

## Point Mutations in HpuB Enable Gonococcal HpuA Deletion Mutants To Grow on Hemoglobin

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Received 6 August 2001/Accepted 20 October 2001

*Neisseria gonorrhoeae* ordinarily requires both HpuA and HpuB to use hemoglobin (Hb) as a source of iron for growth. Deletion of HpuA resulted in reduced Hb binding and failure of growth on Hb. We identified rare Hb-utilizing colonies (Hb<sup>+</sup>) from an *hpuA* deletion mutant of FA1090, which fell into two phenotypic classes. One class of the Hb<sup>+</sup> revertants required expression of both TonB and HpuB for growth on Hb, while the other class required neither TonB nor HpuB. All TonB/HpuB-dependent mutants had single amino acid alterations in HpuB, which occurred in clusters, particularly near the C terminus. The point mutations in HpuB did not restore normal Hb binding. Human serum albumin inhibited Hb-dependent growth of HpuB point mutants lacking HpuA but did not inhibit growth when expression of HpuA was restored. Thus, HpuB point mutants internalized heme in the absence of HpuA despite reduced binding of Hb. HpuA facilitated Hb binding and was important in allowing use of heme from Hb for growth.

Mammalian hosts use iron-binding proteins and iron-sequestering compounds to maintain free iron at a level that is too low for the growth of invading pathogens. Iron bound to hemoglobin (Hb) is a significant constituent of the total iron in the human body, but it is not readily available to pathogens because of its compartmentalization in erythrocytes. Pathogenic neisseriae are able to use Hb, haptoglobin-hemoglobin (Hp-Hb), and heme as a source of iron for growth (12). Neisseriae have two distinct Hb receptors, the bipartite outer membrane Hb receptor, HpuAB, which acquires iron from Hb and Hb-Hp, and HmbR, which utilizes iron from Hb (7, 8, 15, 16, 23). Some meningococci express either HpuAB or HmbR, while others express both (23). HpuAB is most likely the only Hb receptor of gonococci, since all strains tested are capable of expressing HpuAB through phase variation (7, 8), but there is a premature stop codon in *hmbR* of gonococci (23).

Most laboratory and clinical *Neisseria gonorrhoeae* isolates do not express HpuAB and are unable to use Hb as the sole source of iron for growth (Hb<sup>-</sup>). A small population (ca. 0.1%) of every tested Hb<sup>-</sup> *N. gonorrhoeae* isolate is able to express HpuAB and utilize Hb under iron-stressed conditions (Hb<sup>+</sup>). Expression of HpuAB phase varies due to translational frame-shifting resulting from slipped-strand mispairing of a poly(G) tract within the coding sequence of *hpuA* (8). The receptor consists of a lipoprotein, HpuA, and a transmembrane protein, HpuB. Growth on Hb is TonB dependent and requires both HpuA and HpuB (3, 8). In contrast, in the related gonococcal TonB-dependent receptors for transferrin and lactoferrin, the lipoproteins TbpB and LbpB are not absolutely required for growth, although they play a role in facilitating ligand binding (2, 4). HpuA can be isolated with HpuB by binding to Hb-

agarose, but not in the absence of HpuB, indicating physical contact between these two proteins (8).

Pathogenic neisseriae can also use free heme as an iron source for growth. Growth with heme requires neither HpuAB nor TonB (3, 7, 24, 26). The existence of a heme receptor has been suggested (13, 14), but a heme receptor has never been clearly identified in either meningococci or gonococci. Pathways for entry and transport of free heme are not well understood. Construction of *hemA* mutants that cannot synthesize heme proved that meningococci and gonococci can utilize Hb as a heme source and that uptake of heme from either Hb or Hb-Hp requires HpuAB (17, 26). Heme-dependent growth does not require FbpA (26); FbpA is required to shuttle iron from transferrin and lactoferrin through the periplasmic space (1, 6).

As part of an effort to examine the role of HpuA and HpuB in the function of the gonococcal Hb receptor, we constructed a nonpolar *hpuA* deletion ( $\Delta hpuA$ ) mutant that could express HpuB under the control of the *hpuA* promoter. The HpuA<sup>-</sup> HpuB<sup>+</sup> mutant was unable to grow on Hb. We were able to identify two different classes of Hb<sup>+</sup> revertants from this HpuA<sup>-</sup> mutant. The first class had point mutations in *hpuB*, and their growth on Hb depended on the expression of both HpuB and TonB. The second class consisted of mutants that grew on Hb in a TonB- and HpuB-independent manner. Characteristics of the HpuA-independent/HpuB-dependent Hb utilization are reported here.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The parent strain used in this study was FA1090. Plasmids and gonococcal strains constructed and/or used in this study are listed in Table 1. The growth conditions for FA1090, various mutants, and *Escherichia coli* strains were as described (26). Hb<sup>-</sup> isolates were grown on Bacto GC medium base (Difco, Becton Dickinson, Sparks, Md.) plates (GCB plates) containing Kellogg's supplement I, ferric nitrate at 12  $\mu$ M. The off to on (Hb<sup>-</sup> to Hb<sup>+</sup>) phase variation frequency of FA1090 was about 10<sup>-3</sup> (7). The Hb<sup>+</sup> isolates were grown on modified GCB plates (Hb/Des plates) containing

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TABLE 1. Plasmids and gonococcal strains constructed and/or used in this study

Plamid or strain	Relevant characteristics	Source or reference
<b>Plasmids</b>		
PCR2.1-TOPO	Vector for inserting PCR product	Invitrogen Corp.
pHP45 $\Omega$	Source for $\Omega$ (Sp <sup>r</sup> Sm <sup>r</sup> ) cassette	20
pNC40	Source for <i>cat</i> (Cm <sup>r</sup> ) cassette	25
pSM85kE	850-bp <i>ApoI</i> fragment of meningococcal <i>hpu::mTn3erm</i>	15
pIRS756	<i>hmbR</i> from IR1113 (MS11 gene)	23
pIRS756-CAT	<i>hmbR::cat</i>	This study
pIRS756- $\Omega$	<i>hmbR::\Omega</i>	This study
pUNCH173	<i>tonB::\Omega</i>	3
pUNCH1306	<i>hemA::\Omega</i>	26
pUNCH272	pCR2.1-TOPO with coding sequence of mature HpuB	This study
pUNCH277	pCR2.1-TOPO with <i>hpuA</i> promoter region PCR insert	This study
pUNCH278	pCR2.1-TOPO with <i>hpuB</i> PCR insert	This study
pUNCH279	pUNCH278 with <i>cat</i> cassette from pNC40 inserted in <i>hpuB</i>	This study
pUNCH280	Ligated <i>hpuA</i> promoter with <i>hpuB::CAT</i> in pCR2.1-TOPO	This study
<b>Strains</b>		
FA1090	Wild-type parent strain	11
FA1090 Hb <sup>+</sup>	HpuAB phase on variant of FA1090	7
FA6929	<i>hpuB::mTn3erm</i> mutant of FA1090 Hb <sup>+</sup> variant, Em <sup>r</sup> Hb <sup>-</sup>	7
FA6982	<i>hpuA::\Omega</i> mutant of FA1090 Hb <sup>+</sup> Sp <sup>r</sup> Hb <sup>-</sup>	This study
FA6983	<i>hpuA::aphA-3</i> mutant of FA1090 Hb <sup>+</sup> , Km <sup>r</sup> Hb <sup>-</sup>	This study
FA7167	$\Delta$ <i>hpuA hpuB::cat</i> Cm <sup>r</sup> Hb <sup>-</sup>	This study
FA7168	Cm <sup>s</sup> Hb <sup>+</sup> revertant of FA7167	This study
FA7169	$\Delta$ <i>hpuA hpuB</i> <sup>+</sup> derivative of FA7167, Cm <sup>s</sup> Hb <sup>-</sup>	This study
FA7170	Cm <sup>s</sup> Hb <sup>+</sup> revertant of FA7169, <i>hpuB</i> * <sup>650</sup> (G217D)	This study
FA7184	<i>hmbR::\Omega</i> mutant of FA7168, Sp <sup>r</sup> Hb <sup>+</sup>	This study
FA7185	<i>hmbR::cat</i> mutant of FA7169, Cm <sup>r</sup> Hb <sup>-</sup>	This study
FA7185H1	Cm <sup>r</sup> Hb <sup>+</sup> revertant of FA7185, <i>hpuB</i> * <sup>2369</sup> (G790D)	This study
FA7185H2	Cm <sup>r</sup> Hb <sup>+</sup> revertant of FA7185, <i>hpuB</i> * <sup>2369</sup> (G790D)	This study
FA7185H11	Cm <sup>r</sup> Hb <sup>+</sup> revertant of FA7185, <i>hpuB</i> * <sup>2199</sup> (S733R)	This study
FA7185H12	Cm <sup>r</sup> Hb <sup>+</sup> revertant of FA7185, <i>hpuB</i> * <sup>1526</sup> (G509V)	This study
FA7185H13	Cm <sup>r</sup> Hb <sup>+</sup> revertant of FA7185, <i>hpuB</i> * <sup>2378</sup> (R793L)	This study
FA7185H14	Cm <sup>r</sup> Hb <sup>+</sup> revertant of FA7185, <i>hpuB</i> * <sup>770</sup> (A257D)	This study
FA7186	<i>hmbR::\Omega</i> mutant of FA7169, Sp <sup>r</sup> Hb <sup>-</sup>	This study
FA7186H1	Sp <sup>r</sup> Hb <sup>+</sup> revertant of FA7186	This study
FA7186H2	Sp <sup>r</sup> Hb <sup>+</sup> revertant of FA7186, <i>hpuB</i> * <sup>2369</sup> (G790D)	This study
FA7186H16	Sp <sup>r</sup> Hb <sup>+</sup> revertant of FA7186, <i>hpuB</i> * <sup>2369</sup> (G790D)	This study
FA7186H17	Sp <sup>r</sup> Hb <sup>+</sup> revertant of FA7186, <i>hpuB</i> * <sup>2181</sup> (W727C)	This study
FA7187	<i>hmbR::cat</i> mutant of FA7170, Sp <sup>r</sup> Hb <sup>+</sup>	This study
FA7188	<i>hmbR::\Omega</i> mutant of FA7170, Sp <sup>r</sup> Hb <sup>+</sup>	This study
FA7199	<i>tonB::\Omega</i> mutant of FA7168, Sp <sup>r</sup> Hb <sup>+</sup>	This study
FA7200	<i>tonB::\Omega</i> mutant of FA7170, Sp <sup>r</sup> Hb <sup>-</sup>	This study
FA7229	<i>tonB::\Omega</i> mutant of FA7185H1, Sp <sup>r</sup> Hb <sup>-</sup>	This study
FA7242	FA6929 with <i>hpuB</i> * from FA7170, Em <sup>s</sup> Hb <sup>+</sup>	This study
FA7243	FA6929 with <i>hpuB</i> * from FA7185H14, Em <sup>s</sup> Hb <sup>+</sup>	This study
FA7244	<i>hemA::\Omega</i> mutant of FA7170, Sp <sup>r</sup> Hb <sup>+</sup>	This study
FA7245	<i>hemA::\Omega</i> mutant of FA7185H14, Sp <sup>r</sup> Hb <sup>+</sup>	This study
FA7246	<i>hpuB::cat</i> mutant of FA7186H2, Cm <sup>r</sup> Hb <sup>-</sup>	This study
FA7247	<i>hpuB::cat</i> mutant of FA7186H17, Cm <sup>r</sup> Hb <sup>-</sup>	This study
FA7288	<i>hemA::\Omega</i> mutant of FA7169, Sp <sup>r</sup> Hb <sup>-</sup>	This study

human Hb at a concentration of 2  $\mu$ M and deferoxamine mesylate (Desferal) at 100  $\mu$ M instead of ferric nitrate.

Antibiotics were used at the following concentrations: for *E. coli* strains, ampicillin at 100  $\mu$ g/ml, erythromycin at 100  $\mu$ g/ml, chloramphenicol at 30  $\mu$ g/ml, and kanamycin at 30  $\mu$ g/ml; for gonococcal strains, erythromycin at 1  $\mu$ g/ml, chloramphenicol at 1  $\mu$ g/ml, and spectinomycin at 100  $\mu$ g/ml. A stock solution of heme was prepared by dissolving 10 mg of heme (Sigma; bovine hemin chloride) in 1 ml of 0.1 N NaOH. To prepare a stock solution of Hb, 100 mg of Hb (Sigma; human hemoglobin) was dissolved in 10 ml of 10 mM HEPES (pH 7.4), rocked overnight in a 4°C cold room, and sterile filtered through 0.45- $\mu$ m-pore-size Acrodisc (Pall Gelman Laboratory, Ann Arbor, Mich.).

**Reagents, isotope, oligonucleotide, and DNA sequencing.** All chemicals were purchased from Sigma, St. Louis, Mo., unless otherwise indicated. Iodine-125 was purchased from Amersham Pharmacia Biotech, United Kingdom. Oligonucleotides were synthesized at the Lineberger Comprehensive Cancer Center

DNA Synthesis Facility of the University of North Carolina-Chapel Hill. Sequencing was carried out at the Automated DNA Sequencing Facility of the University of North Carolina-Chapel Hill with an Applied Biosystems model 373 DNA sequencer by use of the Taq Dye Terminator cycle sequencing kit (Applied Biosystems, Perkin-Elmer, Foster City, Calif.).

**Construction of *hpuA* deletion mutants which express HpuB under the control of the *hpuA* promoter.** PCR-amplified DNA of the *hpuA* promoter region of the FA1090 Hb<sup>+</sup> variant was inserted into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.) and cloned in *E. coli* DH5 $\alpha$ MCR to generate pUNCH277. The upstream PCR primer uphpuA (5'-TATCGGCCCGTCGCGCAAAACGTGTTCC-3') started at -567 bp from the beginning of the *hpuA* coding sequence and carried an added *EagI* site at the 5' end. The downstream primer hpuApro (5'-ACGCATATGATGTATTAATAATAGTTTGGATTATC-3') started at -17 bp from the ATG site and carried an added *NdeI* site at the 3' end.

Another plasmid, pUNCH278, carrying PCR-amplified DNA of FA1090

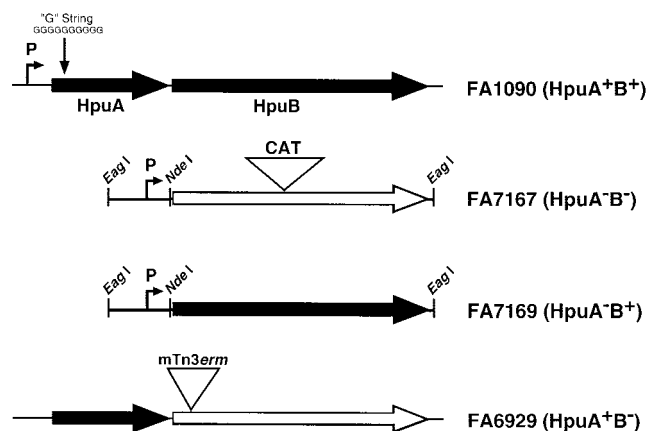


FIG. 1. *hpuA/B* genotype of key strains constructed and/or used in this study. The open arrow indicates that the gene is not expressed. The solid arrow indicates that the gene is expressed.

*hpuB*, including its own ribosome-binding site, was constructed in the same way. The upstream primer *hpuBrbs* (5'-ATACATATGGCAAAGGTTTCTTATGCCATTCC-3') started at -13 bp from the ATG site, and the downstream primer *hpu.61* (5'-TATCGGCCGCGGGCGCGGTGCGACC-3') started at 80 bp after the TAA terminator site. They carried added *NdeI* and *EagI* sites at the 5' and 3' ends, respectively. A 1-kb cassette carrying the gene for chloramphenicol acetyltransferase (*cat* cassette) was inserted at the *Clal* site of pUNCH278, 1,059 bp downstream from the ATG, to make pUNCH279. The *hpuB::cat* fragment of pUNCH279 was isolated by *NdeI-AvaI* double digestion and inserted into the *NdeI-AvaI* double-digested pUNCH277 to produce pUNCH280.

The DNA fragment from pUNCH280 carrying the *hpuA* promoter followed by *hpuB::cat* was isolated with *HindIII* and transformed into the FA1090 Hb<sup>+</sup> variant to make FA7167. One of the *HindIII* sites was at the vector polylinker region, and the other was 687 bp before the TAA terminator site. FA7167 was Hb<sup>-</sup> and chloramphenicol resistant (Cm<sup>r</sup>). Transformation of FA7167 with pUNCH272 carrying the coding sequence for mature HpuB resulted in FA7169 (Fig. 1). The primer pair used to amplify this section of *hpuB* was *hpu.51* (5'-TATACATATGGCAGACCCCGCGCCGAGTC-3') and *hpu.61*. FA7169 was chloramphenicol sensitive (Cm<sup>s</sup>) and Hb<sup>-</sup>, as expected. During the construction, we observed unexpected rare Cm<sup>r</sup> Hb<sup>+</sup> colonies on the Hb/Des plates. Two of the colonies were isolated, characterized, and named FA7168 and FA7170.

**Detection of HpuA and HpuB.** Expression of HpuA and HpuB was examined by Western blots and dot blots. Strains were grown in iron-sufficient and iron-limited media as described previously (8). Crude lysates prepared from iron-stressed and iron-replete cell suspensions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrophoretic transfer to nitrocellulose. Polyclonal antibodies raised in rabbits against either the N terminus of HpuB or the C terminus of HpuA were used for detection of HpuB and HpuA in Western blots (8). Whole-cell dot blots were prepared from wells loaded with 100  $\mu$ l of cell suspensions at an optical density at 600 nm (OD<sub>600</sub>) of 0.2. Dot blots were dried, blocked with 5% dry milk, and probed with rabbit antisera raised against either recombinant HpuB or HpuAB affinity purified on an Hb-agarose column.

**Mutagenesis of *hmbR*, *tonB*, and *hemA*.** The *hmbR* gene of FA7168, FA7169, and FA7170 was mutagenized with either the  $\Omega$  or the *cat* cassette. The constructions started with pIRS756 (a gift from I. Stojiljkovic of Emory University) containing the *hmbR* gene of gonococcal strain MS11. The *cat* cassette was inserted at the *PstI* site and the  $\Omega$  cassette was inserted at the blunted *AgeI* site of *hmbR*. The pCR2.1-TOPO-cloned *hmbR::cat* and *hmbR:: $\Omega$*  fragments were isolated and used in transformations to derive Cm<sup>r</sup> and spectinomycin-resistant (Sp<sup>r</sup>) transformants, respectively. The TonB dependence of Hb<sup>+</sup> revertants was tested by insertional inactivation of *tonB* with the  $\Omega$  cassette. This procedure used the *tonB:: $\Omega$*  plasmid pUNCH173 (3) to transform various  $\Delta$ *hpuA* mutants and selected for Sp<sup>r</sup>. The ability of  $\Delta$ *hpuA* mutants to utilize Hb as a heme source was tested by the construction of *hemA* mutants with the *hemA:: $\Omega$*  plasmid pUNCH1306 described by Turner et al. (26).

**Hb<sup>+</sup> revertants of *hpuA* deletion mutants.** After initial recognition of FA7168 and FA7170, more Hb<sup>+</sup> mutants were selected from the *hmbR* mutants of

FA7169, FA7185 (*hmbR::cat*), and FA7186 (*hmbR:: $\Omega$* ) by plating on Hb/Des plates.

**Primers for PCR and DNA sequencing.** The design of primers was based on analysis of contiguities released from the University of Oklahoma Gonococcal Genome Sequencing Project (21). The sequence of *hpuB* in Hb<sup>+</sup> mutants of  $\Delta$ *hpuA* parents was examined by sequencing PCR-amplified genomic DNA. Amplification of *hpuB* used the primer pair *hpuBrbs* and *hpu.61*. The sequencing used eight primers as needed to read through *hpuB*: *hpu.05* (5'-TCCTTCAAACCCGTATTGGCT-3'), *hpu.09* (5'-ATTCAGCAGCATTACCGCCG-3'), *hpu.07* (5'-GCGGCGCAATACGGCTTAG-3'), *hpu.11* (5'-AGATACGCCCCGTTTACA-3'), *hpu.10* (5'-ATTTCGACATCGCCCTCGGT-3'), *hpu.08* (5'-CCGCCAAGTAAACACATTGT-3'), *hpu.12* (5'-ATGTAGCTGACGTGAGGCC-3'), and *hpu.61*.

**Dependence of Hb-supported growth on HpuB expression.** DNA sequencing showed that one class of Hb<sup>+</sup> mutants had point mutations in *hpuB*, which were designated *hpuB\**. Insertional inactivation of HpuB was carried out in  $\Delta$ *hpuA* *hpuB\** mutants FA7186H2 and FA7186H17 to confirm that point mutation in HpuB was the factor responsible for their Hb<sup>+</sup> phenotype. PCR products prepared from the region flanking the *cat* insert in the *hpuB* of FA7167 were used as the transforming DNA. The upstream primer, *hpu.09*, and the downstream primer, *hpu.08*, covered an *hpuB* fragment of 795 bp, not counting the insertion. The transformants were selected for Cm<sup>r</sup> and scored for inability to grow on Hb/Des plates.

**Moving HpuB point mutants into an HpuA<sup>+</sup> background.** The role of HpuA in gonococcal Hb utilization was examined by comparing the growth of  $\Delta$ *hpuA* *hpuB\** and *hpuA<sup>+</sup> hpuB\** mutants. PCR-amplified *hpuB* was prepared from two *hpuB\** mutants, FA7170 and FA7185H14, and moved into FA6929, which is *hpuA<sup>+</sup> hpuB::mTn3erm*. Transformants were selected for growth on Hb/Des plates and scored for sensitivity to erythromycin (Em<sup>r</sup>). The *hpuB* of selected Hb<sup>+</sup> Em<sup>r</sup> transformants was PCR amplified and sequenced to verify that the *hpuB* point mutations were preserved and the mTn3erm cassette of FA6929 had been replaced.

**Plate assays for Hb and heme utilization and detergent and antibiotic sensitivity.** The phenotype of various mutants was assessed by spreading 100  $\mu$ l of a 1:100 dilution of cell suspensions at an OD<sub>600</sub> of 0.4 onto GCB/Des or GCB plates. To test for growth, wells (0.6 cm in diameter) were cut into the agar and filled with 60  $\mu$ l of heme at 0.1 mg/ml or Hb at 10 mg/ml (26). To test for antimicrobial sensitivity, filter paper disks (0.6 cm in diameter) were placed on the GCB plate surface and 5  $\mu$ l of test solution was dropped at the center of the disks. Chloramphenicol was used at 0.3  $\mu$ g/ $\mu$ l, rifampin was used at 0.1  $\mu$ g/ $\mu$ l, and Triton X-100 was used at a 1:100 dilution.

**Liquid culture assays.** Liquid cultures were started from gonococci grown overnight on Chelex-treated defined medium (CDM) plates containing 5  $\mu$ M ferric nitrate (12). Ten milliliters of liquid CDM was inoculated to approximately 20 Klett units and incubated with shaking at 37°C in a 5% CO<sub>2</sub> atmosphere for 2 h. Once the starting culture reached about 30 Klett units, fresh CDM containing the iron sources of interest was inoculated to a starting Klett unit of about 5 for Hb cultures and about 10 for heme cultures. Hb was used at a final concentration of 1  $\mu$ M, while heme was tested at concentrations varying between 0.05 and 5  $\mu$ M. Desferal was added to all Hb and heme media to 100  $\mu$ M. For iron-stressed growth, only Desferal was added to the liquid culture. Human serum albumin (HSA) was added as indicated to a final concentration of 16  $\mu$ M.

**Hb-binding assays.** Two kinds of Hb-binding assays were carried out using either biotinylated human Hb or iodinated anti-Hb antibody. The biotinylated Hb-whole-cell dot blot assay has been described previously (7). The radioimmunoassay used iodinated goat anti-human Hb antibodies (affinity purified; Bethyl Laboratories, Montgomery, Tex.) instead of Hb because of the difficulty in maintaining the stability of iodinated Hb. Iodination was done in IODO-GEN-coated iodination tubes from Pierce (Pierce Endogen, Rockford, Ill.) and followed the suggested protocol. The assay used MultiScreen plates purchased from Millipore (Bedford, Mass.). CDM solution was the suspension and washing buffer, and 0.5% dry milk was used as the blocker.

The wild type and the  $\Delta$ *hpuA* strains to be tested were grown in iron-replete and iron-stressed cultures and prepared as OD<sub>600</sub> = 0.2 cell suspensions in CDM solution. Hb was added to the final concentration of 0.1  $\mu$ M to 100  $\mu$ l of gonococcal suspensions in the wells. The mixture was incubated at room temperature for 30 min and filtered through the MultiScreen plate. After three washes with CDM solution, labeled goat anti-human Hb antibody was added to approximately 10<sup>6</sup> cpm per well. After another incubation of 30 min, the wells were washed three times again, disks at the bottom of wells were punched out, and radioactivity was counted in a gamma scintillation counter. Specific Hb binding values were derived from the difference between the cpm bound in wells with iron-stressed gonococci and those bound in wells with iron-replete gono-



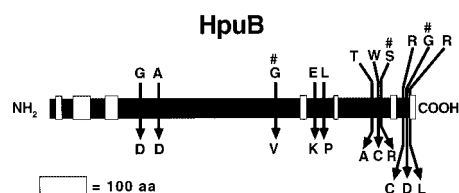


FIG. 2. Illustration of point mutation sites found in FA7170 and  $\Delta hpuA hpuB^*$  mutants derived from FA7185 and FA7186. The arrows indicate alterations of amino acids (aa), with amino acids of the wild type on the top row and those of the mutants on the bottom row. The # indicates sites with multiple occurrences. The corresponding sites of mutation in *hpuB* are bp 650, 770, 1526, 1780, 1838, 2146, 2199, 2181, 2344, 2369, and 2378 from the beginning of coding sequence. The shaded area is the signal sequence, and the boxes are seven regions of homology derived by Lewis et al. (16) from their peptide alignment of meningococcal HpuB- and TonB-dependent outer membrane proteins described by Cornelissen et al. (9).

cocci. Specific counts accumulated from each test strain were then compared with corresponding counts from the wild-type strain using paired Student's *t* test.

## RESULTS

In order to study the roles of HpuA and HpuB in the gonococcal Hb receptor, we constructed a  $\Delta hpuA hpuB^+$  mutant of FA1090, FA7169, as illustrated in Fig. 1. Moving the  $\Delta hpuA hpuB::cat$  DNA fragment from pUNCH280 into the Hb<sup>+</sup> variant of FA1090 resulted in Cm<sup>r</sup> Hb<sup>-</sup> FA7167. Transformation of FA7167 with pUNCH272, which contained the wild-type *hpuB* sequence, removed the *cat* cassette insert and resulted in FA7169. The *hpuB* sequence of FA7169 was identical to that of FA1090. The *hpuB* gene, along with its ribosome-binding site, followed the *hpuA* promoter at a distance equal to that between *hpuA* and the promoter in FA1090, and HpuB was expressed under iron-stressed growth conditions. FA7169 could not grow on Hb as a sole iron source but expressed HpuB, as previously reported for the analogous *hpuA* nonpolar insertion mutant FA6983 (8).

During the construction of FA7169, two mutants, FA7168 and FA7170, were discovered which were phenotypically Cm<sup>s</sup> but grew on Hb/Des plates (Hb<sup>+</sup>). DNA sequencing revealed that both mutants had the expected deletion of *hpuA*. FA7168 was  $\Delta hpuA hpuB::cat$ , while FA7170 was  $\Delta hpuA hpuB^{*650}$  (G217D), with a point mutation 650 bp downstream from the beginning of the coding sequence. Both FA7168 and FA7170 grew on Hb/Des after insertional mutagenesis of *hmbR* (data not shown), and thus HmbR was not responsible for the Hb<sup>+</sup> phenotype of either mutant. Insertional inactivation of *tonB* resulted in an Hb<sup>-</sup> phenotype in FA7170, but a *tonB* mutant of FA7168 was still Hb<sup>+</sup> (data not shown), indicating that they used different mechanisms for Hb-supported growth. FA7168 was unable to grow on plates containing chloramphenicol (1  $\mu$ g/ml) and was highly sensitive to rifampin and Triton X-100, while FA1090, FA7167, FA7169, and FA7170 were able to grow on the same plates (data not shown).

After these initial characterizations, additional Hb<sup>+</sup> mutants were selected from *hmbR* mutants of FA7169, FA7185 (*hmbR::cat*) and FA7186 (*hmbR:: $\Omega$* ), on Hb/Des plates at frequencies of about 10<sup>-6</sup>. DNA sequencing confirmed that there were two classes of Hb<sup>+</sup> revertants. One had single point

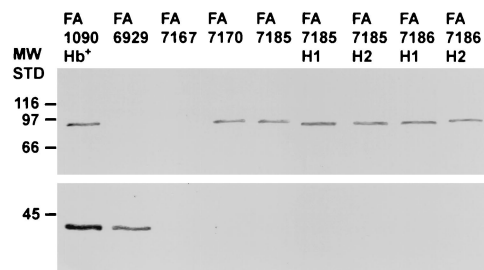


FIG. 3. Western blot analysis of whole-cell lysates prepared from the indicated strains grown under iron-stressed condition. FA7185H1 and FA7185H2 were Hb<sup>+</sup> revertants of FA7185. FA7186H1 and FA7186H2 were revertants of FA7186. FA7186 H1 was an *hgbX* mutant, while the other three were *hpuB*<sup>\*</sup> mutants. The top panel was probed with rabbit antiserum raised against the HpuB N-terminal peptide, and the bottom panel was probed with rabbit antiserum raised against the HpuA C-terminal peptide. The positions of size standards are shown on the left (in kilodaltons).

mutations in *hpuB*, and these were designated  $\Delta hpuA hpuB^*$  mutants. The other class did not have mutations in *hpuB*, but, like FA7168, all were highly sensitive to Triton X-100 and rifampin (data not shown). Mutants of this class were designated  $\Delta hpuA hgbX$  mutants, pending identification of the gene(s) involved in the Hb<sup>+</sup> phenotype. All 15 *hpuB*<sup>\*</sup> mutants contained a single predicted amino acid alteration, which were clustered toward the C-terminal end of HpuB. Three mutations were isolated independently multiple times (Fig. 2).

The Hb<sup>-</sup>  $\Delta hpuA hpuB^+$  FA7169 and the Hb<sup>+</sup>  $\Delta hpuA hpuB^*$  mutants expressed HpuB under iron-stressed conditions (Fig. 3). Insertional inactivation of the altered HpuB in FA7186H2 and FA7186H17 resulted in Hb<sup>-</sup> transformants FA7246 and FA7247 (data not shown). Thus, expression of HpuB was required for the utilization of Hb for growth. Further proof that the point mutations in *hpuB* caused the Hb<sup>+</sup> phenotype was obtained by transforming FA7169 with PCR-amplified *hpuB* DNA from FA7170 or FA7185H14. The *hpuB* gene was sequenced from two Hb<sup>+</sup> transformants of each transformation and confirmed that each carried the same *hpuB* point mutation as the donor (data not shown).

Zones of growth around Hb were smaller for  $\Delta hpuA hpuB^*$  mutants than FA1090 Hb<sup>+</sup> (Fig. 4), suggesting that the *hpuB*<sup>\*</sup> mutants required a higher Hb concentration for growth than the wild type. Free heme also could be used by the *hpuB*<sup>\*</sup>

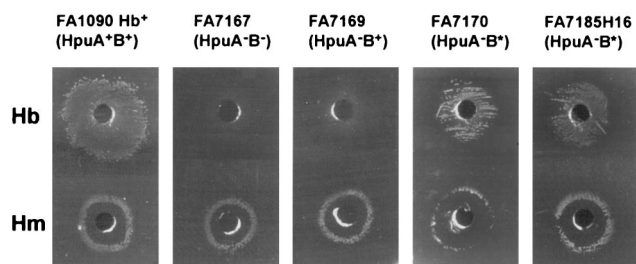


FIG. 4. Plate assays testing the ability of indicated  $\Delta hpuA$  mutants for growth on Hb and heme (Hm). Wells were cut into GCB/Des agar and loaded with Hb (60  $\mu$ l at 10  $\mu$ g/ $\mu$ l) and heme (60  $\mu$ l at 0.1  $\mu$ g/ $\mu$ l). All tested strains grew on heme. The  $\Delta hpuA hpuB^+$  strain FA7169 could not grow on Hb, but the  $\Delta hpuA hpuB^*$  mutants could.

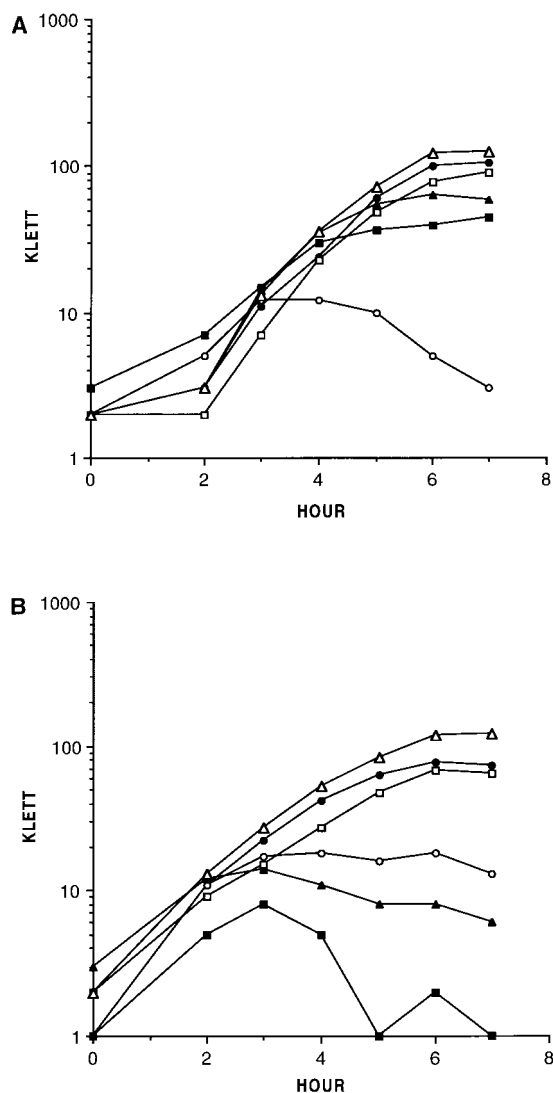


FIG. 5. Growth of  $\Delta hpuA hpuB^*$  mutants and  $hpuA^+ hpuB^*$  mutants in medium supplemented with 1  $\mu\text{M}$  Hb (A) and 1  $\mu\text{M}$  Hb plus HSA at 16  $\mu\text{M}$  (B). FA1090 Hb<sup>+</sup> is the wild-type parent (●). FA6929 is an  $hpuA^+ hpuB^*$  mutant (○). The  $hpuA^+ hpuB^*$  strains FA7242 (□) and FA7243 (△) were derivatives of the  $\Delta hpuA hpuB^*$  mutants FA7170 (■) and FA7185H14 (▲), respectively.

mutants as an iron source, as previously described for FA1090 Hb<sup>+</sup> (26). Both Hb<sup>+</sup> FA1090 and Hb<sup>-</sup> FA7169 showed similar heme growth phenotypes, with an inner zone of inhibition and an outer zone of growth around the well (Fig. 4). The zone of inhibition reflects the sensitivity of these strains to heme; they only grew on a narrow range of heme concentrations.

Growth of  $\Delta hpuA hpuB^*$  mutants in liquid culture containing 1  $\mu\text{M}$  Hb and 100  $\mu\text{M}$  Desferal was inhibited by HSA, while that of FA1090 Hb<sup>+</sup> was not inhibited by HSA (Fig. 5). Thus, growth of these mutants apparently relied on free heme released extracellularly from Hb. The role of HpuA was further investigated by constructing  $hpuA^+$  derivatives of the  $hpuB^*$  strains FA7170 and FA7185H14 (FA7242 and FA7243, respectively). Growth of the  $hpuA^+ hpuB^*$  strains on Hb was

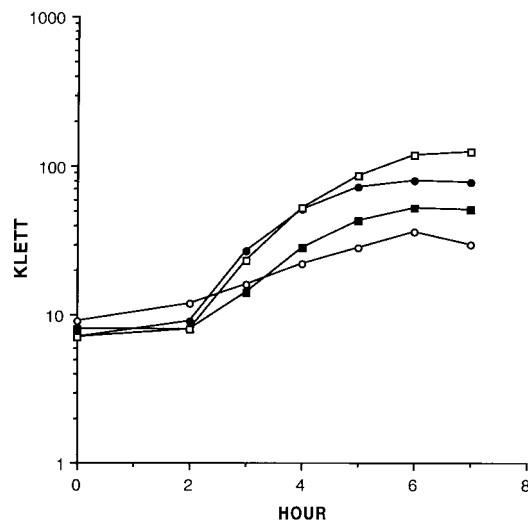


FIG. 6. Growth of the  $\Delta hpuA hpuB^*$  mutant and its  $\Delta hpuA hpuB^+$  parent in medium supplemented with 0.5  $\mu\text{M}$  heme. FA1090 Hb<sup>+</sup> is the wild-type parent (●). FA7167 is the  $\Delta hpuA hpuB^*::cat$  negative control (○). FA7169 (■) is the  $hpuB^+$  parent of FA7170 (□). Results presented in this graph were also true for FA7186H16 and its parent FA7186. Similar results were observed in two other experiments.

not inhibited by HSA (Fig. 5), confirming the importance of HpuA in allowing the use of heme from Hb.

Insertional inactivation of *hemA* in the  $hpuB^*$  mutants FA7170 and FA7185H14 resulted in FA7244 and FA7245, respectively, while the *hemA* mutant of FA7169 was designated FA7288. All of these *hemA* mutants needed  $\delta$ -aminolevulinic acid to grow on GCB plates, but were able to grow on heme/Des (data not shown). Both FA7244 and FA7245 were able to grow on Hg/Des plates, but FA7288 did not grow on the same plate (data not shown). These results demonstrated that Hb was used as a heme source by the  $\Delta hpuA hpuB^*$  mutants, while  $\Delta hpuA hpuB^+$  FA7169 could not use Hb as a heme source.

The uptake of free heme by  $hpuB^*$  mutants was studied by measuring heme-dependent growth in liquid cultures. At heme concentrations of 1 and 5  $\mu\text{M}$ , growth of all  $hpuB^*$  and  $hpuB^+$  strains was indistinguishable and quickly reached a plateau. At heme concentrations of 0.05 to 0.1  $\mu\text{M}$ , all strains grew poorly (data not shown). At 0.5  $\mu\text{M}$  heme, strains expressing HpuB grew better than the HpuB<sup>-</sup> FA7167. The  $hpuB^*$  mutants FA7170 and FA7186H16 grew slightly but reproducibly better than their  $hpuB^+$  parents, FA7169 and FA7186 (Fig. 6 and data not shown). Thus, at limiting heme concentrations,  $hpuB^*$  mutants apparently transported more free heme than their  $hpuB^+$  parents.

In order to evaluate the relative roles of HpuA and HpuB in Hb binding, qualitative and semiquantitative Hb binding assays were carried out. Probing whole-cell dot blots with biotinylated Hb showed that iron-stressed  $\Delta hpuA hpuB^*$  mutants bound Hb, although apparently less than FA1090 Hb<sup>+</sup>. The binding corresponded to the expression of HpuB, as detected by anti-HpuB antibodies (data not shown). Radioimmunoassays further confirmed that Hb bound to all mutants that expressed HpuB regardless of their growth phenotype. FA7169, which was Hb<sup>-</sup>, made HpuB and bound Hb, whereas FA7168, which

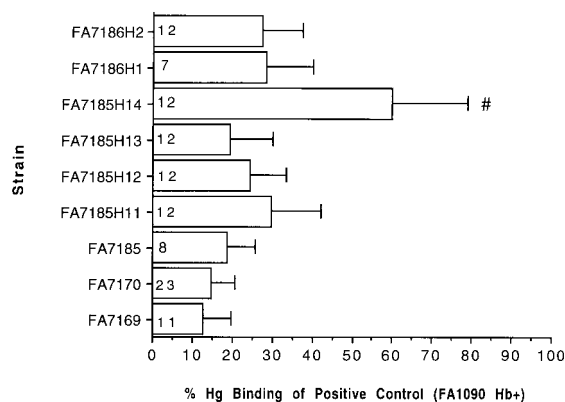


FIG. 7. Results from radioimmunoassays of Hb binding of  $\Delta hpuA$  mutants. FA7169, FA7185, and FA7186H1 are HpuB<sup>+</sup>, while the rest are HpuB<sup>\*</sup>. Hb binding to each mutant was significantly less than that of the wild type ( $P < 0.01$ , paired Student's  $t$  test). # indicates that FA7185H14 bound more Hb than its parent FA7185 ( $P < 0.01$ , Student's  $t$  test). The other FA7185 derivatives and FA7170 did not bind significantly more Hb than their HpuB<sup>+</sup> parents. Since experiments were done over a period of 4 weeks using different batches of iodinated anti-human Hb antibody, binding data for each tested strain are presented as a percentage of that of the corresponding wild-type control (FA1090 Hb<sup>+</sup>). Bars indicate standard deviations. The number at the base of each column represents the number of determinations.

was Hb<sup>+</sup> but could not express HpuB, did not bind Hb (data not shown). Hb binding to each  $\Delta hpuA$  mutant,  $hpuB^+$  or  $hpuB^*$ , was significantly less than that of the wild type ( $P < 0.01$ , paired Student's  $t$  test). With the exception of FA7185H14, all  $\Delta hpuA$  mutants bound less than 30% of the Hb bound to the wild type. Hb binding generally was not increased by the  $hpuB^*$  mutations; FA7185H14 was the only strain that bound significantly more Hb than its  $hpuB^+$  parent (Fig. 7).

## DISCUSSION

*N. gonorrhoeae* ordinarily requires both HpuA and HpuB to use human Hb as the source of iron for growth. Expression of both proteins is controlled at the translational level by the length of a run of guanine residues within sequence encoding the mature HpuA protein (8). As expected, a mutant with an in-frame deletion of *hpuA* that expressed wild-type HpuB was unable to grow on Hb. However, we were able to identify Hb<sup>+</sup> revertants from the  $\Delta hpuA$  mutant at frequencies of about  $10^{-6}$ . Two classes of revertants were identified:  $hpuB^*$  mutants, which had single point mutations in *hpuB*, and *hgbX* mutants, which had mutations in a still unidentified locus or loci. Involvement of HmbR, the single-component, TonB-dependent outer membrane receptor known to bind and acquire iron from Hb in meningococci (22), was ruled out after insertional mutagenesis of *hmbR*.

The neisserial transferrin and lactoferrin receptors are similar to the HpuAB Hb receptor. All are bipartite, TonB-dependent outer membrane receptors, consisting of a lipoprotein and an integral outer membrane protein. The lipoproteins TbpB and LbpB are necessary for full binding of ligand, but are not absolutely required for iron acquisition (2, 4, 10, 19). In contrast, both HpuA and HpuB are required for iron acquisition

from Hb in *N. gonorrhoeae* (8) and *Neisseria meningitidis* (18). However, the current work proves that the requirement for HpuA can be bypassed under certain circumstances by point mutations in HpuB.

Growth on Hb by  $\Delta hpuA$   $hpuB^*$  mutants was TonB and HpuB dependent, indicating that iron acquisition was an energy-dependent process involving HpuB. All  $\Delta hpuA$  mutants bound less Hb than the wild type, confirming the importance of HpuA in the ligand binding. The point mutations in HpuB that restored growth on Hb in the absence of HpuA did not significantly increase Hb binding, with the exception of FA7185H14. None of the mutations in HpuB restored full Hb binding to the cell surface, and therefore growth apparently was not due merely to increased ligand binding. The low Hb binding efficiency of  $\Delta hpuA$   $hpuB^*$  mutants might have contributed to the higher Hb concentration required for growth on Hb.

HSA inhibited Hb-dependent growth of  $hpuB^*$  mutants, suggesting that free heme was released external to the outer membrane before it was internalized, presumably by passing through a pore in the integral outer membrane protein HpuB. Proof that heme released from Hb entered intact was provided by demonstration of Hb-dependent growth in  $\Delta hpuA$   $hpuB^*$  *hemA::Ω* mutants. In these mutants, heme released from Hb was used both as a porphyrin source and as an iron source. The mechanism of heme removal from Hb is currently unknown.

Growth of  $\Delta hpuA$   $hpuB^*$  mutants on Hb was inhibited by HSA, but HSA was not growth inhibitory in  $hpuB^*$  strains that expressed HpuA. These results suggested that HpuA both increased Hb binding and allowed use of heme released externally from Hb. The current results are insufficient to determine precise roles of HpuA or HpuB in ligand binding. HpuA might increase Hb binding by stabilizing a conformation of HpuB that has high affinity for Hb. Apparent protection of heme from HSA binding by HpuA could have been due to steric effects of the Hb receptor, but also could have been due to increased release of heme from HSA.

In liquid cultures with limiting heme concentrations,  $\Delta hpuA$   $hpuB^*$  mutants grew slightly better than their  $\Delta hpuA$   $hpuB^+$  parents did. Thus, the point mutations in HpuB might have altered the transmembrane barrel and increased the ability of free heme to traverse the membrane. However, we were unable to determine the relative contribution of heme binding and heme entry to the heme-dependent growth of  $\Delta hpuA$   $hpuB^*$  mutants. With regard to Hb-dependent growth, our data did not show a general increase of Hb binding in the  $hpuB^*$  mutants in comparison to their  $hpuB^+$  parents. The  $hpuB^*$  mutations could have effected either increased heme release from Hb or increased transport of heme through HpuB or both.

The mutations in HpuB occurred in clusters, particularly near the C terminus, but the meaning of this distribution is not yet clear. None of the mutations was likely to be in the putative N-terminal plug that is characteristic of TonB-dependent siderophore receptors (5). Until a crystal structure is determined for HpuB, the exact location of point mutations in the barrel or outer loops of HpuB will remain conjectural. The point mutations in HpuB that restored function of the receptor in the absence of the otherwise requisite partner HpuA should prove helpful in ultimately understanding the structure-function relationship in HpuAB. Likewise, the other class of mutations,

designated *hgbX*, should prove useful in understanding Hb receptor-independent and TonB-independent mechanisms for heme utilization, and these studies are being actively pursued.

#### ACKNOWLEDGMENTS

We thank the reviewers for their constructive criticisms and suggestions. We thank Gour Biswas, Christopher Elkins, and Paul Turner for helpful suggestions and discussions. We also thank Annice Rountree for technical assistance.

This work was supported by NIH grants AI26837 and AI31496 to P. Frederick Sparling.

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