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-type 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 haemolsins 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.

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

Lucía Fernández,¹ Miguel Prieto² and José A. Guijarro¹

¹Área de Microbiología, Departamento de Biología Funcional, Facultad de Medicina, IUBA, Universidad de Oviedo, 33006 Oviedo, Asturias, Spain

²Laboratorio de Sanidad Animal de Jove, Serida, 33299 Gijón, Asturias, Spain

*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 (*yhlB*), which had been previously identified by *in vivo* expression technology, was further analysed. The gene *yhlB* precedes another ORF (*yhlA*) 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 *yhlA* can be induced under certain *in vitro* conditions similar to those encountered in the host, as deduced from the results obtained by using a *yhlB::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 *yhlB* and *yhlA* 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 *yhlB* and *yhlA* genes in 12 *Y. ruckeri* strains proved once more the genetic homogeneity of this species, since all possessed both haemolytic activity and the *yhlB* and *yhlA* genes.

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 YhIA, 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 2xTY (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⁻¹, 50 μg rifampicin ml⁻¹, 100 μg 2,2'-dipyridyl ml⁻¹ or 100 μg FeCl₃ ml⁻¹, 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 OD₆₀₀ 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, and ligase and calf intestinal alkaline phosphatase were purchased from Perkin-Elmer at the Universidad de Oviedo facilities. The sequences obtained were analysed with the computer program BLASTX.

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 Clal or Sfil, 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 150RyhlB 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'-ATATCCGGGCGCGAAGGC-3' (nt 911 to 927 of yhlA) and A2, 5'-ATTTGCGATCAATGAC-3' (nt 1848 to 1832 of yhlA), for yhlA (938 bp); B1, 5'-ATAACCGGTTGGAATATCA-3' (nt 472 to 488 of yhlB) and B2, 5'-CAGTTATAGTGCAGAT-3' (nt 1206 to 1190 of yhlB), for yhlB (735 bp); BA1, 5'-GCAGAATCTTATATCC-3' (nt 1340 to 1447 of yhlB) and BA2, 5'-GCATGCTGGAAGCAAT-3' (nt 185 to 169 of yhlA), for a region overlapping yhlB and yhlA (510 bp); and O1, 5'-ACAGCAAAATTCGAC-3' (nt 8 to 24 of orf1) and O2, 5'-TTATCCACTGGGTTCA-3' (nt 298 to 282 of orf1), for the ORF 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.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Yersinia ruckeri</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146, 147, 148, 149, 150</td>
<td>Trout-isolated virulent strains (Danish fish farms)</td>
<td>J. L. Larsen (University of Frederikssberg, Denmark)</td>
</tr>
<tr>
<td>35/85, 13/86</td>
<td>Trout-isolated virulent strains (Danish and UK fish farms, respectively)</td>
<td>C. J. Rodgers (University of Tarragona, Spain)</td>
</tr>
<tr>
<td>955 (11.4), 956 (11.29), 43/19</td>
<td>Trout-isolated virulent strains (USA fish farms)</td>
<td>CECT (Spanish Type Culture Collection)</td>
</tr>
<tr>
<td>A800, A102</td>
<td>Trout-isolated virulent strains (Spanish fish farms)</td>
<td>I. Márquez (Laboratory of Animal Health, Asturias, Spain)</td>
</tr>
<tr>
<td>150R</td>
<td>Rif² derivative of 150</td>
<td>Fernández et al. (2002)</td>
</tr>
<tr>
<td>150RiviV</td>
<td>Strain containing fusion between <em>yhlB</em> promoter and <em>lacZ</em>Y</td>
<td>Fernández et al. (2004)</td>
</tr>
<tr>
<td>150RyhlA</td>
<td>Rif² <em>yhlA::pIVET8 Ap</em></td>
<td>This study</td>
</tr>
<tr>
<td>150RyhlB</td>
<td>Rif² <em>yhlB::pIVET8 Ap</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM10::pir</td>
<td>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km::Apir</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLPY5</td>
<td>pIVET8::BglII (yhlA), Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pLPY6</td>
<td>pIVET8::BglII (yhlB), Ap'</td>
<td>This study</td>
</tr>
</tbody>
</table>
In vitro regulation analysis. For promoter expression studies, Y. ruckeri 150RiviV was grown in M9 supplemented with either FeCl₃ or 2,2′-dipyridyl. To determine the influence of temperature, cultures were incubated at 18 °C or 28 °C. Samples from stationary-phase cultures, under these conditions, were collected and their β-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 yhlB (735 bp) and yhlA (938 bp) were amplified by PCR with the following primers: forward primers yhlB1 and yhlA1 (5′-GCAAGAGATCTAAACCCGGTGGAGATCA-3′, nt 472 to 488 in bold type, and 5′-GTCAAGATCTATATCGGGGC-CGAAGGC-3′, nt 911 to 927 in bold type), respectively, and reverse primers yhlB2 and yhlA2 (5′-CTCTAGATCTCAGGGTATGAG-TGCGGTT-3′, nt 1206 to 1190 in bold type, and 5′-GACCGAGATCTATTTCGATCAATAAGC-3′, nt 1848 to 1832 in bold type), respectively. All primers contained a BglII site (in italics) and four additional bases at their 5′ end. The generated amplicons were digested with BglII 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/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 BamHI or CiaI for yhlB and yhlA, 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 yhlA mutant strain and 14 and 4.5 kb in the yhlB 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 BamHI and CiaI sites (Fig. 1), these patterns of hybridization demonstrated that plasmid pLPY5 or pLPY6 (Table 1) harbouring internal fragments of yhlB or yhlA, 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 FeCl₃ or 2,2′-dipyridyl. To determine the involvement of haemolysin production in virulence, 50 % lethal dose (LD₅₀) 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 10⁷ c.f.u. per fish and mortalities were followed up for 7 days. The LD₅₀ 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 BE-2 (blue gill fry) fish cell line. Cell cultures were grown in 24-well plates at 16 °C in Tangle medium (Sigma) supplemented with 1 % (w/v) NaHCO₃ 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 × 10⁵ bacterial cells to each well containing 5 × 10⁵ 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 Axiosvert 200M inverted microscope.

PCR detection of yhlB and yhlA 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 yhlB and yhlA genes were as above.

RESULTS AND DISCUSSION

Sequence analysis of the yhlBA 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,
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 yhlB, consists of 1686 bp and encodes a protein of 561 amino acids which shares a high
identity with ShIB from Serratia marcescens (65 %) (Poole et al., 1988), PhIB 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 YhlB 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 YhlB 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 yhlA, was found 69 bp
downstream of yhlB. 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 ShIB from S. marcescens (Poole et al., 1988)
and PhIB 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), WGGIIG (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 yhlB and yhlA 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 yhlB and yhlA are
cotranscribed (Fig. 2b). On the other hand, the fact that no
mRNA corresponding to yhlA or to the overlapping region is
present in the mutant Y. ruckeri 150RyhlB reveals that this
mutation has a polar effect (Fig. 2c). However, the gene
located downstream of yhlA, orf1, is expressed in both the
parental and the mutant strains, which demonstrates that it
is not affected by the interruption of yhlB (Fig. 2b, c). This
operon structure also occurs for genes encoding other
Serratia-type haemolysins such as PhIB 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 yhlB promoter by iron and
temperature

Since yhlB and yhlA form a single transcriptional unit,
regulation analysis was only performed on the 5’-upstream
region of yhlBA. The strain Y. ruckeri 150RiviV, obtained by
IVET, contains a transcriptional fusion between the yhlB
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 yhlB and yhlA and
the ORF located downstream of yhlA. (a)
Positions of the primers used within the
yhlB, yhlA and orf1 genes. (b, c) RT-PCRs
using RNA from Y. ruckeri 150R (b) and
150RyhlB (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, mole-
cular size markers from 1000 to 100 bp.](image-url)
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 β-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 β-galactosidase activity (expressed as A_{420} ml^{-1} min^{-1} per OD_{600} unit) were 0.47 ± 0.03, 2.83 ± 0.21 and 4.9 ± 0.20 when the cells were incubated in M9 supplemented with FeCl₃, M9 and M9 supplemented with 2,2'-dipiridyl, 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 °C (2.83 ± 0.21 A_{420} ml^{-1} min^{-1} per OD_{600} unit), the infection temperature, than at 28 °C (0.42 ± 0.11 A_{420} ml^{-1} min^{-1} per 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 (150RyhlA and 150RyhlB) (Table 1). Two independent experiments showed that, under the assayed conditions, the percentage of lysed erythrocytes was 41.5 ± 6.9, 21.7 ± 4.8 and 6.6 ± 1.1% for Y. ruckeri 150R, Y. ruckeri 150RyhlA and Y. ruckeri 150RyhlB 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. LD₅₀ experiments indicated that the mutant strains are attenuated in their virulence, because the values obtained with Y. ruckeri 150RyhlA and Y. ruckeri 150RyhlB were approximately 10- and 100-fold higher, respectively, than those of the parental strain. Thus, the means of the LD₅₀ values obtained for the parental strain, mutant yhlA and mutant yhlB were 2.7 × 10⁴, 3.9 × 10⁵ and 3 × 10⁶ c.f.u. per fish, respectively. This relationship between haemolysis 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 haemolysin 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
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 yhlBA 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.

**ACKNOWLEDGEMENTS**

This study was supported by the Spanish MCYT (grants AGL2000-0869 and AGL2003-229). L.F. was the recipient of predoctoral fellowships from the MCYT and Universidad de Oviedo.

We thank J. Méndez for providing the blood for the haemolysin assays, and P. Solano for contributing to the experiments on cytotoxicity. We thank A. F. Braña and T. Fabek for critical reading of the manuscript.

**REFERENCES**


---

**Edited by:** S. C. Andrews