In Vitro and In Vivo Studies of the Yrp1 Protease from Yersinia ruckeri and Its Role in Protective Immunity against Enteric Red Mouth Disease of Salmonids

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Yersinia ruckeri, the etiological agent of the enteric red mouth disease (ERM) of salmonids, produces Yrp1, a serralysin metalloprotease involved in pathogenesis. We describe here the hydrolytic and immunogenic properties of Yrp1. The protease was able to hydrolyze different matrix and muscle proteins as laminin, fibrinogen, gelatine, actin, and myosin but not type II and IV collagens. In addition, the Yrp1 protein, when inactivated by heat and used as an immunogen, was able to elicit a strong protection against the development of ERM. The analysis of different Y. ruckeri strains with (Azo) or without (Azo-) Yrp1 activity showed that all of them contained the yrp1 operon. By using yrp1::lacZ operon fusions, protease production analysis, and complementation studies, it was possible to show that an Azo- strain was blocked at the transcription level. The transcriptional study of the yrp1 operon under different environmental conditions showed that it was regulated by osmolarity and temperature, without pH influence. Finally, when β-galactosidase activity was used as a probe in vivo, the progression of the disease in the fish could be visualized, and the tropism of the bacteria and affected organs could be defined. This system opens a vast field of study not only with regard to fish disease progression but also in pathogen interactions, temporal gene expression, carrier stages, antibiotic resistance selection, etc.

Yersiniosis or enteric red mouth disease (ERM) is a serious infectious disease in salmonids that causes important economic losses in many countries. The etiological agent is the gram-negative bacterium Yersinia ruckeri, which eventually produces hemorrhagic zones around the mouth as a characteristic symptom during the infection process. Once infected, fish grow and survive during weeks or even months without disease symptoms, thus remaining in a carrier stage. Under stress conditions outbreaks occur. Fish that survive may exhibit bacterial shedding of the intestine over long periods (12, 37). In addition, Y. ruckeri can remain infective in the aquatic environment (38), and it also has a biofilm-forming capacity (7). Despite the importance and extensive knowledge of Yersinia species in pathogenesis of mammals, there are few studies about Y. ruckeri, and the precise mechanisms of virulence are practically unknown. Iron-regulated outer membrane proteins (39), iron availability (10), or the presence of a thermolabile factor (16, 17) have been suggested to be involved in pathogenesis. Other authors have described the importance of extracellular products in the virulence of this bacterium (40–42). Based on this, Secades and Guijarro (45) purified the extracellular serralysin metalloprotease Yrp1 and two groups of strains, named Azo- and Azo+, were defined according to the presence or absence of the Yrp1 proteolytic activity, respectively. More recently, Fernandez et al. (15) showed that the gene encoding Yrp1 is part of an operon containing a type I ABC transporter involved in protein secretion, encoded by three genes (yrpD, yrpE, and yrpF), together with gene inh, that encodes a protease inhibitor. Using a trout model, it was possible to show that inactivation of either yrp1 or yrpE by insertion mutagenesis resulted in a significant increase in the 50% lethal dose after inoculation by intraperitoneal injection, indicating the participation of the protease in pathogenesis (15). Thus, although this extracellular protease is a clear virulence factor in Y. ruckeri, there is no description in the literature of the involvement of this kind of enzyme in pathogenesis by other pathogenic Yersinia spp. (Y. enterocolitica, Y. pestis, and Y. pseudotuberculosis), with the exception of the recently described HreP protease in Y. enterocolitica (20). Virulence in these species has been related to the presence of a 70-kb plasmid (9), and chromosomal encoded virulence genes have also been described for different Yersinia species (for a review, see reference 36).

In many cases, virulence factors from a particular pathogen are under the control of environmental conditions. The ivi genes induced exclusively during the infection process are a clear example (29, 30). There are specific host induction factors and others related to environmental conditions, such as temperature, pH, iron availability, osmotic pressure, etc., that influence the expression of virulence genes (for reviews, see references 18, 22, and 31). In Yersinia species, there is an important group of virulence genes that are up- and downregulated by temperature (48). In fish pathogenic bacteria, temperature regulation is particularly important because the production of a specific protein may stop at a temperature corresponding to the upper limit of pathogenicity of the bacteria, which is below the optimal growth temperature (5, 45, 46). However, the level at which this regulation takes place is unknown. Other factors with regulatory effects on gene expression on fish pathogens, such as pH and osmolarity, have not been genetically studied. An additional way to study gene expression is...
in vivo analysis, a powerful technique that enables monitoring biological process through different approaches by coupling the gene of interest or its promoter to a reporter gene (6, 49). Despite the ever-increasing work on bacterial fish pathogens, only an in vivo study with a green fluorescent protein has been carried out in Edwardsiella tarda (25, 26).

On the other hand, good levels of protection against ERM disease have been reached by the use of preventive commercial vaccines made of dead bacterial cells (47). However, fish farm outbreaks that are probably due to the carrier stage mentioned above or to the existence of different serotypes do occur from time to time. For that reason, new approaches based on subunit or DNA vaccines could be used as an additional way to eliminate or minimize these outbreaks. Several proteins from fish bacterial pathogens have been shown to elicit an immune response against the respective infections (13, 23, 28), and DNA vaccines have been mainly studied for fish viral pathogens (14, 19, 27). Thus, a future form of prevention of infectious diseases in aquaculture could be a polyspecific vaccine based on the use of a mixture of antigens or DNA-encoding antigens from different pathogens that would protect against several diseases.

We sought here to study some enzymatic properties, regulation, and in vivo expression of the Yrp1 protease from Y. ruckeri. Thus, by using purified Yrp1 protease we were able to determine its cleavage pattern over different matrix and muscle proteins. Experiments with Yrp1 toxoid were carried out in order to assess the induction of a protective immunity against Y. ruckeri disease. We show by PCR analysis that the presence of the yrp1 operon in all of the tested strains was independent from the Yrp1 phenotype. Complementation studies, together with yrp1::lacZ fusion analysis, showed that the yrp1 operon in an Azo− strain was blocked at the transcriptional level. Furthermore, similar studies showed that the yrp1 operon was regulated at the transcriptional level by osmotic and temperature conditions. Finally, the transcriptional fusion was used as a promoter probe to visualize gene expression in the fish. The yrp1 spatial expression was similar to the one found with other Y. ruckeri promoter fusions. This technique opens a new, wide, and varied way of investigating in vivo colonization, invasion, specific time gene expression, and different fish-pathogen interactions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Y. ruckeri Azo− strains 146, 147, 3585, 955, and 956 and Azo+ strains 148, 149, 150, 4319, and 1386, as well as strain 150RI4, were described previously (45). Strains A100 (Azo+) and A102 (Azo−) (the present study) were isolated from Spanish fish farm outbreaks. Bacterial strains were routinely cultivated on nutrient broth (NB; Difco) or NB with 1.5% (wt/vol) agar at 18°C. Growth in liquid cultures was monitored by determining the absorbance at 600 nm at different times during incubation at 250 rpm.

Yrp1 substrate hydrolysis analysis. Pure Yrp1 protein (0.5 μg), obtained as previously described by Secades and Guijarro (45), was incubated with different protein substrates of human origin (12-μg portions; Sigma Chemical Co.)—including fibrinogen, fibronectin, laminin, gelatine, collagen (types I, II, and IV), and the muscle proteins actin and myosin—after electrophoresis, the gel was stained with Coomassie brilliant blue.

In vitro β-galactosidase determination in the yrp1::lacZ fusions and complementation studies. Y. ruckeri 150RI4 (yrp1::lacZ fusion) was previously obtained by Fernandez et al. (15) by insertional mutagenesis with an internal fragment from the yrp1 gene and the suicide plasmid pVET8 (30). Y. ruckeri 146RI1 (yrp1::lacZ fusion) (the present study) was obtained in a similar way. Portions (100 μl) of overnight cultures of Y. ruckeri 146RI1 and 150RI4 were incubated for 2 h in 0.1 ml of 25 mM Tris-HCl, pH 7.4, containing 5 mM MgCl₂, and then samples were frozen, lyophilized, and resuspended in Laemmli sample buffer (24). Samples were loaded onto a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and, after electrophoresis, the gel was stained with Coomassie brilliant blue.

β-galactosidase activity was assayed in cells by the Miller method (33). For the study of the influence of osmotic pressure, NB was prepared with a 100, 250, or 500 mM concentration of either NaCl, KCl, or D-xylose, 100 μl of the cultures reached the appropriate optical density (OD₆₀₀ nm of 1.4), and the samples were processed as previously described for the analysis of β-galactosidase activity. A similar method was used to assay the pH effect. Medium was buffered with 50 mM morpholine ethanesulfonate for pH 6 or 6.5 and with HEPES for pH 7, 7.5, and 8. In all cases, a standard NB was used as a control.

Complementation of the wild-type Azo− 146 strain was carried out by using plasmids pUK21B, pUK21C, or pUK21T containing yrp1 and inh genes, inh, yrp1, yrpE, and yrpF genes and all of the operon of Y. ruckeri 150, respectively. Y. ruckeri 146 was transformed by electroporation as described by Fernandez et al. (15).

Fish protection studies with Yrp1 toxoid. Rainbow troutons (Oncorhynchus mykiss) weighing between 8 and 10 g were kept in 60-liter tanks at 18 ± 1°C in continuous flowing dechlorinated water with feeding. Groups of 10 fish were injected intraperitoneally with 8 μg of heat-denatured (100°C for 2 min) Yrp1 protease in 0.1 ml of phosphate-buffered saline (PBS), obtained as described by Secades and Guijarro (45). Simultaneously, two control groups of 20 fish were injected. One of them was injected with a 0.1-ml portion of 10² heat-treated Y. ruckeri cells (100°C for 15 min) in PBS, and the other control group was injected with 0.1 ml of PBS. Fish were kept as described previously during 28 to 30 days, and then fish from each group were challenged with 0.1 ml of PBS containing 10⁵ cells of Y. ruckeri 150, an inoculum that was previously found to kill >50% of the untreated fish 7 days after infection. Dead fish from each group were collected everyday and, after 10 days, the relative percent survival—defined as [1 – (% vaccinated mortality/100% control mortality)] × 100—was determined.

PCR detection of the yrp1 and yrpDEF genes in several Y. ruckeri strains. The specific primers designed for the present study from the respective gene sequences (EMBL accession no. AJ188052 and AJ142157) and used for PCR amplification in Y. ruckeri strains were as follows: for yrp1, XA2RP, nucleotides (nt) 269 to 272 upstream from the putative ATG start codon (5′-TATTCAAC TGAAGGTTA-3′), and XA01, nt 672 to 656 (5′-ATAGCTGCTAATACCTG A-3′), generating a 961-bp PCR product; for yrpD, XA10RP, nt 157 to 173 (5′-GATCAGGATGAAAT-3′), and XBSRP, nt 881 to 864 (5′-CAATCGAGT GATCAAATA-3′), generating a 725-bp PCR product; for yrpE, XA2RP2, nt 672 to 688 (5′-ACGGATGACGCGAATCTA-3′), and XAC02, nt 978 to 962 (5′-CCG TACGAGTATCGGGAAGTCA-3′), generating a 581-bp PCR product; and for yrpF, XA2RP, nt 433 to 450 (5′-GGCTGCTAATACCTG A-3′), and XAC05, nt 1013 to 997 (5′-TCCGTCGTAGCTGCCATG-3′), generating a 581-bp PCR product. As a positive control, Y. ruckeri 150 was used. The different strains were grown in nutrient broth at 18°C, and 1 ml of stationary-phase cultures were centrifuged for 5 min at 12,000 × g, the pellet was resuspended in 100 μl of water, and the cells were lysed by boiling them for 10 min. Cell debris was then precipitated by centrifugation for 30 s, and 5-μl aliquots were used as a template DNA in the PCR assays. All PCR components (DNA polymerase, reaction buffer, and deoxynucleoside triphosphates) were provided by Biotools. The amplification reactions (25 cycles) were performed in two groups (yrp1::yrpD and yrpE::yrpF) in a Perkin-Elmer thermal cycler with 94°C for a 5-min initial denaturation, followed by denaturation at 94°C for 30 s, annealing at 40°C for 30 s for yrp1 and yrpD and at 72°C for 30 s for yrpE and yrpF, and a final extension at 72°C for 7 min. The reaction products of the two groups were mixed, and 1.5% (wt/vol) agarose gel electrophoresis was used to separate the generated PCR amplicons.

In vivo β-galactosidase assay. Rainbow troutons weighing 8 to 10 g were intra-peritoneally injected with 10⁶ cells of the Y. ruckeri 150RI4 (yrp1::lacZ fusion) strain or the Y. ruckeri 150 strain as a negative control. The fish were kept at 18°C as described above and, once dead, they were dissected and fixed in 4% paraformaldehyde in PBS for 20 min to 1 h. The fish were washed several times with PBS, followed by the addition of 5 ml of BetaBlue staining kit solution (Nova-gen/CN Biosciences, Inc.) to each fish, which were then incubated at 37°C until a blue color was apparent. Color progression was stopped by washing the samples for 2 min in PBS, followed by the addition of 1 ml of 1% DAB and 10 ml of DAB solution for 15 min. Photographs were taken with a digital Kodak DC 290 camera. Microscopic examinations of gill and intestine tissue samples were car-
rried out by removing them from the fish and observing them with an Olympus BH12 microscope equipped with an Olympus DP12 digital camera. During the in vivo β-galactosidase assay, it was observed that some batches of fish showed a low β-galactosidase background activity in the intestine. Thus, batches of fish had to be checked before being used in the experiments.

Other determinations. Proteolytic activity was assayed by using azocasein (Sigma) as a substrate, according to the method described by Secades and Guijarro (45). After an analysis of variance test, P values of <0.05 were considered significant.

RESULTS

Yrp1 protease has a wide range of substrate proteins. In order to define the role in virulence of the Yrp1 protease, different matrix and muscle proteins were used as possible substrates of the enzyme. As shown in Fig. 1A, lane 1, fibronectin was completely degraded, whereas type I collagen suffered a small hydrolysis (lane 4). In contrast, type II (lane 2) and IV (lane 3) collagens were refractory to hydrolysis. Other proteins (Fig. 1B), such as fibrinogen (lane 1), laminin (lane 2), and gelatine (lane 3), were hydrolyzed to different degrees, whereas gelatine was completely degraded. The muscle proteins actin and myosin (Fig. 1C, lanes 1 and 2, respectively) were extensively degraded by Yrp1.

Yrp1 toxoid confers protective immunity against Y. ruckeri. In order to determine the possible protective immunity properties of the Yrp1 protease on rainbow trout, a toxoid was prepared by inactivation of the protein by heating. The toxoid was then injected intramuscularly into fish. The animals were maintained for 28 to 30 days with feeding, and then they were challenged with intraperitoneal injections of $10^3$ cells of the wild-type strain. The PBS-treated fish used as a control group showed 95% mortality after 10 days, whereas fish treated with toxoid or heat-killed cells showed 20 or 10% mortality, respectively (Fig. 2). Thus, in our experimental conditions, the relative percent survival value of the Yrp1 toxoid-treated fish was 79%, a value closer to that obtained with heat-killed cells ($n = 90$).

Detection of the yrp1 operon in several Y. ruckeri strains and regulation by temperature, osmolarity, and pH. In a previous study (44), Y. ruckeri strains were defined in two groups, Azo$^+$ and Azo$^-$, according to their azocasein degradation capacity, which correlated with the presence and absence of the Yrp1 extracellular protease, respectively. By PCR analysis with specific oligonucleotides designed from yrp1 or yrpD, yrpE, and yrpF gene sequences from the yrp1 operon, it was possible to show, as can be observed in Fig. 3B, that all four genes were present in all of the analyzed strains, even those described as Azo$^-$. In order to determine the reason for the absence of proteolytic activity in the Azo$^-$ strains containing the yrp1 operon, a yrp1::lacZ transcriptional gene fusion Azo$^-$ strain was constructed (146RI1). As shown in Fig. 4A, strain 146RI1 grown at 18ºC presents a low level of β-galactosidase activity during growth. This low transcription level corresponds to an undetectable azocasein hydrolytic activity (Fig. 4B). In contrast, β-galactosidase and proteolytic activity production showed a continuous increase throughout growth in the Azo$^+$ 150RI4 strain (Fig. 4A and B). Complementation studies with Azo$^-$ wild-type strain 146 as a recipient showed that only the strain carrying the yrp1 operon was able to hydrolyze azocasein. However, when the complementation was carried out with the yrp1 gene or the yrpDEF genes, there was no production of proteolytic activity.

Examination of the yrp1::lacZ fusions under different envi-
Environmental conditions showed that, although bacterial growth was better at 28°C (45), the level of yrp1 expression was higher at 18°C than at 28°C (Fig. 4A). These data correspond with the proteolytic activity found at both temperatures (Fig. 4B). In addition, as the osmotic pressure of the medium increased, a slight decrease in transcription of the yrp1 operon was observed, with a significant inhibition of the transcription at either 500 mM NaCl (P = 0.004) or KCl (P = 0.001) (Fig. 4C). Incubation of the bacterium in the presence of the sugar D-xylose, which is not metabolized, caused greater repression (P = 0.002) (Fig. 4C). On the contrary, no major changes in transcription levels were observed at pH values between 6 and 8 (P = 0.669) (data not shown).

Visualization of yrp1 expression in fish by using yrp1::lacZ fusion. In studies with strain 150RI4, which contains the yrp1::lacZ transcriptional gene fusion (15), it was possible to monitor the in vivo expression of the yrp1 virulence gene promoter driving β-galactosidase activity. We found that fish that were intraperitoneally injected with the Y. ruckeri 150RI4 strain showed two clear macroscopic and defined β-galactosidase activity zones, corresponding with the gill and intestine tissues (Fig. 5A). A low and diffuse blue-green color could be visualized through the enlarged spleen and liver (Fig. 5A). In Fig. 5, a clear picture of the gills (Fig. 5B) and intestines (Fig. 5D) may be observed. A microscopic picture of the yrp1::lacZ fusion expression in gills can be seen in Fig. 5C, where the gill arches and filaments seem to be completely covered by the bacterium. In addition, a similar picture is evident in the intestine tissue, which shows an intense color along the capillary system (Fig. 5E).

In order to determine whether the pattern of yrp1 expression in vivo was specific for this particular gene, different Y. ruckeri promoters were cloned by shotgun cloning in plasmid pIVET8, and the respective insertional mutant strains were generated as described by Fernandez et al. (15). All of them showed an expression similar to that of yrp1, both in intensity as well as in distribution in fish tissues (data not shown).

DISCUSSION

Degradation studies with Yrp1 protease showed that the protein digests a wide variety of matrix and muscle proteins. This behavior shares some similarities with the degradation pattern obtained with Fppl protease from the fish pathogenic bacterium Flavobacterium psychrophilum (46) and other metalloproteases related to tissue damage or invasion (for reviews, see reference 34). Invasive processes involve the degradation of extracellular matrix and basement membranes. It is particularly interesting the fact that laminin, a major component of basement membranes, was digested by this protease. Although the proteins used in these experiments were not of fish origin, all of them have conserved sequences among vertebrates. This,
together with the clear preference for the hydrolysis of laminin in relation to the other assayed proteins, suggests that fish laminin could be one of the major natural substrates of this protease. Thus, this degradation may be the cause of membrane alterations leading to erosion and pores in capillary vessels, which results in the leakage of blood through micro-hemorrhages in particular areas such as the mouth and intestine, which is characteristic of this disease. The fact that a yrp1-deficient mutant seems to produce attenuated symptoms supports this hypothesis (data not shown). These results, together with the fact that the Yrp1 protease is a virulence factor not essential for disease development and growth of the bacterium (15), allowed us to speculate that this protein could be involved in the invasion of different tissues during progression of the infection and may also have a nutritional role.

According to the extracellular location of the Yrp1 protease and its involvement in virulence, it was reasonable to expect some protection against the ERM disease when a toxoid of the protease was used as an immunogen. Thus, the protection against ERM was made possible by using active immunization with the Yrp1 toxoid through intramuscular injections. The efficacy of protection was found to be high, confirming the role of the Yrp1 toxoid as a subunit immunogen. The level of protection obtained with dead \textit{Y. ruckeri} cells as a vaccine is similar to the one described by Altinok et al. (2), with 95% mortality in unvaccinated fish. Several proteins from bacterial fish pathogens have been shown to elicit protective immunity against the respective disease. Such are the cases of the OspA lipoprotein antigen of \textit{Piscirickettsia salmonis} (23), a porin of \textit{Aeromonas salmonicida} (28), and an adhesin of \textit{Aeromonas hydrophila} (13). However, until now there has never been a description of subunit vaccine made of a toxoid protease in fish. According to this result, it would be interesting to study the application of Yrp1 toxoid through an immersion bath in order to facilitate its utilization together with the commercial vaccine. At the same time, a putative DNA vaccine using a part of the yrp1 gene encoding an immunogenic nontoxic peptide could be assayed.

The Azo\(^-\) phenotype seems to be a widespread phenomenon in \textit{Y. ruckeri} (45). The fact that all of the analyzed Azo\(^-\) strains had the yrp1 operon was surprising. Based on this finding it can be concluded that this operon is present in \textit{Y. ruckeri} as a genotypic characteristic. In spite of this, a basal level of expression of this operon occurs in Azo\(^-\) strains. When one of the Azo\(^-\) strains was analyzed by transcriptional fusion, it was observed that there was a low level of transcription of the yrp1 operon, which apparently is not enough for the detection of proteolytic activity, indicating that a transcriptional blockade was the cause of the Azo\(^-\) phenotype. Complementation studies showed that only when the whole operon, carrying either its own promoter or the lacZ promoter from the plasmid, was introduced in the Azo\(^-\) strain was there azocasein hydrolysis. All of these results indicate that the whole yrp1 operon is transcriptionally inactive or with very low expression levels in this Azo\(^-\) strain, and this in turn suggests that transcriptional level is a major regulation mechanism of the yrp1 operon. Although this result could not be extrapolated to all of the Azo\(^-\) strains, it does suggest, in accordance with the presence of the operon in all of them, that transcriptional regulation is the basis for the protease-negative phenotype. The phe-
were treated with a X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution as described in Materials and Methods. Control yrp1::lacZ) in the right column. (B and C) Macroscopic and microscopic details of intestine, respectively. (D and E) Macroscopic and microscopic details of gills, respectively.

FIG. 5. Macroscopic and microscopic observation of yrp1::lacZ gene expression in fish. Fish were infected by intraperitoneal injection with 10^4 cells of Y. ruckeri 150RI4 (yrp1::lacZ) and, after death, they were treated with a X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution as described in Materials and Methods. Control fish were injected with the Y. ruckeri 150RI4 strain (sh. Fish were infected by intraperitoneal injection with 10^3 cells of Y. ruckeri 150RI4 (yrp1::lacZ) and, after death, they were treated with a X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution as described in Materials and Methods. Control fish were injected with the Y. ruckeri 150RI4 strain (sh). Panels B, C, D, and E (left column) show the 150 wild-type strain (control fish) in the left and the 150RI4 strain (yrp1::lacZ) in the right column. (B and C) Macroscopic and microscopic details of gills, respectively. (D and E) Macroscopic and microscopic details of intestine, respectively.

The production of the Yrp1 protease by Y. ruckeri is temperature dependent (45). The activation of the yrp1 promoter occurred at the end of the growth phase, and it was repressed when bacteria were grown at 28°C. Thus, Yrp1 production is regulated, in part, in response to environmental signals such as temperature. Maximal Yrp1 production was consistent with the temperature at which the bacterium causes the disease, a usual temperature in many phases of the salmonid’s production cycle in aquaculture. Yrp1 production stopped abruptly at the temperature optimal for bacterial growth, which is lethal for the salmonid’s life. Thus, Yrp1 induction may be an environmental adaptation to the optimal temperature conditions for efficient infection and colonization. This regulatory behavior was already phenotypically observed for siderophore synthesis in Vibrio salmonicida (5), Fp1 protease production in F. psychrophilum (45), lipopolysaccharide changes in A. hydrophila (1), and the expression of aprX protease (3) and lipA lipase genes (50) of Pseudomonas fluorescens. A well-studied system of adaptive gene expression to the environmental temperature for an efficient infection is the one developed by other Yersinia species (Y. enterocolitica, Y. pseudotuberculosis, and Y. pestis), wherein two types of regulatory effects influence the expression of genes encoding some proteins involved in infection. Thus, some genes are downregulated and others are upregulated at 37 and 26°C, respectively (8, 9, 48). In that sense, the Yrp1 protease from Y. ruckeri is a new example of the expression of a virulence gene that is regulated by specific environmental conditions, being highly expressed at temperatures found in the host and repressed at higher temperatures. A related case of regulation by temperature is the one described for splA (44), yst (32), inv (35), and are (11) virulence genes in Y. enterocolitica, among others, which are repressed at temperatures below that of the host.

The effect of osmolarity on virulence gene expression has only been studied to a limited degree. This is particularly important in the case of fish pathogens because osmolarity could define in some cases the range of fish species (saltwater or freshwater fish) that a pathogen such as Y. ruckeri could infect. The expression of yrp1 was maximal in NB without added NaCl, KCl, or xylose. The fact that a significant decrease of expression of the yrp1 operon took place at a low D-xylose concentration (100 mM), together with the low level of expression detected with 500 mM NaCl or KCl, indicates that the yrp1 operon is influenced by the osmotic pressure of the medium, and this probably limits the capacity to generate disease to saltwater fish. In A. hydrophila, virulence is related to osmolarity in an opposite way. Thus, it was more virulent for fish when it was grown at high osmolarity, increasing, at the same time, its caseinolytic and hemolytic extracellular activities (1). Osmolarity also affects the regulation of some Y. enterocolitica genes such as yst (32) and fleABC (21) or the type IV pilus gene cluster of Y. pseudotuberculosis (4).

The use of an operon fusion with β-galactosidase activity as a label was very useful in determining the in vivo expression of the yrp1 gene. This approach has been used in different organ-
isms but, as far as we know, this is the first time that it has been applied to fish. Clearly, the yrp1 gene expression occurred mainly in the gill and intestine tissue. The fact that different bacterial genes were expressed in the same location strongly suggests that, more than a specific pattern of gene expression in tissues, this result shows the location of Y. ruckeri in fish. This result also shows that other tissues and organs are less invaded. It is not clear why Y. ruckeri has such a tropism, but it may be related to the presence in both tissues of an accessible superficial capillary system. More studies using immersion experiments should be done for defining the entry site, colonization, and invasion to show the importance of the gastrointestinal tract as a portal of entry, as suggested by Ross et al. (43) for this bacterium and as has been shown for E. tarda (25). This technique could be a useful tool for studying temporal gene expression, as well as for performing experiments on strain competition, the development of infections and colonization, routes of infection, etc.

Taking these results as a whole, we conclude that the Yrp1 protease, the first virulence factor defined in Y. ruckeri, presents similarities in its mode of regulation to other virulence genes from mammal pathogenic Yersinia and plays a nonsential, although relevant, role during infection and ERM disease development in salmonids.

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REFERENCES


