

# The iron- and temperature-regulated haemolysin YhlA is a virulence factor of *Yersinia ruckeri*

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*Yersinia ruckeri* causes the enteric redmouth disease or yersiniosis, an important systemic fish infection. In an attempt to dissect the virulence mechanisms of this bacterium, a gene encoding a putative protein involved in the secretion/activation of a haemolysin (*yhIB*), which had been previously identified by *in vivo* expression technology, was further analysed. The gene *yhIB* precedes another ORF (*yhIA*) encoding a *Serratia*-type haemolysin. Other toxins belonging to this group have been identified in genomic analyses of human-pathogenic yersiniae, although their role and importance in pathogenicity have not been defined yet. In spite of its being an *in vivo*-induced gene, the expression of *yhIA* can be induced under certain *in vitro* conditions similar to those encountered in the host, as deduced from the results obtained by using a *yhIB* : *lacZY* fusion. Thus, higher levels of expression were obtained at 18 °C, the temperature of occurrence of disease outbreaks, than at 28 °C, the optimal growth temperature. The expression of the haemolysin also increased under iron-starvation conditions. This confirmed the decisive role of iron and temperature as environmental cues that regulate and coordinate the expression of genes encoding extracellular factors involved in the virulence of *Y. ruckeri*. LD<sub>50</sub> and cell culture experiments, using *yhIB* and *yhIA* insertional mutant strains, demonstrated the participation of the haemolysin in the virulence of *Y. ruckeri* and also its cytolytic properties against the BF-2 fish cell line. Finally, a screening for the production of haemolytic activity and the presence of *yhIB* and *yhIA* genes in 12 *Y. ruckeri* strains proved once more the genetic homogeneity of this species, since all possessed both haemolytic activity and the *yhIB* and *yhIA* genes.

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## INTRODUCTION

The Gram-negative bacterium *Yersinia ruckeri* is the aetiological agent of the enteric redmouth disease, which affects mainly salmonid fish. Despite the general administration of a quite effective vaccine, outbreaks still occur, produced mainly by certain strains (Austin *et al.*, 2003; Fouz *et al.*, 2006). Therefore, this pathology continues to cause important economic losses in the aquaculture industry in many countries.

Early work showed that some extracellular products of *Y. ruckeri*, including several enzymic activities such as protease, lipase and haemolysin, may play an important role in the development of pathogenesis. Romalde & Toranzo (1993) observed that the injection of these products into fish reproduced some characteristic symptoms of yersiniosis such as haemorrhages in the mouth and intestine. Nevertheless, it was only recently that several studies started

to shed light on the specific pathogenicity mechanisms of the enteric redmouth bacterium. For example, it has been demonstrated that the protease Yrp1, produced by the so-called Azo<sup>+</sup> strains, as well as ruckerbactin, a catecholate siderophore, are involved in virulence (Fernández *et al.*, 2002, 2003, 2004).

With the aim of achieving a better understanding of the precise virulence factors possessed by this micro-organism, Fernández *et al.* (2004) used an *in vivo* expression technology (IVET) system to identify genes induced during the infection of fish by *Y. ruckeri*, which would very likely be related to virulence. This technique allowed the identification of 14 different *in vivo*-induced (*ivi*) genetic loci. One of these *ivi* genes encodes a protein putatively involved in the secretion and activation of a *Serratia*-type haemolysin (Fernández *et al.*, 2004). It is well known that haemolysins participate in the pathogenicity of Gram-positive and Gram-negative bacteria and that they sometimes also show a cytolytic activity against different types of nucleated cells. For this reason, it seemed interesting to carry out further analyses on this *in vivo*-induced haemolysin.

Abbreviation: IVET, *in vivo* expression technology.

The GenBank/EMBL/DDBJ accession number for the sequences reported in this paper is AY576533.

This paper reports the study of two genes required for the production of an *in vivo*-induced extracellular cytolysin named YhlA, concerning its regulation by temperature and iron, transcriptional analysis, implication in virulence and cytotoxicity, and presence in *Y. ruckeri* strains of different origins.

METHODS

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in 2 × TY (tryptone/yeast extract) broth and agar, and *Y. ruckeri* strains in nutrient broth (NB) and agar (NA) (Pronadisa). When required, the following compounds were added to the media: 100 µg ampicillin ml<sup>-1</sup>, 50 µg rifampicin ml<sup>-1</sup>, 100 µg 2,2'-dipyridyl ml<sup>-1</sup> or 100 µg FeCl<sub>3</sub> ml<sup>-1</sup>, all of them from Sigma Aldrich. Liquid cultures were incubated at 37 °C for *E. coli* and 18 °C or 28 °C for *Y. ruckeri* in orbital shakers at 250 r.p.m. Growth was monitored by determining the OD<sub>600</sub> at different times during incubation and bacterial concentrations were determined by serial dilutions and plate counts. Experiments on regulation of gene expression were performed using minimal medium M9, prepared as described by Romalde *et al.* (1991). All the glassware needed for the preparation of this medium was previously treated with 1 M HCl to remove iron traces and rinsed with double-distilled water.

**Genetic techniques.** DNA isolation and manipulation were performed as described by Sambrook & Russell (2001). Phage T4 DNA ligase and calf intestinal alkaline phosphatase were purchased from Roche, restriction enzymes from Amersham Pharmacia Biotech, DNA polymerase from Biotools, and oligonucleotides from Sigma Aldrich.

Nucleic acid sequencing was performed by the dideoxy chain-termination method with the DR Terminator kit (Applied Biosystems) in an ABI Prism 310A automated DNA sequencer from Perkin-Elmer at the Universidad de Oviedo facilities. The sequences obtained were analysed with the computer program BLASTX.

The sequence adjacent to the partial fragment of *yhlB* present in clone *iviV* was obtained by inverse PCR. Briefly, genomic DNA from *Y. ruckeri* 150 was digested with *Cla*I or *Sall*, and the generated fragments were religated. The ligation mixture was then used as template DNA for a PCR, using the Long Amplification kit (Biotools) and oligonucleotides corresponding to the known DNA sequence. The reaction was performed in a Perkin-Elmer 9700 GeneAmp thermocycler.

**RT-PCR.** Total RNA was obtained from 3 ml late-exponential-phase cultures of parental strain 150R and mutant 150R*yhlB* grown in M9 supplemented with 2,2'-dipyridyl. RNA was isolated using an RNeasy mini kit (Qiagen) and was treated with RNase-free DNase (Promega) to eliminate traces of DNA. Reverse transcription (RT)-PCRs were performed using Superscript One-Step with Platinum *Taq* (Invitrogen Life Technologies); 20 ng RNA was used in each reaction. Control PCRs using DNA polymerase (Biotools) were performed to determine whether RNA was free of contaminant DNA. The primers used were: A1, 5'-ATATCCGGGCCGAAGGC-3' (nt 911 to 927 of *yhlA*) and A2, 5'-ATTGTCGATCAATAAGC-3' (nt 1848 to 1832 of *yhlA*), for *yhlA* (938 bp); B1, 5'-ATAACCGGTGGAGATCA-3' (nt 472 to 488 of *yhlB*) and B2, 5'-CAGTTATGAGTGCGGT-3' (nt 1206 to 1190 of *yhlB*), for *yhlB* (735 bp); BA1, 5'-GCAGAATACTTTATCGC-3' (nt 1430 to 1447 of *yhlB*) and BA2, 5'-GCTGAAGGTGTCACAAT-3' (nt 185 to 169 of *yhlA*), for a region overlapping *yhlB* and *yhlA* (510 bp); and O1, 5'-ACAGGCAAATTATGGAC-3' (nt 8 to 24 of *orf1*) and O2, 5'-TTATCAACTGGGGTTCA-3' (nt 298 to 282 of *orf1*), for the ORF located downstream of *yhlA* (293 bp).

Table 1. Bacterial strains and plasmids

All the *Y. ruckeri* strains belong to serotype O1, the most virulent, except 956, which belongs to serotype O2.

Strain or plasmid	Characteristics	Source or reference
<i>Yersinia ruckeri</i>		
146, 147, 148, 149, 150	Trout-isolated virulent strains (Danish fish farms)	J. L. Larsen (University of Frederiksberg, Denmark)
35/85, 13/86	Trout-isolated virulent strains (Danish and UK fish farms, respectively)	C. J. Rodgers (University of Tarragona, Spain)
955 (11.4), 956 (11.29), 43/19	Trout-isolated virulent strains (USA fish farms)	CECT (Spanish Type Culture Collection)
Al00, Al02	Trout-isolated virulent strains (Spanish fish farms)	I. Márquez (Laboratory of Animal Health, Asturias, Spain)
150R	Rif <sup>r</sup> derivative of 150	Fernández <i>et al.</i> (2002)
150R <i>iviV</i>	Strain containing fusion between <i>yhlB</i> promoter and <i>lacZY</i>	Fernández <i>et al.</i> (2004)
150R <i>yhlA</i>	Rif <sup>r</sup> <i>yhlA</i> ::pIVET8 Ap <sup>r</sup>	This study
150R <i>yhlB</i>	Rif <sup>r</sup> <i>yhlB</i> ::pIVET8 Ap <sup>r</sup>	This study
<i>Escherichia coli</i>		
SM10λ <i>pir</i>	<i>thi thr leu tonA lacY supE recA</i> ::RP4-2-Tc::Mu Km::λ <i>pir</i>	Simon <i>et al.</i> (1983)
<b>Plasmids</b>		
pIVET8	Ap <sup>r</sup> , oriR6K, mob <sup>+</sup>	Mahan <i>et al.</i> (1995)
pLPY5	pIVET8::BglII ( <i>yhlA</i> ), Ap <sup>r</sup>	This study
pLPY6	pIVET8::BglII ( <i>yhlB</i> ), Ap <sup>r</sup>	This study

**In vitro regulation analysis.** For promoter expression studies, *Y. ruckeri* 150RiviV was grown in M9 supplemented with either FeCl<sub>3</sub> or 2,2'-dipyridyl. To determine the influence of temperature, cells were incubated at 18 °C or 28 °C. Samples from stationary-phase cultures, under these conditions, were collected and their  $\beta$ -galactosidase activity measured as described by Miller (1972). The results were then submitted to an analysis of variance test and *P* values <0.05 were considered significant.

**Construction of insertion mutants.** Internal fragments of the predicted ORFs of *yhIB* (735 bp) and *yhIA* (938 bp) were amplified by PCR with the following primers: forward primers *yhIB*1 and *yhIA*1 (5'-GCAGAGATCTATAACCGGTGGAGATCA-3', nt 472 to 488 in bold type, and 5'-GTCAAGATCTATATCCGGGC-GGAAGGC-3', nt 911 to 927 in bold type), respectively, and reverse primers *yhIB*2 and *yhIA*2 (5'-CTGTAGATCTCAGGTTATGAG-TGCGGT-3', nt 1206 to 1190 in bold type, and 5'-GACCAGATCTATTGTCGATCAATAAGC-3', nt 1848 to 1832 in bold type), respectively. All primers contained a *Bgl*II site (in italics) and four additional bases at their 5' end. The generated amplicons were digested with *Bgl*II and ligated into pIVET8, previously digested with the same enzyme and dephosphorylated. The ligation mixture was utilized to transform by electroporation competent cells of *E. coli* SM10 $\lambda$ pir.

Clones containing the vector with each insert were used to transfer the recombinant plasmids to *Y. ruckeri* 150R by filter mating, as described by Fernández *et al.* (2002). The mutation of the target gene in the transconjugants was verified by Southern blot analysis. Probe labelling, hybridization and developing were performed with the DIG DNA labelling and detection kit from Roche, following the manufacturer's instructions. To check that gene interruption had taken place as expected, DNA from the mutant and parental strains was prepared and digested with *Bam*HI or *Clal* for *yhIB* and *yhIA*, respectively. After separation of the restriction fragments in an agarose gel, these were transferred to a nylon membrane and hybridized with probes corresponding to each gene. This digestion produced bands of approximately 10 and 4 kb in the *yhIA* mutant strain and 14 and 4.5 kb in the *yhIB* mutant when hybridizing with the respective digoxigenin-labelled probes, instead of the 4 and 16 kb single fragments that appear, respectively, in the parental strain. Since pIVET8 contains internal *Bam*HI and *Clal* sites (Fig. 1), these patterns of hybridization demonstrated that plasmid pLPY5 or pLPY6 (Table 1) harbouring internal fragments of *yhIA* or *yhIB*, respectively, was integrated in the chromosome by a single crossover event and, therefore, that the desired mutations had occurred.

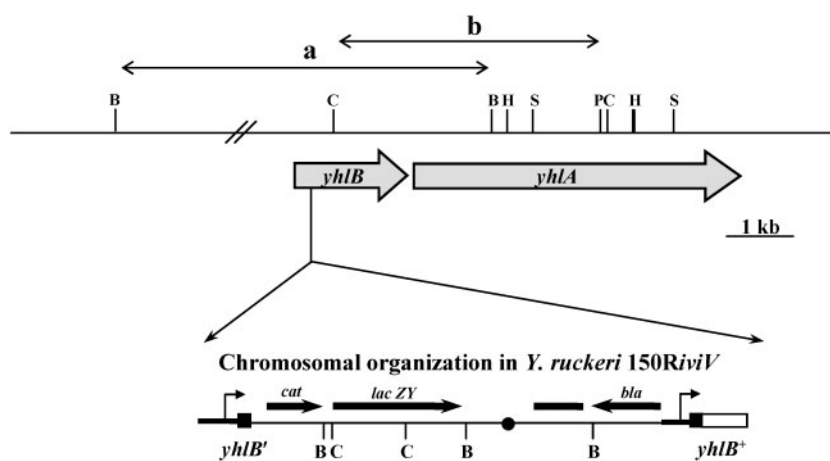
**Phenotypic characterization of the mutant strains.** Mutant clones were tested for production of haemolytic activity, using defibrinated sheep blood (Biomedics SL) as described for *Photobacterium luminescens* (Brillard *et al.*, 2002), as well as for their ability to grow in M9 supplemented with either FeCl<sub>3</sub> or 2,2'-dipyridyl. To determine the involvement of haemolysin production in virulence, 50% lethal dose (LD<sub>50</sub>) experiments with the parental and the mutant strains were carried out as described by Fernández *et al.* (2002). The doses injected ranged from 10 to 10<sup>7</sup> c.f.u. per fish and mortalities were followed up for 7 days. The LD<sub>50</sub> values were calculated by the method of Reed & Muench (1938). Additionally, the cytotoxicity of the mutant and parental strains was studied using cultures of the BF-2 (blue gill fry) fish cell line. Cell cultures were grown in 24-well plates at 16 °C in Tagle medium (Sigma) supplemented with 1% (w/v) NaHCO<sub>3</sub> and 10% (v/v) bovine fetal serum. Bacterial cultures were grown in M9 medium with 2,2'-dipyridyl. Before inoculation, bacterial cells were washed three times with PBS. The inoculation was carried out in a 1:1 proportion (5 × 10<sup>5</sup> bacterial cells to each well containing 5 × 10<sup>5</sup> eukaryotic cells, approximately, as determined with a haemocytometer). Control wells were inoculated with PBS. After 48 h incubation at 18 °C, the cultures were observed with a Zeiss Axiovert 200M inverted microscope.

**PCR detection of *yhIB* and *yhIA* in different *Y. ruckeri* strains.** The production of haemolytic activity in cultures of 12 *Y. ruckeri* strains was examined following the method described above (Brillard *et al.*, 2002). The primers used for the detection of *yhIB* and *yhIA* genes were as above.

## RESULTS AND DISCUSSION

### Sequence analysis of the *yhIBA* loci

The application of IVET technology to *Y. ruckeri* led to the identification of 14 different clones, carrying *in vivo*-induced transcriptional fusions between *Y. ruckeri* promoters and the promoterless *cat* and *lacZY* genes (Fernández *et al.*, 2004). The analysis of one of these clones, *Y. ruckeri* 150RiviV, revealed the presence of the initial 292 nt of an ORF, encoding a protein putatively involved in the activation and secretion of a *Serratia*-type haemolysin (Fig. 1). In order to examine more closely the function of this toxin throughout the infectious process by *Y. ruckeri*,



**Fig. 1.** Chromosomal arrangement and restriction map of the region containing genes *yhIB* and *yhIA* in *Y. ruckeri* 150. The organization of the transcriptional fusion between the *yhIB* promoter and *lacZY* in *Y. ruckeri* 150RiviV is represented from its insertion point in *yhIB*. Arrows indicate the direction of transcription. C, *Clal*; B, *Bam*HI; H, *Hind*III; S, *Sal*I; P, *Pst*I; a, 16 kb *Bam*HI fragment hybridizing with *yhIB* probe; b, 4 kb *Clal* fragment hybridizing with *yhIA* probe; *yhIB'*, initial 292 nt of *yhIB* gene; *yhIB*<sup>+</sup>, complete copy of *yhIB* gene; *cat*, chloramphenicol acetyltransferase gene (promoterless); *lacZY*, genes for lactose fermentation (promoterless); *bla*, ampicillin resistance gene.

the adjacent region of this genetic locus was amplified by means of inverse PCR. This technique, and subsequent DNA sequencing, allowed the identification of two genes thought necessary for the production of the haemolysin (Fig. 1).

The upstream gene, named *yhIB*, consists of 1686 bp and encodes a protein of 561 amino acids which shares a high identity with ShlB from *Serratia marcescens* (65 %) (Poole *et al.*, 1988), PhlB from *P. luminescens* (59 %) (Brillard *et al.*, 2002) and a putative haemolysin activation protein from *Yersinia pestis* CO92 (51 %) (CAC93188). According to the SignalP program, the protein YhIB carries a signal peptide of 18 amino acids. In addition, the analysis of its amino acid sequence with the program PSLPred, which predicts the subcellular location of proteins, suggested that YhIB would be probably located in the outer membrane. This is in agreement with the secretory function of these proteins, being necessary for the transport of the respective toxins through the outer membrane as well as for their activation during this process (Schönherr *et al.*, 1993).

Another ORF, of 4893 bp, designated *yhIA*, was found 69 bp downstream of *yhIB*. The product of this gene has high sequence identity with haemolysins of the *Serratia*-type pore-forming toxins. The identity was 52, 48 and 45 % with the haemolysins ShlA from *S. marcescens* (Poole *et al.*, 1988) and PhlA from *P. luminescens* (Brillard *et al.*, 2002), and a hypothetical protein from *Y. pestis* CO92 (NP\_407172), respectively. These toxins typically have a large molecular size and are secreted to the extracellular milieu by means of a type V or two-partner secretion system (TPSS). The deduced amino acid sequence has a putative signal peptide of 30 amino acid residues and contains the conserved motifs which are characteristic of this kind of protein: QLAG (92 to 95), ILNEV (111 to 115), NPNG (140 to 143), CGFIN (149 to 153), LWGNP (159 to 164), WGGIGG (553 to 558) and LQGT (1259 to 1262) (Hirono *et al.*, 1997). According to Schönherr *et al.* (1993), the most relevant motif is NPNG, which seems to be involved in the secretion of the haemolysin as well as in the exertion of the haemolytic activity. These toxins bind to the membrane of erythrocytes

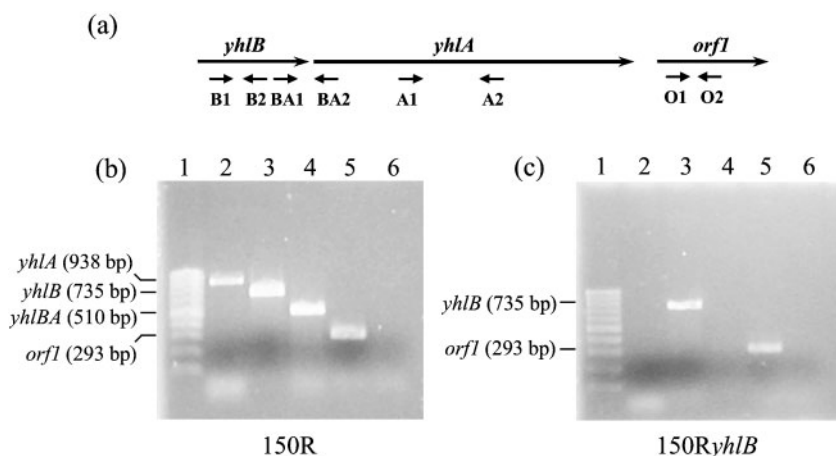
and other cell types and are able to create pores. In addition, they have been related to invasive properties (Hertle *et al.*, 1999). Interestingly, these haemolysins have also been found in genomic analyses of other *Yersinia* species, though their function is still to be determined (Deng *et al.*, 2002; Chain *et al.*, 2004). Therefore, as far as we know, this is the first study on the role of this group of toxins in the genus *Yersinia*.

### Transcriptional analysis

RT-PCRs were carried out to confirm the prediction made by sequence analysis that genes *yhIB* and *yhIA* form an operon. The results obtained with this analysis are represented in Fig. 2 and show that a region overlapping the two genes can be amplified when using RNA from the parental strain and, therefore, that *yhIB* and *yhIA* are co-transcribed (Fig. 2b). On the other hand, the fact that no mRNA corresponding to *yhIA* or to the overlapping region is present in the mutant *Y. ruckeri* 150R*yhIB* reveals that this mutation has a polar effect (Fig. 2c). However, the gene located downstream of *yhIA*, *orf1*, is expressed in both the parental and the mutant strains, which demonstrates that it is not affected by the interruption of *yhIB* (Fig. 2b, c). This operon structure also occurs for genes encoding other *Serratia*-type haemolysins such as PhlA from *P. luminescens* (Brillard *et al.*, 2002), although it is not a general rule. For example, the genes responsible for the production of the EthA haemolysin from *Edwardsiella tarda* are transcribed independently (Hirono *et al.*, 1997).

### Regulation of the *yhIB* promoter by iron and temperature

Since *yhIB* and *yhIA* form a single transcriptional unit, regulation analysis was only performed on the 5'-upstream region of *yhIB*. The strain *Y. ruckeri* 150R*iviV*, obtained by IVET, contains a transcriptional fusion between the *yhIB* promoter and the *lacZY* genes (Fig. 1), which was used to study the regulation of the expression of this gene in response to two important environmental signals, namely iron and temperature. These factors are well studied, given



**Fig. 2.** RT-PCR analysis of *yhIB* and *yhIA* and the ORF located downstream of *yhIA*. (a) Positions of the primers used within the *yhIB*, *yhIA* and *orf1* genes. (b, c) RT-PCRs using RNA from *Y. ruckeri* 150R (b) and 150R*yhIB* (c). The following primers were used: lanes 2, A1 and A2; lanes 3, B1 and B2; lanes 4, BA1 and BA2; lanes 5, O1 and O2; lanes 6, control reaction with Biotools DNA polymerase. Lanes 1, molecular size markers from 1000 to 100 bp.



the significance they have for the pathogen to sense the transition into the host, which generally involves a change in temperature and a limitation in the amount of accessible iron (Mekalanos, 1992). Studies on the regulation of *Serratia*-type haemolysins have demonstrated the role of iron availability in regulation of expression (Hirono *et al.*, 1997; Brillard *et al.*, 2002). The influence of temperature, however, is still to be determined.

The results obtained by the determination of  $\beta$ -galactosidase activity in cultures grown in different conditions showed that both iron and temperature exert an important influence on the transcription levels of *yhlB*, which is repressed by iron and induced by temperature downshift. Thus, the levels of  $\beta$ -galactosidase activity (expressed as  $A_{420} \text{ ml}^{-1} \text{ min}^{-1}$  per  $\text{OD}_{600}$  unit) were  $0.47 \pm 0.03$ ,  $2.83 \pm 0.21$  and  $4.9 \pm 0.20$  when the cells were incubated in M9 supplemented with  $\text{FeCl}_3$ , M9 and M9 supplemented with 2,2'-dipyridyl, respectively ( $P=0.002$ ). This induction under iron-restricted conditions also occurred with the haemolysins produced by *S. marcescens* (Schiebel *et al.*, 1989), *P. luminescens* (Brillard *et al.*, 2002) and *Ed. tarda* (Hirono *et al.*, 1997). These results agree with the hypothesis that haemolysin production might be a way of releasing iron from erythrocytes so that it can be used by the bacterial cell (Litwin & Calderwood, 1993). In addition, the expression from the *yhlB* promoter was approximately sevenfold higher at  $18^\circ\text{C}$  ( $2.83 \pm 0.21 A_{420} \text{ ml}^{-1} \text{ min}^{-1}$  per  $\text{OD}_{600}$  unit), the infection temperature, than at  $28^\circ\text{C}$  ( $0.42 \pm 0.11 A_{420} \text{ ml}^{-1} \text{ min}^{-1}$  per  $\text{OD}_{600}$  unit), the optimal growth temperature of this micro-organism ( $P=0.003$ ). This matches the results obtained for gene regulation of the protease Yrp1 and ruckerbactin production (Fernández *et al.*, 2003, 2004) and reinforces the importance of temperature as an environmental signal regulating the virulence of *Y. ruckeri*. The temperature-dependent modulation of virulence genes tends to trigger the expression of these in conditions mimicking those encountered in the host. This is not, however, a general rule and each case must be analysed independently. In the case of the genus *Yersinia*, the regulation of virulence genes by temperature is a well-characterized phenomenon (Straley & Perry, 1995; Konkel & Tilly, 2000).

### Phenotypic characterization and virulence determination of strains with mutations in *yhlB* and *yhlA*

Independent mutations in *yhlB* and *yhlA* were generated (as described in Methods) to allow studies on the function and importance of the haemolysin, YhlA, in *Y. ruckeri*.

The haemolytic activity of YhlA was barely detectable in blood agar plates, as with other haemolysins of this type (Braun *et al.*, 1985; Brillard *et al.*, 2002). This phenomenon could be a consequence of the low diffusion of these proteins into the culture medium due to their high molecular mass and/or to the adherence of the protein to the cell surface. For this reason, haemolytic activity was measured using liquid

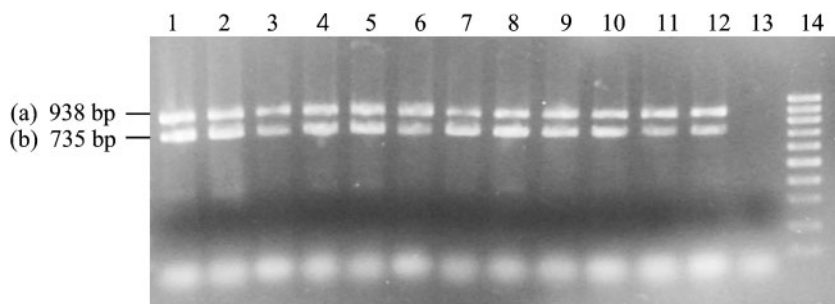
cultures corresponding to the parental (150R) and the mutant strains (150R*yhlA* and 150R*yhlB*) (Table 1). Two independent experiments showed that, under the assayed conditions, the percentage of lysed erythrocytes was  $41.5 \pm 6\%$ ,  $21.7 \pm 4\%$  and  $6.6 \pm 1\%$  for *Y. ruckeri* 150R, *Y. ruckeri* 150R*yhlA* and *Y. ruckeri* 150R*yhlB* supernatants, respectively.

The growth of the *yhlA* and *yhlB* mutant strains was not retarded relative to that of the parental strain, in either iron-rich or iron-depleted conditions (data not shown). This indicates that the mutations do not cause any defect in the growth ability and, therefore, that the differences in virulence could be due to the specific involvement of these proteins in the *in vivo* conditions. In some cases, the production of haemolysins has been related to the metabolism of iron in the cell, which implies that their main function would be the release of the iron bound to the haem group of erythrocytes (Poole *et al.*, 1988).

Once the main characteristics of the mutant strains *in vitro* had been analysed, their behaviour in a fish infection model and in cell cultures was studied.  $\text{LD}_{50}$  experiments indicated that the mutant strains are attenuated in their virulence, because the values obtained with *Y. ruckeri* 150R*yhlA* and *Y. ruckeri* 150R*yhlB* were approximately 10- and 100-fold higher, respectively, than those of the parental strain. Thus, the means of the  $\text{LD}_{50}$  values obtained for the parental strain, mutant *yhlA* and mutant *yhlB* were  $2.7 \times 10^4$ ,  $3.9 \times 10^5$  and  $3 \times 10^6$  c.f.u. per fish, respectively. This relationship between haemolytic activity and virulence was not found in other fish pathogens such as *Edwardsiella ictaluri* (Williams & Lawrence, 2005). Cytotoxicity assays demonstrated that the deficient production of the haemolysin YhlA, caused by the mutations, led to a significant reduction of the cytopathic effects produced by this *Y. ruckeri*. Thus, microscopic analysis of BF-2 cell cultures infected with the mutant and parental strains revealed that only the latter was able to lyse the eukaryotic cells after 2 days incubation, whereas the tissue organization remained unaltered not only in the control wells inoculated with PBS, but also in the wells infected with the mutant strains (data not shown). This cytotoxic effect has already been demonstrated for other haemolysins of this type produced by micro-organisms such as *Haemophilus ducreyi* (Palmer *et al.*, 1996), *Ed. tarda* (Strauss *et al.*, 1997) and *S. marcescens* (Hertle *et al.*, 1999).

### Presence of *yhlB* and *yhlA* in *Y. ruckeri* strains of different origins

By using the liquid culture assay, previously described for the analysis of mutant strains, the supernatants of 12 *Y. ruckeri* strains from different sources were tested for the production of haemolytic activity. All of them showed the ability to lyse erythrocytes, with percentages of lysis ranging from 40 to 75%. Likewise, all the strains gave a positive result in the PCR detection analysis of genes *yhlB* and *yhlA* (Fig. 3), in which the amplification of two bands of 735 and



**Fig. 3.** PCR detection of *yhIA* and *yhIB* in 12 *Y. ruckeri* strains of different origins. After incubating the strains in NB medium, two different PCR reactions were performed. The fragments obtained were mixed and separated in 1.5% agarose gel. The amplicons generated were (a) *yhIA* (938 bp) and (b) *yhIB* (735 bp). The strains corresponding to each lane are: 1, 146; 2, 147; 3, 148; 4, 149; 5, 150; 6, 13/86; 7, 35/85; 8, 43/19; 9, 955; 10, 956; 11, AI00; 12, AI02; 13, negative control; 14, molecular size markers from 1000 to 100 bp.

938 bp, respectively, indicated the presence of these two genes. This result confirms that *Y. ruckeri* is a highly homogeneous species at the genetic level, as several authors have pointed out before on the basis of fingerprinting (Romalde *et al.*, 1993) and multilocus sequence typing (Kotetishvili *et al.*, 2005) analyses. The results obtained by Fernández *et al.* (2003, 2004) also showed that all the strains tested harbour the genes necessary for the production of the protease Yrp1 and the siderophore ruckerbactin.

## Conclusion

According to the data obtained in the present work, it can be concluded that the haemolysin YhlA plays an active role in the pathogenicity of *Y. ruckeri*. This effect is very likely related to its cytopathic activity and, perhaps, to its contribution to the acquisition of iron from the host cells. The expression of the *yhIBA* genes is regulated by iron and temperature, which also modulates the production of ruckerbactin and the protease Yrp1. Even so, further studies are needed to unveil the regulatory cascades involved in the induction of virulence genes in this micro-organism.

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