An iron-regulated LysR-type element mediates antimicrobial peptide resistance and virulence in *Yersinia pseudotuberculosis*

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During the course of its infection of the mammalian digestive tract, the entero-invasive, Gram-negative bacterium *Yersinia pseudotuberculosis* must overcome various hostile living conditions (notably, iron starvation and the presence of antimicrobial compounds produced *in situ*). We have previously reported that *in vitro* bacterial growth during iron deprivation raises resistance to the antimicrobial peptide polymyxin B; here, we show that this phenotype is mediated by a chromosomal gene (*YPTB0333*) encoding a transcriptional regulator from the LysR family. We determined that the product of *YPTB0333* is a pleiotropic regulator which controls (in addition to its own expression) genes encoding the Yfe iron-uptake system and polymyxin B resistance. Lastly, by using a mouse model of oral infection, we demonstrated that *YPTB0333* is required for colonization of Peyer's patches and mesenteric lymph nodes by *Y. pseudotuberculosis*.

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INTRODUCTION

Iron is an essential nutrient for almost all micro-organisms, since it is present in the active site of a number of key enzymes involved in essential metabolic pathways (such as the tricarboxylic acid cycle, the respiratory chain and DNA synthesis) (Andrews *et al.*, 2003). In mammals, limiting access to iron is a critical line of host defence against bacterial pathogens and is achieved by the deployment of specific proteins which bind ferric iron (the usual state of iron under aerobic, neutral-pH conditions) with high affinity (Hentze *et al.*, 2004). Hence, in order to replicate *in vivo*, a bacterial pathogen needs to take up ferric ions from the host's protein complexes. This is performed by iron-acquisition systems, the most common of which are the siderophores (Ratledge & Dover, 2000). After bacterial

internalization and entry into the cytosol, insoluble ferric cations are reduced to soluble ferrous cations (Vartivarian & Cowart, 1999). Additionally, iron starvation signals to a pathogen that it has entered the mammalian host and triggers production of virulence factors (such as exotoxins) that allow microbial tissue colonization (Calderwood & Mekalanos, 1987; Leong & Murphy, 1985; Lory, 1986). Gene expression induced by iron limitation is often controlled by global regulators, including the Fur ferric uptake regulator (Bagg & Neilands, 1987).

The Gram-negative bacterium *Yersinia pseudotuberculosis* is a pathogen of the digestive tract of animals (including humans), and causes ileitis and mesenteric adenitis after ingestion of contaminated food or water. During the course of infection in the gut, this entero-invasive micro-organism must not only overcome iron starvation but also resist antimicrobial peptides termed defensins (cryptdins in mice) (Hancock & Diamond, 2000) produced in large amounts in the crypts of the intestinal lumen by epithelial cells, i.e. Paneth cells and enterocytes (Ayabe *et al.*, 2000; Bevins, 2004). After binding to LPS on the bacterial surface through electrostatic interactions, antimicrobial peptides permeabilize the bacterial membranes and potentially lead to cell death (Shai, 2002; Yeaman & Yount, 2003). It has been demonstrated in different studies that in order to

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Abbreviation: MFS, major facilitator superfamily.

A supplementary table, listing putative additional *YPTB0333*-regulated genes, and a supplementary figure, showing promoter analysis of the *YPTB0331* and *YPTB0333* genes, are available with the online version of this paper.

circumvent the microbicidal activity of these host compounds, bacteria have developed a range of strategies that are reminiscent of those involved in antibiotic resistance (Nizet, 2006). One stratagem involves producing bacterial proteases that degrade antimicrobial peptides (Stumpe *et al.*, 1998). Another incorporates positively charged groups into the lipid A, reducing the net anionic charge of the bacterial envelope and thus lowering the affinity of the latter for antimicrobial peptides (Groisman *et al.*, 1997; Gunn *et al.*, 1998, 2000). Additional resistance mechanisms involve preventing the antimicrobial peptides from accessing their target via extracellular trapping by secretory proteins (Jin *et al.*, 2004) or actively pumping the peptides back across the cytoplasmic membrane (Shafer *et al.*, 1998). Hence, bacterial determinants that support antimicrobial peptide resistance (the expression of which is frequently induced by environmental cues) can be viewed as virulence factors per se (Garcia Vescovi *et al.*, 1996; Wösten *et al.*, 2000).

In a previous study (Marceau *et al.*, 2004), we observed an increase in *Y. pseudotuberculosis* resistance to polymyxin B (the benchmark cationic antimicrobial peptide) when bacteria were grown under iron-starvation conditions: to the best of our knowledge, such a phenotype had not been reported for any bacteria. We have now identified

the genetic determinant of inducible resistance to polymyxin B in *Y. pseudotuberculosis* and demonstrate that one of the genes involved contributes to bacterial virulence and promotes intestinal colonization by the enteropathogen.

METHODS

Bacterial strains and growth conditions. The main characteristics of the plasmids and bacterial strains used in this study are listed in Tables 1 and 2. *Escherichia coli*, *Y. pseudotuberculosis* and *Yersinia enterocolitica* strains were grown aerobically in Luria–Bertani (LB) broth or on agar, at 37 °C for the first-mentioned species and at 28 °C for the last two. Ampicillin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), irgasan (4 µg ml⁻¹) and sucrose (10%) were added to media for bacterial selection when necessary. IPTG (1 mM) and X-Gal (200 µg ml⁻¹) were used for blue/white colony screening.

Reagents. The iron chelator 2,2'-dipyridyl and polymyxin B were purchased from Sigma. Dansyl-polymyxin B was obtained from Molecular Probes.

Molecular biology techniques. Standard procedures were used for genomic DNA extraction, small-scale plasmid preparation, endonuclease digestion, DNA ligation, agarose gel electrophoresis, elution of DNA fragments and *E. coli* transformation (Sambrook & Russell, 2001). Large-scale plasmid DNA preparations were purified on

Table 1. Strains used in this study

Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; s, susceptible; r, resistant.

Strain	Relevant characteristics	Reference or source
<i>E. coli</i> strains		
DH5α	F ⁻ Φ80dlacZΔM15 Δ(lacZYA–argF)U169 recA1 endA1 hsdR17 (r _k ⁻ m _k ⁻) supE44 λ ⁻ thi-1 gyrA relA1; host for DNA cloning	Invitrogen
EC100	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC)Φ80dlacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara,leu)7697 galU galK l ⁻ rpsL nupG relA1; host for DNA cloning	Epicentre Biotechnologies
BL21-DE3	F ⁻ ompT hsdS (r ⁻ m ⁻) gal dcm (DE3); host for efficient recombinant protein expression	Invitrogen
SY327 λpir	Δ(lac pro) argE (Am) recA rif ^r nalA λpir; host for replication of suicide plasmids pCVD442 and derivatives	Miller & Mekalanos (1988)
SM10 λpir	thi thr leu sup tonA lacY recA::RP4-2Tc::MuKm λpir; host for replication of suicide plasmids pCVD442 and derivatives	Simon <i>et al.</i> (1983)
<i>Y. pseudotuberculosis</i> strains		
IP32777	Wild-type strain, harbouring the virulence plasmid pYV; Ap ^s , Km ^s	Sebbane <i>et al.</i> (2001)
YPTB0331-0332	IP32777 mutant, engineered with plasmid pCVD0331-0332-Aph	This work
YPTB0333	IP32777 mutant, engineered with plasmid pCVD0333-Aph	This work
YPTB0333c	IP32777 mutant, cis-complemented with plasmid pCVD0333	This work
YPTB0331-0333	IP32777 mutant, engineered with plasmid pCVD0331-0333-Aph	This work
<i>Y. enterocolitica</i> strains		
IP383	O9 serogroup wild-type strain; Ap ^r , Cm ^s	
5.1	IP383 complemented with plasmid pSA5.1	This work
5.1.1	IP383 complemented with plasmid pSA5.1.1	This work
5.1.2	IP383 complemented with plasmid pSA5.1.2	This work
5.1.3	IP383 complemented with plasmid pSA5.1.3	This work
5.1.4	IP383 complemented with plasmid pSA5.1.4	This work

Table 2. Plasmids used in this study

Ap^r, Km^r, Cm^r, resistance to ampicillin, kanamycin and chloramphenicol, respectively. Ω , *in vitro* insertion; F, forward; R, reverse.

Plasmid	Relevant characteristics	Reference or source
pUC18	Cloning vector; Ap ^r	Appligene
pCVD442	Suicide vector containing the counter-selectable marker <i>sacB</i> ; Ap ^r	Donnenberg & Kaper (1991)
pUC4K	Source of aminoglycoside phosphotransferase <i>aphA-1a</i> gene; Km ^r	Amersham Pharmacia
pET24a (+)	Expression vector; Km ^r	Novagen
pSA	pACYC184 derivative with <i>lacZ'</i> and the multiple cloning site from pUC18; Cm ^r	Flamez <i>et al.</i> (2008)
pSA5.1	pSA Ω 7923 bp (<i>Y. pseudotuberculosis</i> IP32777 chromosomal fragment located at position 392 816 to 400 738)	This work
pSA5.1.1	pSA Ω 2709 bp (fragment generated by <i>Bam</i> HI and <i>Bgl</i> II digestion of pSA5.1)	This work
pSA5.1.2	pSA Ω 4633 bp (fragment generated by <i>Nde</i> I digestion of pSA5.1 and self-ligation of the remaining plasmid)	This work
pSA5.1.3	pSA Ω 4664 bp (fragment generated by <i>Pst</i> I digestion of pSA5.1)	This work
pSA5.1.4	pSA Ω 6296 bp (fragment generated by <i>Nco</i> I digestion of 5.1 region and self-ligation of the remaining plasmid)	This work
pCVD0331-0332-Aph	pCVD442 Ω 2022 bp (1245 bp <i>aphA-1a</i> inserted at position 560 in a 777 bp <i>Sac</i> I/ <i>Sac</i> I PCR fragment generated with primer sets F4/R4 and F5/R5 and derived from the <i>YPTB0331</i> and <i>YPTB0332</i> genes of <i>Y. pseudotuberculosis</i>)	This work
pCVD0333-Aph	pCVD442 Ω 2190 bp (1245 bp <i>aphA-1a</i> inserted at position 345 in a 945 bp <i>Sac</i> I/ <i>Sac</i> I PCR fragment generated with primer set F1/R1 and derived from the <i>YPTB0333</i> gene of <i>Y. pseudotuberculosis</i>)	This work
pCVD0331-0333-Aph	pCVD442 Ω 2047 bp (1245 bp <i>aphA-1a</i> inserted at position 394 in a <i>Sac</i> I/ <i>Sac</i> I PCR fragment generated with primer sets F21/R21 and F22/R22 and derived from the <i>YPTB0331</i> and <i>YPTB0333</i> genes of <i>Y. pseudotuberculosis</i>)	This work
pCVD0333	pCVD442 Ω 1269 bp (<i>Sac</i> I/ <i>Sac</i> I PCR fragment generated with primer set F2/R2 and derived from pSA5.1)	This work
pET-YPTB0333	pET24a (+) Ω 980 bp (<i>Nde</i> I/ <i>Xho</i> I PCR fragment generated with F3/R3 and derived from pSA5.1)	This work

Qiagen columns. Recombinant plasmid DNA was introduced into *Y. pseudotuberculosis* and *Y. enterocolitica* by mating or electroporation (Conchas & Carniel, 1990). Southern blots were performed according to standard procedures. The ECL direct nucleic acid labelling and detection system (Amersham Biosciences) was used for nucleic acid hybridization. DNA was sequenced with the dideoxy-chain-termination method using the ABI PRISM Dichlororhodamine Dye Terminator Sequencing kit with AmpliTaq DNA polymerase FS (Perkin-Elmer). The nucleotide sequences obtained were analysed with Sequencher v4.2.2 software.

Oligonucleotide primers (Table 3) were custom-synthesized (Eurogentec and MWG Biotech) for the PCR generation of DNA fragments used in cloning or probing. PCR amplification was performed as described previously (Sebbane *et al.*, 2001) with AmpliTaq Gold polymerase (Perkin-Elmer Applied Biosystems) and PCR products were purified using a Qiaquick PCR purification kit (Qiagen).

Total RNA was extracted with the RNeasy kit (Qiagen). mRNAs were reverse-transcribed into cDNA from 1 μ g nucleic acid by using specific primers and Transcriptor reverse transcriptase, according to the manufacturer's instructions (Roche). The resulting cDNAs were amplified using the primer sets described in Table 3 and the Power SYBR Green PCR master mix. Amplimers were detected on a Prism 7300 detection system (Applied Biosystems). The 16S *rrn* gene was used for normalization. On completion of PCR amplification, a DNA melting curve analysis was performed to confirm the presence of a single amplicon. Relative mRNA levels ($2^{-\Delta\Delta C_t}$) were determined by comparing (i) the PCR cycle thresholds (Ct) for the gene of interest

and 16S *rrn* (ΔC), and (ii) Ct values in the absence and presence of iron ($\Delta\Delta C$).

Gene mutagenesis and complementation. pUC18 plasmid derivatives containing an inactivated copy of the microbial gene of interest were generated as follows: briefly, each construct was obtained by fusing the upstream and downstream regions of the PCR-amplified target gene with the 5' and 3' ends of the kanamycin-resistance gene *aphA-1a*, respectively. DNA inserts were then subcloned into the suicide vector pCVD442 (Donnenberg & Kaper, 1991). Allelic exchange was carried out by mating *E. coli* SM10 λ pir with *Y. pseudotuberculosis*. *Yersinia* transconjugants were screened on LB medium supplemented with irgasan. Concomitantly, ampicillin and kanamycin were also added in order to select the first recombination event. To select for the second recombination event, the presence of pCVD442 (which bears the levane sucrose-encoding gene *sacB*) was counter-selected on LB agar plus 10% sucrose. Ampicillin-susceptible and kanamycin-resistant clones were isolated, and the expected mutations were checked by Southern blot analysis with appropriate DNA probes. Mutants were *cis*-complemented with wild-type genes borne on the suicide plasmid pCVD442, with merodiploids that resulted from homologous recombination being selected on LB agar plus ampicillin.

Genomic library construction. Partially digested *Sau*3AI fragments (5–10 kb) from the *Y. pseudotuberculosis* IP32777 genome were inserted into the *Bam*HI restriction site of pSA, a low-copy-number derivative of pACYC184; this avoids the cloning of genes that are

Table 3. *Yersinia*-specific oligonucleotide primers used in this study

F, Forward; R, reverse.

Amplified gene	Primer designation	Primer sequence (5'→3')*	Primer position†	Reference or source
16S <i>rrn</i>	F10	AGCCAGCGGACCACATAAAG	1244–1263	This work
	R10	AGTTGCAGACTCCAATCCGG	1275–1294	
<i>inv</i>	F9	GACGGTTAATTTACGGCAGAT	2057–2078	This work
	R9	GGTGTGGAGACGGTGAAGCTG	2102–2122	
<i>pmrF</i>	F11	CGGCGGACGACAGAGATT	576–593	This work
	R11	GGCTATATTTGGAGTTGCCAAATT	616–639	
	F15	CGCTTAGATATATTTATTGGC	–329–349	
<i>yfeA</i>	R15	TAATAAATCCCTTAGGACACC	–1–21	This work
	F8	GAATATCCGCAAAGCGTTAGTTG	35–54	
	R8	CATAGGCTTGCGCGTTACG	902–921	
<i>yopH</i>	YopH 1	CATCGTCAGGTATCTCGA	102–119	Carnoy <i>et al.</i> (2000)
	YopH 2	CAATCAGTTGCGCAGTAC	1303–1320	
YPTB0331	F4	AAAAGAGCTCATGTTGCTTGTGGTGACACC	–495–476	This work
	R4	GCAGTCGCTGATCACCTCATGCATAATAACCGCAGGTAAAGG	48–65	
	F6	GCCTACTACTGGGACGATTTTCG	1011–1032	
	R6	GAACGTCTCCAACAGGTAACTTTATC	1056–1082	
	F13	AAAACCCGGGTCTTCGAGCCAGTTGCTGCG	–462–443	
	R13	AAAAGGTACCTTTAACTCGGGCCAGCTCA	1–20	
	F17	ATAGGCTTATTATATAAG	–281–264	
	F18	TCCCTGTTACCTTTTATC	–69–52	
	F21	AAAAAAGCTTGAGCTCCAGCTTGCTTTTGGAGGTGC	–24–5	
	R21	AAAACCTGCAGAAACCAGCAATTATGCCCCC	351–370	
YPTB0332	F5	CCTTTACCTGCGGTTATTATGCATGAGGTGATCAGCGACTGC	147–164	This work
	R5	AAAAGAGCTCCAAATCTCATTATCCTGACG	345–364	
YPTB0333	F1	AAAAGAGCTCGCATTTAGCGCAAAATAAATAAT	24–47	This work
	R1	AAAAGAGCTCATGAGATTTGATAATCTGCTCTCC	952–975	
	F2	CGACATCATACTGTGTAAG	–226–246	
	R2	AGGCTCCTTCCAACCTGCTTG	1005–1024	
	F3	AAAACATATGTCAATAGATGAAAACAC	3–19	
	R3	AAAACCTCGAGGGATAATGAGATTTGATAATCTGC	957–980	
	F7	GCGCGCCAACCTCTTTT	252–268	
	R7	TGCCCCTGTGGGTGGTTAT	307–325	
	F12	AAAACCCGGGCCCCAGTTCTTACCTAAATA	–465–446	
	R12	AAAAGGTACCAAGTGTTCATCTATTGACA	1–20	
	F19	GTTCTTACCTAAATATTGCG	–460–441	
	F20	AATCAGTACAGTTATCAGA	–425–407	
	F22	AAAAGAGCTCAACACTTTTGCATTTAGCGC	–34–15	
	R22	AAAACCTGCAGCTACCGATAAACAGACATCG	404–423	

*Targeted gene-specific sequences are in upper-case type and additional nucleotides at the 5' end recognized by a restriction endonuclease (*Hind*III for F21; *Kpn*I for R12 and R13; *Nde*I for F3; *Pst*I for R3, R21 and R22; *Sac*I for F1, R1, F4, F21 and F22; *Sma*I for F12 and F13) are underlined.
†Relative to the start codon.

potentially deleterious to bacteria when present in a high copy number. Thereafter, *E. coli* EC100 was transformed with recombinant plasmids and the transformants were screened on IPTG-X-Gal agar. Plasmids were extracted from white colonies and introduced (by electroporation) into *Y. enterocolitica* IP383. Recombinant bacteria were selected on LB agar with chloramphenicol.

Polymyxin B activity. The bactericidal assay was performed with *Yersinia* cells from cultures in LB broth (which naturally contains iron) at 28 °C for 16–18 h (OD₆₂₀ 0.3), followed by an additional 3 h

incubation at 37 °C in LB broth with or without 300 µM of the iron chelator 2,2'-dipyridyl. First, 10 µl of serial dilutions in distilled water of polymyxin B (50 mg ml^{–1} in sterile distilled water, 0.1 % BSA, 0.01 % acetic acid) was distributed into microtitre plate wells. Thereafter, 100 µl *Yersinia* cell suspension was added to each well (final bacterial concentration 2.5 × 10⁶ c.f.u. ml^{–1}). As a control, bacterial suspensions were concomitantly added to wells containing 100 µl incubation medium lacking polymyxin B. Microtitre plates were shaken gently and then incubated at 37 °C for 2 h. To assess *Yersinia* viability, 50 µl mixture from each well was serially diluted in

sterile distilled water. Diluates were plated on LB and incubated at 28 °C for 48–72 h. Bacterial survival was defined as the ratio of the number of viable bacteria after a 2 h contact with polymyxin B to the number of viable bacteria in the absence of the antimicrobial peptide.

The polymyxin-binding assay was performed by using dansyl-polymyxin B (Moore *et al.*, 1986). Bacteria were grown at 37 °C for 3 h. After centrifugation, the cell pellets were suspended in sterile water and 2×10^6 *Yersinia* were incubated for 15 min at 37 °C with 1 µg dansyl-polymyxin B. Bacteria were examined with a Zeiss microscope equipped with suitable filters for fluorescence emission at 485 nm. Fluorescence intensity was quantified using ImageJ software.

Measurement of antimicrobial peptide MICs. MICs of reported antimicrobial peptides (Friedrich *et al.*, 1999, 2000; Gudmundsson *et al.*, 1996; Miyata *et al.*, 1989; Selsted *et al.*, 1992) were determined using a standard microtitre dilution assay in Mueller–Hinton (MH) medium. Briefly, cells were grown overnight at 28 °C in MH broth and diluted in the same medium. Twofold serial dilutions (in 0.01 % acetic acid/0.2 % BSA) of the peptides were added to the microtitre plates and then bacterial suspensions were added (final inoculum $1\text{--}5 \times 10^5$ c.f.u. ml⁻¹). Plates were incubated at 28 °C for 24 h and the MIC was determined as the lowest peptide concentration that inhibited bacterial growth.

Purification of His-tagged recombinant proteins. The recombinant BL21-DE3 *E. coli* strain harbouring pET-YPTB0333 was grown in 1 l LB medium supplemented with kanamycin (50 µg ml⁻¹). When the OD₆₀₀ reached 0.8, expression of the gene encoding the recombinant protein was induced with 1 mM IPTG for 1 h. The cells were harvested by centrifugation and then resuspended in 5 ml lysis buffer (300 mM NaCl, 50 mM Na₂HPO₄, 5 mM imidazole, pH 8) per gram fresh weight. Cells were lysed using a French press at a pressure of 1000 p.s.i. (6900 kPa). The lysate was clarified by centrifugation at 10 000 g for 20 min. The supernatant was loaded on a Hi-Trap HP chelating column (GE Healthcare). The column was washed first with standard lysis buffer (containing 5 mM imidazole) and then with lysis buffer containing 20–50 mM imidazole. Elution was performed with lysis buffer containing 200 mM imidazole. The collected fractions were analysed by SDS-PAGE, using a 10 % polyacrylamide gel and Coomassie Blue staining, and by Western blot analysis with an anti-polyhistidine antibody (H1029, Sigma). The purified YPTB0333 protein was dialysed against the DNA-binding buffer [10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 5 % (v/v) glycerol, 10 mM NaCl, 1 mM MgCl₂, 100 mg BSA ml⁻¹].

Electrophoretic mobility shift assay. Purified PCR-generated DNA fragments were incubated for 20 min at room temperature with purified protein in DNA-binding buffer in a 20 µl volume (Heroven & Dersch, 2006). Next, the reaction mixture was loaded onto an 8 % polyacrylamide gel and subjected to electrophoresis. The DNA was revealed by ethidium bromide staining.

Mouse infection. Experiments with 6-week-old, female BALB/c mice (purchased from Charles River) were performed in an accredited establishment (the Institut Pasteur de Lille) in accordance with current European legislation and guidelines (number 86/609/EEC) and following approval by the local ethics committee (file number 04-10-003). For each experimental murine infection with *Y. pseudotuberculosis*, the presence of the pYV virulence plasmid in mutant and wild-type strains was confirmed by PCR on total bacterial DNA using YopH 1 and YopH 2 primers (which correspond to sequences within *yopH*, a gene located on virulence plasmid pYV) (Carnoy *et al.*, 2000). Bacterial inocula were prepared from overnight cultures in LB broth at 28 °C. The cultures were centrifuged and the bacterial pellets were washed once and suspended in distilled water or PBS (Invitrogen). Mice were challenged either intragastrically (0.2 ml bacterial suspension in sterile

distilled water) using a gastric tube or intravenously (0.3 ml bacterial suspension in sterile PBS). Mice were fasted for 18 h prior to gastric inoculation. Infected animals were kept in positive-pressure cabinets throughout the experiment. Mortality was monitored twice daily for up to 21 days post-challenge. The survival kinetics and survival rates for mutant- and wild-type-infected mice were compared statistically using the log-rank test. In order to assess bacterial colonization of the digestive tract, five infected animals were killed at day 3 post-inoculation and *Yersinia* were counted by spreading dilutions of Peyer's patches and mesenteric lymph node homogenates on LB agar. The c.f.u. numbers in lymphoid tissues of mutant- and wild-type-infected animals were compared using the Mann–Whitney statistical test.

RESULTS

Polymyxin B resistance is induced by iron starvation in *Y. pseudotuberculosis* but not in *Y. enterocolitica*

We had previously observed that when exponentially growing *Y. pseudotuberculosis* IP32777 was inoculated into growth medium supplemented with the iron chelators desferral or 2,2-dipyridyl, the resistance of the bacterium to polymyxin B increased about 20-fold (Marceau *et al.*, 2004). In the present study, we assessed whether this phenotype could also be observed in the closely related enteropathogenic species *Y. enterocolitica* (Bottone, 1997) under iron availability limitation conditions. Since this micro-organism is more susceptible to polymyxin B than *Y. pseudotuberculosis* (Bengoechea *et al.*, 1998), we used lower concentrations of the antibiotic in our *in vitro* assay. As shown in Fig. 1(a), the polymyxin B resistance of *Y. enterocolitica* IP383 did not change according to whether iron was complexed or not in the culture medium. We thus hypothesized that *Y. pseudotuberculosis*, but not *Y. enterocolitica*, possesses a polymyxin B-resistance mechanism involving one or more genes whose expression is induced by iron starvation.

To identify these genes, we complemented *Y. enterocolitica* IP383 with a random genomic library from *Y. pseudotuberculosis* IP32777 in low-copy-number plasmid pSA, a pACYC184 derivative that did not change the *Y. enterocolitica* polymyxin B-resistance phenotype (results not shown). The resulting recombinant bacteria were assessed for polymyxin B susceptibility in the presence and absence of iron. Of the approximately 10 000 transformants tested, one (designated Ye5.1) displayed a 50-fold increase in polymyxin B resistance in iron-depleted growth medium. Furthermore, the intrinsic resistance level of the transformant was around 1000-fold higher than that of the *Y. enterocolitica* wild-type strain (Fig. 1b).

Adjacent *Y. pseudotuberculosis* chromosomal genes YPTB0331–0332 and YPTB0333 confer inducible polymyxin B resistance in *Y. enterocolitica*

Plasmid 5.1 was extracted from *Y. enterocolitica* and the 5' and 3' ends of the *Y. pseudotuberculosis* DNA insert were

Fig. 1. Polymyxin B resistance of iron-starved *Y. pseudotuberculosis* and *Y. enterocolitica*. (a) *Y. pseudotuberculosis* IP32777 and *Y. enterocolitica* IP383 were incubated at 37 °C under normal (white bars) or iron-restricted (hatched bars) conditions, with various amounts of polymyxin B. Bacterial survival was assessed after a 2 h incubation period. Each bar represents the mean value (+SD) of three independent experiments. *Y. pseudotuberculosis* and *Y. enterocolitica* survival rates in the presence of polymyxin B were compared using the Mann–Whitney test. Asterisks indicate significant differences ($P < 0.05$). (b) Recombinant *Y. enterocolitica* strains harbouring pSA or pSA5.1 were incubated at 37 °C under normal (white bars) or iron-restricted (hatched bars) conditions with 3125 or 12500 μg polymyxin B ml^{-1} . Bacterial survival was assessed after a 2 h incubation period. Each bar represents the mean value (+SD) of three independent experiments. Survival rates in the presence of polymyxin B were compared using a Mann–Whitney test. Asterisks indicate significant differences ($P < 0.05$).

sequenced. Nucleotide sequence alignment of the insert termini with the whole genome of *Y. pseudotuberculosis* strain IP32953 (Chain *et al.*, 2004) revealed that the *Y. pseudotuberculosis* 5.1 region was a 7.5 kb fragment located on the bacterial chromosome from nucleotide position 392 841 through to 400 738. On the basis of restriction endonuclease analyses, we determined that the fragment is organized in the same way as in strain IP2353 and comprises nine genes (*YPTB0327* to *YPTB0335*), with those at each extremity being truncated (Fig. 2). *YPTB0327* to *YPTB0330* belong to a polycistronic unit (composed of 18 other genes that were not included in the 5.1 insert) encoding the chromosomal type III secretion system Ysa (Chain *et al.*, 2004; Foultier *et al.*, 2002). *YPTB0331* and *YPTB0332* overlap and are probably co-transcribed, as suggested by promoter prediction analysis. *YPTB0333* is adjacent to *YPTB0331* and *YPTB0332* but is transcribed in the opposite direction.

In order to define which of these genes are required for inducible polymyxin B resistance under iron-starvation conditions, we subcloned the 5.1 region into plasmid pSA and phenotyped the five resulting *Y. enterocolitica* subclones. Plasmid pSA5.1.3 (including *YPTB0331–0332–0333*) was

necessary and sufficient for iron-induced resistance to polymyxin B to the same extent as the entire 5.1 region (Fig. 2). In contrast, neither plasmid pSA5.1.1 (containing *YPTB0331–332*) nor pSA5.1.2 (harbouring *YPTB0333*) conferred such a phenotype. *YPTB0331* encodes a putative transporter component with 10 transmembrane segments and shares homology with efflux pump proteins of the major facilitator superfamily (MFS) (Pao *et al.*, 1998). The product of *YPTB0332* belongs to a protein family of unknown function, whereas that of *YPTB0333* displays similarities with transcriptional regulators from the LysR family (Maddocks & Oyston, 2008; Schell, 1993).

***Y. enterocolitica* harbouring *YPTB0331–0333* does not bind polymyxin B**

One of the main bacterial targets of polymyxin B is LPS (Schindler & Osborn, 1979; Vaara & Viljanen, 1985), and so we decided to compare polymyxin B binding to wild-type *Y. enterocolitica* and *Y. enterocolitica* harbouring plasmid pSA5.1. This was achieved by fluorescence microscopy analysis of microbial cells incubated with dansyl-polymyxin B, a polymyxin B derivative that

Fig. 2. Polymyxin B resistance of *Y. enterocolitica* 5.1 subclones under iron-limitation conditions. The genetic organization of the 7.5 kb *Y. pseudotuberculosis* region 5.1 is shown and the inducible polymyxin resistance conferred by the chromosomal segment is mapped.

demonstrates an increase in (green) fluorescence after it binds to LPS but is weakly fluorescent when free in solution. As depicted in Fig. 3, wild-type cells fluoresced after incubation with dansyl-polymyxin B, whereas recombinant 5.1 cells did not. From this experiment, we conclude that *YPTB0331*, *YPTB0332* and *YPTB0333* may modify the *Yersinia* surface and thus decrease the binding of polymyxin B.

Only the *YPTB0333* knockout is associated with impaired polymyxin B resistance in *Y. pseudotuberculosis*

To confirm the involvement of *YPTB0331–0332* and *0333* in *Y. pseudotuberculosis* polymyxin B resistance, two mutants were engineered from strain IP32777 via (i) deletion of both *YPTB0331* and *YPTB0332* and (ii) disruption of *YPTB0333*. Their differential susceptibility to polymyxin B (presented in Fig. 4) indicated that only *YPTB0333* inactivation leads to impaired bacterial resistance to this compound. *cis*-Complementation of the *YPTB0333* mutant (resulting from chromosomal integration of pCVD442, bearing *YPTB0333*) restored the wild-type phenotype and thus ruled out a polar effect of a *YPTB0333* mutation. Secondly, a triple mutant (in which *YPTB0331–0332* and *YPTB0333* had been inactivated) displayed the same polymyxin B-resistance phenotype as the *YPTB0333* single mutant strain (data not shown). Interestingly, antimicrobial resistance in the *Y. pseudotuberculosis* *YPTB0333* mutant was still inducible by iron

starvation, suggesting that other iron-inducible resistance gene(s) may exist in this species. Thus, our results indicate that in contrast to *YPTB0331–0332*, *YPTB0333* plays a significant role in resistance to polymyxin B in *Y. pseudotuberculosis*. Nevertheless, the three genes *YPTB0331–0332–0333* are all required for polymyxin B resistance in *Y. enterocolitica*.

***YPTB0333* regulates the *YPTB0331–0332* operon and its own expression in an iron-dependent manner**

We assumed that expression of *YPTB0331–0332* and *YPTB0333* was triggered by iron starvation, and therefore used quantitative RT-PCR (qRT-PCR) to evaluate gene transcript levels in *Y. pseudotuberculosis* under these conditions. Concomitantly, we studied two transcription controls: (i) the iron transporter-encoding gene *yfeA*, which is induced by iron restriction (Bearden *et al.*, 1998), and (ii) the invasin-encoding gene *inv*, which is iron-independent (Heroven *et al.*, 2007). As shown in Fig. 5(a), the *YPTB0331–0332* and *YPTB0333* genes were upregulated when *Y. pseudotuberculosis* was grown in the presence of the iron chelator 2,2'-dipyridyl. We suspected that *YPTB0333* mediates transcription of the upstream operon in iron-starved *Y. pseudotuberculosis*, since it is contiguous with the *YPTB0331–0332* operon (but has opposite polarity) and encodes a product that shares motifs with transcriptional regulators from the LysR family. The strong observed reduction in *YPTB0331–0332* expression after

Fig. 3. Dansyl-polymyxin B binding to *Y. enterocolitica* complemented (or not) with the 5.1 region from *Y. pseudotuberculosis*. Bacteria grown in iron-depleted LB medium were incubated for 15 min with 1 μ g dansyl-polymyxin B and examined by fluorescence microscopy. The figure shows representative photographs of *Y. enterocolitica* bearing (a) pSA and (b) pSA5.1 (i.e. including the *Y. pseudotuberculosis* 5.1 locus) exposed to UV light (upper panels) or not (lower panels). Green dots are assumed to correspond to dansyl-polymyxin B aggregates. Mean fluorescence intensity values ($\pm$ SD) from three independent experiments are indicated in the upper panels.

inactivation of *YPTB0333* (Fig. 5b) was consistent with this hypothesis. Furthermore, it was demonstrated that up-regulation of *yfeA* under iron-starvation conditions was diminished in the *YPTB0333* mutant. Transcriptional regulatory activity of the *YPTB0333* product was definitively proven by demonstrating its binding to the

YPTB0331–0332 promoter (Fig. 5c). Since LysR-type regulators autoregulate their expression (Schell, 1993), we monitored the ability of *YPTB0333* to bind to the *YPTB0333* promoter. As shown in Fig. 5(c) by using an electrophoretic mobility shift assay, *YPTB0333* recognized this promoter in a dose-dependent manner.

Fig. 4. Polymyxin B susceptibility of *YPTB0333* and *YPTB0331–0332* *Y. pseudotuberculosis* mutants. *Yersinia* were incubated at 37 °C in normal (white bars) or iron-starved (shaded bars) Roswell Park Memorial Institute (RPMI) medium with 50 μ g polymyxin B ml⁻¹. Bacterial survival was assessed 2 h later. Each bar represents the mean value ($\pm$ SD) of three independent experiments. Wild-type and mutant survival after polymyxin B treatment was compared using the Mann–Whitney test. *P* values < 0.05 are indicated by asterisks.

Fig. 5. Regulation of *YPTB0331*–*0332* and *YPTB0333* genes. (a) *YPTB0331* and *YPTB0333* expression in iron-starved wild-type *Y. pseudotuberculosis*. Total RNA from wild-type *Y. pseudotuberculosis* grown in standard or iron-depleted LB broth was extracted and reverse-transcribed into cDNA specific for *YPTB0331*, *YPTB0333*, *yfeA* and *inv* genes using the reverse primers (R6, R7, R8 and R9, respectively) described in Table 3. cDNAs were then amplified by real-time PCR with primer sets F6/R6, F7/R7, F8/R8 and F9/R9. Data were normalized against the 16S *rrn* values obtained with primers F10 and R10. Results are presented as the fold increase in gene expression under iron-starvation conditions versus standard conditions. Each bar represents the mean value (+sd) of three independent experiments. Gene expression was compared with that of *yfeA* using the Mann–Whitney test, and asterisks indicate significant differences ($P < 0.05$). (b) *YPTB0331*, *yfeA* and *inv* expression in iron-starved wild-type and YPTB0333-deficient *Y. pseudotuberculosis*. Total RNA from wild-type (white bars) and YPTB0333-deficient (grey bars) *Y. pseudotuberculosis* cultured in standard or iron-depleted LB broth was extracted and reverse-transcribed to cDNA specific for *YPTB0331*, *yfeA* and *inv* with primers R6, R8 and R9, respectively. Next, the cDNA was amplified by real-time PCR with primer sets F6/R6, F8/R8 and F9/R9. The data were normalized against the respective 16S *rrn* values obtained with primers F10 and R10. Results are presented as the fold increase in gene expression under iron-restriction conditions versus standard conditions. Each bar represents the mean value (+sd) of three independent experiments. A Mann–Whitney test was used to compare the two strains in terms of *YPTB0331* and *yfeA* expression, and asterisks indicate a significant difference ($P < 0.05$). (c) YPTB0333 binding to the promoter region of the *Y. pseudotuberculosis* *YPTB0331* and *YPTB0333* genes. Fragments (500 bp) encompassing the putative *YPTB0331* and *YPTB0333* promoters (see Supplementary Fig. S1) were PCR-generated with primers F12/R12 and F13/R13. The figure shows electrophoretic mobility of the ethidium bromide-stained 500 bp fragment alone or mixed with 0.1, 0.2 or 0.4 µg purified YPTB0333. The arrow indicates the highest-molecular-mass YPTB0333–DNA complex. No binding of YPTB0333 to an irrelevant DNA fragment (a 400 bp internal fragment from *Y. pseudotuberculosis* *mltB*) was detected (control) and an excess of the irrelevant DNA fragment did not inhibit YPTB0333 binding to the *YPTB0331* and *YPTB0333* promoters (results not shown). Additionally, a YPTB0333-free protein fraction obtained for the purification procedure of the recombinant protein was unable to alter the mobility of the DNA fragment encompassing the *YPTB0333* promoter (not shown). As shown, YPTB0333 bound specifically to the DNA segment encompassing the putative promoter of the *YPTB0331* and *YPTB0333* genes.

YPTB0333 binds to the promoter region of the *pmrF* operon

One common bacterial surface modification governing polymyxin B resistance in Gram-negative bacteria (including *Yersinia*) is L-4-aminoarabinose substitution of LPS, which contributes to a reduction in the net negative charge of lipid A. This substitution is mediated by a seven-gene polycistronic unit (*pmrHFIJKLM*, the *pmrF* operon) (Gunn *et al.*, 1998). Therefore, we hypothesized that decreased binding of polymyxin B to the cell envelope of iron-restricted *Yersinia* was associated with *pmrF* upregulation. Indeed, in *Y. enterocolitica* trans-complemented with YPTB0331–0332–0333 (i.e. strain Ye5.1, in which dansyl-polymyxin binding was impaired, see Fig. 3), we observed that transcription of the *pmrF* gene of the operon was sevenfold greater than in *Y. enterocolitica* bearing the empty vector pSA (data not shown). Conversely, expression of this gene was diminished by nearly threefold in *Y. pseudotuberculosis* after inactivation of YPTB0333. Although this reduction was not statistically significant [most likely because *pmrF* transcription has been shown to be regulated by other means, such as the two-component system regulator PhoP (Marceau *et al.*, 2004)], YPTB0333

is nevertheless likely to regulate the *pmrF* operon by binding upstream of *pmrH* (Fig. 6).

YPTB0333 mutation decreases *Y. pseudotuberculosis* pathogenicity

In the digestive tract of its host, the enteropathogen *Y. pseudotuberculosis* encounters both iron-limiting conditions and high quantities of antimicrobial peptides. Therefore, we assessed whether inactivation of the above-mentioned polymyxin B-resistance genes could alter *Y. pseudotuberculosis* virulence *in vivo*. Using a murine model of oral infection, we found that inactivation of YPTB0333, but not of YPTB0331–0332, significantly reduced bacterial-induced lethality in mice (Fig. 7a). The mechanism was thought to be due to an increased susceptibility to killing by cationic antimicrobial peptides, as the YPTB0333 mutant demonstrated increased susceptibility to a range of such peptides (Table 4). However, as previously reported (Marceau *et al.*, 2004), the *Y. pseudotuberculosis* *pmrF* operon is dispensable for mouse infection. In contrast, the YPTB0333 mutant (as well as the YPTB0331–0332 mutant) was as virulent as the parent strain when administered

Fig. 6. Regulation of *Y. pseudotuberculosis* *pmrF* gene expression by YPTB0333. (a) *pmrF* expression in iron-starved wild-type and YPTB0333-deficient *Y. pseudotuberculosis*. Total RNA from wild-type and YPTB0333-deficient *Y. pseudotuberculosis* cultured in standard or iron-depleted LB broth was extracted and reverse-transcribed into *pmrF*-specific cDNA with primer R11. Next, the cDNA was amplified using real-time PCR (primers F11/R11). The data were normalized against the 16S *rrn* values obtained with primers F10 and R10. Results are presented as the fold increase in gene expression under iron-restriction conditions versus standard conditions. Each bar represents the mean value (+SD) of three independent experiments. A Mann–Whitney test was used to compare the two strains in terms of *pmrF* expression. (b) YPTB0333 binding to the promoter region of the *Y. pseudotuberculosis* *pmrF* operon. A 400 bp fragment encompassing the putative *pmrF* promoter region was PCR-generated with primers F15/R15. The figure shows the electrophoretic mobility of the ethidium bromide-stained 400 bp fragment alone or mixed with 0.1, 0.2 or 0.4 µg purified YPTB0333. The arrow indicates the highest-molecular-mass YPTB0333–His–DNA complex. An excess of the irrelevant DNA fragment did not inhibit YPTB0333 binding to the *pmrF* promoter (data not shown).

Fig. 7. Oral virulence in the mouse of *YPTB0331–0332* and *YPTB0333* *Y. pseudotuberculosis* mutants. BALB/c mice were fed with 5×10^8 *Yersinia*. (a) The survival kinetics and survival rate for 12 individuals infected by either the wild-type or the mutant strain are depicted and were compared statistically using the logrank test. (b) Bacterial colonization of Peyer's patches and mesenteric lymph nodes at day 3 post-inoculation. Individual bacterial counts are shown (bars represent the mean value) and were compared using the Mann–Whitney test. *P* values <0.05 are indicated by asterisks.

intravenously (data not shown). The decreased oral virulence of the *YPTB0333* mutant was due to its lower ability to colonize the Peyer's patches and the mesenteric lymph nodes at an early stage of gut infection, since 1000-fold fewer bacteria were recovered from intestinal lymphoid tissues (Fig. 7b). Taken together, our results indicate that the *YPTB0333* gene product is a *Y. pseudotuberculosis* virulence factor.

DISCUSSION

It has been known for a decade that the two human pathogenic species *Y. pseudotuberculosis* and *Y. enterocolitica* have differential susceptibility to bactericidal polycationic peptides, with the latter being more susceptible (Bengoechea *et al.*, 1998). However, the genetic differences supporting this phenotypic feature have remained obscure. In the present study, we describe a previously uncharacterized *Y. pseudotuberculosis* chromosomal locus contain-

ing two potential effector genes (along with their cognate transcriptional regulatory gene) and show that this locus is involved in polymyxin B resistance. It also contributes to resistance to α -helical antimicrobial peptides and polyphemusin. Transfer of this locus into *Y. enterocolitica* rendered this bacterium significantly more resistant to the action of polymyxin B.

In line with this observation, an inter-species genomic comparison revealed that *YPTB0331* and *YPTB0332* are present (but strongly altered) in *Y. enterocolitica*. In the O8 serogroup strain 8081 sequenced genome (Thomson *et al.*, 2006), *Ye0314* (the *YPTB0332* counterpart) is intact but *Ye0313* (the *YPTB0331* counterpart) is truncated at its 5' end. Furthermore, no potential *YPTB0333* orthologue has been detected. Identical results were observed in PCR assays (using primers defined from the strain 8081 genomic sequence) carried out on a broad range of *Y. enterocolitica* representatives, including the IP383 (O9 serogroup) strain used in this study (data not shown). *In silico* analysis of the

Table 4. Susceptibility of *Y. pseudotuberculosis* *YPTB0333* mutant to six antimicrobial peptides

Strain	MIC ($\mu\text{g ml}^{-1}$)					
	CP26	CP29	LL-37	Polyphemusin	Bac2A	Indolicidin
Wild-type	>512	64	64	8	>512	16
<i>YPTB0333</i> mutant	64	2	32	1	>512	16

Ye0313 promoter region also revealed that its sequence differs from that of YPTB0331, raising the possibility that Ye0313 and Ye0314 are not transcribed at all; the fact that the complete *Y. pseudotuberculosis* locus (instead of YPTB0333 alone) is required to give *Y. enterocolitica* strain IP383 a polymyxin B-resistance level similar to that observed in *Y. pseudotuberculosis* is consistent with this hypothesis.

Polymyxin B resistance in *Y. pseudotuberculosis* can be mediated by the *pmrF* operon (Marceau *et al.*, 2004). This polycistronic unit comprises seven genes (*pmrHFJKLM*) required for the substitution of lipid A with L-4-aminoarabinose (Gunn *et al.*, 1998). The products of the *pmrH*, *-F*, *-I*, *-J* and *K* genes catalyse the biosynthesis and attachment of this cationic sugar to the lipid A of LPS. The *pmrL* and *pmrM* products belong to the drug/metabolite exporter family and have been recently shown to form a heterodimer that transfers the L-4-aminoarabinose linked to the undecaprenyl phosphate of the lipid from the cytoplasmic to the periplasmic side of the inner membrane. In *E. coli*, *pmrL* and *pmrM* inactivation is not enough to abolish L-4-aminoarabinose export to the periplasm (Yan *et al.*, 2007); additional, unidentified drug/metabolite exporters may thus exist. YPTB0331 is predicted to contain 10 membrane-spanning peptide segments and transporter-specific motifs, and thus falls into the MFS, which contains members capable of exporting molecules of various sizes through the inner membrane (Pao *et al.*, 1998). This raises the possibility that YPTB0331 participates in the *pmrF* operon-driven polymyxin B-resistance mechanism as an accessory L-4-aminoarabinose exporter. In *Y. pseudotuberculosis*, both *pmrF* and YPTB0331/2 operons are regulated by YPTB0333 in a coordinated manner, suggesting that the mechanisms driven by these two distant gene sets may be closely associated in the performance of a common physiological function. However, as we previously reported (Marceau *et al.*, 2004), the former operon is essential for polymyxin B resistance in *Y. pseudotuberculosis*, whereas the latter (as shown in this study) appears to be dispensable. This observation suggests that, in this species, the genetic information supplied by YPTB0331 and YPTB0332 is redundant, due to the existence of either paralogues or genes that rescue the resistance process in other ways. In contrast, this situation was not observed in *Y. enterocolitica*: *pmrF*-dependent resistance to antimicrobial peptides absolutely required YPTB0331 and YPTB0332, and the consequences of the *pmrF* inactivation were detectable after *trans*-complementation with the complete locus but not with the regulatory gene YPTB0333 alone (data not shown). In addition, our preliminary results revealed that *pmrF* transcription levels are similar (and PhoP–PhoQ dependent) in both species under the conditions tested here. Taken as a whole, these data rule out the hypothesis of less efficient transcription of the *pmrF* operon in *Y. enterocolitica*, but raise the possibility that products of the *pmrF* operon and those of YPTB0331 and YPTB0332 participate in a common antimicrobial

resistance process. In *Y. enterocolitica*, PmrL- and PmrM-like proteins are less than 80 % identical to their respective *Y. pseudotuberculosis* counterparts and thus may be less efficient in the former species. This is consistent with the possibility that YPTB0331 and YPTB0332 are involved in a supplementary means of L-4-aminoarabinose export through the inner membrane.

Resistance mechanisms to antimicrobial peptides are often activated in response to potentially membrane-damaging environmental conditions, such as abnormal pH, temperature and metal ion concentrations. The effects of Mg^{2+} scarcity on regulation of polymyxin B resistance pathways have been extensively studied, especially in *Salmonella enterica*. More recently, it has been reported that an excess of Fe^{3+} is associated with greater antimicrobial resistance in this bacterial model (Wösten *et al.*, 2000). Like the process induced by Mg^{2+} deprivation, the high-iron-induced polymyxin B resistance process has been shown to require the *pmrF* operon (Gunn *et al.*, 1998), and to involve complex and partially overlapping regulatory mechanisms. Here, for the first time to our knowledge, we provide evidence to suggest that low iron levels also trigger *pmrF*-mediated bacterial resistance to antimicrobial peptides. The fact that the genetic locus in question is absent or only partially present in sequenced bacterial genomes (including *Salmonella*) suggests that this mechanism is available in only a limited number of *pmrF* operon-harbouring species.

Based on the presence of a helix–turn–helix motif and a typical substrate-binding domain, YPTB0333 can be classified within the LysR transcriptional regulator family, along with the family prototype (YPTB3046), RovM (YPTB2588) and 17 other putative regulators detected in the *Y. pseudotuberculosis* strain IP2953 genome. The most similar homologous protein (found in another enterobacterial species, *Citrobacter koseri*) displays only 50 % identity with YPTB0333, suggesting that this regulator is at least specific to yersiniae if not unique to *Y. pseudotuberculosis* and its derivative species *Yersinia pestis*. According to our data, the activity of YPTB0333 depends strongly on iron levels. Amino acid sequence analysis of the putative YPTB0333 substrate-binding domain revealed a particular ExxE motif that is also present in several iron-binding eukaryotic proteins and thought to be involved in iron recognition in PmrB, the sensor of the bacterial two-component system PmrA–PmrB (Wösten *et al.*, 2000). This finding, combined with the fact that YPTB0333 transcription and autoregulation depend on iron, raises the hypothesis of a direct interaction between this regulator and the metal in question (which, in such a situation, would behave as an anti-inducer). YPTB0333 is able (at least indirectly) to positively regulate *yfe* operon transcription, whereas Fur represses this polycistronic unit. This observation indicates that these two regulators have overlapping spheres of influence (at least in this respect) and that YPTB0333 might function as a Fur antagonist. Experiments seeking to demonstrate such a property are currently in progress.

YPTB0333 controls the transcription level of four separate loci that mediate at least two apparently distinct physiological processes (i.e. iron uptake and resistance to antimicrobial peptides), and hence may be considered to be a pleiotropic regulator. The RSAT DNA-pattern tool (<http://rsat.bigre.ulb.ac.be/rsat/>) predicted a consensus DNA-binding motif (CCAkGAAGGy) by alignment with YPTB0333-targeted promoters. A search for this motif within the *Y. pseudotuberculosis* IP2953 genome sequence revealed the existence of at least eight other genes (two of which are absent in the *Y. enterocolitica* genome) putatively controlled by YPTB0333 (see Supplementary Table S1). A comparative transcriptome analysis of the wild-type and the YPTB0333 mutant might provide more information on this point.

Since inactivation of YPTB0333 attenuates bacterial colonization in mice infected by the oral route, it is also a virulence factor; however, YPTB0333 is not required for bacterial systemic infection. This finding might be related to differences in gut and blood antimicrobial peptide contents. Indeed, cryptdins are secreted in large amounts in the intestinal lumen of mice (Ayabe *et al.*, 2000), whereas these defensins are not produced by murine polymorphonuclear leukocytes (Eisenhauer & Lehrer, 1992), phagocytes which are essential for host defence against *Y. pseudotuberculosis*. As we previously reported (Marceau *et al.*, 2004), the *Y. pseudotuberculosis* *pmrF* operon is dispensable for mouse infection and, consistently, the results presented here show that YPTB0331 and YPTB0332 are also not necessary. A contribution of the YPTB0333-regulated *yfe* operon [required for deep organ colonization by *Y. pestis* (Bearden *et al.*, 1998)] to systemic infection can also be ruled out, since the YPTB0333 mutant is as virulent as the wild-type after an intravenous challenge; however, involvement of *yfe* in oral infection is still an open question. It now remains to identify virulence genes belonging to the YPTB0333 regulon.

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