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Review article:

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6 Molecular virulence mechanisms of the fish pathogen *Yersinia ruckeri*

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25

1 **Abstract**

2

3 *Yersinia ruckeri* is the causative agent of enteric redmouth disease or yersiniosis,
4 which affects mainly salmonids. This microorganism has been consistently causing
5 economic losses in the aquaculture industry since its first description; but the early
6 development of a vaccine allowed a relative control of the disease. This might be the
7 reason why the specific pathogenicity mechanisms of this bacterium remained elusive
8 until recently, when the results obtained with traditional microbiology have been
9 complemented with those provided by molecular biology. The data obtained by using
10 these novel techniques, which are the main subject of this review, have started to shed
11 light on the virulence of this pathogen. Thus, iron acquisition by the siderophore
12 ruckerbactin, proteolytic and haemolytic activities, and resistance to immune
13 mechanisms, were proved to be involved in the virulence of this bacterium.
14 Additionally, these data will, in the long term, help clarify the controversial taxonomic
15 status of this microorganism and allow the development of novel ways to prevent
16 outbreaks, which is particularly interesting nowadays, given that commercial vaccines
17 seem to be ineffective against some new isolates.

1 **1. Introduction**

2
3 *Yersinia ruckeri* is the aetiological agent of enteric redmouth (ERM) disease or
4 yersiniosis, a general septicaemia affecting mainly salmonids. Although generally well
5 controlled by means of vaccination and antibiotic treatment, this disease has kept on
6 causing outbreaks, especially in endemic areas. In these cases, the losses can be as
7 important as 30 to 70 % of the stock (Horne and Barnes, 1999). Little is known,
8 however, about the mechanisms of pathogenicity possessed by this microorganism
9 (Horne and Barnes, 1999). There is an additional intriguing aspect about *Y. ruckeri*: the
10 ongoing dilemma on its belonging to the genus *Yersinia* (Bercovier and Mollaret, 1984;
11 Kotetishvili et al., 2005). Over the last years, the use of molecular techniques has open
12 new possibilities to go deeper into these two issues (Fernandez et al., 2004; Fernandez
13 et al., 2007; Coquet et al., 2002; Coquet et al., 2005). This review summarises the
14 different techniques used throughout the years to study the virulence mechanisms of this
15 microorganism as well as the current knowledge on this topic, giving special importance
16 to the most recent studies. (For a comprehensive review on the characteristics of *Y.*
17 *ruckeri* and ERM see: Horne and Barnes, 1999).

19 **2. Taxonomic position and intraspecies classification of *Y. ruckeri***

20
21 The allocation of this gram-negative bacterium to a genus within the family
22 *Enterobacteriaceae* is still an open debate. DNA homology studies indicated a similar
23 proximity, sharing approximately 30% homology rates, to species of the genera *Serratia*
24 and *Yersinia*; but its final placement in the latter was made on the basis of the guanine
25 plus cytosine (G+C) content of the DNA (Ewing et al., 1978). Thus, this bacterium with

1 47.5-48% G+C (de Grandis et al., 1988) differs clearly from *Serratia* species (52-60%
2 G+C) and is closer to other *Yersinia*e (46-50% G+C). Nevertheless, this classification
3 has always been controversial and several authors suggested that it should be revised
4 and that this microorganism could even constitute a new genus (Bercovier and Mollaret,
5 1984; Bottone et al., 2005). More recently, multilocus sequence typing (MLST) and
6 rRNA 16S analysis have shown that *Y. ruckeri* was the most distant species within the
7 genus *Yersinia* and, therefore, that a re-evaluation of its taxonomic status would be
8 necessary (Kotetishvili et al., 2005). The species is nevertheless included in the genus
9 *Yersinia* in the most recent edition of Bergey's Manual of Systematic Bacteriology
10 (2005).

11 Additionally, this analysis (Kotetishvili et al., 2005) confirmed the high
12 homogeneity of this species, at least at a genetic level, also pointed out by other
13 researchers (Schill et al., 1984; García et al., 1998; Fernández et al., 2007). In spite of
14 this uniformity, isolates from different areas can be classified according to diverse
15 criteria. One of the currently accepted nomenclatures proposed the existence of 6
16 serovars (I to VI) on the basis of whole-cell serological reactions, although serovar IV
17 was later excluded (Stevenson et al., 1993). Another intraspecies classification of *Y.*
18 *ruckeri*, and one of the most accepted to date, is the one proposed by Romalde et al.
19 (1993) based on studies using absorbed sera and antigenic determinants
20 (lipopolysaccharide and membrane proteins). Among the four serotypes distinguished
21 by these authors, it is serotype O1 which includes the most virulent strains. A
22 comparison between these two classifications can be seen in Table 1. Another
23 classification based on biochemical properties differentiates between two biotypes:
24 biotype 1, comprising strains positive for motility and phospholipase activity, and
25 biotype 2, negative for both tests (Davies and Frerichs, 1989).

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3. ERM: a potential re-emergent disease?

ERM was first isolated and described in Idaho (USA) in the 1950s from farmed rainbow trout (*Oncorhynchus mykiss*) (Ross et al., 1966; Rucker, 1966) and has been isolated since then, in many different countries, affecting not only salmonids but also fish from diverse families.

Diagnosis of ERM is based on the observation of the clinical signs of the disease as well as the subsequent isolation of the pathogen from the fish internal organs. However, molecular detection by means of PCR analysis is more and more used as a complement in routine diagnosis due to its sensibility and speed (Gibello et al., 1999; Temprano et al., 2001; del Cerro et al., 2002).

The use of correct handling techniques is very important in the prevention of the ERM. In addition, a vaccine containing inactivated whole cells is generally administered in fish farms (For review see Stevenson, 1997). Although this is quite effective, it cannot avoid the appearance of outbreaks under severe stress conditions, especially in endemic areas, due to the spreading through the water of a large number of bacteria from the faeces of carrier and ill fish (Hunter et al., 1980). Moreover, some recently isolated strains in UK, Spanish and USA fish farms seem to be unaffected by commercial vaccines (Austin et al., 2003; Fouz et al., 2006; Arias et al., 2007). All these strains lack motility, but whereas the strains from Southern England have been classified as a new biogroup (Austin et al., 2003), the Spanish and USA strains belong to serotype O1, biotype 2 (Fouz et al., 2006; Arias et al., 2007). It is difficult to determine the reason why there is not cross-protection, especially because the major

1 antigens present in the vaccine are unknown. Nevertheless, it might be possible that
2 some surface proteins involved in motility, i.e. flagellins, could be necessary to trigger
3 the immune response. Until recently, most isolates from outbreaks were motile, but
4 perhaps vaccination exerted a selective pressure that enabled the emergence of non-
5 motile variants resistant to commercial vaccines. This is a critical point because these
6 emergent non-motile vaccine-resistant strains if spreading could originate an epidemic
7 of catastrophic consequences. In that sense, epidemic care as well as vaccination studies
8 are necessary.

9

10 **4. Models and techniques used to study the pathogenesis of yersiniosis**

11

12 Despite the initial lack of knowledge regarding *Y. ruckeri* pathogenicity, it was
13 expected that its virulence mechanisms shared traits of other members of the family
14 *Enterobacteriaceae* as well as certain characteristics from fish pathogenic
15 microorganisms. For this reason the start point for researchers was the combination of
16 molecular biology techniques commonly applied in other gram-negative bacteria with
17 fish infection models.

18

19 *4.1. Animal and cell culture models*

20

21 Animal models have been limited to the salmonids because of the narrow host range
22 of *Y. ruckeri*. Experimental infections of fish, in most cases rainbow trout, have been
23 carried out to test the importance of some putative virulence factors, differences in
24 virulence among different strains, or the toxicity of the injection of the extracellular
25 products (ECP) for fish (Romalde and Toranzo, 1993), etc. Sometimes the aim was to

1 study several isolates from different areas, mainly from outbreaks (Furones et al., 1990;
2 Davies, 1991a; Romalde and Toranzo, 1993). In other cases, these assays allowed the
3 determination of the involvement of mutations in different genetic systems in the
4 virulence of this microorganism (Fernández et al., 2002; Fernández et al., 2004;
5 Fernández et al., 2007). All of these approaches contributed to the knowledge of the
6 pathogenic mechanisms of this bacterium. However, the recent application of genetic
7 and molecular techniques provided a more extensive and accurate information. In
8 animal models it must be also considered that the route of infection is an important
9 factor, and different results could be obtained depending on the route of challenge even
10 for a well-defined virulence factor. Thus, injection models, although more artificial, are
11 more reproducible and easy to carry out than immersion trials and they are a good
12 approach to initially determine the role in virulence of a specific gene or protein. Once
13 again, it seems necessary the standardisation of some procedures in order to compare
14 results from different authors.

15 The analysis of the production of β -galactosidase activity in fish injected
16 intraperitoneally with a strain harbouring a transcriptional fusion between a *Y. ruckeri*
17 promoter and the *lacZ* gene allowed the detection of the presence of the bacterial cells
18 in different organs, especially in the gills and intestine (Fernández et al., 2003). More
19 recently, Welch and Wiens (2005) constructed a virulent, green fluorescent protein-
20 tagged *Y. ruckeri* and carried out the detection of this strain in rainbow trout after
21 intraperitoneal injection and immersion challenge, detecting a high number of bacteria
22 in spleen, kidney and peripheral blood. Green fluorescent protein seems to be a more
23 sensitive and easy approach to the study of bacterial distribution in fish and
24 transcriptional fusions could be useful for studies concerning the progress of infections,

1 the interactions between this bacterium and its natural host, and the expression of
2 specific genes during the infection process in certain tissues.

3 Apart from fish infection experiments, some authors also carried out assays using
4 fish cell line cultures in order to analyse the ability to adhere to and invade several cell
5 lines (Santos et al., 1990; Romalde and Toranzo, 1993) and to determine the
6 involvement of a haemolysin in cytotoxicity (Fernández et al., 2007). The results
7 obtained in these experiments will be discussed later in this review. It is also remarkable
8 that the possibility of using cell line cultures for some experiments about pathogenicity,
9 i.e. selection and analysis of mutants, is a good alternative to the use of animal models.

10

11 4.2. *In vivo expression technology (IVET)*

12

13 In addition to the methods traditionally used for the analysis of virulence factors,
14 some novel techniques have been conducted in order to achieve a better understanding
15 of the pathogenicity mechanisms of this microorganism. Fernández et al., (2004)
16 recently applied IVET (Mahan et al., 1993), which is a promoter trap that allowed the
17 identification of genes specifically expressed in the host and, therefore, of putative
18 virulence factors. In the case of *Y. ruckeri*, these genes were selected by means of the
19 administration of the antibiotic chloramphenicol (Mahan et al., 1995) and it led to the
20 isolation of 14 different clones (Fernández et al., 2004). Some of them contained
21 putative ORFs similar to known virulence genes from other bacteria like one involved
22 in a type IV secretion system, a haemagglutinin, a haemolysin, genes required for iron
23 acquisition by a catecholate siderophore and finally an ORF belonging to a cluster
24 related to tight adherence.

25 The study of the IVET-identified genes together with the application of a

1 complementary *in vivo* technique, such as Signature Tagged Mutagenesis (STM), could
2 give an overview of the main pathogenic mechanisms of this bacterium.

3 4 *4.3. Proteomic analysis*

5
6 Analysis by means of 2DE electrophoresis allows determining differences in the
7 protein content in different strains or environmental conditions. In the case of *Y.*
8 *ruckeri*, the first study of this type compared the outer membrane profile in several
9 environmental and reference strains and tried to establish a relationship between the
10 presence of certain proteins and adherence to solid supports and flagellum-mediated
11 motility (Coquet et al., 2002). Later, Coquet et al., (2005) compared the outer
12 membrane proteins of free and immobilized *Y. ruckeri* cells of the same strain. These
13 studies are not related directly to virulence but showed the presence of some proteins
14 related to adherence that could be important during the initial steps of the infection.
15 Nevertheless, 2DE studies have some limitations for the study of virulence factors
16 because it is not possible to compare the *in vivo* and *in vitro* protein profiles.

17 18 **5. Pathogenic mechanisms of *Y. ruckeri***

19 20 *5.1. Survival in the aquatic environment*

21
22 The ability to survive outside the host in nutrient-limiting environments for long periods
23 of time facilitates transmission and it is therefore important for the pathogenesis,
24 leading to problems in aquaculture. If the bacteria released from ill and carrier fish are
25 able to survive in the environment for longer periods, they will remain in the tanks and

1 eventually cause new outbreaks. According to the results obtained by Romalde et al.,
2 (1994), *Y. ruckeri* stressed or nutrient-starved cells may enter a state of dormancy
3 remaining infective but unable to grow on normal laboratory media. This bacterium is
4 able to survive in unsupplemented water with salinities of 0-20 p.p.t. for at least 4
5 months, whereas this survival time is notably reduced in water with salinities of 35
6 p.p.t. (Thorsen et al., 1992). This different survival times probably contribute to the low
7 incidence of the disease in salty water. On the other hand, the ability to survive for long
8 periods in water with low salinities could be an important factor that makes it difficult
9 to control the disease in salmonid freshwater aquaculture.

10

11 5.2. *Biofilm forming capacity, adherence and invasiveness*

12

13 Biofilms are known to be important for the survival in the environment, adherence
14 to solid supports, but also for the adherence to the host tissues. They also enhance
15 resistance to antibiotics. The role of *Y. ruckeri* biofilms in either host-pathogen
16 interaction or viability in the environment is still to be determined. The formation of
17 biofilm structures in *Y. ruckeri* was first reported by Coquet et al., (2002) in isolates
18 from a French fish farm. This isolates showed an increased resistance to oxolinic acid
19 and this biofilm forming capacity was correlated with flagellum-mediated motility.

20 In a later work, proteomic analysis revealed differences in OMP pattern between
21 immobilised and planktonic cells affecting proteins involved in motility, transport,
22 metabolism, adaptation, etc (Coquet et al., 2005). It is important to consider that the
23 differences between these two physiological states cannot be extrapolated to other more
24 complex situations during the infection process. The results of the assays of adherence
25 and invasiveness of *Y. ruckeri* in fish cell line cultures were dependent on the line used

1 (Santos et al., 1990; Romalde and Toranzo, 1993). The molecular mechanisms involved
2 in these processes remain, however, unknown. PCR and sequencing analysis revealed
3 the presence of a gene homologous to the *inv* gene from other *Yersinia* species involved
4 in bacterial adherence to and penetration into the host cells (Fernández and Guijarro,
5 unpublished data). It would be interesting to study the mechanisms used by this
6 microorganism in order to adhere to and to invade the host tissues, as well as compare
7 them to the systems used by other fish pathogens and other *Yersinia*.

8 9 5.3. Iron acquisition

10
11 Two studies dating from 1991 analysed the ability of *Y. ruckeri* to grow under iron-
12 limiting conditions showing that this bacterium was able to grow in the presence of a
13 chelator compound. The results were contradictory and only one of them showed the
14 production of a high-affinity iron uptake system by this microorganism (Romalde et al.,
15 1991). However, both papers report the induction of some OMPs in iron-depleted
16 conditions (Davies, 1991b; Romalde et al., 1991).

17 More recently, Fernández et al., (2004) found a group of *in vivo* induced genes
18 necessary for the synthesis and utilisation of a catecholate siderophore, named
19 ruckerbactin which, very likely, corresponds to the activity detected by Romalde et al.,
20 (1991). Some of these genes were located in the same chromosomal region constituting
21 a gene cluster having a similar organization to *E. coli* enterobactin gene cluster.
22 Nevertheless, the chemical structure of the compound is probably more akin to that of
23 *Erwinia chrysanthemi* chrysobactin rather than to enterobactin given that the outer
24 membrane receptor shares a much higher homology to chrysobactin receptor.
25 Ruckerbactin was proved to be involved in iron acquisition in iron-depleted conditions

1 *in vitro*, but during the infection as well. Thus, LD₅₀ of mutant strains deficient in
2 siderophore production and uptake was higher than that of the wild-type strain
3 (Fernández et al., 2004). Therefore, the ability to scavenge iron from the environment
4 is an essential requirement for the development of the infection process by *Y. ruckeri*.

6 5.4. Extracellular toxins: haemolysin/cytolysin *YhlA* and metalloprotease *Yrp1*

8 Romalde and Toranzo (1993) showed that the intraperitoneal injection of the
9 extracellular products of *Y. ruckeri* were highly toxic for the fish and reproduced some
10 external signs of the infection like darkening of the skin and haemorrhagic and necrotic
11 areas at the injection site. These products included lipase, protease and haemolytic
12 activity against erythrocytes from different origins and cytotoxic properties in some cell
13 line cultures, although the genes and proteins associated to these activities were not
14 identified. One of the genes isolated by IVET technology had a high homology to
15 genes encoding proteins involved in the secretion and activation of *Serratia*-type
16 haemolysins (Fernández et al., 2004). Further studies on this locus revealed that this
17 gene as well as the adjacent ORF, which coded for a haemolysin of this kind, was
18 responsible for the production of haemolytic activity. Besides, experiments with
19 insertional mutants in both genes demonstrated that this toxin was not only involved in
20 virulence, but also in cytotoxicity (Fernández et al., 2007). Both activities could be
21 essential for the progression of the disease although, it is possible, that *Y. ruckeri*
22 produces other haemolysins.

23 Romalde and Toranzo (1993) already showed the presence of proteolytic activity in
24 the extracellular products of *Y. ruckeri*. Years later, Secades and Guijarro (1999)
25 purified and characterised an extracellular 47-kDa protease produced by some strains,

1 the so-called Azo⁺ strains. The gene responsible for the production of this protease,
2 named Yrp1, was subsequently cloned and analysed showing that it encodes a protein
3 belonging to the serralyisin family and that it was secreted via the type I machinery
4 (Fernández et al., 2002). LD₅₀ determination experiments indicated that this enzyme is
5 involved in the virulence of this bacterium, since mutants defective in the production or
6 secretion of Yrp1 showed a 100-fold increase in LD₅₀ (Fernández et al., 2002). An
7 additional work analysed different matrix and muscle proteins as possible substrates of
8 this protease (Fernández et al., 2003) demonstrating that it is able to degrade a wide
9 range of them, particularly laminin which is a major component of basement
10 membranes. This result together with the fact that Yrp1 coding gene is expressed in the
11 fish seems to corroborate the role of this protein in the virulence of *Y. ruckeri*
12 (Fernández et al., 2003). Interestingly, this work also reports that heat-inactivated
13 protease Yrp1 confers a protective immunity against ERM. All these data indicate that
14 Yrp1 plays an important role during the pathogenic process.

15

16 5.5. Resistance to innate immune mechanisms

17

18 Stave et al., (1987) observed a decrease in the presence of reactive oxygen species in
19 striped bass (*Morone saxatilis*) macrophages. Most strains showing this response
20 belonged to serovar I and harboured the 70 MDa plasmid. Most strains of this serovar
21 contained this large plasmid, whose involvement in pathogenicity is still to be
22 determined and which does not share any homology with the virulence plasmid of
23 human pathogenic *Yersinia* (de Grandis and Stevenson, 1982; Toranzo et al., 1983;
24 Stave et al., 1987; García et al., 1998). However, one serovar II strain, which did not
25 carry this plasmid, had the same behaviour. Other authors demonstrated the production

1 by serovar I isolates of superoxide dismutases besides catalases (Horne and Barnes,
2 1999). These enzymes allow bacteria to survive inside macrophages by avoiding
3 phagocytic killing and, as a result, are considered to be important virulence factors. In
4 the case of *Y. ruckeri*, it is still to be determined whether they are plasmid- or
5 chromosomally encoded.

6 As for serum resistance, the results obtained by Davies (1991b) indicated that there
7 was a relationship between this property and virulence, since virulent serotype O1
8 strains were serum resistant and avirulent serotype O1 and other serotypes strains were,
9 with exceptions, serum sensitive. This interesting correlation should be studied more in-
10 depth. In that sense, Furones et al., (1990) also suggested that the presence of a heat
11 sensitive factor (HSF) in virulent serotype I strains could be involved in conferring
12 resistance to serum bactericidal activity or phagocytic killing. This factor could be a
13 good indicator of virulent strains and work on it should be taken up again.

14 On the other hand, there is a heritable genetic variation in rainbow trout resistance to
15 *Y. ruckeri* (Henryon et al., 2005). This finding indicates some variation in the host
16 physiology (i.e. lack of an adhesin) or innate immune system. Additionally, it should be
17 considered that these fish resistant to *Y. ruckeri* infection could be in the long term a
18 selection system that causes the apparition of new strains with different virulence
19 determinants able to produce the disease in these fish. May be this could be the cause
20 for the appearance of the new biotypes identified recently, which were described in a
21 previous section of this article.

22 Finally, taking into account the efficiency of the *Y. ruckeri* vaccine, which indicates
23 a protective response by the fish immune system, *Y. ruckeri* should be considered as a
24 model for understanding pathogen-host immune system interactions.

25

1 5.6. Regulation of virulence gene expression

2
3 5.6.1. Modulation of gene expression by environmental factors

4
5 Virulence is the result of the effect of several factors contributing to the different
6 steps in the infection process. Therefore, it is very important that their expression is
7 tightly coordinated and regulated in response to host environmental factors. To address
8 whether *Y. ruckeri* virulence genes are coordinately regulated by environmental factors,
9 we constructed transcriptional fusions between virulence gene promoter and the *lacZ*
10 gene. We measured LacZ enzymatic conversion of a synthetic substrate, ONPG, as a
11 surrogate measure of virulence factor transcription and examined promoter activity
12 under varying iron (Fernández et al., 2004; Fernández et al., 2007),
13 temperature(Fernández et al., 2003; Fernández et al., 2004; Fernández et al., 2007), pH
14 (Fernández et al., 2003), and osmolarity (Fernández et al., 2003) conditions. The results
15 from these studies are summarised in Table 2. LacZ activity, from all reporter constructs
16 was increased at 18 °C in comparison to 28 °C, the optimal growth temperature. The
17 regulation by temperature of all these virulence factors suggests the existence of a very
18 interesting regulation system controlled by this environmental factor. The extent of the
19 regulatory response triggered by the infection temperature in *Y. ruckeri* should be
20 analysed by using 2D protein gel electrophoresis or microarrays. The response to other
21 factors was, however, variable depending on the gene tested and sometimes could be
22 explained considering the function of the encoded protein, i.e. gene *rucC*, involved in
23 siderophore biosynthesis, is regulated by iron availability.

24

25

1 5.6.2. Quorum sensing

2
3 Cell density regulates gene expression by means of the production of a small
4 molecule called autoinducer (N-acylhomoserine lactone or AHL), which is synthesised
5 by the product of a gene homologue to *luxI*. This compound interacts with a response
6 regulator encoded by the homologue of *luxR*. Production of AHLs by *Y. ruckeri* was
7 demonstrated by cross complementation of *Chromobacterium violaceum* mutant strains
8 (Temprano et al., 2001; Bruhn et al., 2005). In addition, Temprano et al., (2001) cloned
9 and sequenced genes *yruR* and *yruI*, *Y. ruckeri* counterparts of *luxR* and *luxI*,
10 respectively. A recent article pointed out that quorum sensing in *Y. ruckeri* is complex
11 and could involve several regulatory systems (Kastbjerg et al., 2007). This study also
12 indicated that exogenous acyl homoserine lactones and known quorum-sensing
13 inhibitors did not have any effect on protease production. Further studies on this system
14 will give information about the processes, especially those related to virulence and
15 survival in the environmental conditions, which are controlled by quorum sensing in
16 this microorganism.

17 18 5.6.3. RpsO sigma factor

19
20 The alternative sigma factor RpoS is known to play an important role not only as a
21 stress-response regulator, but also inducing some virulence genes (Mahan et al., 1996).
22 To date, it has only been reported the presence of a gene homologous to *rpoS* in *Y.*
23 *ruckeri* but studies concerning the specific genes regulated by this system as well as its
24 importance in this microorganism are still to be carried out (Martínez-García *et al.*,
25 2001).

1

2 **6. Conclusion and future directions**

3

4 In a previous review on vaccination against yersiniosis Stevenson (1997), ten years
5 ago, had already pointed out the necessity of understanding the virulence mechanisms
6 and specific bacterial antigens of *Y. ruckeri* in order to design more specific and
7 effective vaccines. The appearance of new biotypes resistant to the long-term used
8 vaccine confirms the importance of that indication.

9 The pathogenicity mechanisms of the fish pathogen *Y. ruckeri* have started to be
10 unravelled thanks to the application of new molecular techniques. New and up-to-date
11 approaches to the study of its virulence factors allowed the identification of several
12 genes involved in pathogenesis, which, in this microorganism, is a multifactorial
13 process involving different types of factors. One major environmental cue involved in
14 the regulation of virulence is temperature, given that these genes were highly induced
15 under temperatures outbreaks. Another important conclusion of these studies is that the
16 virulence mechanisms used by this bacterium are different with relation to other
17 *Yersinia* species. The DNA sequence of the genes in common are, however, more
18 similar in general to other *Yersiniae* than to other genera. This might indicate a closer
19 relation to this genus than to others, but a significantly different evolution, perhaps
20 because of the different hosts and environments.

21 Nevertheless, all the interesting results included in this paper are but a first glance at
22 the pathogenicity of this bacterium and further research is still needed in order to picture
23 the different mechanisms involved in the different stages of the infectious process by *Y.*
24 *ruckeri*. In that sense, the currently available data on *Y. ruckeri* virulence are very basic
25 in comparison to other pathogens, including the human pathogenic *Yersiniae*. Thus, the

1 number of genetic systems known to be involved in virulence is still very scarce and the
2 interaction between these systems is completely unknown. There is also a general lack
3 of information about regulatory pathways in this species. All of this is understandable,
4 given that the studies on molecular mechanisms of pathogenicity in *Y. ruckeri* are still in
5 an early stage.

6 An important help for future work could be the complete genome sequence which
7 will open new and definitive approaches to study the virulence of this fish pathogen.
8 Therefore, the use of microarrays, silico experiments, 2D maps of induced and
9 repressed proteins under definite conditions, and identification of virulence regulation
10 genes will allow having a more complete description of the systems involved in
11 pathogenesis. In addition, the controversial taxonomic status of *Y. ruckeri* with relation
12 to other *Yersinia* species could be solved. Subtractive hybridization as well as molecular
13 base epidemiologic studies will help to understand the relationship between strains,
14 outbreaks and vaccination. Finally, more sensitive methods such as microchip
15 technology are necessary for the detection of bacteria in carrier fish and water in order
16 to prevent outbreaks.

17

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19

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1 Table 1: Equivalence between serotype and serovar classifications

Serotype	Subgroup	Serovar	Designation of serovar
O1	a	I	Hagerman
	b	III	Australian
O2	a, b, c	II	Oregon
O3	-	V	Colorado
O4	-	VI	Ontario

2 -, no subgroups.

3

Table 2: Regulation of *Y. ruckeri* virulence genes by environmental factors

Gene	Function	Temperature	Iron	pH	Osmolarity	Reference
<i>yrpI</i>	Protease	+ 18° C	ND	NR	+ ↓ osmolarity	Fernández et al., (2003)
		- 28° C			- ↑ osmolarity	
<i>rucC</i>	Siderophore-mediated iron acquisition	+ 18° C	+ ↓ iron availability	ND	ND	Fernández et al., (2004)
		- 28° C	- ↑ iron availability			
<i>rupD</i>	Siderophore-mediated iron acquisition	+ 18° C	+ ↓ iron availability	ND	ND	Fernández et al., (2004)
		- 28° C	- ↑ iron availability			
<i>yhlB</i>	Haemolysin	+ 18° C	+ ↓ iron availability	ND	ND	Fernández et al., (2007)
		- 28° C	- ↑ iron availability			

ND, not tested; NR, not regulated by this factor.