fur-independent regulation of the *Pasteurella multocida* hbpA gene encoding a haemin-binding protein

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INTRODUCTION

Bacterial species have developed different strategies for iron uptake. Some of them (e.g. *Escherichia coli* and *Pseudomonas aeruginosa*) produce small molecules, known as siderophores, which are secreted from the cells and which can chelate iron present in the environment (Ratledge & Dover, 2000). Other bacteria have outer-membrane proteins which are specific receptors for iron-binding host molecules, such as transferrin, lactoferrin, haemoglobin or haem (Ratledge & Dover, 2000). After iron has been captured by these molecules, its transport into the bacterial cell requires the activity of the *exbB, exbD* and *tonB* gene products which supply the energy necessary for this process (Braun, 1995).

In bacteria, two families of global regulators involved in the control of genes whose products participate in several pathways of iron uptake have been described (Hantke, 2001). The Fur family has the Fur, PerR and Irr proteins as members, while the DtxR family includes the DtxR and IdeR proteins. Of these regulators, Fur seems to be present in all classes of proteobacteria (Radledge & Dover, 2000; Hantke, 2001). The *irr* gene has only been described so far in *Bradyrhizobium japonicum* (Hamza *et al.*, 1998), a member of the *α*-Proteobacteria, although in this organism the *fur* gene is also present (Hamza *et al.*, 1999). Likewise, the Fur protein has also been identified in some Gram-positive bacteria such as *Bacillus subtilis* (Bsat *et al.*, 1998). The rest of the regulatory proteins have so far only been described for Gram-positive bacteria (Hantke, 2001), with the exception of PerR, whose presence has been reported in *Campylobacter jejuni* (van Vliet *et al.*, 2002).

The Fur protein has been studied in several bacterial species,
whereas much less information is available about the product of the *B. japonicum* *irr* gene. The Fur protein, which has a size of about 17 kDa, exhibits Fe²⁺-dependent DNA-binding activity (Escolar et al., 1999). Genes under Fur control require the presence in their promoters of at least three contiguous NATA/TAT-like hexamers in either direct or inverse orientations to which this protein binds, repressing transcription, when the iron concentration is high (Escolar et al., 1999). This sequence, known as the Fur box, is widespread in bacteria because it has been detected in the promoters of iron-regulated genes of several species belonging to families as diverse as the *Enterobacteriaceae*, *Pseudomonadaceae*, *Neisseriaceae*, *Pasteurellaceae* and *Bacillaceae* (Hantke, 2001). Thus, treatment of cultures of these organisms with iron-chelating agents like 2,2'-dipyridyl (DPD) induces expression of genes negatively regulated by the Fur protein. Nevertheless, it has also been demonstrated that Fur can act as a positive regulator, although the exact mechanism by which it stimulates gene expression has not been definitively established (Dubrac & Touati, 2000).

*Pasteurella multocida* is responsible for causing diseases in many species of mammals and birds, originating important economic losses in farms. The presence of several iron-binding proteins regulated by the Fur protein has been reported for this organism (Bosch et al., 2001, 2002a, b). In this context, a fur-knockout mutant of *P. multocida* has been constructed in our laboratory (Bosch et al., 2001). This mutant shows constitutive expression of high-molecular-mass proteins which have been associated with iron-uptake processes (Snipes et al., 1988; Choi et al., 1991). We noted the presence of two proteins that are strongly induced in DPD-treated cultures of *P. multocida*. In this work, we have shown that these two proteins are encoded by the same gene, *hbpA*, that both gene products bind haemin, and that expression of *hbpA* is regulated by iron in a Fur-independent manner.

**METHODS**

**Bacterial strains and growth conditions.** Bacteria used in this study are listed in Table 1. *E. coli* strains were grown in LB medium (Sambrook et al., 1989). *P. multocida* was grown in brain-heart infusion (BHI) or buffered peptone water (BPW) liquid medium and on sheep-blood agar (SBA) plates (Fernández de Henestrosa et al., 1997). Unless otherwise indicated, the concentration of DPD, when used, was 150 μM. Antibiotic concentrations were as reported by Cardenas et al. (2001). In the Fur-titration assay (FURTA) (Stojilkovic et al., 1994), Lac-EMBO agar plates supplemented with 1 mM FeSO₄ (Miller, 1992) were used. β-Galactosidase activities were measured as previously reported (Jordan et al., 1996).

**DNA and RNA techniques.** DNA methodology, including Southern blotting experiments, and DNA sequence analyses were as described by Cardenas et al. (2001). A 1970 bp DNA fragment of *P. multocida* chromosomal DNA including the *hbpA* gene and its surrounding region was isolated using the primers indicated in Table 2. The entire nucleotide sequence of this fragment was determined for both DNA strands by the dideoxy method on an ALF Sequencer (Pharmacia Biotech). Total cellular RNA extraction and competitive reverse transcriptase (RT)-PCR analyses were performed as reported by Jordan et al. (1996).

**Genetic methods.** To obtain the *hbpA–lacZ* fusion used in this work, a 339 bp DNA fragment containing 266 bp of the upstream region, as well as 73 bp of the coding sequence of the *hbpA* gene, was isolated by PCR amplification with the primers indicated in Table 2. This DNA fragment was then cloned into pUA949 which

<table>
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<th>Table 1. Bacterial strains and plasmids used in this work</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>DH5α</td>
</tr>
<tr>
<td>H1717</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
</tr>
<tr>
<td>PM25</td>
</tr>
<tr>
<td>PM1002</td>
</tr>
<tr>
<td>PM1056</td>
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<tr>
<td><strong>Plasmids</strong></td>
</tr>
<tr>
<td>pGEM-T</td>
</tr>
<tr>
<td>pRK2013</td>
</tr>
<tr>
<td>pHRP309</td>
</tr>
<tr>
<td>pUA1034</td>
</tr>
<tr>
<td>pET22-b</td>
</tr>
<tr>
<td>pUA1035</td>
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<tr>
<td>pUA1036</td>
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<td>pUA949</td>
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had been digested with *Eco*RI and *Bam*HI. The plasmid obtained was then digested with *Sal*I and *Bam*HI, and the 2.6 kb fragment containing the *hbpA* promoter and a cassette encoding Km resistance was cloned upstream of the *lacZ* gene of the promoter-probe vector pHRP309, which had previously been digested with *Sal*I and *Bam*HI. Taking advantage of the fact that pHRP309 is a broad-host-range plasmid which is stable in *P. multocida* cells (Parales & Harwood, 1993), its derivative carrying the *hbpA-lacZ* fusion was introduced into this organism by triparental mating as reported by Bosch *et al.* (2002b) using the pRK2013 plasmid as the mobilizing system (Ditta *et al.*, 1985).

**Protein and immunoblot analysis.** Outer-membrane proteins from the *P. multocida* wild-type or *fur* strains were extracted from cultures grown under the desired conditions as described by Bosch *et al.* (2001). Briefly, cultures were centrifuged at 48 000 g and pellets were resuspended in 0.1 M acetate buffer/0.2 M lithium chloride at pH 5.8, incubated for 2 h at 45°C in a shaking water-bath, and passed through a 21-gauge needle. These suspensions were then centrifuged at 10 000 g, and the pellets were discarded. Membrane fragments were obtained from the supernatant by centrifugation at 30 000 g for 2.5 h, and the pellet was resuspended in distilled water. The protein concentration of outer-membrane samples was determined by the Lowry method, and their profiles were examined by 12% PAGE in the presence of SDS (Laemmli, 1970).

To identify both the 60 and 40 kDa proteins, SDS-PAGE gels were electroblotted onto polyvinylidene difluoride membranes (Bio-Rad), and stained with Coomassie Brilliant Blue. Both proteins were then recovered from the membrane and their N-terminal amino acid sequences were determined by Edman degradation using Protein Sequencer 477A (Applied Biosystems).

The antigenicity of the 60 and 40 kDa proteins was determined by Western blot analysis. Crude extracts of *E. coli* BL21 cells over-expressing these proteins were subjected to SDS-PAGE. Gels were transferred to Immobilon-P membranes (Millipore) using a Hoesch miniVe (Amersham Pharmacia Biotech) TransBlot Cell. Membranes were air-dried for 20 min and blocked for 2 h in blocking solution (10 mM Tris/HCl pH 8, 150 mM NaCl, 0.4 g Block Reagent and 0.2 ml Tween 20 brought up to 200 ml H2O). Transferred proteins were immunostained overnight with specific antisera at a dilution of 1/100 in blocking solution. Following this, membranes were washed three times (10 min each) with PBS and incubated in a 1/30 000 dilution in blocking solution of anti-mouse IgG, Fc-specific (Sigma), for 1 h. Afterwards, the membranes were washed three times with PBS and reactive polypeptides were visualized in alkaline phosphate buffer (100 mM NaCl, 50 mM Tris/HCl, 5 mM MnCl2) containing 4-nitro blue tetrazolium chloride and X-phosphate-5-bromo-4-chloro-3-indolyl phosphate (BCIP, 4-toluidine salt), as recommended by the supplier (Roche Diagnostics). All procedures were carried out at room temperature.

**Protection studies.** Two groups of five female 3-week-old Swiss mice (obtained from Harlan Iberica; Barcelona, Spain) were injected intraperitoneally with either 3 µg HbpA protein recovered from polyacrylamide gels or 0.5 µg outer-membrane proteins from *P. multocida* wild-type cells. A third group of five mice was injected with PBS as the negative control. After 2 weeks, a second immunization was carried out. The challenge was made 3 weeks later by intraperitoneal inoculation of 100 × LD50 of the *P. multocida* wild-type strain.

The number of animals which were alive 24, 48 and 72 h post-inoculation was recorded, and the virulence power was calculated as reported by Reed & Muench (1938). These animals were afterwards used to obtain serum for Western blot assays. To perform this, mice were bled from the vena cava and the blood was incubated at 37°C for 2 h, and then kept overnight at 4°C, to facilitate clot formation. Following this, the blood was centrifuged at 2000 g for 15 min and the serum was recovered and maintained at 4°C.

### Table 2. Primers used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Position</th>
<th>Application</th>
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<tbody>
<tr>
<td>Fhpup</td>
<td>5′-GAATTCGCCACTTTAGACTG-3′</td>
<td>−266†</td>
<td>Upper primer to obtain the promoter of the <em>hbpA</em> gene and the entire 1970 bp <em>hbpA</em> gene</td>
</tr>
<tr>
<td>Fhprr</td>
<td>5′-GGATCCCTAAAGCTACTTG-3′</td>
<td>+73†</td>
<td>Lower primer to obtain the promoter of the <em>hbpA</em> gene</td>
</tr>
<tr>
<td>Hbexpup</td>
<td>5′-CATAGAAGTGGACAACCAAC-3′</td>
<td>+1†</td>
<td>Upper primer to obtain the entire and truncated encoding region of the <em>hbpA</em> gene</td>
</tr>
<tr>
<td>Hbexprrp1</td>
<td>5′-GCGGCCGCTTTATTAATCC-3′</td>
<td>+1 704†</td>
<td>Lower primer to obtain the entire encoding region of the <em>hbpA</em> gene and the entire 1970 bp <em>hbpA</em> gene</td>
</tr>
<tr>
<td>Hbexprrp2</td>
<td>5′-GCGGCCGACATCAATGATGAGTG-GTTTATCGGTAGCATA-3′</td>
<td>+960†</td>
<td>Lower primer to obtain the truncated coding region of the <em>hbpA</em> gene</td>
</tr>
<tr>
<td>RThbprntup</td>
<td>5′-CCTATACGAGGTATC-3′</td>
<td>+379†</td>
<td>Upper primer to detect the <em>hbpA</em> transcript</td>
</tr>
<tr>
<td>RThbprntrp</td>
<td>5′-GGCTAAGGTTGTTGCG-3′</td>
<td>+1 080†</td>
<td>Lower primer to detect the <em>hbpA</em> transcript</td>
</tr>
<tr>
<td>RTrecAintup</td>
<td>5′-ATGGAGTTCAACATGGC-3′</td>
<td>−15§</td>
<td>Upper primer to detect the recA transcript</td>
</tr>
<tr>
<td>RTrecAintrp</td>
<td>5′-ATATGCCTTCTAAGC-3′</td>
<td>+1 057§</td>
<td>Lower primer to detect the recA transcript</td>
</tr>
<tr>
<td>hbpintup</td>
<td>5′-CCTATACGAGGTATC-3′</td>
<td>+379†</td>
<td>Upper primer to obtain the <em>hbpA</em> internal fragment used as a probe in Southern blot</td>
</tr>
<tr>
<td>hbpintrp</td>
<td>5′-AATTTGCCATACGTGTCC-3′</td>
<td>+759†</td>
<td>Lower primer to obtain the <em>hbpA</em> internal fragment used as a probe in Southern blot</td>
</tr>
</tbody>
</table>

*When present, added restriction sites are shown in italics.
†Position of the 5′-end of the oligonucleotide with respect to the translational starting point of the *P. multocida* *hbpA* gene.
‡Position of the 5′-end of the oligonucleotide with respect to the translational starting point of the *P. multocida* recA gene.
To eliminate the antibodies against *E. coli* that could be present in recovered serum, this was incubated overnight at 4 °C with a sediment of *E. coli* cells harbouring the pET22-b vector alone. Haemin binding of *E. coli* cells expressing the *P. multocida* wild-type and truncated *hbpA* genes. Haemin binding was analysed as described by Genco et al. (1994). *E. coli* BL21 (DE3) cells carrying the pUA1035 or pUA1036 plasmid containing the whole or the truncated *hbpA* gene, respectively, were grown in LB medium and harvested after IPTG (1 mM) addition. The cells were washed with PBS and adjusted to an OD<sub>550</sub> of 1-0, and 0-8 ml aliquots of the cell suspension in this buffer were mixed with 0-2 ml haemin to a concentration of 10 mM. Samples were incubated at 37 °C for 1 h and centrifuged. Afterwards, the A<sub>400</sub> of the supernatant was measured. Haemin diluted in PBS was incubated under the same conditions as an appropriate control. The binding of haemin was determined by the decrease of the absorbance of the supernatant compared to that of control samples, which were set as being 100%.

### RESULTS

**Two proteins induced in DPD-treated cells are products of the same gene whose expression is Fur-independent**

Two proteins of approximately 60 and 40 kDa are induced in the presence of DPD in both *P. multocida* wild-type and fur-defective cells (Fig. 1). These proteins are located in the outer membrane of *P. multocida*. To identify these proteins, their N-terminal amino acid sequences were determined in order to clone their respective genes. Surprisingly, the N-terminus of both proteins was identical: SNKTFINVSRAP. A BLAST search of the GenBank database found total identity of this sequence with the N-terminus of the ORF PM0592 of the *P. multocida* genome. The gene encoding PM0592 is monocistronic, since its transcriptional direction is opposite to its two flanking ORFs, encoding a putative protein of unknown function and a ThrS-like protein (Fig. 2). A detailed analysis of the PM0592 gene sequence revealed a hexanucleotide (AAAAAA) beginning at position 943 (codon 315), with respect to its putative translational initiation codon (Fig. 2). It has been reported that the presence of polyA tracts can produce translational frame-shifts, which can give rise to premature translational termination (Chandler & Fayet, 1993; Baranov et al., 2002). It must be noted that either 14 or 40 bp downstream of the polyA tract (positions 957 and 983, respectively) there is an opal stop codon, which could be read if a translational frameshift of one or two bases occurred in this hexanucleotide sequence (Fig. 2). Furthermore, the size of these two prematurely terminated polypeptides is in accord with the truncated 40 kDa polypeptide of the PM0592 protein detected in Fig. 1. Another possibility could be that in the chromosome of the *P. multocida* PM25 strain there are both a complete and a truncated copy of the PM0592 gene. However, Southern blot analysis using a 381 bp internal fragment of PM0592 as a probe, which is 186 bp upstream of the AAAAAA hexanucleotide, demonstrated that *P. multocida* PM25 cells have only one copy of this gene (data not shown).

![Fig. 1. SDS-PAGE profiles of outer-membrane proteins from wild-type (lanes 1 and 2) and fur (lanes 3 and 4) strains of *P. multocida* grown in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of DPD at 150 μM. White arrows indicate the 60 and 40 kDa proteins induced by DPD but not by the inactivation of the fur gene.](image1)

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![Fig. 2. Genetic organization of the *P. multocida* chromosome region containing the PM0592 ORF (*hbpA* gene). The distance between each of the three ORFs is shown in bp. Arrows indicate the transcriptional direction of each gene. The hexanucleotide at which a translational frameshift is predicted to occur, as well as the opal stop codons, are in bold and underlined. The translational initiation and termination codons of the *hbpA* gene are in italics.](image2)

**Fig. 2.** Genetic organization of the *P. multocida* chromosome region containing the PM0592 ORF (*hbpA* gene). The distance between each of the three ORFs is shown in bp. Arrows indicate the transcriptional direction of each gene. The hexanucleotide at which a translational frameshift is predicted to occur, as well as the opal stop codons, are in bold and underlined. The translational initiation and termination codons of the *hbpA* gene are in italics.
Identical regulation of PM0592 in the wild-type and a fur mutant indicated that expression of the gene is Fur-independent. Indeed, upstream of the PM0592 gene no Fur-binding site could be detected. In further agreement with this fact, the PM0592 promoter was negative when analysed in a FURTA assay, which enables the detection of Fur-regulated promoters (data not shown).

Identification of PM0592 (HbpA) as a haemin-binding protein

The product of the PM0592 ORF includes the consensus amino acid sequence (D/E)TXVX(A/S) (where X is variable), which is characteristic of the TonB-dependent receptor proteins (Lundrigan & Kadner, 1986), which has been proposed to be a putative haemin-binding protein (May et al., 2001). To confirm this possibility, a haemin-binding test was performed with E. coli cells carrying the pET22-b expression vector containing either the wild-type PM0592 gene or the PM0592 gene truncated at position 957 (Fig. 3). The results indicate that E. coli cells expressing either of these two proteins can bind haemin, whereas those that only carry the pET22-b vector can not (Fig. 3). For this reason, and since to our knowledge this is the first protein of P. multocida in which a haemin-binding activity has been experimentally demonstrated, the gene was redesignated hbpA (for haemin-binding protein).

Analysis of hbpA expression showed that synthesis of hbpA mRNA is induced in the presence of DPD as determined by competitive RT-PCR using the P. multocida recA mRNA as a control (Fig. 4) (Cardenas et al., 2001). Therefore, the effect of DPD on hbpA expression occurs at the transcriptional level. It is also conceivable that the half-life of the hbpA mRNA could be increased by DPD treatment, as has been described for other genes (Rosner et al., 2002).

To establish which one of these two possibilities was responsible for the DPD-dependent induction of hbpA expression, a transcriptional fusion between the hbpA promoter region and the lacZ gene was constructed using the broad-host-range promoter-probe vector pHRP309 (Parales & Harwood, 1993). It can be seen that hbpA expression in P. multocida cells is increased not only by DPD, but also when other chelating agents such as EDTA or EGTA are used (Fig. 5). The results in Fig. 5 also indicate that addition of either Fe$^{2+}$ or Mn$^{2+}$ decreases expression of hbpA in DPD-treated P. multocida cells. However, the simultaneous presence of both cations recovers the basal expression of hbpA. In the same way, the addition of haemin decreases the basal level of hbpA promoter expression, as well as eliminates the stimulatory effect of the chelating agent (Fig. 5). The same results were obtained with the fur mutant (data not shown), confirming that the Fur protein does not participate in the control of hbpA gene expression. These findings also demonstrate that enhanced expression is not mediated by DPD, since other chelating agents also have the same effect. All of these data strongly suggest that the effect of DPD on hbpA expression is at the transcriptional level.

Results obtained with the hbpA–lacZ fusion were confirmed when the profile of the outer-membrane proteins was analysed (Fig. 6), since either the addition of both Fe$^{2+}$ and Mn$^{2+}$ or the addition of haemin abolished the production of the 60 and 40 kDa proteins in the DPD-treated cultures.

Analysis of the immunogenicity of the HbpA protein

Since HbpA is an outer-membrane protein inducible under iron- and haemin-starvation conditions, and these limitations are naturally found in the animal host, it was decided to study its antigenicity and its potential protective ability.

![Fig. 3. Haemin binding to E. coli cells expressing either the whole HbpA protein (■) or its derivative truncated at the hexanucleotide AAAAAA located at nucleotide position 957 of the hbpA gene (▲). The percentage of haemin bound by E. coli BL21(DE3) cells carrying the pTE22-b expression vector alone is also shown as a control (●).](http://mic.sgmjournals.org)
Thus serum obtained from Swiss mice inoculated with *P. multocida* wild-type cells was used in Western blot analysis of *E. coli* cell extracts carrying plasmids pUA1035 and pUA1036 overexpressing the wild-type HbpA protein or its truncated 40 kDa derivative, respectively (Fig. 7a). The fact that in both cases there were immunoreactive bands demonstrates that both proteins are immunogenic (Fig. 7b), as expected by their superficial localization. Nevertheless, when purified HbpA protein was inoculated into Swiss mice previous to a challenge treatment with wild-type *P. multocida* cells, no protective effect was obtained, contrary to what happens when a sample of whole outer-membrane proteins of this micro-organism is used in the immunization procedure (data not shown). This result suggests that either other haemin-binding proteins are present in *P. multocida* cells or blocking HbpA by antibodies is not sufficient to prevent the infective process of the mice cells. This is presumably because other iron receptors present in this bacterium are able to acquire the necessary amount of iron for survival (see below).

**DISCUSSION**

The presence of haem-binding systems negatively regulated by both iron and haem has been reported for many Gram-negative bacteria (Stojiljkovic & Hantke, 1992; Letoffe *et al*., 1994; Mills & Payne, 1995; Thompson *et al*., 1999; Ochsner *et al*., 2000; Henderson *et al*., 2001). In all of these cases, the iron-dependent control is mediated through the Fur protein. In the present work, it has been clearly established that the *P. multocida* hbpA gene, encoding a haemin-binding protein, is regulated by iron, manganese and haemin through a Fur-independent mechanism. To our knowledge, this is the first report of iron-mediated regulation of haem receptors where Fur is not involved.

The existence of Fur-independent iron-regulatory mechanisms has been demonstrated in a few Gram-negative bacterial species. Thus 2-D gel electrophoresis analysis of a *Yersinia pestis fur* strain revealed the presence of several unidentified proteins, either iron-repressible or -inducible (Staggs *et al*., 1994). Likewise, a catalase and an alkyl hydroperoxide reductase (encoded by the *katA* and *ahpC* genes, respectively) are negatively regulated by iron in *C. jejuni fur* cells through the PerR protein (van Vliet *et al*., 1998, 2002). Moreover, the *ptxR* gene of *Pseudomonas aeruginosa*, which encodes a regulator of exotoxin A
production, seems to be under iron control through a fur-independent mechanism in cells growing under aerobic but not under microaerobic conditions (Vasil et al., 1998). However, transcription of this ptXR gene requires the presence of an alternative sigma factor encoded by pvdS, which is directly regulated by Fur (Vasil et al., 1998). In this way, it cannot be definitively confirmed that iron-mediated control of ptXR is absolutely independent of the Fur protein. On the other hand, the iron regulation of the Bradyrhizobium japonicum hemB gene, whose product participates in the haem biosynthesis pathway, is not mediated by Fur, but rather by Irr (Hamza et al., 2000). In fact, this is the only protein different from Fur for which a role in iron-regulated gene expression has been demonstrated. It must be noted that a TBLASTN search in the P. multocida genome sequence using the B. japonicum Irr protein as a query has not revealed the presence of any Irr-like protein. Therefore, an orthologue of this protein is not responsible for hbpA regulation in P. multocida.

Recently, a novel iron regulator, RirA, has been identified as controlling iron assimilatory gene function in Rhizobium leguminosarum (Todd et al., 2002). No protein showing similarity to RirA has yet been identified in the genome of P. multocida.

An unexpected result obtained in this work is the negative effect that the presence of Mn2+ has on the transcription of the P. multocida hbpA gene. The importance of this cation in the virulence of Salmonella typhimurium has recently been demonstrated (Boyer et al., 2002). This bacterial species has a Mn2+-dependent gene network which is regulated by the product of the mntR gene (Patzer & Hantke, 2001). Nevertheless, a mntR-like gene does not seem to be present in P. multocida, as shown by TBLASTN analysis carried out by us. Therefore, the participation of Mn2+ in P. multocida hbpA control must be through an unknown regulatory protein different from MntR.

Furthermore, we have also found that a programmed translational frameshift modulates termination of hbpA mRNA translation in vivo. It has been proposed that these kinds of strategies are often used by pathogenic bacteria to allow escape from the host defence system (Dorman & Smith, 2001) or to adapt to variations in the supply and amount of various iron sources (Lewis et al., 1997; Schryvers & Stojiljkovic, 1999). However, this seems unlikely to be the case with P. multocida HbpA, since both the wild-type and the truncated HbpA proteins bind haemin (Fig. 3) and are recognized by serum obtained from animals previously infected with this organism (Fig. 7b).

Moreover, it should be noted that use of the HbpA haemin-binding protein in immunization assays is not able to protect mice against a challenge with virulent P. multocida cells. These data are in agreement with the existence in P. multocida of several putative haem- or haemoglobin-binding proteins (May et al., 2001). In accord with this prediction, PM0040, PM0236, PM0741, PM1081, PM1282 and PM1428 ORFs from P. multocida have been cloned in our laboratory, and, after overexpression in E. coli, we have found that all of them bind haem in vitro (unpublished observations). Finally, from the perspective of using iron-binding proteins for vaccination, our data on HbpA suggest that strategies other than the inoculation of a single type of protein should be used in those bacteria.
which, like *P. multocida*, present more than one kind of receptor.

**ACKNOWLEDGEMENTS**

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