An iron-regulated outer-membrane protein of *Proteus mirabilis* is a haem receptor that plays an important role in urinary tract infection and in *in vivo* growth

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Proteus mirabilis, a common cause of urinary tract infections, expresses iron-regulated outer-membrane proteins (OMPs) in response to iron restriction. It has been suggested that a 64 kDa OMP is involved in haemoprotein uptake and that this might have a role in pathogenesis. In order to confirm this hypothesis, this study generated a *P. mirabilis* mutant strain (P7) that did not express the 64 kDa OMP, by insertion of the *TnphoA* transposon. The nucleotide sequence of the interrupted gene revealed that it corresponded to a haemin receptor precursor. Moreover, *in vitro* growth assays showed that the mutant was unable to grow using haemoglobin and haemin as unique iron sources. The authors also carried out *in vivo* growth and infectivity assays and demonstrated that P7 was not able to survive in an *in vivo* model and was less efficient than wild-type strain Pr 6515 in colonizing the urinary tract. These results confirmed that the *P. mirabilis* 64 kDa iron-regulated OMP is a haem receptor that has an important role for survival and multiplication of these bacteria in the mammalian host and in the development of urinary tract infection.

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INTRODUCTION

Iron plays a central role in bacterial metabolism and pathogenesis. In the mammalian host, iron is unavailable for bacterial uptake (Litwin & Calderwood, 1993) as most of it is located intracellularly, stored in ferritin or linked to haem or haem-containing compounds, whilst extracellular iron is bound to transferrin, lactoferrin, haemopexin and haptoglobin (Braun, 2001).

To overcome this problem, Gram-negative pathogenic bacteria have evolved iron-regulated systems to acquire this element in the host. These include the direct binding of iron-containing proteins to outer-membrane receptors and the secretion of siderophores or haemophores (Wandersman & Stojiljkovic, 2000).

Transport of haem, iron-binding proteins and Fe^{3+} -siderophore complexes towards the cytoplasm represents an active process. Bacteria have developed a system formed by an outer-membrane receptor and a cytoplasmic membrane complex composed of ExbB, ExbD and TonB proteins that transduces the proton-motive force of the cytoplasmic membrane to the high-affinity outer-membrane receptors,

known as TonB-dependent receptors (Olczak *et al.*, 2005; Llamas & Bitter, 2006).

Proteus mirabilis is an important cause of urinary tract infections (UTIs) in humans, especially in patients with urinary catheters and structural abnormalities. It shows a predilection for the upper urinary tract where it can cause serious renal damage (Stamm *et al.*, 1977; Senior, 1978). Several potential virulence factors have been described for *P. mirabilis*, including haemolysin production and iron-uptake systems (Ròzalski *et al.*, 1997).

Piccini *et al.* (1998) studied the effect of iron deprivation on the expression of outer-membrane proteins (OMPs) in uropathogenic isolates of *P. mirabilis*. They found that under low-iron conditions, expression of three OMPs with molecular masses ranging from 66 to 75 kDa increased and that a 64 kDa OMP was synthesized *de novo*. They also suggested that the 64 and 66 kDa OMPs were involved in haemin (Hm) and haemoglobin (Hb) uptake. Moreover, Burall *et al.* (2004) identified four *P. mirabilis* ORFs that are involved in iron uptake as potential *in vivo*-expressed virulence determinants, three of which are potential OMPs.

Here, we describe the generation and characterization of a *P. mirabilis* insertion mutant lacking the 64 kDa OMP, and the role of this protein in bacterial growth, *in vivo* survival and infectivity. Growth assays using different iron sources

Abbreviations: Amp, ampicillin; EDDHA, di-*o*-hydroxyphenylacetic acid; GaPPIX, gallium protoporphyrin IX; Hb, haemoglobin; Hm, haemin; IPC, intraperitoneal chamber; Km, kanamycin; OMP, outer-membrane protein; Tet, tetracycline; UTI, urinary tract infection.

were performed to evaluate the ability of the mutant strain to grow using Hm or Hb as the sole iron source. Moreover, we studied the role of the 64 kDa OMP in the pathogenicity of *P. mirabilis* through *in vivo* experimental UTI models in mice and during *in vivo* growth using an intraperitoneal chamber (IPC) model.

METHODS

Bacterial strains, plasmids, transposon and growth conditions.

Bacterial strains, plasmids and the transposon used in this study are listed in Table 1. Bacterial strains were grown in Luria–Bertani (LB) broth, LB agar, nutrient broth (NB) or nutrient agar (NA). When necessary, the media were supplemented with 100 µg ampicillin (Amp) ml⁻¹, 15 µg tetracycline (Tet) ml⁻¹ and 40 µg kanamycin (Km) ml⁻¹. Iron-limited conditions were obtained by adding di-*o*-hydroxyphenylacetic acid (EDDHA) to the medium at a final concentration of 100 µM.

Animals. Female CD-1 outbred mice weighing 20–25 g and Sprague–Dawley outbred rats weighing 220–250 g from the breeding facilities at IIBCE, Uruguay, were provided with food pellets and tap water *ad libitum*. All animal experiments were conducted in accordance with procedures authorized by IIBCE.

Transposon mutagenesis and isolation of mutant strains.

TnphoA was introduced into the *P. mirabilis* strain Pr 6515 chromosome by conjugation. To this end, *Escherichia coli* SM10λpir was mated with Pr 6515 for 6 h at 37 °C on LB agar plates supplemented with 100 mM MgCl₂. Bacteria were scraped from the plate, suspended in PBS and plated on LB agar (lacking NaCl and solidified with 2 % agar to inhibit swarming motility) supplemented with Tet, Km, 40 µg 5-bromo-4-chloro-3-indolyl phosphate ml⁻¹ and 2 µg gallium protoporphyrin IX (GaPIX) ml⁻¹. Plates were incubated overnight at 37 °C and selected clones were restreaked on Amp plates to discard clones containing plasmid co-integrates.

OMP extraction. *P. mirabilis* Pr 6515 and the mutant strain P7 were inoculated into NB and NB-EDDHA and incubated at 37 °C for 24 and 48 h, respectively. OMP extraction was performed as described by Piccini *et al.* (1998).

SDS-PAGE. OMP-enriched pellets were suspended in electrophoresis loading buffer and heated at 100 °C for 5 min. The samples were

analysed by SDS-PAGE using a 10 % acrylamide resolving gel according to the method described by Sambrook *et al.* (1989) and stained with 0.1 % Coomassie brilliant blue.

Chromosomal DNA extraction and Southern blotting. Clones P3, P7, P14 and wild-type strain Pr 6515 were analysed by Southern blot hybridization using the entire transposon as a probe. Chromosomal DNA from each strain was obtained using an agarose-embedded DNA extraction procedure, as described by Maskell *et al.* (1988), and was subjected to simple digestion with the restriction enzyme *Bam*HI or *Hind*III and double digestion with both enzymes. The restriction reactions were incubated overnight at 37 °C. Southern blot analysis was carried out according to Sambrook *et al.* (1989). Hybridization was carried out overnight at 55 °C using AlkPhos Direct hybridization buffer (Amersham) and the *TnphoA* transposon was labelled with AlkPhos Direct (Amersham) when used as a probe. The expected hybridization pattern for a single *TnphoA* insertion was two bands for simple digestion with *Bam*HI and three bands for simple digestion with *Hind*III (one of about 3.5 kb, the expected size of the internal fragment). In the double digestion, the expected hybridization pattern was five bands, two having approximate sizes of 1.5 and 2 kb.

DNA amplification, sequencing and analysis. In order to identify the mutated gene, *TnphoA* flanking regions were amplified by two rounds of PCR according to the protocol described by Gallagher & Manoil (2001). Briefly, *P. mirabilis* Pr 6515 and P7 chromosomal DNA was obtained and a first-round PCR was performed using the primer pair Tn1 (5'-GTGCAGTAATATCGCCCTGAG-3', nt 93–73 of *TnphoA*) and CEKG2C (5'-GGCCACGCGTCGACTAGTAC-NNNNNNNNNNGATAT-3', a partially random primer that hybridizes at different sites in the *P. mirabilis* genome) for the 5' end. For amplification of the 3' end, Tn2 (5'-CGATGAAGAGCAGAAAG-TTATC-3', nt 7620–7640 of the *TnphoA* gene) and CEKG2C were used. The annealing temperature was 37 °C for both pairs of primers. PCR products obtained from both strains were compared in an agarose gel, and a band present in P7 but not in Pr 6515 was excised, purified with a Concert Rapid Gel Extraction System kit (Life Technologies) and used as a template for the second-round PCR. For the second round, Tn1 or Tn2 was used in combination with CEKG4 (5'-GGCCACGCGTCGACTAGTAC-3'), which hybridizes to the sequence introduced at the 5' end of primer CEKG2C, using an annealing temperature of 52 °C. The obtained PCR product was purified using a Concert Rapid Gel Extraction System kit. DNA sequencing was performed at the Centro Técnico de Análisis Genéticos, Facultad de Ciencias, Universidad de la República, Uruguay, using an Applied Biosystems Model 377 Automated DNA

Table 1. Bacterial strains, plasmids and transposon used in this study

Strain/plasmid/transposon	Relevant characteristics	Source or reference
Strains		
<i>P. mirabilis</i>		
Pr 6515	Wild-type strain isolated from a patient with symptomatic UTI	Piccini <i>et al.</i> (1998)
P7	Mutant strain generated from Pr 6515 by insertion of <i>TnphoA</i> . Does not express 64 kDa OMP	This study
<i>E. coli</i>		
SM10λpir	RP4-Tc::Mu Km ^R thi thr leu su _u /pRT733	Simon <i>et al.</i> (1983)
XL-1 Blue	recA1 endA gyrA96 thi-1 hsdR17 supE44 relA1 lac [F'proABlacI ^q lacZΔM15Tn10(Tet ^R)]	Bullock <i>et al.</i> (1987)
Plasmids/transposon		
pUC19	pBR322ori, lacZ, Amp ^R	Yanisch-Perron <i>et al.</i> (1985)
pRT733	Ori6K, Amp ^R , tra ⁻ , pir ⁻ , mob ⁺ <i>TnphoA</i>	Taylor <i>et al.</i> (1989)
<i>TnphoA</i>	Tn5 IS50 _L :: <i>phoA</i> , Km ^R	Manoil & Beckwith (1985)

sequencer. The obtained transposon flanking sequences were searched in the genome sequence from *P. mirabilis* strain HI4320. These sequence data were produced by the *P. mirabilis* Sequencing Group at the Sanger Institute and are available at <ftp://ftp.sanger.ac.uk/pub/pathogens/pm>. Sequence analysis was carried out using Artemis software (Rutherford *et al.*, 2000). The DNA sequence was also searched in the protein sequences derived from the patent division of GenBank (PAT) database at NCBI using the BLASTN tool.

Growth curves. Pr 6515 and P7 were incubated overnight in NB-EDDHA at 37 °C in order to promote the consumption of intracellular iron. Cells were then suspended in PBS and inoculated at a final concentration of 1×10^5 c.f.u. ml⁻¹ into the following media: NB, NB-EDDHA, NB-EDDHA supplemented with 5 mg Hm ml⁻¹ and NB-EDDHA supplemented with 5 mg Hb ml⁻¹. These cultures were incubated at 37 °C with vigorous shaking. Samples were taken at different times and the number of c.f.u. ml⁻¹ was determined by bacterial plate counts on NA at 37 °C. Each growth assay was repeated at least three times.

Growth assays with different iron sources. The ability of Pr 6515 and P7 to grow using different iron sources was assessed. Thus 1×10^5 c.f.u. Pr 6515 or P7 ml⁻¹ from NB-EDDHA overnight cultures was seeded onto NA-EDDHA. Sterile paper discs were placed onto the plates and soaked with 15 µl of one of the following iron sources: 5 mg Hb ml⁻¹, 5 mg Hm ml⁻¹ or 50 mM ferric citrate. A disc soaked in sterile water was used as a negative control. Plates were incubated at 37 °C for 48 h and the diameters of the growth zones around the discs were determined. Each growth assay was repeated at least three times.

Bacterial growth in urine. To evaluate the ability of the strains to grow in human urine, urine samples from five healthy female donors were pooled and filter-sterilized. *P. mirabilis* strains Pr 6515 and P7 from overnight cultures on NB-EDDHA at 37 °C were inoculated in 20 ml fresh urine at a final density of 1×10^4 c.f.u. ml⁻¹. Samples were taken at different times and cells were counted after overnight culture on NA (Zunino *et al.*, 1999). Each growth assay was repeated at least three times.

Haemolysis assays. Evaluation of the haemolytic activity of Pr 6515 and P7 was performed as described previously (Mobley & Chippendale, 1990; Zunino *et al.*, 1999). The haemolytic titre was defined as the highest dilution in which no visible pellet was observed.

Bacterial growth in IPCs in rats. Growth of Pr 6515 and P7 in IPCs was monitored. Diffusion chambers were prepared as described previously (Zunino *et al.*, 1999). Two chambers were introduced through a 1 cm longitudinal incision into the peritoneal cavity of rats anaesthetized previously by intramuscular injection of ketamine hydrochloride (88 mg kg⁻¹) and xylazine (4 mg kg⁻¹). The abdomen was sutured with 0000 vicryl and the skin with 000 nylon (Quessy *et al.*, 1994). After 12, 24, 48 and 72 h, the rats were killed (three rats per sampling point) and the chambers were removed aseptically. Bacteria were removed from the chamber using a syringe and viable bacterial counts were determined by plating serial dilutions on NA.

***P. mirabilis* UTI models in mice.** The ascending UTI mouse model was performed as described previously by Zunino *et al.* (2001). Briefly, Pr 6515 and P7 bacteria were suspended in PBS at a concentration of 1×10^8 c.f.u. ml⁻¹. Mice were anaesthetized with xylazine (10 mg kg⁻¹) and ketamine (50 mg kg⁻¹) and their bladders were emptied by gentle abdominal massage. A soft polyethylene catheter (outer diameter 0.61 mm) was inserted through the urethra and 0.05 ml bacterial suspension (5×10^6 c.f.u. per mouse) was slowly introduced into the bladder over a period of not less than 30 s to avoid vesicourethral reflux. Moreover, in order to observe the ability

of both strains to compete *in vivo*, a co-challenge experiment was performed. For these assays, mice were treated as described above and a group of mice was challenged with a 1:1 mixture of wild-type Pr 6515 and mutant P7.

When the haematogenous model was used, bacterial suspensions (0.1 ml, 1×10^7 c.f.u. per mouse) were injected into a tail vein (Zunino *et al.*, 1994).

In every experiment, mice were sacrificed 7 days after infection by cervical dislocation. Kidneys and bladder were removed aseptically and homogenized in 10 ml PBS, and viable bacterial counts were determined on NA. In transurethral co-challenge experiments, recovery of the mutant strain from the bladder and kidneys of infected mice was determined by viable bacterial counts on NA-Km, whereas recovery of the wild-type strain was determined as the difference between the counts on NA and NA-Km. In haematogenous infection assays, animals were recorded as infected when bacteria were recovered from one or both kidneys.

Statistical analysis. Mann-Whitney one-tailed non-parametric analysis was used to compare bacterial colonization levels in kidney and bladder. A χ^2 test with Yates correction for small sample sizes was used to compare the number of animals infected with Pr 6515 and P7. Differences were considered significant for $P < 0.05$.

RESULTS AND DISCUSSION

Generation and selection of a *P. mirabilis* mutant strain deficient in a haem receptor

The mechanism of Hm or Hb utilization as an iron source by *P. mirabilis*, the protein receptors involved in iron uptake and their participation in pathogenesis are poorly understood. In a previous study, Piccini *et al.* (1998) demonstrated that *P. mirabilis* could use Hm and Hb as unique iron sources and they suggested that the 64 kDa OMP was involved in Hm and Hb uptake. In order to confirm the role of the 64 kDa OMP as the Hm or Hb receptor, in the present study we used the clinical *P. mirabilis* strain Pr 6515 to generate a mutant strain that did not express the 64 kDa OMP.

Mutant strains were generated by insertion of the *TnphoA* transposon, which was transferred by conjugation through a suicide plasmid. *TnphoA* was randomly inserted in the chromosome generating fusions between target genes and the *E. coli* gene for alkaline phosphatase. This genetic tool was chosen as it allowed simple selection and enrichment of mutant strains of genes for secreted, transmembrane and periplasmic proteins by selecting those clones that showed alkaline phosphatase activity (Manoil & Beckwith, 1985).

Selection of transconjugant clones was also based on their resistance to GaPPIX, a structural analogue of the haem group but with gallium instead of iron conjugated to the protoporphyrin group. Gallium is not able to substitute for iron in biological reactions and for that reason this compound is toxic to bacteria that use haem or haemoprotein uptake systems, causing cell death by the production of reactive oxygen radicals. Therefore, mutant strains lacking haem or haemoprotein receptors would be

resistant to GaPPIX (Stojiljkovic *et al.*, 1999). After conjugation, those insertion mutants that were able to grow on the selective media were chosen. This strategy allowed the isolation of 15 GaPPIX-resistant clones, but just three (P3, P7 and P14) were sensitive to Amp. These results showed a high frequency of plasmid co-integration at the transposon insertion site. This phenomenon has been reported by other authors (Ali *et al.*, 2000) and is one of the obstacles described for the use of this Tn5-derived transposon (Taylor *et al.*, 1989; Ahmed, 1991).

Among the three Amp-sensitive selected clones, only P7 showed blue colonies when 5-bromo-4-chloro-3-indolyl phosphate was used as the alkaline phosphatase substrate. Moreover, when the OMP patterns of Pr 6515 and mutant strain P7 grown on iron-restricted media were analysed by SDS-PAGE (Fig. 1), it was observed that the selected clone lacked the band corresponding to the 64 kDa OMP, indicating that P7 had the transposon inserted in the structural gene encoding the 64 kDa OMP.

Genetic analysis of the *P. mirabilis* mutant lacking the 64 kDa OMP

Clones P3, P7 and P14 were analysed by Southern blot hybridization using the entire transposon as a probe. The results showed that P7 exhibited the expected hybridization pattern for a single *TnphoA* insertion, whilst the hybridization pattern of P3 and P14 mutant strains did not show a unique insertion of the transposon (data not shown). The wells corresponding to Pr 6515 did not show any signal. For these reasons, P7 was selected for further characterization studies.

The DNA sequence of the P7 mutated gene was obtained by semi-random PCR of chromosomal DNA (Gallagher & Manoil, 2001). When the 5' end of the transposon

sequence was analysed, we always found *TnphoA*-related sequences. It has been shown that *Tn5*, from which *TnphoA* derives, transposes by a replicative pathway and promotes IS50 insertions frequently accompanied by inversions of adjacent DNA (Ahmed, 1991). This particular behaviour of *Tn5* and derivatives could explain why DNA sequences obtained from the 5' end of the transposon corresponded to *TnphoA* sequences.

The DNA sequence obtained from the transposon 3' end was compared with the *P. mirabilis* genome sequence and was found to match the predicted gene PMI1426, which encodes a Hm receptor (M. Sebahia, personal communication). The 3' *TnphoA* flanking sequence was also compared with the PAT database at NCBI using the BLASTN tool, which recovered the sequence 310 from patent US 6605709 (GenBank accession no. AR375304.1; E-value 2×10^{-5} , identity 100%), described as 'nucleic acid and amino acid sequences related to *Proteus mirabilis* for diagnostics and therapeutics'. The sequences of PMI1426 and US 6605709 were analysed using the BLASTX tool from NCBI and both retrieved various Hm receptor precursors, outer-membrane receptors and TonB-dependent outer-membrane receptors from various bacteria. The protein with the highest amino acid identity (58%, E-value 0.0) was a Hm receptor precursor from *Photobacterium luminescens* (GenBank accession no. NP_929867).

In addition, the *P. mirabilis* ORF was subjected to a search for conserved domains employing the CDART tool (NCBI), which determined that the sequence represented a TonB-dependent/ligand-gated channel domain. It is well documented that Hm, Hb, siderophore and haemophore receptors are TonB-dependent proteins (Braun & Braun, 2002) and that in most Gram-negative pathogenic bacteria that have been examined, a functional TonB protein is required for haem and Hb utilization (Braun, 1995).

Taken together, these data strongly indicated that P7 had a single in-frame transposon insertion in the structural gene encoding the 64 kDa OMP. The nucleotide sequence of the interrupted gene revealed that it corresponded to a Hm receptor precursor.

In vitro growth assays

When Pr 6515 and P7 *in vitro* growth curves were determined in NB (Fig. 2), P7 showed a lag phase slightly longer than that of Pr 6515 and reached a lower final bacterial count (about tenfold). As NB is a complex medium containing meat extract, which is a source of haem, the slower growth of P7 could be due to its inability to grow using haem as its iron source. In contrast, Pr 6515 can use the inorganic iron present in the medium as well as the haem, giving it a growth advantage. It was noticed that neither Pr 6515 nor P7 were able to sustain growth in iron-restricted medium (NB-EDDHA). However, P7 was still unable to grow in NB-EDDHA supplemented with Hb or Hm as the sole iron source, whilst Pr 6515 presented a

Fig. 1. OMP profile of Pr 6515 (a) and the 64 kDa OMP-deleted mutant P7 (b). OMPs were obtained from bacteria grown in NB (lane 1) and in NB supplemented with 100 μ M EDDHA (lane 2). IROMPs, iron-regulated outer-membrane proteins.

Fig. 2. Representative growth curves of *P. mirabilis* strain Pr 6515 (filled symbols) and mutant strain P7 (open symbols) in NB (■, □), NB-EDDHA (●, ○), NB-EDDHA with Hm (5 µg ml⁻¹) (▲, △) and NB-EDDHA with Hb (5 µg ml⁻¹) (▼, ▽). Growth was monitored by plate counts on NA.

growth curve similar to that observed in NB when grown in NB-EDDHA supplemented with Hb or Hm. Moreover, when we carried out *in vitro* growth assays on NA-EDDHA plates supplemented with different iron sources spotted onto sterile discs placed on the medium, it was observed that P7 was unable to grow around the Hb-supplemented discs (diameter of growth zone: 0.0±0.0 mm) and grew weakly around the Hm discs (diameter of growth zone: 2.7±0.47 mm), but grew normally using ferric citrate as the only iron source (diameter of growth zone: 13.3±1.53 mm). Pr 6515 could grow using all three different iron sources (diameter of growth zones: 11.3±1.15, 14.3±1.53 and 16.3±0.58 mm, respectively). These results supported our experimental findings indicating that P7 has a *TnphoA* insertion in an OMP gene related to haem uptake and that its insertion prevents Hm or Hb utilization.

Growth of Pr 6515 and P7 in urine was also tested (Fig. 3a). The results showed that, in contrast to the growth observed in NB, both strains were able to grow in urine to similar levels. The different growth patterns of P7 observed in NB and urine, when compared with Pr 6515, could be attributed to the higher levels of iron sources present in the culture medium. Relatively high levels of iron are excreted in normal urine, although it is not available as it is associated with transferrin or lactoferrin (Shand *et al.*, 1985). It has been shown that *P. mirabilis* is not able to utilize transferrin or lactoferrin as an iron source (Piccini *et al.*, 1998) and that this pathogen does not produce conventional siderophores (Coker *et al.*, 2000). Moreover, it was reported that the enzyme amino acid deaminase may play a role in iron acquisition (Massad *et al.*, 1995).

Fig. 3. Representative growth curves of *P. mirabilis* Pr 6515 (■) and mutant P7 (▲) in human urine (a) and in IPCs in rats (b). Growth was monitored by plate counts on NA.

Therefore, the iron acquisition from urine by *P. mirabilis* could be mediated by the action of the enzyme amino acid deaminase or another uncharacterized system that could involve other *P. mirabilis* iron-regulated OMPs. Uropathogens have functional redundancy in their iron-uptake systems and there are general hypotheses that may explain the existence of such redundancy, such as the maximization of possibilities to obtain iron from different sources and increases in efficacy of iron acquisition in different media (Russo *et al.*, 2002).

In vivo assays

In a previous study, it was observed that sera obtained from animals infected with *P. mirabilis* Pr 6515 reacted with the 64 kDa OMP, suggesting that this protein is expressed *in vivo* during infection (Piccini *et al.*, 1998). It

was reported that *P. mirabilis* is able to grow and survive *in vivo* in an IPC model, and it was also suggested that *P. mirabilis* OMPs are potential specialized virulence factors for survival and may play an important role in UTIs (Moayeri *et al.*, 1991; Zunino *et al.*, 1999).

In this work, *in vivo* bacterial growth was studied using IPCs filled with a suspension of Pr 6515 or P7 and implanted into the peritoneal cavity of rats. Viable counts of bacteria were performed at different periods of time (Fig. 3b). It was observed that, whereas Pr 6515 was able to multiply 1000-fold after 24 h and to maintain levels of 10^8 c.f.u. ml⁻¹ over the period of time evaluated, the number of P7 c.f.u. drastically decreased after 12 h. As iron is not available in the mammalian host as a free nutrient but is linked to carrier proteins (Braun, 2001), it is possible that the absence of the 64 kDa OMP in P7 prevented its growth *in vivo*, confirming the importance of this OMP for *P. mirabilis* survival in this *in vivo* environment.

The role of the 64 kDa OMP in urovirulence was assessed using different models of ascending and haematogenous UTI in the mouse. The ascending UTI model (Hagberg *et al.*, 1983) resembles the natural route of infection in humans and allowed us to evaluate the colonization of the urinary tract. For transurethral infection, bacteria recovered from the kidneys and bladder of mice challenged with strain Pr 6515 or P7 were enumerated (Fig. 4a). In the simple experimental transurethral infection, no significant differences were observed between kidney and bladder viable counts of P7 and Pr 6515 ($P > 0.05$).

However, when the ability of Pr 6515 and P7 to compete directly *in vivo* was assessed by performing an ascending co-infection UTI assay, P7 was significantly outcompeted by Pr 6515 and colonization of the kidneys and bladder of mice was significantly affected ($P < 0.05$) (Fig. 4b). Co-infection assays provide a model to compare the colonization ability of different strains, as they avoid individual host differences and permit the detection of subtle differences between competing strains (Nagy *et al.*, 2002). In our co-infection experiments, P7 was probably impaired in its ability to colonize the urinary tract, an aspect that was not apparent in the simple infection model. From our results, it could be hypothesized that the role of bacterial haem/Hb uptake mechanisms, particularly those mediated by OMP receptors, is more evident in later steps of infection, contributing to bacteria survival and invasion (Warren *et al.*, 1987; Zunino *et al.*, 1999).

When we carried out intravenous infection assays, we observed that 63 % (10/16) of mice inoculated with Pr 6515 and 20 % (3/15) of mice inoculated with P7 were infected. Therefore, a significant difference between infectivity in mice inoculated with Pr 6515 and P7 was observed ($P < 0.05$). Considering that, in this experimental infection route, bacteria reach the urinary tract through the bloodstream, where haem is the main iron source, and that it was proposed that haem is the dominant iron source in kidneys (Burall *et al.*, 2004), this result suggested that the

Fig. 4. Virulence of wild-type strain Pr 6515 and mutant strain P7 in a simple ascending UTI model (a) and a co-infection ascending UTI model (b) in mice. The median (horizontal bar) is indicated for each group. The detection limit was 1×10^2 c.f.u. per kidney or bladder. P values were determined by the Mann–Whitney non-parametric test.

absence of the 64 kDa OMP in P7 impaired the development of infection by avoiding the access of this strain to haem. Moreover, considering that the production of functional haemolysin in *P. mirabilis* is regulated at the transcriptional level by iron concentration (Lukowski *et al.*, 1991), we suggest that the utilization of haem delivered from eukaryotic cells by the action of haemolysin could be an important strategy for obtaining iron during an infection. We were able to determine that Pr 6515 and P7 presented similar haemolytic activity (haemolytic titres of 1:4 for both bacteria after 3 h of incubation). This

could indicate that the attenuation of the mutant strain in intravenous infection was exclusively due to the lack of the 64 kDa OMP.

In conclusion, we confirmed that the 64 kDa iron-regulated OMP is a haem receptor of *P. mirabilis* and has an important role in the growth of these bacteria in the mammal host and in UTIs. Further work assessing the amino acid sequence of this OMP by mass spectrometry is currently under way.

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