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HmbR Outer Membrane Receptors of Pathogenic *Neisseria* spp.: Iron-Regulated, Hemoglobin-Binding Proteins with a High Level of Primary Structure Conservation

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We have recently cloned and characterized the hemoglobin receptor gene from *Neisseria meningitidis* serogroup C. *N. meningitidis* cells expressing HmbR protein were able to bind biotinylated hemoglobin, and the binding was specifically inhibited by unlabeled hemoglobin and not heme. The HmbR-mediated hemoglobin binding activity of *N. meningitidis* cells was shown to be iron regulated. The presence of hemoglobin but not heme in the growth medium stimulated HmbR-mediated hemoglobin binding activity. The efficiency of utilization of different hemoglobins by the HmbR-expressing *N. meningitidis* cells was shown to be species specific; human hemoglobin was the best source of iron, followed by horse, rat, turkey, dog, mouse, and sheep hemoglobins. The phenotypic characterization of HmbR mutants of some clinical strains of *N. meningitidis* suggested the existence of two unrelated hemoglobin receptors. The HmbR-unrelated hemoglobin receptor was shown to be identical to Hpu, the hemoglobin-haptoglobin receptor of *N. meningitidis*. The Hpu-dependent hemoglobin utilization system was not able to distinguish between different sources of hemoglobin; all animal hemoglobins were utilized equally well. HmbR-like genes are also present in *N. meningitidis* serogroups A and B, *Neisseria gonorrhoeae* MS11 and FA19, *Neisseria perflava*, and *Neisseria polysaccharea*. The hemoglobin receptor genes from *N. meningitidis* serogroups A and B and *N. gonorrhoeae* MS11 were cloned, and their nucleotide sequences were determined. The nucleotide sequence identity ranged between 86.5% (for *N. meningitidis* serogroup B *hmbR* and MS11 *hmbR*) and 93.4% (for *N. meningitidis* serogroup B *hmbR* and *N. meningitidis* serogroup C *hmbR*). The deduced amino acid sequences of these neisserial hemoglobin receptors were also highly related, with overall 84.7% conserved amino acid residues. A stop codon was found in the *hmbR* gene of *N. gonorrhoeae* MS11. This strain was still able to use hemoglobin and hemoglobin-haptoglobin complexes as iron sources, indicating that some gonococci may express only the HmbR-independent hemoglobin utilization system.

The survival of bacterial pathogens in the host depends on the ability of the microorganism to scavenge iron, a rate-limiting element for successful bacterial replication (9, 32, 49). Heme is the most abundant source of iron in mammalian bodies; it is found in erythrocytes as a component of hemoglobin (33). Utilization of heme iron is widespread among pathogenic bacteria but has only recently become an intensive area of research. Coulton and Pang were the first to show that the heme uptake in *Haemophilus influenzae* requires energy and that both the porphyrin ring and iron are internalized by the cell (8). The recently characterized heme utilization in *Yersinia enterocolitica* was shown to be very similar to the utilization of siderophore-iron complexes in gram-negative bacteria. Heme is transported into bacterial periplasm by the heme-specific outer membrane receptor (43). The transport is driven by the energy of the cytoplasmic membrane that is delivered to the receptor by the TonB-ExbBD proteins (21–23, 37, 43). Once in the periplasm, heme is shuttled into the cytoplasm through the heme-specific periplasmic binding protein-dependent transport mechanism. The *Y. enterocolitica* HemS protein was postulated to be a heme oxygenase involved in the release of iron from porphyrin and in the protection of cells against heme-induced toxicity (43, 44). Similar heme utilization systems have recently been identified in many gram-negative bacteria: *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Yersinia pestis*, shigellae, *Escherichia coli*, *Seratia marcescens*, *Plesiomonas shigelloides*, *Porphyromonas gingivalis*, *H. influenzae*, and *Haemophilus ducreyi* (5, 8, 10, 15, 18–21, 24, 34, 38, 46, 47, 51). Some pathogens have abilities to utilize different heme-containing compounds, such as hemoglobin (Hb), heme-hemopexin complexes, and Hb-haptoglobin (Hb-Hpt) complexes, and therefore to circumvent the host's heme-scavenging defenses (4, 11–14, 25, 27–29, 45, 50). Moreover, the identification of a TonB-independent mechanism of heme utilization in *H. influenzae* suggests that similar systems may exist in other gram-negative bacteria (38).

One among the microorganisms best adapted to the hostile environment in host fluids and surfaces is *Neisseria meningitidis*, a causative agent of bacterial meningitis. Neisseriae are able to bind and extract iron from the host iron-binding proteins transferrin and lactoferrin and the heme-containing proteins Hb and Hb-Hpt complexes and can utilize the heme moiety alone but not heme bound to human serum albumin (2, 6, 7, 11, 25, 26, 28–30, 35, 36). We have recently characterized the Hb receptor of *N. meningitidis* (45). Hb receptor-negative mutants were still able to use heme as an iron source, indicating the existence of a separate heme-utilizing system in this organism.

In this report, we show that the binding of biotinylated Hb to *N. meningitidis* cell surfaces is HmbR dependent and that the expression of this activity is iron regulated and not heme regulated. The HmbR-dependent Hb binding was inhibitable with

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
N. meningitidis		
IR1072	Serogroup C, 8013#6	45
IR1073	Serogroup A, ATCC 13011	ATCC[a]
IR1074	Serogroup B, clinical isolate	Laboratory collection
IR1075	Serogroup C, clinical isolate	Laboratory collection
IR1077	IR1074 *hmbR*::Km^r	This study
IR1078	IR1075 *hmbR*::Km^r	This study
IR1097	*hmbR* deletion derivative of IR1072	This study
IR2036	IR1078 but *hpu*::mTn*3*Erm	This study
N. gonorrhoeae		
IR1113	MS11	Laboratory collection
IR1114	FA19	Laboratory collection
N. polysaccharea		Laboratory collection
N. perflava		Laboratory collection
B. catarrhalis		Laboratory collection
Plasmids		
pCRII	Amp^r Km^r	Invitrogen
pSUSK/KS	Chl^r, pAT15 replicon	42
pBluescript	Amp^r	Stratagene
pWKS30	Amp^r	48
pIRS523	pUC19, *hmbR′*	45
pSM85kE	*hpu*::mTn*3*Erm	28
pVIV10	pIRS523 but Δ*Mlu*I-*Pst*I	This study
pIRS744	*hmbR* from IR1074, cloned into pCRII	This study
pIRS747	*hmbR* from IR1074, cloned into pSUSK	This study
pIRS748	*hmbR* from IR1074, cloned into pBluescript	This study
pIRS749	*hmbR* from IR1073, cloned into pSUKS	This study
pIRS756	*hmbR* from IR1113, cloned into pWKS30	This study
pIRS747.2	Km^r cassette (KSAC; Pharmacia) inserted into *hmbR Pst*I site of IRS747	This study
pIRS749.1	Km^r cassette (KSAC; Pharmacia) inserted into *hmbR Pst*I site of IRS749	This study
pMS11.INTERNAL	*Taq* DNA polymerase-generated PCR product carrying part of MS11 *hmbR* gene	This study

[a] ATCC, American Type Culture Collection.

excess unlabeled Hb and not heme, indicating that the HmbR protein recognizes the globin moiety of Hb. Human Hb was the best source of iron for HmbR-expressing cells, followed by horse, dog, sheep, goat, turkey, rat, and mouse Hbs. We present evidence that some clinical strains of *N. meningitidis* possess two unrelated systems involved in Hb utilization. The second, HmbR-unrelated Hb utilization system, which is also involved in utilization of Hb-Hpt complexes, was shown to be identical to the recently described Hpu protein of *N. meningitidis* (28, 29). This system utilized all tested Hbs equally well, suggesting that it recognizes a heme moiety on Hb. Finally, we present data indicating the widespread occurrence of *hmbR*-related sequences in neisseriae and a high level of amino acid conservation between HmbR proteins from *N. meningitidis* serogroups A, B, and C.

MATERIALS AND METHODS

Plasmids, bacteria, and media. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in Luria broth. The meningococci were grown on GCB (Difco) agar containing supplements as described previously (45) and were incubated at 37°C with 5% CO_2. Transformation of meningococci was performed as previously described (45). When necessary, the antibiotics chloramphenicol (20 mg/liter) and carbenicillin (100 mg/liter) were used in work with *E. coli*. For neisseriae, kanamycin (100 mg/liter) and erythromycin (3 mg/liter) were used when needed.

To test the ability of neisserae to use various iron-containing proteins as sole iron sources, a suspension of neisseriae was plated onto GCB agar containing 75 μM deferoxamine mesylate (Desferal; Ciba-Geigy). Filter discs (1/4 in. [ca. 0.6 cm]; Schleicher & Schuell, Inc., Keene, N.H.) impregnated with test compounds (10 μl of 5-mg/ml stock solutions unless otherwise stated) were placed on these plates. Zones of growth around the discs were recorded after overnight incubation at 37°C in the presence of 5% CO_2. Different animal Hbs (human, rat, dog, goat, garter snake, sheep, and mouse Hbs and the modified *N,N′*-dimethylated bovine Hb) were obtained from Sigma Chemical Co., St. Louis, Mo. Hemin (bovine), Hpt (human), and lactoferrin (human; 90% iron saturated) were also obtained from Sigma. The concentrations of all animal Hbs used in the study were adjusted to 5 mg/ml with an Hb diagnostic kit (Sigma). Human transferrin (90% iron saturated) was obtained from Boehringer Mannheim Biochemicals.

Comparison of the efficiencies of utilization of various Hbs by *N. meningitidis* IR1072. Bacteria grown overnight were resuspended in 1 ml of GCB medium (without supplements), and 100 μl was plated onto GCB plates supplemented with 75 μM Desferal. Filter paper discs soaked with different amounts of various freshly prepared animal Hbs were placed on the plate together with the disc soaked with a standard amount of human Hb (3 μl of a 5-mg/ml solution). The zones of growth around Hb discs were scored after 20 h of incubation at 37°C in the presence of 5% CO_2. Six independent experiments were performed.

Binding of biotinylated Hb to whole cells and outer membranes. Bacteria grown overnight were diluted in fresh GCB medium (1:30), supplemented with different concentrations of iron (ferric nitrate), heme (hemin chloride), or Hb (human Hb), and grown for approximately 3 h at 37°C in the presence of 5% CO_2. The bacteria were transferred into a microcentrifuge tube, spun down (3,000 × *g*, 5 min), washed once with TBS (50 mM Tris HCl, 100 mM NaCl, pH 7.5), and resuspended in 200 μl of TBS. Bacteria were transferred onto a nitrocellulose-cellulose membrane (Millipore HAHY 10710) by using a dot blot manifold (Schleicher & Schuell Minifold I) and air dried. Unspecific binding sites were blocked by incubating the membrane in a blocking solution (TBS, 5% skim milk, 0.025% Tween 20) at room temperature. Hb, prepared by using the Gibco BRL protein biotinylation kit, was applied to the membrane in the binding solution (blocking solution plus biotinylated Hb) in a final concentration of 50 nM for 1 h at room temperature. The membrane was rinsed twice for 5 min each with 50 ml of rinsing solution (TBS plus 0.15% Tween 20) and then rinsed once with 50 ml of TBS alone. The membrane was incubated at 37°C for 45 min in 50 ml of blocking solution containing 15 μl of a streptavidin-alkaline phosphatase complex (Boehringer Mannheim). The membrane was rinsed twice for 5 min each in TBS containing 0.15% Tween 20 and once with TBS alone. The membrane was equilibrated in alkaline phosphatase buffer (100 mM NaCl, 100 mM

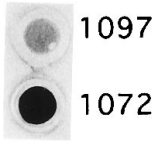

FIG. 1. Binding of biotinylated Hb by *N. meningitidis* cells expressing the HmbR protein. Iron-restricted wild-type *N. meningitidis* (IR1072) cells, which possess only one Hb utilization mechanism (HmbR), were able to bind biotinylated Hb when immobilized on a nitrocellulose membrane. The *hmbR* deletion mutant (IR1097) was unable to bind biotinylated human Hb.

Tris Cl, 5 mM $MnCl_2$, pH 9.5) for 5 min. The membrane was developed by adding 45 μl of nitroblue tetrazolium and 35 μl of X-phosphate in 10 ml of equilibration buffer. The reaction was stopped by rinsing the membrane in deionized water. In some experiments a streptavidin-POD complex and a BM blue POD substate (Boehringer, Mannheim, Germany) were used for the detection of bound biotinylated human Hb.

Recombinant DNA techniques. Standard methods for plasmid DNA preparation, restriction endonuclease analyses, and ligations were carried out as described by Sambrook et al. (39). Southern blot analysis was done with a digoxigenin nonradioactive DNA labeling and detection kit (Genius system; Boehringer) under high-stringency conditions (60°C, then two washes with 2× SSC, [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–sodium dodecyl sulfate (SDS) at room temperature followed by two washes with 0.1× SSC–0.1% SDS at 60°C). The following PCR primers were used in PCRs: OLIGO147, 5′ AAACAGGTCTCGGCATAG3′; *Eco*RI-OLIGO147, 5′ CGCGAATTCAAAC AGGTCTCGGCATAG3′; HmbR-COOH.A primer, 5′ CGCGAATTCAAAA ACTTCCATTCCAGCGATACG.3′; and *Eco*RI-HmbR-COOH.B primer, 5′ TAAAACTTCCATTCCAGCGATACG3′. Oligonucleotide primers MS11-internal-A (5′ GGGAATTCGCTTTCAGGTTGGGATTGGG 3′) and MS11-internal-B (5′ GGGAATTCAACGACAAGCACCGCATC 3′) were used to amplify an internal fragment of the MS11 *hmbR* gene which contained a putative stop codon.

Isolation of *N. meningitidis* outer membranes. *N. meninigitidis* cells grown on a GCB plate were resuspended in 10 ml of GCB medium containing 10 μM Desferal and incubated for 3 h in a shaker. The bacteria were spun down and resuspended in 2 ml of Li acetate buffer (200 mM Li acetate, 5 mM EDTA, pH 6.0) and incubated for 3.5 h at 37°C with gentle agitation. The culture was then passed through a 21-guage needle seven times and pelleted at 8,000 × *g* (40 min). The supernatant was transferred into a clean tube, and the membranes were pelleted at 28,000 × *g* (3 h). Concentrations of outer membranes were standardized by using a bicinchoninic acid protein reagent kit (Pierce).

Cloning of the *hmbR* genes from *N. meningitidis* serogroups A and B and *Neisseria gonorrhoeae* MS11. The Hb receptor gene from the *N. meningitidis* serogroup A isolate was amplified with *Pfu* polymerase and cloned into an *Eco*RV-linearized pSUKS vector, creating plasmid pIRS749. The *hmbR* gene from the *N. meningitidis* serogroup B isolate was PCR amplified with both *Taq* (Boehringer) and *Pfu* (Stratagene) DNA polymerases. The *Pfu* DNA polymerase-generated fragment was cloned into an *Sma*I-linearized pSUKS, vector creating the plasmid pIRS747. The *Taq* DNA polymerase-generated fragment was cloned into pBluescript after digestion of the DNA fragment with *Bam*HI and *Eco*RI restriction endonucleases, creating pIRS748. The *hmbR* receptor gene from *N. gonorrhoeae* MS11 was amplified with the *Taq* DNA polymerase, cut with the *Eco*RI restriction enzyme, and cloned into pWKS30, creating the plasmid pIRS756. An internal fragment of the *hmbR* gene from strain MS11 carrying a putative stop codon was independently amplified with *Taq* DNA polymerase and cloned into pCRII, creating plasmid pMS11.INTERNAL.

Construction of the *hmbR* deletion mutant and the *N. meningitidis* serogroup B *hmbR* mutant. The internal 0.88-kb *Mlu*I-*Pst*I *hmbR* fragment was deleted from the plasmid pIRS523. The resulting plasmid, pVIV10, was linearized and transformed into *N. meningitidis* IR1072. Approximately 1,000 colonies were picked and investigated for the ability to grow on Hb. One isolate, IR1097, was unable to use Hb but was proficient in both transferrin and heme utilization. Southern hybridization with an *hmbR* probe revealed a deletion of the predicted size in the *hmbR* gene of strain IR1097 (data not shown).

In order to inactivate the Hb receptor gene in *N. meningitidis* serogroup B, plasmid pIRS747 was partially digested with *Pst*I and ligated with a *Pst*I-digested Km^r (KIXX; Pharmacia) cassette. Plasmid pIRS747.2 contained the Km^r cassette inserted in the internal *Pst*I site and was used for insertional inactivation of the chromosomal copy of *hmbR*. Plasmid pIRS747.2 was retransformed into *N. meningitidis* serogroup B, and several Km^r colonies were investigated for their abilities to grow on Hb and heme as sources of iron. All transformants were deficient in the utilization of Hb as a source of iron.

DNA sequence determination. The DNA sequences of *hmbR* genes from different neisserial isolates were determined by the dideoxy chain termination method with the AutoRead kit and ALF automatic sequencer (Pharmacia).

RESULTS

Construction of the *hmbR* deletion mutation in *N. meningitidis* IR1072. The *N. meningitidis* 8013#6 *hmbR* mutant is unable to use Hb but is able to use heme as a sole source of iron. The *hmbR* gene was inactivated by introducing a kanamycin cassette into the *Not*I restriction site located at the 5′ end of the gene (45). Distal to this *Not*I site, a second potential start codon in the *hmbR* nucleotide sequence, coding for an open reading frame with a potential signal sequence, was discovered (data not shown). In order to completely exclude the possibility that the *hmbR*::Km mutant produces a truncated Hb receptor capable of utilizing heme, a deletion mutation of *hmbR* was constructed (see Materials and Methods). The *N. meningitidis* 1097 *hmbR* deletion mutant was unable to use Hb but still able to use heme as a sole source of iron.

HmbR-expressing cells and their outer membranes bind biotinylated Hb in vitro. In order to investigate whether HmbR mediates Hb binding, a dot blot assay with biotinylated Hb and whole *N. meningitidis* cells was performed. The wild-type strain IR1072 and the *hmbR* deletion derivative IR1097 were used in these experiments. In order to increase the expression of the *hmbR* gene, both strains were grown in GCB medium containing 5 μM Desferal, and approximately 10^7 cells were applied to nitrocellulose filters. As can be seen in Fig. 1, biotinylated Hb binds very efficiently to the wild-type *N. meningitidis* cells (1072) but is not able to bind cells of the HmbR deletion mutant (1097). The outer membranes of strain IR1072 were also able to bind biotinylated Hb, and the binding was inhibitable by excess unlabeled human A(0) Hb but not heme alone (Fig. 2).

Expression of the HmbR-dependent Hb binding activity is regulated by iron availability. Outer membrane proteins involved in iron assimilation are often regulated by iron. The less iron in the medium, the stronger is the expression of genes encoding different outer membrane proteins involved in iron assimilation (1, 9, 17, 33). This regulation is mediated by the Fur repressor, which binds to the promoter regions (Fur boxes) of iron-regulated genes and represses their expression (3, 17). We have identified a putative Fur box nucleotide sequence in the promoter of the *hmbR* gene by using a "Fur box scanner" (42, 45). The dot blot Hb binding assay was used to assess the degree of iron regulation of HmbR expression. Since IR1072 expresses only the HmbR-dependent Hb binding activity, the degree of Hb binding was taken as a measure of HmbR expression. The expression of the HmbR-dependent Hb binding activity was clearly repressed when the cells were grown with high concentrations of iron (Fig. 3a). Densitometric analysis of the blot shown in Fig. 3a revealed that a sevenfold increase in the amount of iron in the medium results in a threefold decrease in the expression of Hb binding activity. Similarly, the presence of an iron chelator in the growth medium resulted in approximately threefold induction of HmbR-dependent Hb binding by the *N. meningitidis* 1072 cells (Fig. 3b). These results

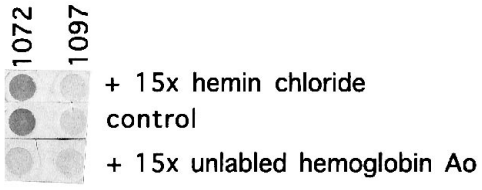

FIG. 2. HmbR-mediated Hb binding is inhibited by unlabeled Hb and not by heme. IR1072 is the wild-type strain expressing only HmbR. IR1097 is the *hmbR* deletion mutant unable to bind and utilize Hb.

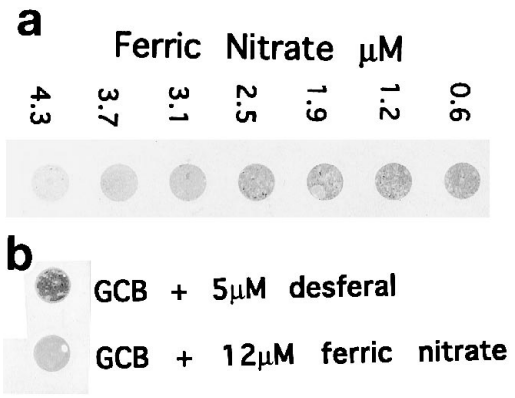

FIG. 3. Binding of biotinylated Hb to HmbR-expressing *N. meningitidis* cells is regulated by the availability of iron. (a) IR1072 cells were grown at different iron concentrations, and their ability to bind biotinylated Hb was assessed. (b) Induction of HmbR-mediated Hb binding by *N. meningitidis* IR1072 cells grown in the presence of the iron chelator Desferal.

clearly showed that the expression of the HmbR-mediated Hb binding activity is repressed by iron, proving the iron-regulated expression of *hmbR*.

Expression of the HmbR-dependent Hb binding activity is stimulated by Hb and not by heme. The Hb and transferrin binding activities of *H. influenzae* and *H. ducreyi* were recently shown to be regulated by heme, and not iron availability (12, 13, 31). In order to investigate the roles of heme and Hb availabilities in on the expression of Hb binding activity by HmbR-expressing IR1072 cells, the dot blot Hb binding assays were performed with cells pregrown in the presence of iron alone, heme and iron, and Hb and iron. The degrees of Hb binding activity by IR1072 cells pregrown in the presence of iron and of iron and heme were indistinguishable. These experiments showed that heme availability does not regulate the expression of Hb binding activity by *N. meningitidis* cells (Fig. 4). However, Hb binding activity was stimulated by growing the bacteria in the presence of Hb and iron (Fig. 4). The difference between the Hb binding activities of the cells grown with iron alone and those grown with Hb and iron varied from 125 to 300%, depending on the growth conditions of the bacteria (data not shown). Since the expression of HmbR is repressed by iron, supplementation of the growth medium with Hb should have resulted in the repression, and not stimulation, of HmbR expression. It is unlikely that the increase of Hb binding by IR1072 cells grown in the presence of Hb was due to the de novo expression of an *hmbR*-unrelated Hb-binding protein, since the *hmbR* deletion mutant was not able to bind Hb under similar grown conditions (see above). Additional experiments are needed in order to clearly establish whether HmbR-expressing cells are able to respond to the presence of Hb in the medium by upregulating the expression of the *hmbR* gene.

Human Hb is the best source of iron for HmbR-expressing *N. meningitidis* cells. Neisseriae are highly adapted organisms, capable of causing a full clinical picture of bacterial meningitis or gonorrhea only in humans. It is well established that neisserial transferrin receptors are capable of binding and utilizing only human transferrin (40). We have studied the ability of neisseriae to use a variety of animal and modified Hbs as their sole sources of iron. These experiments were performed with the *N. meningitidis* IR1072 strain, which possesses only the HmbR Hb receptor. In order to measure the abilities of different Hbs to serve as an iron source, the quantities of different Hbs needed to promote the same amount of growth under iron restriction conditions were compared. The growth promotion assay is a very sensitive method for assessing the abilities of bacteria to utilize different iron sources, and even very small differences in growth zones around a compound indicate large differences in the efficiencies of utilization of these compounds.

As can be seen from Fig. 5, there is a significant difference in the abilities of some animal Hbs to serve as iron sources to neisseriae. Snake Hb cannot serve as an iron source (not shown), while all other animal Hbs tested, except horse Hb, were needed in nearly twofold-higher concentrations in order to enable the growth stimulation accomplished by the human Hb. A modified Hb, *N,N′*-dimethyl Hb, was the least efficient iron source for *N. meningitidis*. The preference of HmbR-expressing cells for human Hb indicates that the HmbR protein is able to discriminate between Hbs from different animal sources.

Distribution of Hb receptor-like genes in different neisseriae. Mickelsen and Sparling have investigated the distribution of neisserial isolates able to utilize Hb as an iron source. They found that more then 95% of *N. meningitidis* strains and 60% of *N. gonorrhoeae* strains were able to use Hb as an iron source (30). Recently, Lee and Hill showed by a dot blot assay that isolates from *N. meningitidis* serogroups A, B, C, X, Y, and W135 possess iron-regulated Hb binding activity in their outer membranes (25).

With an *hmbR* gene as a probe, the presence of *hmbR*-like sequences in *N. meningitidis* serogroup A, B, and C isolates; in the MS11 and FA19 *N. gonorhoeae* strains; and in *Neisseria perflava* and *Neisseria polysaccharea* was identified by Southern blot hybridization (Fig. 6). All tested strains contained only one copy of an *hmbR*-like sequence; the two hybridizing bands found in the lane containing *N. gonorrhoeae* DNAs were due to the presence of an internal restriction site for the enzyme used in the digest of chromosomal DNA. All neisserial strains examined in the Southern blot experiment were also able to use Hb as an iron source (Fig. 7). The *N. meningitidis* serogroup C (IR1075) and A (IR1073) isolates, as well as the *N. gonorrhoeae* FA19 and MS11 laboratory strains, used Hb-Hpt complexes. Conversely, the *N. meningitidis* IR1072 and the *N. meningitidis* serogroup B (IR1074) isolates were not able to use Hb-Hpt complexes as iron sources (Fig. 7). The *Branhamella catarrhalis* strain was able to use both heme and Hb as iron sources, although its DNA did not hybridize with the *hmbR* DNA probe under the high-stringency conditions. This limited study showed a widespread occurrence of Hb utilization capa-

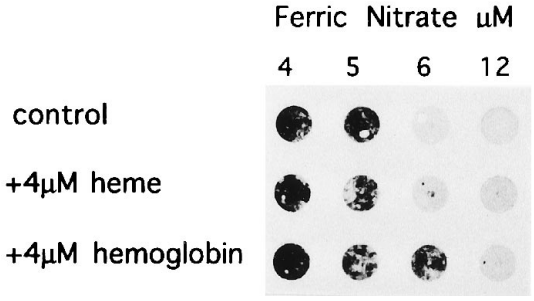

FIG. 4. Influence of different growth conditions on the expression of HmbR-mediated Hb binding activity by *N. meningitidis* IR1072 cells. Wild-type *N. meningitidis* cells were pregrown in the presence of iron alone, iron plus 4 μM heme, or iron plus 4 μM Hb. The ability of whole IR1072 cells to bind Hb was assessed by using biotinylated Hb and an alkaline phosphatase-streptavidin complex.

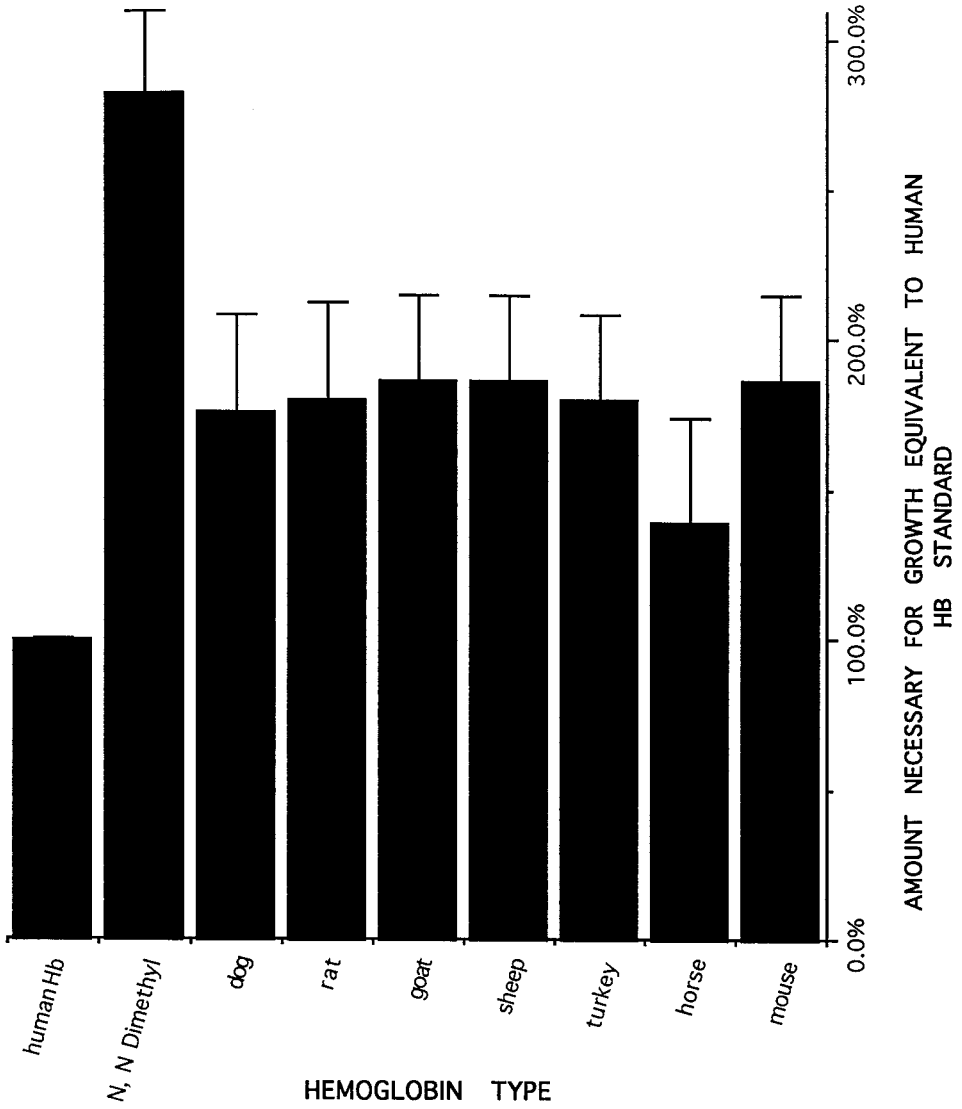

FIG. 5. Abilities of different animal and modified Hbs to serve as an iron source for HmbR-expressing *N. meningitidis* IR1072 cells. The actual concentrations of all tested Hbs were determined with the Drabkin reagent (Sigma) and standardized at 5 mg/ml prior to the experiment. The bars indicate the amount of a particular Hb that was necessary in order to produce a bacterial growth zone identical to the one produced by human Hb. Six independent measurements were performed. Error bars indicate standard error of the mean.

bilities as well as *hmbR*-like sequences in both pathogenic and nonpathogenic neisserial isolates.

Characterization of the *N. meningitidis* serogroup A and B and *N. gonorrhoeae* MS11 *hmbR* genes. In order to assess the level of conservation between Hb receptors identified in different neisserial isolates, the HmbR-encoding genes from *N. meningitidis* serogroups A and B and *N. gonorrhoeae* MS11 were cloned, and their nucleotide sequences were determined. Comparisons of their amino acid and nucleotide sequences are presented in Fig. 8. Nucleotide differences were spread throughout the *hmbR* genes, and no large patches (>5 nucleotides) of disimilarity were found. The *hmbR* nucleotide sequences from serogroups C and B have the highest degree of conservation (93.4% identity), while the sequences from MS11 and serogroup B are the most distant (86.5% identity) (Fig. 8). The deduced amino acid sequences of all four receptors are also highly homologous: overall, 87.4% of all amino acid residues are shared among all four receptors, with the largest difference between the *N. gonorrhoeae* MS11 putative receptor and the *N. meningitidis* serogroup B receptor. Interestingly, the presence of an adenine at position 1014 in the MS11 *hmbR* nucleotide sequence created a stop codon in the coding sequence. Although the MS11 *hmbR* gene was cloned by PCR, it is unlikely that the observed insertion was due to an error in PCR amplification, because the same stop codon was detected in the sequences of two independently PCR-amplified products. Since *N. gonorrhoeae* MS11 utilizes Hb as an iron source (Fig. 7), the most likely explanation for this finding is that this strain contains another, *hmbR*-unrelated Hb receptor. The ability of MS11 to utilize Hb-Hpt complexes (Fig. 7) indicated that the second, HmbR-unrelated Hb receptor might be identical to the Hpu receptor.

Some *N. meningitidis* strains possess two unrelated systems for Hb utilization. Cloning of the Hb receptor genes from different neisseriae allowed us to assess the role that these genes have in the utilization of Hb. The *hmbR* gene of the *N.*

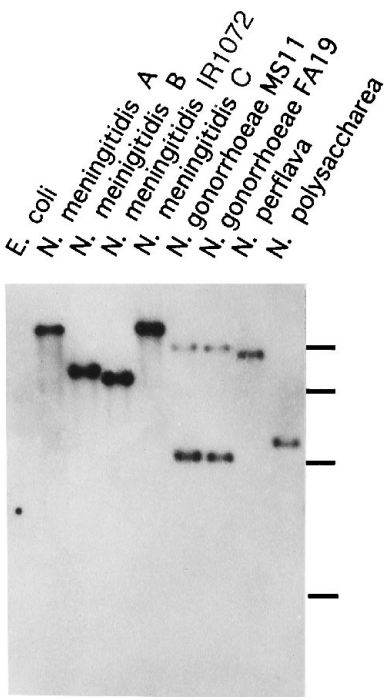

FIG. 6. Distribution of *hmbR* Hb receptor genes in different neisseriae. A 3-kb *Bam*HI-*Sal*I fragment carrying the *N. meningitidis* IR1072 *hmbR* gene was used as a probe. Different chromosomal DNAs were digested with *Cla*I and hybridized with a digoxigenin-labeled *hmbR* probe under high-stringency conditions.

meningitidis serogroup C clinical isolate (IR1075) was inactivated by the approach described previously (45). Southern blot hybridizations confirmed the existence of only one *hmbR*-like sequence in the parent strain (Fig. 6) and confirmed the inactivation of the *hmbR* chromosomal copy in the mutant IR1078 (data not shown). Surprisingly, this mutant was still proficient in Hb utilization (Fig. 7). Both the mutant and the parent strain were able to use Hb-Hpt complexes as iron sources. These data suggest that the IR1075 isolate possesses two different Hb utilization mechanisms: one involving the HmbR protein and the second one most probably involving the Hb-Hpt receptor, Hpu (28, 29).

In order to prove that the second Hb utilization system is indeed Hpu dependent, the inactivation of the *hpu* gene in IR1078 was carried out. Plasmid pSM85KE, carrying the *hpu*::mTn*3*Erm construct, was transformed into *N. meningitidis* IR1078. The *N. meningitidis* 1078 *hmbR hpu* double mutant was unable to use Hb as an iron source but was still proficient in the utilization of heme (Fig. 7).

The inactivation of *hmbR* in the *N. meningitidis* serogroup B isolate IR1074 resulted in an Hb utilization-negative phenotype of the mutant. Interestingly, several Hb utilization-positive colonies appeared around the Hb disc after prolonged incubation (Fig. 7). The most likely explanation for these data is that strain IR1074 contains two Hb utilization systems and that the expression of the HmbR-unrelated Hb utilization system (i.e., Hpu) is under a phase variation control.

Inability of the second *N. meningitidis* Hb utilization system to distinguish between different animal Hbs as sources of iron. The construction of an *hmbR* mutation in the strain (IR1078) that expresses two Hb utilization systems enabled us to investigate whether the Hpu-dependent Hb utilization system is able to distinguish between different animal Hbs as sources of iron. Different animal and modified Hbs were applied to GCB-Desferal plates seeded with the IR1075 parent strain and the IR1078 *hmbR* mutant, and degrees of growth stimulation were determined. The two strains were equally efficient in the utilization of all Hbs used in the experiments. These results indicate that the Hpu-dependent Hb utilization system expressed in the IR1075 strain does not discriminate between different sources of Hb.

DISCUSSION

The HmbR outer membrane receptor is responsible for the Hb utilization by some *N. meningitidis* serogroup C isolates (45). The initial study did not differentiate between the direct involvement of HmbR in Hb binding and utilization and an alternative scenario in which the second protein interacts with Hb and shuttles heme from Hb to the HmbR receptor. Such systems were recently described for *H. influenzae* and *S. marcescens*, in which secretable proteins (HxuA and HasA, respectively) extract heme from hemopexin-heme and Hb and deliver it to a second protein, which transports heme into bacterial interior (4, 5, 27). In order to resolve this issue, we have shown that HmbR-expressing cells and their outer membranes are able to bind biotinylated Hb. The binding activity was shown to be inhibitable by an excess of Hb, proving that the HmbR protein is an essential component of the HmbR-mediated Hb utilization system in neisseriae. The binding of biotinylated Hb to outer membranes of HmbR-expressing cells was not inhibitable by an excess of heme, suggesting that HmbR recognizes the globin chain and not heme on the Hb molecule.

The recently described *H. ducreyi* Hb receptor HgbA was also shown to mediate binding of iodinated Hb. This binding activity was inhibitable by different animal Hbs but not by globin or heme alone (12). Similar results were obtained in a study of an Hb binding activity of *H. influenzae* cells (14). Conversely, Lee and Hill (25) have described an Hb binding

	Heme	Hb	Hb-Hpt	HTr
IR1072 (hmbR+)	++	++	-	++
IR1073 (sero. A)	++	++	++	ND
IR1074 (sero. B)	++	++	-	ND
IR1075 (sero. C)	++	++	++	ND
IR1072 (hmbR::Km^r)	++	-	-	ND
IR1077 (B/hmbR::Km^r)	++	-/+	-	ND
IR1078 (C/hmbR::Km^r)	++	++	++	ND
IR1097 (ΔhmbR)	++	-	-	++
IR2035 hmbR::Km^r hpu::Erm^r	++	-	-	ND
N. gonorrhoeae				
MS11	++	++	++	++
FA19	++	++	++	++
N. polysaccharea	++	++	ND	++
N. perflava	++	++	ND	-
B. catarrhalis	++	++	ND	ND

FIG. 7. Abilities of different neisserial isolates to use heme-containing compounds as sources of iron. ND, not done; ++, large (>10-mm) growth zone around the disc containing a heme compound (Hb, 5 mg/ml; heme, 5 mg/ml; Hb-Hpt [1:1], 5 mg/ml; human transferrin [HTr, 10 μg/ml]); −, no growth zone; −/+, no growth zone but only a few colonies growing around the disc. sero., serogroup.

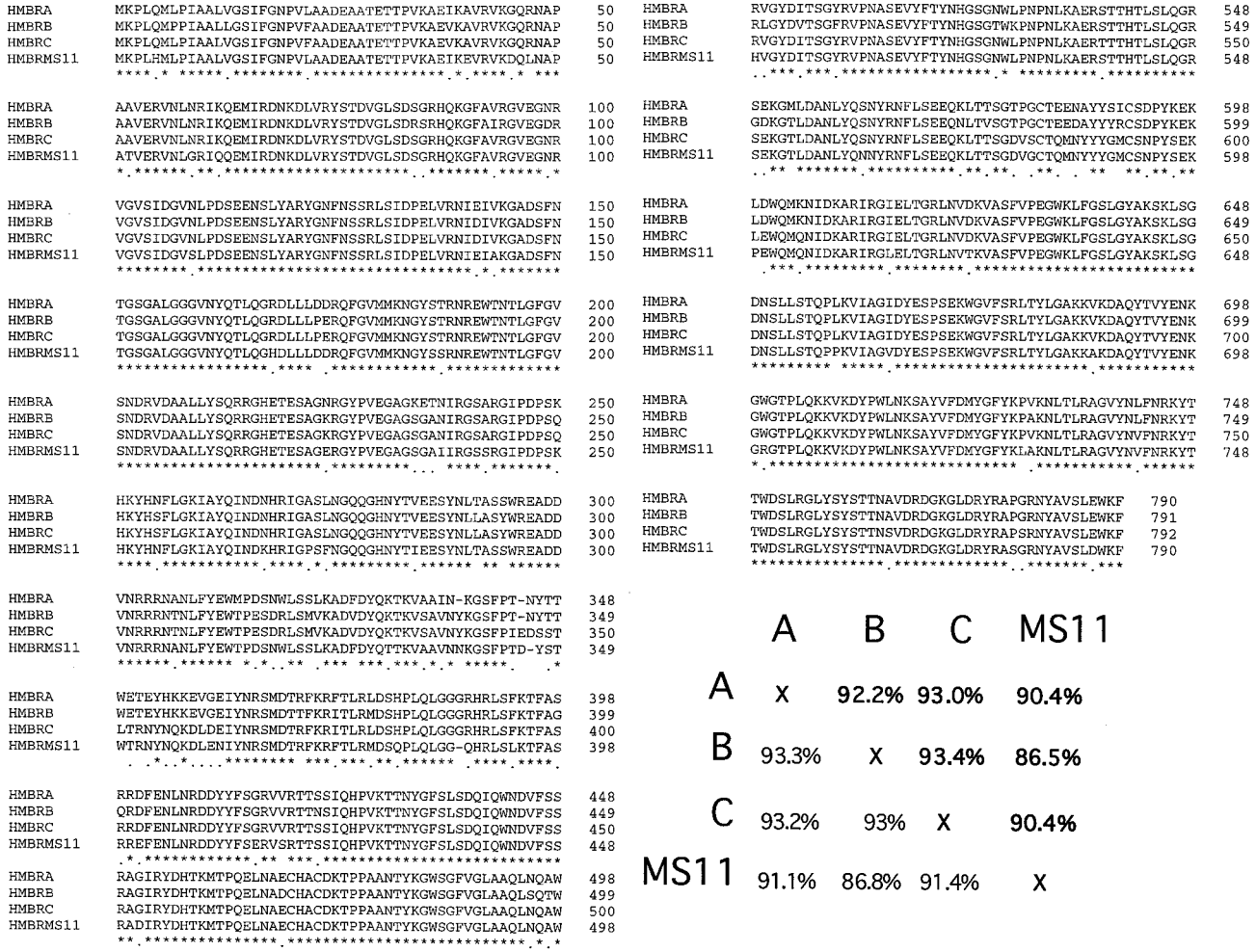

FIG. 8. Comparison of Hb receptor primary structures from *N. meningitidis* serogroup C (HMBRC), serogroup B (HMBRB), and serogroup A (HMBRA) and *N. gonorrhoeae* MS11 (HMBRMS11). The amino acid sequence of the MS11 HmbR protein was deduced from the MS11 *hmbR* nucleotide sequence lacking the stop codon at position 1014. Asterisks, identical residues; dots, similar residues. All four HmbR receptors had 84.7% identical and 11.4% similar amino acid residues. The comparison was done with the CLUSTAL program (PC/GENE software; Intellegenetics Inc.). At the bottom is a comparison of the percent identities of nucleotide (boldface) and amino acid sequences of *hmbR* genes isolated from different neisserial isolates.

activity in the outer membranes of *N. meningitidis* B16B6 which was inhibitable by bovine and human Hbs, by bovine catalase, and by the heme moiety alone. The degree of inhibition differed drastically depending on the nature of the competitor: an approximately 50% reduction in binding of biotinylated Hb (50 nM) was achieved with only 1 μM concentrations of bovine and human Hbs, whereas approximately 60 μM catalase or heme was necessary to achieve the same degree of inhibition (25). The observed inhibition of Hb binding achieved with a 1,200-fold excess of heme might be unspecific rather then specific. Another explanation for these results is that heme did not affect the binding of Hb to the HmbR protein but instead inhibited the binding of Hb to the HmbR-unrelated Hb receptor expressed in the same strain, and this inhibition resulted in an overall reduction of Hb binding. Indeed, the strain used in that study was shown to possess two different Hb binding activities (25).

The expression of heme-Hb utilization operons of *Y. enterocolitica*, *V. cholerae*, and *S. marcescens* was shown be mediated by the availability of iron (20, 27, 43). In contrast, the expression of the *H. ducreyi* Hb receptor gene is regulated by the availability of heme and not iron (12, 13). Similarly, the expression of *H. influenzae* transferrin and Hb receptors was found to be regulated by heme availability (14, 31). Previously, we have identified a Fur box sequence in front of the *hmbR* gene (45). The finding that Hb binding by the HmbR-expressing cells is regulated by the availability of iron indicates that the *hmbR* gene is another member of a Fur regulon of neisserae. Our data also suggest that *N. meningitidis* cells grown in the presence of Hb stimulate the expression of Hb binding activity. Whether neisserae possess a system which specifically respond to the presence of Hb at the bacterial surface is unclear at present and requires further study, although it would be advantageous to the pathogen to express a particular iron utilization system only when the substrate is present.

The comparison of the nucleotide sequences of *hmbR* genes from *N. meningitidis* serogroups A, B, and C and *N. gonorrhoeae* MS11 revealed a degree of identity ranging between 86.5% (for serogroup B *hmbR* and MS11 *hmbR*) and 93.4% (for serogroup B *hmbR* and serogroup C *hmbR*). The deduced amino acid sequences of all four characterized neisserial Hb receptors showed a relatively high degree of relatedness, with overall 84.7% identical amino acid residues. Studies that compared nucleotide and amino acid sequences of transferrin re-

ceptors from different *N. meningitidis* serogroups and *N. gonorrhoeae* isolates found much higher degrees of divergence (7, 26). The determination of nucleotide sequences from *N. meningitidis* and *N. gonorrhoeae* lactoferrin receptor-encoding genes revealed that these receptors have 94 to 95% identical amino acid residues (2, 35, 36). Conservation of the primary structures of Hb and lactoferrin receptors makes these proteins attractive vaccine targets (41).

All neisserial strains used in the study were able to use Hb, whereas only a few utilized Hb-Hpt complexes, as a sole iron source. The phenotype of *hmbR* mutants constructed from the clinical strains of *N. meningitidis* serogroups C and B as well as the finding of the stop codon in the MS11 *hmbR* sequence indicated the existence of a second Hb utilization mechanism in some neisserial isolates. The existence of a second Hb utilization system correlated with the ability of strains to utilize Hb-Hpt complexes. Lewis and Dyer have recently described an *N. meningitidis* Hpu protein that is involved in the utilization of Hb-Hpt complexes and Hb alone (28). The Hb-Hpt receptor of *N. meningitidis* shares only 25% identical amino acids with the HmbR protein from *N. meningitidis*, which clearly shows that neisseriae possess at least two different mechanisms of Hb utilization (29).

The ability of a pathogen to discriminate between iron-binding proteins from different species is a common phenomenon. The Hb utilization system of *H. influenzae* was shown to be species specific, since the binding of biotinylated human Hb was only partially inhibited by excess unlabeled rat Hb (14). Moreover, neisseriae, strict human pathogens, are able to use only human transferrin, while a bovine pathogen, *Actinobacillus pleuropneumonieae*, is able to use only bovine transferrin as an iron source (16, 40).

The HmbR-expressing cells could distinguish between human and nonhuman sources of Hb and could utilize the human Hb most efficiently. The preference for human Hb was not absolute, since animal Hbs were still able to support growth of HmbR-expressing neisseriae under iron-limiting growth conditions. In contrast, the Hpu-dependent Hb utilization system of *N. meningitidis* could not distinguish between different sources of Hb; all animal and modified Hbs were utilized equally well. These results could be explained by the hypothesis that the HmbR-unrelated receptor (Hpu) recognizes a heme moiety on Hb while the HmbR receptor recognizes and binds to a globin part of the Hb molecule.

The existence of multiple systems for the utilization of heme-containing compounds in neisseriae clearly indicates the importance of heme-containing compounds for the bacterium. The heme utilization systems together with transferrin- and lactoferrin-specific receptors make these pathogens extremely well adapted for colonization and survival on mucosal surfaces of the host.

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