB::erm</i> derivatives			<i>hmbR::kan</i> derivatives		
	Strain	<i>hmbR</i> ^a	<i>hpuB</i> ^a	PH ^b	Strain	No. of G ^c	PH	Strain	PH
C	2857	+	+	LW	3272	8	SC	3303	LW
	2858	+	+	LW	3273	13	SC	3304	LW
	2859	+	+	LW	3274	12	LW	3305	SC
	2860	+	+	LW	3275	10	SC	3306	LW
	2861	+	+	LW	NT ^d	*	NA	NT	NA
	2862	+	+	LW	3276	13	SC	3307	LW
	2863	+	+	LW	3277	13	SC	3308	LW
A	2848	+	+	LW	3265	9	LW	NT	NA
	2849	+	+	LW	NT	9	NA	3299	SC
	2850	-	+	LW	3266	*	0	NA	LW
	2851	+	+	LW	3267	9	LW	3300	SC
	2852	-	+	LW	3268	*	0	NA	LW
	2853	-	+	LW	3269	*	0	NA	LW
	2854	-	+	LW	NT	*	NA	NA	LW
	2855	+	+	LW	3270	8	SC	3301	SC
	2856	+	+	LW	3271	9	LW	3302	SC
Y	2843	+	+	LW	NT	10	NA	NT	NA
	2844	-	+	0	3262	*	0	NA	0
	2845	-	+	LW	3263	*	0	NA	LW
	2846	-	+	LW	3264	*	0	NA	LW
	2847	+	+	LW	NT	10	NA	NT	NA
B	1074	+	-	LW	NA ^e	9	LW	3297	0
	2781	+	+	SC	3261	8	SC	3298	SC

^a Presence (+) or absence (-) of homologous sequence determined by Southern blot hybridization.

^b The Hb utilization phenotype (PH) of the strain is as follows: LW, confluent lawn; SC, single colony; NA, not applicable; 0, no growth.

^c Number of consecutive G residues in the poly(G) tract of *hmbR*. An asterisk indicates that no sequence was obtained.

^d NT, no transformants obtained.

^e NA, not available.

hpuB::erm derivative 3267 (Fig. 4) reinforced earlier observations that these strains contain a phase-on copy of *hmbR* (Fig. 2 and Table 2). Strains 3261, 3272, 3273, 3275, and 3277 all hybridized with the *hmbR* probe (Fig. 2) but were all in the off phase (Table 2). None of these strains gave a signal when probed with anti-HmbR antibody (Fig. 4). However, the phase-on revertants of these strains (3287, 3289, 3290, 3291, and 3292, respectively) all gave strong signals with the anti-HmbR antibody (Fig. 4). Conversely, the outer membrane preparations of the *hpuB::erm hmbR::kan* double mutants (3313, 3315, 3316, 3317, and 3318, respectively) gave no signals with the antibody. This result confirmed that the Hb⁺ phenotype of the phase-on *hmbR* revertants was dependent on HmbR expression.

Determination of the rate of switching of the *hmbR* gene.

The rate at which *hmbR* switches from phase off to phase on was determined for each of the six *hmbR* phase-off strains (*hpuB::erm* derivatives of wild-type clinical isolates). The switching rates for strains 3272, 3273, and 3275 ranged from 4.2×10^{-4} to 5.5×10^{-4} . Strain 3277 switched at a lower rate of 3.4×10^{-5} . Strain 3261 switched at a rate that was 40-fold lower than those of 3272, 3273, and 3275, namely, 7.4×10^{-6} . Strain 3270 switched at a rate 70-fold higher than those of strains 3272, 3273, and 3275, namely, 2.0×10^{-2} . All rates are median values for eight independent measurements.

DISCUSSION

It has been previously shown that 95% of tested meningococci and 60% of gonococci were able to use Hb, while all tested strains used free heme as a source of iron (27). The ability of Hb⁺ pathogenic neisseriae to bind Hb in vitro indi-

cated the existence of cell surface Hb receptor(s) (20). This hypothesis has been confirmed by the independent discovery of two outer membrane Hb-binding proteins, HmbR and HpuB (22, 23, 42). Data presented in this study confirmed the universal ability of meningococci to use Hb and heme: only one serogroup Y isolate (2844) was unable to use Hb as a source of iron, although it contained *hpuB*-like nucleotide sequences. More interestingly, hybridization data showed that 64% (16 of 25) of strains possessed both receptor genes, indicating that the redundancy of Hb receptors is a common phenomenon in meningococci. The analysis established that 23 of 25 strains of meningococci possessed *hpuB*, while 7 of 25 isolates, clustered among serogroups A and Y, lacked the *hmbR* gene. This distribution pattern may indicate the relative importance of Hb receptors, since the more prevalent HpuAB receptor uses both Hpt-Hb and Hb, while HmbR is restricted to Hb use (22, 23, 42). The lack of *hmbR* and the high prevalence of *hpuB* in commensals suggest more recent acquisition of *hmbR* by meningococci.

The ability to use Hb has been shown to promote virulence of meningococci, thus categorizing Hb receptors as potential virulence factors (37, 42). The overlap in the functions of HmbR and HpuB is not unusual, and the functional redundancy of virulence factors has been observed in several bacterial pathogens (18, 31, 32, 40). The possession of redundant virulence factors by bacteria compromises the ability of the host immune system to control infection. If this were the main purpose of redundancy, bacteria should be able to regulate the expression of redundant virulence factors in order to minimize their exposure to immune response. One way of controlling the expression of redundant virulence functions in bacteria is by

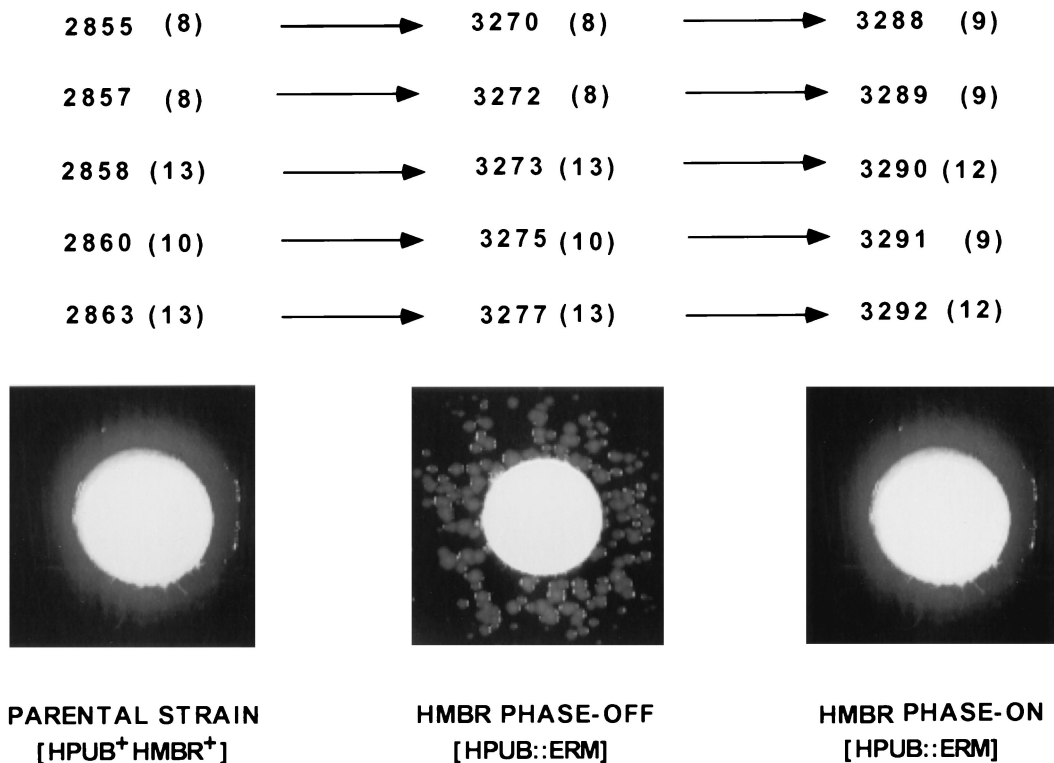


FIG. 3. Phase variation of *hmbR*. The number of G residues in the poly(G) tract of *hmbR* is shown in parentheses for each strain analyzed. The photographs shown are examples of the phenotypes seen for each group of strains. Parental strains (left) all demonstrate a phase-on phenotype. After inactivation of *hpuB*, the phase-off phenotype of *hmbR* can be seen as a single-colony phenotype (middle). These single colonies, representing rare phase-on cells, show a confluent lawn phenotype when tested again for Hb utilization (right). The changes in the Hb utilization phenotypes were accompanied by changes in the number of G residues in the *hmbR* poly(G) tracts.

phase variation. Phase variation is the alteration of the gene expression between on and off phases as a result of reversible changes at the DNA level. It is a common mechanism of controlling the expression of surface-exposed virulence factors in many mucosal pathogens including *Neisseria* spp., *H. influenzae*, and possibly *Haemophilus pylori* (1, 9, 15, 16, 30, 31, 35, 40, 50). A variety of different mechanisms, including DNA inversion, recombination, and SSM have been proposed to account for phase variation in bacteria (21, 25, 26, 41, 53). SSM occurs as a result of illegitimate annealing of DNA containing multiple short tandem repeats. Subsequent replication or repair of this mispaired sequence may result in insertion or deletion of integral repeat units (21). Short repetitive sequences within coding regions can undergo SSM resulting in translational frameshifts, whereas SSM of repeats in promoter elements can result in various levels of transcription. Short polypurine or polypyrimidine tracts are capable of mediating SSM (21). The repetitive element (5' CTCTT 3')_n has been implicated in the phase variation of several genes both transcriptionally and translationally (i.e., opacity proteins in *N. gonorrhoeae* and class V proteins and Opc in *N. meningitidis*) (4, 19, 28). Homopolymeric tracts are conducive to SSM as well. Poly(G) tracts mediate phase variation of several genes encoding surface-exposed molecules in neisseriae (15, 17, 18, 34, 47, 52).

In the course of studying *hmbR* and its role in Hb utilization, it was recognized that some strains that hybridized with an *hmbR*-specific probe did not express HmbR in the outer membranes. These observations led to the hypothesis that the expression of *hmbR* undergoes phase variation. Examination of Hb utilization phenotypes, HmbR expression, and nucleotide

sequence in 15 strains representing four serogroups confirmed the role of the poly(G) tract in the phase variation of *hmbR*. While *hmbR* transcript levels were not examined, the position of the poly(G) tract in the middle of the *hmbR* coding region indicates translational regulation. In strains expressing func-

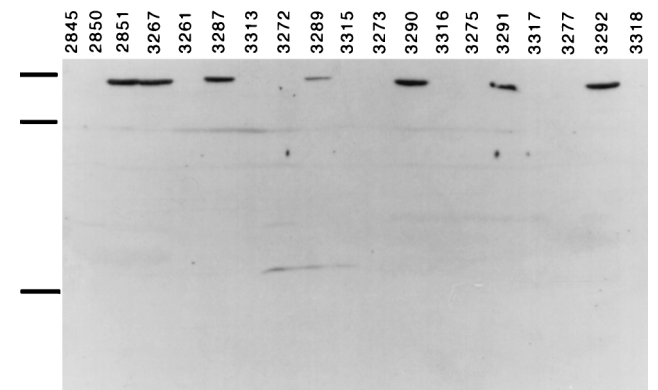


FIG. 4. Western blot analysis of *N. meningitidis* outer membrane preparations isolated from strains with different HmbR-dependent Hb utilization phenotypes. Strains 2845 and 2850 are both HmbR negative controls. Strain 2851 and its *hpuB::erm* derivative 3267 are *hmbR* phase on. Strains 3261, 3272, 3273, 3275, and 3277 are *hmbR* phase off. Strains 3287, 3289, 3290, 3291, and 3292 are *hmbR* phase-on revertants of strains 3261, 3272, 3273, 3275, and 3277, respectively. Strains 3313, 3315, 3316, 3317, and 3318 are *hmbR::kan* derivatives of strains 3287, 3289, 3290, 3291, and 3292, respectively. The positions of 97.4-, 68-, and 18.4-kDa marker bands are indicated by the black bars to the left of the gel (from the top to the bottom).

tionally redundant Hb receptors, it would be advantageous for bacteria to phase vary both receptors. Indeed, the second Hb receptor, HpuAB, has been shown to phase vary in *N. gonorrhoeae* (9). The data presented in this study suggest that the expression of meningococcal HpuAB also undergoes phase variation. Another mucosal pathogen, *H. influenzae*, possesses at least two independent surface-exposed Hb or Hb-Hpt receptors, the expression of which are under the control of SSM-mediated phase variation (31, 32). Therefore, phase variation may be a common mechanism by which mucosal pathogens regulate the expression of surface-exposed molecules with redundant functions. Interestingly, 2 of 10 strains (2855 and 2781) examined had both receptors in off phase, while none of the strains had both receptors switched on. Despite having both receptors switched off, strain 2855 gave confluent growth phenotype around Hb discs due to the very high rate of switching of both genes.

The rates at which many of the phase-varying genes switch between different phases of expression has been determined to be $\sim 10^{-4}$ (4, 15, 28, 46). The rate of *hmbR* switching in all four serogroup C isolates tested was on the order of 10^{-4} to 10^{-5} . However, serogroup A and B isolates gave significantly different rates, 10^{-2} and 10^{-6} , respectively. Though the rate of phase-on to -off transition could not be measured, it is reasonable to assume that it occurs at a similar rate. One explanation for the different rates of switching in serogroup A and B isolates could be small differences in nucleotide sequence surrounding the poly(G) tract of *hmbR*. Some sequences may be more conducive to SSM than others. However, inspection of approximately 600 bp surrounding the poly(G) tract of *hmbR* revealed 94% identity between the strains of different serotypes. Another plausible explanation for differences in rates of switching between strains could be a variation in levels of *hmbR* transcription. Indeed, a recent study has indicated that promoter strength may influence the rate at which genes phase vary (5). The serogroup A strain found to have a higher rate of switching may have higher levels of *hmbR* transcription. Likewise, the low rate of switching of *hmbR* in the serogroup B isolate may be due to decreased transcription. However, Western blot analysis of outer membrane preparations from the serogroup B isolate did not show any decrease in HmbR expression.

We hypothesize that the differences in the rate of switching of *hmbR* among meningococcal isolates is attributed to different genetic backgrounds of these strains. *N. meningitidis* may possess a mechanism that can modulate the rate of switching of phase-varying genes. Strains with higher rates of switching would be able to adapt to the hostile environment of the host more successfully, leading to higher colonization, disease, and transmission rates. Additional work must be done to prove the connection between the rate of switching and virulence of meningococci, however.

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