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Characterization of the *Pasteurella multocida hgbA* Gene Encoding a Hemoglobin-Binding Protein

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Reverse transcriptase PCR analyses have demonstrated that open reading frames (ORFs) PM0298, PM0299, and PM0300 of the animal pathogen *Pasteurella multocida* constitute a single transcriptional unit. By cloning and overexpression studies in *Escherichia coli* cells, the product of ORF PM0300 was shown to bind hemoglobin in vitro; this ORF was therefore designated *hgbA*. In vitro and in vivo quantitative assays demonstrated that the *P. multocida hgbA* mutant bound hemoglobin to the same extent as the wild-type strain, although the adsorption kinetics was slightly slower for the *hgbA* cells. In agreement with this, the virulence of *P. multocida hgbA* cells was not affected, suggesting that other functional hemoglobin receptor proteins must be present in this organism. On the other hand, *P. multocida* mutants defective in PM0298 and PM0299 could be isolated only when a plasmid containing an intact copy of the gene was present in the cells, suggesting that these genes are essential for the viability of this bacterial pathogen. By adapting the recombinase-based expression technology in vivo to *P. multocida*, we also demonstrated that the transcriptional PM0298-PM0299-*hgbA* unit is iron regulated and that its expression is triggered in the first 2 h following infection in a mouse model. Furthermore, hybridization experiments showed that the *hgbA* gene is widespread in *P. multocida* strains regardless of their serotype or the animal from which they were isolated.

Iron is an essential compound for almost all living organisms. Bacterial species have evolved several strategies for the uptake of this element. Some bacteria produce and release into the environment small molecules of various chemical structures, known as siderophores, which are able to chelate free iron (20, 25). Binding of iron-loaded siderophores to specific protein receptors located in the bacterial outer membrane enables iron uptake. Other bacteria have several proteins in the outer membrane which are specific receptors for the different iron-binding host molecules, such as heme, hemoglobin, transferrin, and lactoferrin.

Bacterial transferrin- and lactoferrin-binding proteins have been studied mainly in pathogenic members of the families *Pasteurellaceae* and *Neisseriaceae* (9, 31). Typically, they are formed by two different subunits: TbpA and TbpB for transferrin receptors and LbpA and LbpB for lactoferrin receptors (25). TbpB and LbpB are lipoproteins anchored to the outer membrane and are the proteins which more directly interact with the substrate. TbpA and LbpA are inserted into the outer membrane and seem to serve as channels through which iron penetrates to the periplasmic space (25). Moreover, there are two different kinds of hemoglobin receptor proteins (25, 31). Some gram-negative bacteria secrete a hemoglobin-binding protein which extracts the heme group from hemoglobin and delivers it to an outer membrane-specific protein to introduce it into the cell. Other gram-negative bacteria have, anchored in the outer membrane, a specific receptor able to bind hemoglobin directly and afterwards promote internalization of either iron or the heme group. However, the transport of iron or heme across the outer membrane by all of these high-affinity mechanisms requires the products of the *exbB, exbD*, and *tonB* genes (1). These TonB-dependent outer membrane proteins have several preserved regions; the most characteristic region is the region which is located at the N-terminal end, which is known as the TonB box and which can be represented as (D/E)TXXVXA(A/S), where X is variable (17). By corollary, the presence of a TonB box in the amino acid sequence of a bacterial protein suggests that it may be involved in the uptake of several nutrients, including iron. The increasing number of bacterial genome sequences, together with comparative genomic analysis, allows identification of gene products which may potentially be TonB-dependent receptors. However, any predictions must be confirmed by experimental data to definitively assign a definite biological function.

Pasteurella multocida is a gram-negative bacterium that is able to cause infectious diseases in a wide spectrum of animals, including several mammal and bird species. As a consequence of this, *P. multocida* infections result in substantial economic losses worldwide. Nevertheless, and despite its importance, little is known about the iron uptake mechanisms of this pathogen. Determination of the *P. multocida* genome sequence has

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant features	Source or reference
E. coli strains		
DH5α	F′ *supE4 lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Clontech
HB101	*supE4 hsdS20 recA13 ara-1 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Clontech
MC1061 (λ*pir*)	*hsdR mcrB araD139* Δ(*araABC-leu*) *7679* Δ*lacX74 galI galK rpsL thi* lysogenized with λ*pir* bacteriophage	Our laboratory
BL21 (λDE3)	F^- *ompT* $hsdS_B$ (r_B^- r_B^+) *dcm gal* λ(*DE3*)	38
P. multocida strains		
PM25	Wild type	Our laboratory
PM1002	Same as PM25, but Rif^r Spc^r	Our laboratory
PM1077	Same as PM1002, but carrying pUA961	This study
PM1078	Same as PM1002, but *hgbA*::pUA967	This study
PM1079	Same as PM1002, but PM0298 defective and carrying pUA964	This study
Plasmids		
pGEM-T	PCR cloning vector, Ap^r	Promega
pRK2013	*rep* (ColE1), Mob$^+$ Tra$^+$ Km^r	5
pHRP309	Broad-host-range cloning vector for transcriptional *lacZ* fusion construction, Gm^r Mob$^+$	23
pUA826	Mob$^+$, R6K replicon, Ap^r Str^r Spc^r	2
pET-22b	Overexpression plasmid, Ap^r	Novagen
pGH436	Same as pBR322, but carrying the resl-tet-resl construction	35
pIVET5mut	R6K replicon containing *tnpR* and *lacZY* promotorless genes	15
pUA958	Same as pUA826, but carrying the promotorless *tnpR* gene	This study
pUA959	Same as pGH436, but carrying the Cm cassette	This study
pUA960	Same as pUA958, but carrying the *res1*-Cm-*res1* construction	This study
pUA961	Same as pUA960, but carrying a fusion between the *P. multocida hgbA* 5′ end and the *tnpR* gene	This study
pUA962	Same as pGEM-T, but carrying the *P. multocida* PM0298-PM0299-*hgbA* operon	This study
pUA963	Same as pET-22b, but carrying a fusion between the *P. multocida hgbA* promoterless gene and the T7 promoter	This study
pUA964	Same as pHRP309, but carrying *P. multocida* ORFs PM0298 and PM0299	This study
pUA965	Same as pUA826, but carrying a 169-bp internal fragment of *P. multocida* ORF PM0298	This study
pUA966	Same as pUA826, but carrying a 258-bp internal fragment of *P. multocida* ORF PM0299	This study
pUA967	Same as pUA826, but carrying a 832-bp internal fragment of *P. multocida hgbA* gene	This study

revealed the presence in this organism of several genes encoding potential TonB-dependent receptor proteins (18, 24). Nevertheless, there are no experimental data on the function of the product of any of these genes. One example is the hypothetical protein product of the PM0300 open reading frame (ORF), which lies immediately downstream of two other putative ORFs (PM0298 and PM0299) (Fig. 1A).

In the present work, the transcriptional organization of the DNA region containing these three ORFs and the role of the product of the PM0300 gene in *P. multocida* hemoglobin uptake in vivo and in vitro were investigated. Furthermore, after the recombinase-based in vivo expression technology (RIVET) was adapted to *P. multocida* characteristics, expression of these three *P. multocida* ORFs in vitro and during animal infection in a mouse model was studied.

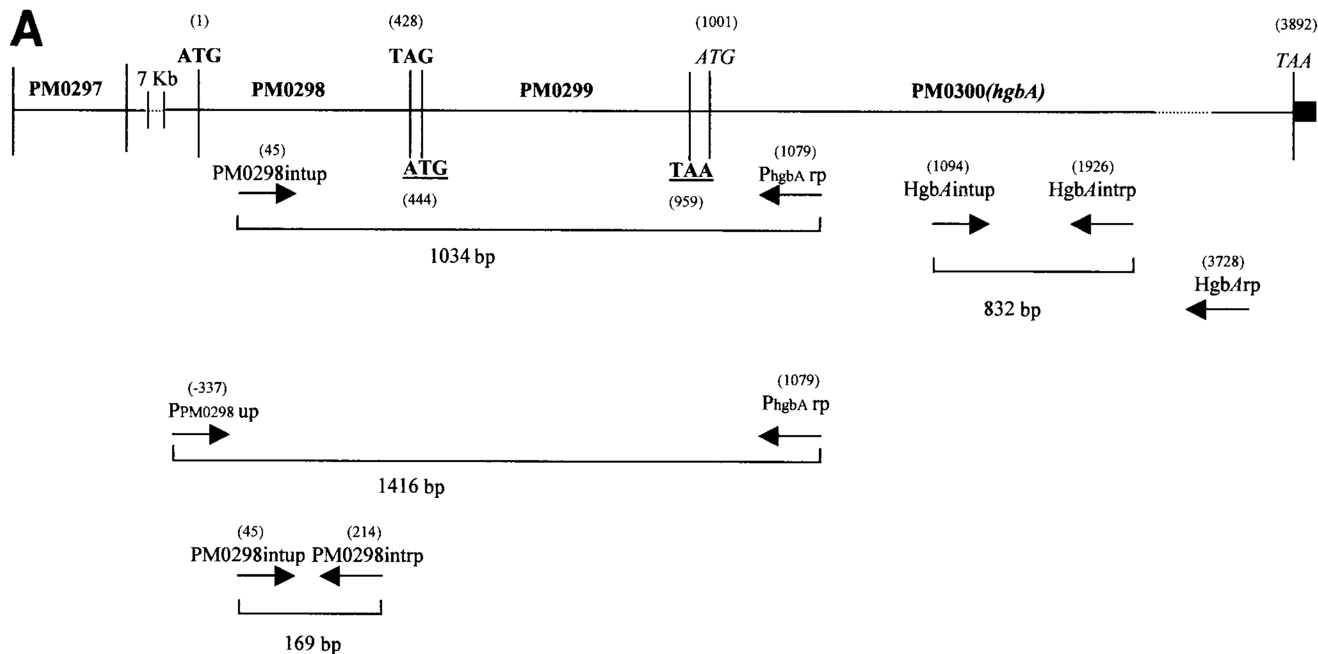
MATERIALS AND METHODS

Bacterial strains, growth conditions, and DNA and RNA techniques. Bacteria used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani medium (19). *P. multocida* was cultured in either buffered peptone water (BPW) or brain heart infusion (BHI) liquid medium or on BHI or sheep blood agar plates. The antibiotic concentrations used have been described previously (3). The DNA method used was also described previously (30). Oligonucleotide primers used in this work are listed in Table 2. The entire nucleotide sequence of *P. multocida* ORFs PM0298, PM0299, and PM0300 was determined for both DNA strands by the dideoxy method with an ALF sequencer (Pharmacia Biotech). Total cellular RNA extraction and reverse transcriptase PCR (RT-PCR) analysis were performed as reported by Jordan et al. (12).

Genetic methods. *P. multocida* mutants were obtained by insertional mutagenesis by using the strategy described previously (3). Briefly, an internal fragment of the gene to be inactivated was inserted into the suicide plasmid pUA826, which is unable to replicate in host strains lacking the R6K-specified Π product of the *pir* gene (3). pUA826 derivatives harboring the internal *P. multocida* gene fragments were then introduced by triparental mating, using pRK2013 as the mobilizing plasmid, into *P. multocida* wild-type cells, and ampicillin-resistant exconjugants were selected. The presence of the desired mutations in these clones was confirmed by PCR analysis by using primers shown in Table 2. When done, biparental mating was performed by using the *E. coli* S-17 mobilizing system (32).

Virulence assays. Female Swiss mice that were 3 to 8 weeks old, were obtained from Harlan Iberica Inc. (Barcelona, Spain), and were housed under specific-pathogen-free conditions were used for studies of virulence. Bacteria were grown for 16 h in BHI medium prior to infection. The 50% lethal doses of the strains were determined in triplicate as previously reported (8). Basically, groups of three mice were injected intraperitoneally with 0.1-ml portions of serial 10-fold dilutions of bacteria in BPW. The concentrations of the original bacterial suspensions were determined by the plate count method. The numbers of animals which were alive at 24, 48, and 72 h postinoculation were recorded, and the 50% lethal dose was calculated as described previously (26). The stability of the *hgbA* mutation in vivo was analyzed in cells recovered from the hearts of dead animals. Heart samples were homogenized in 1 ml of BPW, and 0.1-ml portions of diluted homogenates were plated on BHI medium plates. Following this, the presence of the mutation in 100 recovered colonies was determined by both PCR analysis and replica plating on BHI medium plates in the presence and absence of ampicillin (50 μg/ml).

In vitro and in vivo expression assays of the PM0298-PM0299-*hgbA* operon. An overnight culture of the *P. multocida* PM1077 strain carrying the pUA961 plasmid in its chromosome harboring both the PM0298-PM0299-*hgbA*::*tnpR* transcriptional fusion and the *res1*-Cm-*res1* construction and grown in BHI liquid medium containing streptomycin (75 μg/ml) and chloramphenicol (50 μg/ml)



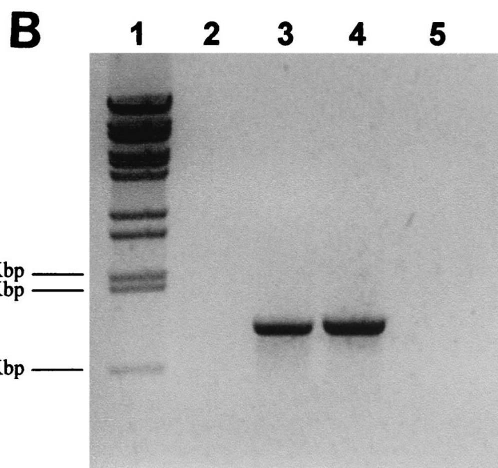


FIG. 1. (A) Genetic organization of the *P. multocida* chromosomal region containing the PM0298-PM0299-PM0300 (*hgbA*) transcriptional unit. The translational start and stop codons for the PM0298, PM0299, and PM0300 ORFs are indicated by boldface type, by boldface type and underlining, and by italics, respectively. The positions of primers (Table 2) used to show the existence of a single mRNA by RT-PCR, as well as in the several constructions used in this study, are indicated. The numbers in parentheses indicate the positions with reference to the ORF PM0298 translational start point. The location of ORF PM0297 immediately upstream of PM0298 is also shown. The solid box downstream of ORF PM0300 (*hgbA*) represents the putative rho-independent transcriptional terminator of the operon. (B) RT-PCR analysis of the PM0298-PM0299-PM0300 (*hgbA*) transcript in *P. multocida* cells performed with PM0298intup and PhgbArp as the upper and lower primers, respectively, in the presence of RNA (lane 3). As a positive control, the PCR fragment obtained when *P. multocida* chromosomal DNA was used as the template (lane 4) was also examined. The negative controls were a preparation containing RNA template but no RT (lane 2) and a preparation lacking both RNA and DNA (lane 5). Lane 1 contained *Bst*EII-digested λ DNA employed as the molecular size marker.

was diluted 1:100 into BHI medium containing streptomycin and supplemented or not supplemented with either 2,2-dypiridyl (DPD), DPD plus $FeSO_4$, or DPD plus hemoglobin. Samples were removed periodically, and serial dilutions of the samples were plated on BHI agar containing 75 μg of streptomycin per ml. The percentage of Cm^s colonies of the resultant Str^r colonies was then calculated by replica plating colonies onto BHI agar supplemented with 50 μg of chloramphenicol per ml and incubating the preparations at 37°C for 24 h.

To analyze the in vivo expression, a sample of an overnight culture of *P. multocida* PM1077 in BHI medium containing chloramphenicol and streptomycin was diluted in BHI medium to obtain a concentration of approximately 10^3 CFU/ml. Portions (100 μl) of this suspension were injected intraperitoneally into 3- to 8-week-old female Swiss mice. To recover the viable bacteria from the peritoneum after different postinoculation periods, 2 ml of BPW was inoculated into the peritoneal cavity of each Swiss mouse used in the trials. Before the bacteria were recovered, the animals were sacrificed by intrapulmonary inoculation of 0.5 ml of T-61 (Hoechst Roussel Vet). The maximum amount of BPW was collected after the abdominal compartment of each mouse was massaged. Serial dilutions of the cleaning fluid, which was maintained at 4°C until examination, as well as serial dilutions of the overnight culture used for the inoculum, were plated on BHI agar containing streptomycin. The percentage of Cm^s clones was calculated for both the inocula and the peritoneally grown bacteria by replica plating as described above. Loss of Cm^r was due to TnpR-mediated excision of the *res1*-Cm-*res1* construction of the *P. multocida* PM1077 chromosome.

Digoxigenin labeling of hemoglobin. Digoxigenin was purchased from Roche, and the labeling procedure used was the procedure recommended by the supplier. Briefly, bovine hemoglobin (5 mg/ml; Sigma, St. Louis, Mo.) was dissolved in phosphate-buffered saline (pH 7.4), and digoxigenin-3-*O*-succinyl-ε-aminocaproic acid-*N*-hydroxysuccinamide ester was dissolved at a concentration of 2 mg/ml in dimethyl sulfoxide. To 1 ml of the hemoglobin solution 215 μl of the digoxigenin solution was added, and after incubation for 2 h at room temperature, unbound digoxigenin was removed by passage through a Sephadex G-25 column (Roche Laboratories). The amount of labeled hemoglobin was determined by measuring the absorption at 280 nm (1 U of A_{280} was equivalent to 0.55 mg of protein/ml).

Expression of HgbA in *E. coli*. The pUA962 plasmid containing ORFs PM0298, PM0299, and PM0300 was used as a template in PCR amplifications with primers NdehgbA and NothgbA (Table 2), and the 3-kb fragment obtained was cloned in pGEM-T. The ATG translational start codon of PM0300 is part of the *Nde*I restriction site of the NdehgbA primer, which allows isopropyl-β-D-thiogalactopyranoside (IPTG)-mediated overexpression of the PM0300 product.

TABLE 2. Oligonucleotide primers used in this work

Primer	Sequence[a]	Position	Aplication
PM0298intup	5′-TTACCTGACGAGTTTGTTCG-3′	+45[b]	Upper primer to detect the present of the single transcrit in RT-PCR experiment; also used as upper primer to obtain the 169-bp internal fragment of *P. multocida* ORF PM0298
PhgbArp	5′-TCTAGAACTATCCGCCAAAATGGCC-3′	+1079[b]	Lower primer to detect the present of the single transcrit in RT-PCR experiment; also used as lower primer to obtain the 360-bp *P. multocida hgbA* 5′ end
PPM0298up	5′-GAGCATCAAATTAGGTCTG-3′	−337[b]	Upper primer to obtain a fragment containing the *P. multocida* PM0298-PM0299-*hgbA* operon
HgbAextrp	5′-CGCTAGCCGATCTCTAATCC-3′	+4026[b]	Lower primer to obtain a fragment containing the *P. multocida* PM0298-PM0299-*hgbA* operon
NdehgbA	5′-CATATGCGTACACAACAACAATAAAAATTTCTGC-3′	+1001[b]	Upper primer to obtain the 3-kb fragment used to overexpress the *P. multocida hgbA* gene
NothgbA	5′-GCGGCCGCCGCTAGCCGATCTCTAATCC-3′	+4026[b]	Lower primer to obtain the 3-kb fragment used to overexpress the *P. multocida hgbA* gene
HgbAintup	5′-CTGAACTTGATACGATTACC-3′	+1094[b]	Upper primer to obtain the 832-bp internal fragment of the *P. multocida hgbA* gene
HgbAintrp	5′-CCAAAATGGCGTAACAG-3′	+1926[b]	Lower primer to obtain the 832-bp internal fragment of the *P. multocida hgbA* gene
HgbArp	5′-CGGTTTAATATAACCAACC-3′	+3728[b]	Primer to confirm disruption of the *P. multocida hgbA* gene by insertion of the pUA967 plasmid
PM0298intrp	5′-GTTCTGGAAATAGTTGACGC-3′	+214[b]	Lower primer to obtain the 169-bp internal fragment of *P. multocida* ORF PM0298
PM0299intup	5′-TCGGTGAAGATGGTAATCCC-3′	+538[b]	Upper primer to obtain the 258-bp internal fragment of *P. multocida* ORF PM0299
PM0299intrp	5′-TTGCTTTGAGTGCCTCAACG-3′	+796[b]	Lower primer to obtain the 258-bp internal fragment of *P. multocida* ORF PM0299
PhgbAup	5′-TCTAGAATCTTTTGATGCGGTTGCGCG-3′	+719[b]	Upper primer to obtain the 360-bp containing the *P. multocida hgbA* 5′ end
Aad	5′-CGGCGATCACCGCTTCCC-3′	+2[c]	Plasmid primer to confirm disruption of *P. multocida* ORFs PM0298 and *hgbA* by insertion of the pUA965 and pUA967 plasmids, respectively
RTHgbAup	5′-TATTGTGGGGTCATTTTGGCG-3′	+1052[b]	Upper primer to detect *hgbA* transcript
RTHgbArp	5′-TGAATATCTTCTTTCACTGGG-3′	+2343[b]	Lower primer to detect *hgbA* transcript
RecAup	5′-GCTCTATTATGAAATTGGGCG-3′	+100[d]	Upper primer to test PM1078 RNA integrity
RecAdw	5′-CTAACCATTTCATCGCG-3′	+957[d]	Lower primer to test PM1078 RNA integrity

[a] When present, added restriction sites are underlined.
[b] Position of the 5′ end of the oligonucleotide with respect the translational start point of *P. multocida* ORF PM0298.
[c] Position of the 5′ end of the oligonucleotide with respect the translational start point of the *aad* gene of the pUA826 plasmid.
[d] Position of the 5′ end of the oligonucleotide with respect the translational start point of the *P. multocida recA* gene.

After digestion with *Nde*I and *Not*I, the PM0300 ORF was cloned into the pET22b expression vector, and the ligation mixture was transformed into DH5α competent cells, resulting in pUA963. After confirmation by sequencing that no mutation had been introduced into the PM0300 ORF contained in pUA963, this plasmid was transformed into *E. coli* BL21(λDE3) for overexpression of the PM0300 protein. Hemoglobin binding to either whole or sonicated *E. coli* BL21(λDE3)/pUA963 cells was tested by a dot blot assay. These cells, grown to the mid-exponential phase in Luria-Bertani medium supplemented with appropriate antibiotics, were treated with 1 mM IPTG for 3 h and then harvested and resuspended in phosphate-buffered saline to a concentration of about 2×10^6 CFU/ml. In a dot blot manifold (Bio-Rad), 150 μg of cell suspension was filtered through nitrocellulose membranes. The membranes were then air dried for 20 min and blocked for 1 h in Tris-buffered saline (TBS) containing 5% skim milk and 0.025% Tween 20. Then 50 nM digoxigenin-labeled hemoglobin was added. After 1 h of incubation, the membranes were washed three times (10 min each) with TBS and incubated for 1 h in TBS containing 5% skim milk, 0.025% Tween 20, and 7.5 U of antidigoxigenin Fab fragments (Roche). Following this, the membranes were washed three times with TBS, and the contents were revealed in alkaline phosphatase buffer (100 mM NaCl, 50 mM Tris-HCl, 5 mM $MnCl_2$) containing 4-nitroblue tetrazolium chloride and X-phosphate-5-bromo-4-chloro-3-indolylphosphate (BCIP, 4-toluidine salt), as recommended by the supplier (Roche). All procedures were carried out at room temperature. When necessary, sonication was performed in an NaCl-ice bath for a total of 3 min in 30-s bursts with 30 s between bursts. Sonicates (750 μg/ml) were filtered onto nitrocellulose membranes in a dot blot manifold, and the membranes were probed and developed as described above. In vitro hemoglobin binding to *P. multocida* was analyzed like in vitro hemoglobin binding to *E. coli*.

In vivo binding of hemoglobin by *P. multocida*. Quantitative analysis of hemoglobin adsorption by *P. multocida* cells was performed basically as described previously (22). Ten milliliters of an overnight culture of *P. multocida* was harvested by centrifugation, washed with 50 mM acetate buffer (pH 6.0), and resuspended in the original volume of the same buffer. The optical density at 650 nm of the cell suspension was adjusted to 0.7. Then 7.3 ml of the cell suspension was mixed with 2.7 ml of bovine hemoglobin (at a concentration of 1 mg/ml in the same buffer). The mixture was incubated at 37°C, and at the desired times samples were removed and centrifuged at $10,000 \times g$ for 15 min. The absorbance of the supernatant at 410 nm was then measured. Adsorbed hemoglobin was evaluated by the decrease in absorbance of the supernatant.

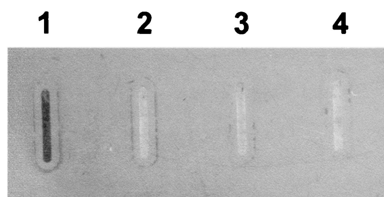


FIG. 2. Solid-phase assay for binding of bovine hemoglobin to sonicated and intact *E. coli* BL21(λDE3)/pUA963 cells (lanes 1 and 2, respectively). Sonicated *E. coli* BL21(λDE3)/pET22b cells in the presence and absence of IPTG were the negative controls (lanes 3 and 4, respectively).

RESULTS

Cloning and transcriptional analysis of ORFs PM0298, PM0299, and PM0300. ORFs PM0298, PM0299, and PM0300 were amplified from total DNA of the *P. multocida* PM25 strain by using the oligonucleotide primers shown in Table 2 and designed by using the *P. multocida* genome sequence (18). The 4.2-kb PCR fragment obtained was cloned into the pGEM-T vector and sequenced to confirm that no mutation was introduced during the amplification reaction. To confirm that the close proximity of PM0298, PM0299, and PM0300 implies that they are transcriptionally linked, RT-PCRs were performed with total RNA by using primers PM0298intup and PhgbArp (Fig. 1A), which amplified a 1-kb DNA fragment if an mRNA spanning the three ORFs was present. Figure 1B shows that the expected band was obtained, indicating that, effectively, the three ORFs constitute a single transcriptional unit.

Hemoglobin binding by the product of PM0300 in *E. coli*. As described above, PM0300 contains the consensus sequence which is characteristic of the TonB-dependent receptor proteins, including hemoglobin-binding proteins. A dot blot assay was therefore used to determine whether *E. coli* cells carrying a plasmid containing ORF PM0300 were able to bind hemoglobin. Sonicated *E. coli* BL21(λDE3) cells containing the pUA963 plasmid, a derivative of the pET22b expression vector in which ORF PM0300 had been cloned, bound digoxigenin-labeled bovine hemoglobin after IPTG induction (Fig. 2, lane 1). However, whole cells of IPTG-induced *E. coli* BL21(λDE3)/pUA963 did not bind hemoglobin (Fig. 2, lane 2). This negative result was probably due to the fact that the recombinant protein cannot be exported to the surface of *E. coli* cells. Moreover, sonicated *E. coli* BL21(λDE3) cells containing the pET22b vector alone were unable to bind hemoglobin in either the presence or the absence of IPTG (Fig. 2, lanes 3 and 4). These data clearly indicate that the product of ORF PM0300 is able to bind hemoglobin. For this reason and since to our knowledge this is the first protein of *P. multocida* in which hemoglobin-binding ability has been demonstrated, the gene was designated *hgbA* (for hemoglobin-binding protein).

Construction of *P. multocida* PM0298, PM0299, and *hgbA* mutants. To further characterize the role of the products encoded by the PM0298-PM0299-*hgbA* transcriptional unit, we attempted to obtain mutants with mutations in each of the three ORFs by insertion of a derivative of the pUA826 suicide plasmid containing an internal region of the corresponding

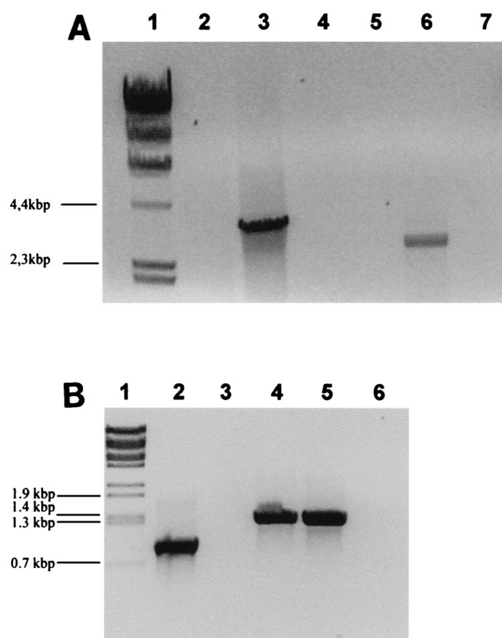


FIG. 3. (A) PCR analysis of *P. multocida* mutants. Chromosomal DNA from *hgbA* (lane 3) and PM0298 (lane 6) mutants were subjected to PCR analysis with the Aad oligonucleotide (Table 2) as the upper primer and the HgbArp and HgbAintrp oligonucleotides (Fig. 1A), respectively, as the lower primers. PCRs performed with chromosomal DNA from the wild-type strain (lanes 2 and 5) and these primer pairs and PCRs performed with preparations lacking DNA template (lanes 4 and 7) were the negative controls. Lane 1 contained *Hin*dIII-digested λ DNA as the molecular size marker. (B) RT-PCR study of the *P. multocida hgbA* mutant. RNA from *hgbA* (lane 3) and wild-type (lane 4) cells were subjected to RT-PCR analysis with the RTHgbAup and RTHgbArp oligonucleotides (Table 2). The control for RNA integrity was an RT-PCR amplification performed with RNA from *hgbA* cells and oligonucleotides RecAup and RecAdw belonging to the internal sequence of the *P. multocida recA* gene (lane 2). PCR performed with chromosomal DNA from the wild-type strain and oligonucleotides RTHgbAup and RTHgbArp (lane 5) and RT-PCR amplification of preparations containing the same primers but lacking both DNA and RNA templates (lane 6) were the product size and negative controls, respectively. Lane 1 contained *Bst*EII-digested λ DNA as the molecular size marker.

ORF. An *hgbA* mutant was obtained, and its structure was confirmed by PCR amplification of the junction segment of the vector with the chromosomal DNA by using an internal primer for *hgbA* and the Aad plasmid primer (Fig. 3A, lane 3). Likewise, an RT-PCR analysis performed with oligonucleotides flanking the insertion point of the pUA967 plasmid (RTHgbAup and RTHgbArp as the upper and lower primers, respectively) clearly demonstrated the absence of the *hgbA* mRNA in the *P. multocida hgbA* mutant (Fig. 3B). In contrast to the *hgbA* results, attempts to isolate either PM0298 or PM0299 insertional mutants were unsuccessful despite the fact that four independent experiments were carried out. This result could indicate that these two ORFs are essential for normal cell growth of *P. multocida*. To test this possibility, a *P. multocida* merodiploid strain containing the pUA964 plasmid, a derivative of the broad-host-range pHRP309 plasmid which is stable in *P. multocida* (23), and carrying a 1.4-kb fragment that included ORFs PM0298 and PM0299 was used as a recipient in

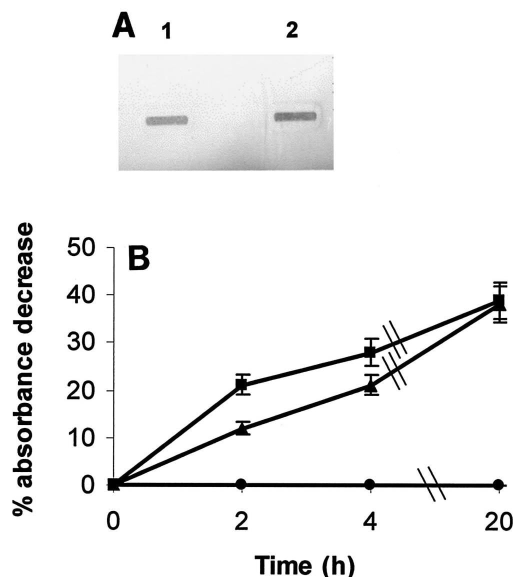


FIG. 4. (A) Solid-phase assay for in vitro binding of bovine hemoglobin to whole *P. multocida* wild-type (lane 1) or *hgbA* mutant (lane 2) cells. (B) In vivo quantitative analysis of hemoglobin adsorption to *P. multocida* wild-type (■) and *hgbA* (▲) cells. The behavior of *E. coli* cells (●) was used as a negative control. All values are the means of three experiments (each performed in triplicate), and the standard error of any value was never greater than 10%.

new insertional mutagenesis experiments. In this case, strains with an insertional mutation in the chromosomal copy of ORF PM0298 were obtained (Fig. 3A, lane 6). These data strongly suggest that mutations in either ORF PM0298 or ORF PM0299 are lethal. However, the level of hemoglobin binding of the *P. multocida hgbA* mutant seemed to be the same as that of the wild-type strain (Fig. 4A), although in vivo quantitative assays revealed that the adsorption kinetics of *hgbA* cells were slightly slower than those of wild-type cells (Fig. 4B). Furthermore, the growth rate and virulence of the *P. multocida hgbA* mutant were not affected compared to the growth rate and virulence of the wild-type strain (data not shown).

Expression of the *hgbA* transcriptional unit in vitro and during infection. To study the iron starvation effect on transcription of the *hgbA* operon, as well as the pattern of temporal expression in a mouse model, the RIVET method was used. In this technique, a suicide plasmid containing a fusion between the promoter to be analyzed and the region encoding the resolvase gene (*tnpR*) from Tnγδ is constructed (2). This plasmid is then introduced into a bacterial strain in which a copy of the *res1-tet-res1* construction is present in the chromosome. In this way, after integration of the plasmid harboring the promoter-*tnpR* fusion, expression of the promoter produces an increase in the intracellular level of resolvase, which facilitates specific recombination between the two *res1* sequences, resulting in a loss of tetracycline resistance (2). Alternatively, the *res1-tet-res1* construction may also be introduced into the same plasmid which contains the promoter-resolvase fusion (34).

To develop a RIVET system for *P. multocida*, the *tnpR*-encoding region was amplified by PCR from the pIVET5mut plasmid (15) and ligated into the pUA826 suicide plasmid to obtain pUA958. Since *P. multocida* isolates are often tetracycline resistant (13) and because the RIVET system could be generally used with any strain of this organism, we decided to replace the *tet* marker of the *res1-tet-res1* construction with the Cm cassette of plasmid pHP45ΩCm. Accordingly, the Cm cassette was isolated from the pHP45ΩCm plasmid by *Hin*dIII digestion and inserted into plasmid pGH436 which had previously been digested with *Acc*I and *Sph*I to delete the *tet* region. Cm cassette and pGH436 plasmid ends were previously made blunt by Klenow fragment treatment. This yielded plasmid pUA959, from which the *res1*-Cm-*res1* fragment was recovered by *Nde*I-*Sca*I digestion and, after end filling, ligated into the *Sma*I-digested pUA958 plasmid to obtain pUA960 containing both the *tnpR*-encoding region and the target region (*res*1-Cm-*res*1) of the resolvase. Finally, a 360-bp PCR fragment which contained 71 bp of the 5′ end of the *hgbA* gene and 241 bp of the 3′ end of PM0299 and was obtained by amplification with the PhgbAup and PhgbArp primers was digested with *Xba*I and cloned into the *Xba*I restriction site present upstream of the *tnpR* region of the pUA960 plasmid, giving rise to pUA961 (Fig. 5). The structure of the desired construction was then confirmed by PCR and restriction analysis (data not shown). This 360-bp fragment belonging to the last gene of the transcriptional unit, *hgbA*, was used rather than another fragment containing the immediate upstream region of PM0298 in order to be certain that transcription of the whole operon was really being measured.

The pUA961 plasmid was then introduced into *P. multocida* by mating, streptomycin- and chloramphenicol-resistant clones were selected, and insertion of the plasmid into the expected point between the PM0299 and *hgbA* genes (Fig. 5) was confirmed by PCR and Southern analysis (data not shown). One of the clones, PM1077, was kept and used for subsequent experiments. Analysis of the development of a number of chloramphenicol-sensitive cells after mouse infection with the PM1077 strain indicated that the *hgbA* operon is rapidly induced after intraperitoneal inoculation. Thus, 35% of the cells recovered from the peritoneal cavity lost chloramphenicol resistance within 2 h after challenge (Fig. 6A). A similar loss of chloramphenicol resistance was also observed in vitro in the presence of the iron chelator DPD (Fig. 6B). Moreover, this induction could be strongly suppressed by addition of $FeSO_4$ or hemoglobin (Fig. 6B).

Presence of the *hgbA* gene in several *P. multocida* serotypes. Genomic DNA was extracted from 34 isolates belonging to several *P. multocida* serotypes and obtained from different animal sources, and a hybridization dot blot analysis was performed to investigate the presence of the *hgbA* gene in these strains with a digoxigenin-labeled 989-bp internal fragment of *hgbA* as the probe. As Fig. 7 shows, DNA from most of the *P. multocida* strains strongly hybridized with the *hgbA* probe; the only exception was the nontypeable P-628 strain. No hybridization was detected when chromosomal DNA from *E. coli* (Fig. 7) or *Mannhemia haemolytica* (data not shown) was used. These results indicate that the *hgbA* gene may be widely present in *P. multocida* strains regardless of the specific serotype.

DISCUSSION

In this paper we report that ORFs PM0298, PM0299, and PM0300 of *P. multocida* constitute a single transcriptional unit.

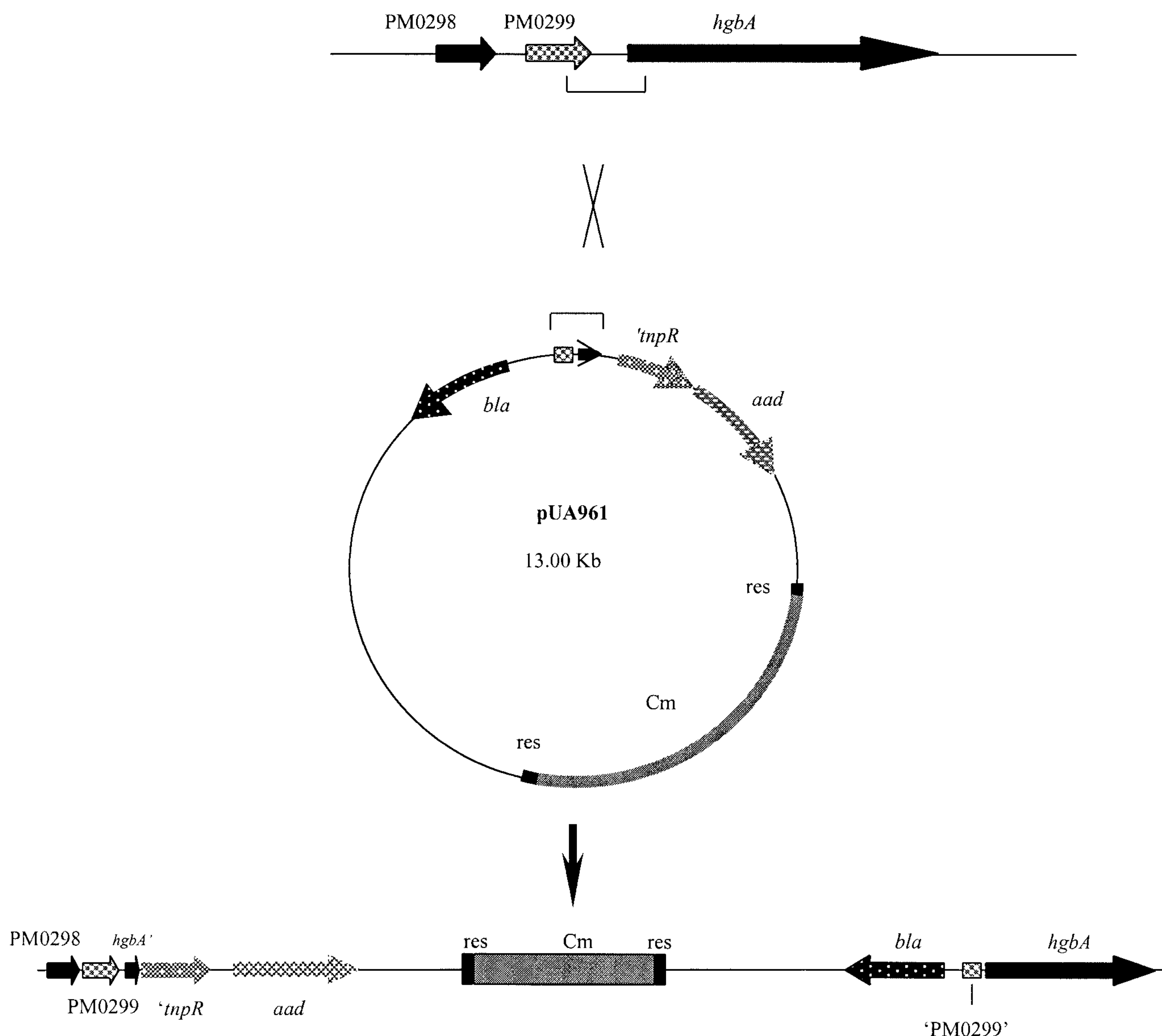


FIG. 5. Schematic representation of specific single recombination between the pUA961 plasmid and the *P. multocida* chromosome through the 370-bp internal fragment of the PM0298-PM0299-*hgbA* transcriptional unit cloned upstream of the *tnpR* gene-encoding region.

Mutation analysis by plasmid insertion showed that strains containing inactivated ORF PM0298 or PM0299 could be obtained only when another copy of the inactivated ORF is present. This fact suggests that PM0298 and PM0299 are essential for *P. multocida* cell viability. The biological role of PM0298 and PM0299 is unknown, although these ORFs show 49 and 61% identity with products of the *Plesiomonas shigelloides hugX* and *hugZ* genes, respectively. These *P. shigelloides* genes seem to be necessary to prevent heme toxicity, as demonstrated by complementation assays performed with *E. coli* strains growing in media with heme as the only iron source (10). Nevertheless, there are no data on the importance of these two genes in *P. shigelloides* since construction of derivative mutants of this bacterial species has not been reported. Our data concerning the lack of viability of *P. multocida* PM0298 and PM0299 mutants are consistent with a detoxifying role under certain growth conditions. In contrast, our results showed that a *P. multocida* PM0300 mutant was viable and did not show any significant defect in the ability to grow either in vitro or during animal infection.

Cloning and overexpression in *E. coli* of ORF PM0300 demonstrated that it is a hemoglobin receptor, and PM0300 was therefore designated *hgbA*. However, the extent of hemoglobin binding by the *P. multocida hgbA* mutant was similar to that of the wild-type strain, although the hemoglobin adsorption kinetics of *hgbA* cells were a little slower than those of the wild-type strain. This fact suggests that other functional hemoglobin receptor proteins must be present in *P. multocida* cells, which can compensate for the loss of HgbA. In fact, when other *P. multocida* ORFs, such as PM0741, PM0745, and

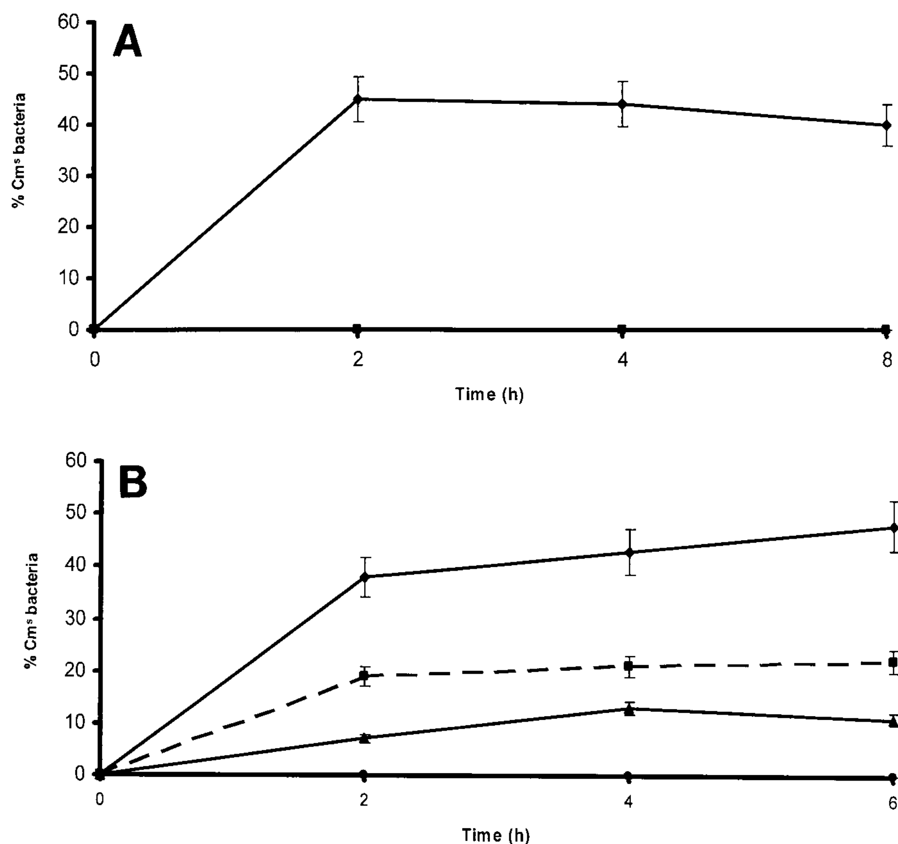


FIG. 6. (A) Kinetics of transcription of the PM0298-PM0299-*hgbA* operon in mice inoculated intraperitoneally with the *P. multocida* PM1077 strain, measured as the proportion of Cm^s bacteria recovered from the peritoneal cavity (♦). The absence of Cm^s bacteria in cultures of the *P. multocida* PM1077 strain growing in BPW medium without chloramphenicol demonstrates the genetic stability of the construction (■). Three animals were used for each point, and each Cm^s percentage is the average of three determinations for each of the inoculated mice. In all cases, the standard error of any value was never greater than 10%. (B) Kinetics of transcription of the PM0298-PM0299-*hgbA* operon in the *P. multocida* PM1077 strain measured in vitro as the proportion of Cm^s bacteria recovered from BPW medium (●) or BPW medium supplemented with either DPD (50 μg/ml) (♦), DPD and $FeSO_4$ (1 mM) (▲), or DPD and hemoglobin (40 mM) (■). All values are the means of at least three experiments (each performed in triplicate), and the standard error of any value was never greater than 10%.

PM1282 present a TonB box, are used as a query in a TBLASTN analysis performed with the GenBank database, the results obtained predict the presence of putative hemoglobin-binding motifs in them (data not shown). Additionally, the presence of several hemoglobin receptor proteins able to support cell growth when one of them is inactivated has also been demonstrated for *Haemophilus influenzae* (27). Thus, this pathogen possesses three hemoglobin receptor proteins encoded by the *hgpA, hgpB*, and *hgpC* genes (21). Mutants of *H. influenzae* defective in either one or two of these three genes show the same behavior as the wild-type strain, whereas triple mutants show a reduced ability to use hemoglobin (21, 27). The presence of more than one gene encoding hemoglobin receptors may be useful to bacterial cells either because it increases the level of hemoglobin binding to the cell or because it prevents the negative effects of mutations. It is worth noting that *H. influenzae hgpA*, *hgpB*, and *hgpC* contain several tandem repeats of the CCAA tetranucleotide, which can introduce frameshifts due to the loss or gain of one or more of these repeats, giving rise to a high frequency of phase variation in these genes (28). This changing ability has been proposed to be related to mechanisms that enhance evasion of the host immune response (11). If there are several genes encoding the same biological function (in this case a hemoglobin receptor), variability is strongly increased. However, the *P. multocida hgbA* gene does not show any of the several tandem repeats which are implicated in phase variation (28), suggesting that its expression is not under this kind of regulation. Moreover, the virulence of some bacteria, such as *Neisseria meningitidis* (37) and *Porphyromonas gingivalis* (33), was significantly reduced when a single gene encoding a hemoglobin receptor protein was mutagenized. These data suggest that the presence of more than one gene for hemoglobin receptors is not a general feature of all bacterial species.

By using RIVET, we were able to show that expression of the PM0298-PM0299-*hgbA* transcriptional unit is triggered within 2 h following intraperitoneal inoculation of mice. In addition, this operon is regulated by iron, as shown by the decrease in its transcription when $FeSO_4$ is added to cultures growing in the presence of DPD (Fig. 6B). Likewise, these results are consistent with preliminary data obtained in microarray analyses for the pattern of *P. multocida* whole-ge-

Strain	Serotype	Source	position	
			row	lane
P-207	A:3	Ovine	A	1
P-208	D:3	Ovine	A	2
P-209	D:3	Ovine	A	3
P-210	-:3	Ovine	A	4
P-215	A:-	Ovine	A	5
P-217	D:-	Ovine	A	6
P-218	A:3	Ovine	A	7
P-399	A:4,7,12	Rabbit	A	8
P-400	A:4,7,12	Rabbit	A	9
P-402	F:3	Rabbit	A	10
P-403	A:3	Rabbit	A	11
P-430	A:4,7,12	Rabbit	A	12
P-431	A:4,7,12	Rabbit	B	1
P-432	A:3	Rabbit	B	2
P-438	D:4,7,12	Pig	B	3
P-440	D:4,7,12	Pig	B	4
P-441	D:4,7,12	Pig	B	5
P-442	A:3,4,7,12	Pig	B	6

Strain	Serotype	Source	position	
			row	lane
P-453	F:3,4,7,12	Rabbit	B	7
P-580	A:3,12	Pig	B	8
P-582	A:3,12	Pig	B	9
P-609	B:7,5	Ovine	B	10
P-617	A:7,12	Rabbit	B	11
P-619	D:4	Rabbit	B	12
P-620	D:3,12	Rabbit	C	1
P-621	D:3,12	Pig	C	2
P-623	A:12	Pig	C	3
P-624	D:3,7,12	Goat	C	4
P-625	D:3,7,12	Bovine	C	5
P-626	D:3,7,12	Poultry	C	6
P-628	D:-	Ovine	C	7
P-633	A:12	Poultry	C	8
P-635	D:12	Poultry	C	9
P-636	D:7,12	Poultry	C	10
None	-	-	C	11
E.coli	-	-	C	12

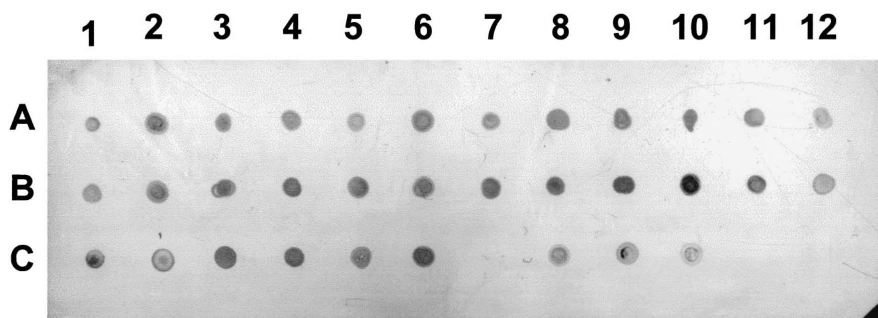


FIG. 7. Presence of the *hgbA* gene in *P. multocida* strains: dot blot hybridization of chromosomal DNA from 34 *P. multocida* strains obtained from different animal sources and belonging to several serotypes with a digoxigenin-labeled 989-bp internal fragment of the *hgbA* gene. The DNA position corresponding to each of the strains is indicated above the nitrocellulose membrane. Equal amounts of chromosomal DNA (1 μg) of all strains were applied to the nitrocellulose membrane, and hybridization was performed under high-stringency conditions.

nome expression when preparations are treated with chelating agents (24). Nevertheless, the rapid induction of these genes in infected mice is consistent with the low concentrations of free iron present in mammalian tissues. However, to our knowledge, this is the first case in which temporal expression of iron-regulated genes has been analyzed in vivo in a nonenteric bacterial species (14, 15). It is known that iron regulates bacterial gene expression through the Fur protein, which in the presence of this element specifically recognizes and binds a given sequence (Fur box), blocking transcription (36). The Fur box is composed of at least three contiguous NAT(A/T)AT-like hexamers in either direct or inverse orientations (7). One putative copy of this motif, which responds to the TATTATC AATATTGATAAT sequence, is found 114 bp upstream of the PM0298 translational start codon. Similar iron-dependent regulation of the transcription of genes encoding hemoglobin receptor proteins has been described for other pathogenic bacteria, such as *H. influenzae, P. gingivalis*, and *N. meningitidis* (27, 33, 37). Finally, our data also indicate that the *hgbA* gene is widespread in *P. multocida* strains regardless of the serotype analyzed or the source of the isolate. This suggests that the *hgbA* gene may be useful as a specific probe to detect *P. multocida* in field samples. It has been demonstrated that some TonB-dependent iron receptors, such as transferrin- and lactoferrin-binding proteins, are able to induce protection when they are used as prophylactic immunogens (4, 6, 16, 29). Further work is required to determine whether the widely distributed HgbA protein may also be useful as an antigen that provides protection against virulent *P. multocida* strains.

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