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Early pathogenesis of *Yersinia ruckeri* infections in rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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CFU	colony forming units
CHSE-214	chinook salmon embryo cell line
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
ECP	extracellular product
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERM	enteric redmouth disease
FCS	fetal calf serum
FHM	fathead minnow epithelial cell line
GFP	green fluorescent protein
H&E	hematoxyline and eosin
IFAT	immunofluorescence antibody technique
IHC	immunohistochemistry
i.p.	intraperitoneal
LPS	lipopolysaccharide
MEM	minimum essential medium
MOI	multiplicity of infection
NB	nutrient broth
NEAA	non essential amino acids
OD	optical density
OMP	outer membrane protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p.i.	post infection
R1	rainbow trout liver cell line
RFLP	restriction fragment-length polymorphism
ROS	reactive oxygen species
SDS-PAGE	sodiumdodecylsulphate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
TFSS	type four secretion system
TTSS	type three secretion system
WST-1	water-soluble tetrazolium salt

GENERAL INTRODUCTION

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Introduction

Yersinia ruckeri was initially isolated from rainbow trout (*Oncorhynchus mykiss*, Walbaum), in the Hagerman valley of Idaho, USA, in the 1950s (Rucker 1966) and is now widely found in fish populations throughout North America, Australia, South Africa and Europe. This bacterium is the causative agent of yersiniosis or enteric redmouth disease (ERM), previously called 'Hagerman redmouth', and causes significant economic losses in the salmonid farming industry. Although infection with this agent has been reported in other fish species, salmonids and especially rainbow trout are most susceptible to ERM (Furones *et al.* 1993).

In the first part of this review, the relatedness of *Y. ruckeri* with other yersiniae and its taxonomic position is discussed, followed by the characteristics of *Y. ruckeri* including morphology and antigenic determinants. Then, the features of the disease caused by this pathogen, including pathogenesis, clinical signs, immune response, diagnostic procedures and current approaches for prevention and treatment are discussed. As much more is known about the *Yersinia* species which elicit disease in humans, the most important pathogenic mechanisms of these species are described, pointing out the possibility of similar mechanisms in *Y. ruckeri*.

1. The micro-organism

1.1. Taxonomic position

The taxonomic position of *Y. ruckeri* and its relatedness with other yersiniae is still an open debate. DNA sequencing analysis generally shows more similarity to other *Yersinia* species than to other genera. Indeed, different DNA homology studies showed approximately 30% homology of *Y. ruckeri* to species of the *Serratia* and *Yersinia* genus. Moreover, considering the guanine plus cytosine (G + C) content of the DNA, *Y. ruckeri* with 47.5 – 48% G + C seems to be closer related to other yersiniae (46 – 50% G + C) than to *Serratia* species (52 – 60% G + C) (Ewing *et al.* 1978; De Grandis *et al.* 1988). But multilocus sequence typing (MLST) and rRNA 16S analysis have demonstrated that *Y. ruckeri* was the most distant species within the genus *Yersinia* (Kotetishvili *et al.* 2005). The virulence mechanisms used by this pathogen are different with relation to other *Yersinia* species as discussed below. Nevertheless, more research and knowledge about *Y. ruckeri* and the pathogenesis is needed to get more insight in the taxonomic status of *Y. ruckeri* with relation to other *Yersinia* species.

1.2. Characteristics of *Y. ruckeri*

Yersinia ruckeri belongs to the family *Enterobacteriaceae*. The cells are Gram-negative rods with rounded ends. Actively growing cells are approximately 0.75 µm in diameter and between 1.0 and 3.0 µm in length. This non-spore-forming bacterium does not possess a capsule, but often has flagella (Ross *et al.* 1966). As flagella are not always present, *Y. ruckeri* strains show variable motility (Davies & Frerichs 1989). As the other members of the *Enterobacteriaceae* family, *Y. ruckeri* is glucose-fermentative, oxidase-negative and nitrate-reductive (Ross *et al*. 1966). Biochemical tests can be used to distinguish *Y. ruckeri* from other species as *Y. ruckeri* strains are fairly homogeneous in biochemical reactions. Distinguishing phenotypic characteristics of *Y. ruckeri* are the presence of β-galactosidase, lysine decarboxylase and ornithine decarboxylase, whereas H_2S and indole are not produced. *Y. ruckeri* ferments glucose and mannitol in contrast to inositol, rhamnose, sucrose, melibiose and arabinose which are not used (Frerichs 1993). Based on these biochemical reactions, *Y. ruckeri* can be positively identified from diagnostic tables as well as from test kits, such as the API 20E system (Frerichs 1993).

1.3. Typing of *Y. ruckeri* strains

Strains of *Y. ruckeri* can be classified on the basis of biotype, serotype and outer-membrane protein type.

Y. ruckeri strains can be divided into two biotypes, biotype 1 and 2, based on their capability to ferment sorbitol, differences in the hydrolysis of Tween 20 and Tween 80, and in motility (Davies & Frerichs 1989). However, the major subdivisions of *Y. ruckeri* are based on whole-cell serological reactions, and in this way six serovars have hitherto been distinguished. Whole-cell serological typing uses the combined recognition of a variety of cell surface associated antigen structures, including O-antigens, flagellar antigens and envelope antigens. Serovar I or 'Hagerman' strains are the most frequently isolated. It is also the most virulent serovar (Ross *et al*. 1966). Serovar II was described by O'Leary (1977) when he isolated a new sorbitol-fermenting strain of *Y. ruckeri* from Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). However, the ability to ferment sorbitol is not typical for serovar II, because the strains belonging to serovar V and some of serovar III also ferment this sugar. An Australian isolate was designated as representative for serovar III as it did not react with antiserum to either serovar I or II (Bullock *et al.* 1978). On the contrary, this Australian isolate did cross-react with serovar I antiserum and was serologically identical to the

'salmonid blood spot bacterium' that had been isolated in Australia before and had been designated as serovar I' on the basis of partial cross-reactions with serovar I strains. Both strains were combined as representatives of serovar III. Two Ontario isolates that did not react with antisera prepared against any of the known serovars were designated serovars IV (Stevenson & Airdrie 1984) and VI (Daly *et al.* 1986). A new strain was isolated from a diseased rainbow trout in Colorado (Stevenson & Airdrie 1984). As the antiserum prepared against this strain reacted only weakly with other isolates, this strain was designated serovar V. Davies (1990) distinguished five different O-serovars (O1, O2, O5, O6 and O7) based on heat-stable O-antigens. It is suggested that the Australian isolate previously described as serovar III is a rough-type mutant of serovar O1 and that the other isolates described in the literature as serovar III have been incorrectly serotyped and are, in fact, serovar O1. De Grandis *et al.* (1988) showed that the strains of serovar I, II, III (currently assimilated to serovar O1), V and VI can clearly be considered as *Y. ruckeri*. On the other hand, they proposed that serovar IV strains and strains which could not be classified in one of these serovars and were capable of fermenting arabinose and rhamnose could not be considered as *Y. ruckeri*, but were probably *Hafnia alvei*.

In 1993, Romalde *et al.* (1993) proposed a new typing scheme and distinguished four different O-serovars, taking the hypothesis of De Grandis *et al.* (1988) into account. Serovar O1 can be subdivided into two subgroups O1a (previously serovar I) and O1b (previously serovar III). Serovar O2 (serovar II) is divided into three subgroups O2a, O2b and O2c. The remaining serovars are designated as serovar O3 (serovar V) and serovar O4 (serovar VI).

The outer-membrane protein (OMP) patterns of different isolates of *Y. ruckeri* have been studied by several authors. Davies (1991b) identified five OMP-types among 135 isolates by examination of the outer membrane proteins by sodiumdodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Similarly, heterogeneity of the OMP-profiles was detected in other *Y. ruckeri* isolates and other OMP-types have been identified based on variation of the OMPs (Romalde *et al*. 1993; Sousa *et al.* 2001).

A combination of biotyping, serotyping and OMP-typing is useful for discriminating between strains of *Y. ruckeri* and was carried out by Davies (1991a) to demonstrate relatedness between isolates and to identify clonal groups. The widely spread serovar O1 isolates can be subdivided into six clonal groups, but only two are associated with major disease outbreaks in Europe (Davies 1991a).

2. The disease

2.1. Pathogenesis

Research into the pathogenic mechanisms of *Y. ruckeri* is very limited. Much more is known about the bacterium-host interactions of the three *Yersinia* species which cause disease in humans: *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. pestis* is the causal agent of bubonic and pneumonic plague, an often lethal disease transmitted by fleas or aerosols infecting regional lymph nodes or lungs. *Y. enterocolitica* and *Y. pseudotuberculosis* are enteric pathogens mainly transmitted through contaminated food or water, causing gastrointestinal disorders (Viboud & Bliska 2005). *Y. enterocolitica* infections result in acute enteritis, enterocolitis and mesenteric lymphadenitis. *Y. pseudotuberculosis* causes mesenteric lymphadenitis and occasionally inflammation of the terminal ileum and caecum (Viboud & Bliska 2005).

A handful of researchers have tried to reveal similar pathological mechanisms between the human *Yersinia* species and *Y. ruckeri* and have investigated the presence of common virulence factors. Despite the fact that these agents cause different clinical signs and utilize different modes of transmission, several common virulence factors have been identified (Revell & Miller 2001).

2.1.1. Transmission

Y. ruckeri infections spread between fish by direct contact with infected animals or carriers. Rucker (1966) already recognized the carrier state for *Y. ruckeri* as he isolated this agent from a surviving fish two months after experimental exposure. Busch & Lingg (1975) recovered the bacterium from different organs of asymptomatic carrier trouts, infected by intraperitoneal (i.p.) injection or immersion. They demonstrated that up to 25% of the fish in a rainbow trout population could carry *Y. ruckeri* in their lower intestines. The intestinal shedding of the pathogen caused recurrent infection and mortality within the population on a cyclic basis (Busch & Lingg 1975).

Infection through carrier fish is especially important under stress conditions. Indeed, Hunter *et al.* (1980) observed that carriers transmitted *Y. ruckeri* to clinically healthy fish when the temperature was raised to 25°C, whereas unstressed carrier fish did not. The pathogen has been isolated from the faeces of carrier fish two months after an ERM outbreak. *Y. ruckeri* is able to survive and remains infective in the aquatic environment and periodic shedding of the

bacterium in the faeces is of great importance in the spread of the disease (Busch & Lingg 1975). It is now well recognized that *Y. ruckeri*, as with many bacterial species in aquatic environments, is associated with surfaces and sediments. Coquet *et al.* (2002) isolated a *Y. ruckeri* strain that displayed the ability to form biofilms on solid supports, including materials commonly found in fish farm tanks. Adhesion is required for the formation of biofilms on surfaces. The overexpression of flagellar proteins is one of the phenotypic characteristics of bacteria that display high adhesion efficiency. Genetic screening analyses of mutants, which were defective in biofilm formation, have shown that the initial reaction with the surface is promoted by pili and flagella. The bacterial cells consequently use either pili or flagella to move along the surface to encounter other bacteria and form or enlarge microcolonies (O'Toole & Kolter 1998; Pratt & Kolter 1998; Watnick & Kolter 1999). Moreover, biofilm bacteria have been shown to be strongly resistant to oxolinic acid, a frequently used antibiotic in the treatment of yersiniosis (Coquet *et al.* 2002). These biofilms may be a source of recurrent infection in rainbow trout farms. The transmission mode of *Y. ruckeri* has also been related to other putative vectors such as aquatic invertebrates and birds (Willumsen 1989).

Whether vertical transmission occurs from brood fish to offspring is not certain. Sauter *et al.* (1985) recovered *Y. ruckeri* from disinfected nonfertilized eggs of Chinook salmon whose offspring experienced low mortality from fertilization to 12 weeks on feed. They suggest that *Y. ruckeri* can be vertically transmitted, although no further proof has been provided.

2.1.2. Portal of entry

Knowledge about the portal of entry is important in bacterial pathogenesis. This provides information about the early stage of disease development and may lead to the development of efficient therapeutic strategies. How *Y. ruckeri* enters its host has not yet been determined, however, the gut has been proposed as main site. This is based on the isolation of *Y. ruckeri* from the intestine of fish after experimental infection and natural outbreaks (Busch & Lingg 1975; Valtonen *et al.* 1992).

The gills, the gut and the skin have been identified as important routes of infection for other fish pathogens. All these tissues are covered with a mucus layer and therefore, the mucus is the first physical barrier that has to be crossed by the pathogen to initiate infection.

The mucus may have an important role in the host defence against pathogens. Antibacterial activity has been recorded in the mucus of several fish species, but the extent of activity seems to vary between species and may be specific for the microorganism (Svendsen & Bøgwald 1997). Some pathogens, however, have been found to efficiently adhere to mucus

components such as mucin and glycoconjugates, penetrate through the mucous layer and reach the underlying epithelium (Chen *et al.* 2008).

The gills are in constant contact with the aquatic environment and therefore, directly accessible to pathogen attack. Different bacteria have been shown to penetrate the gill epithelium and reach the capillary vessels of the secondary lamellae (Smith *et al.* 1999; Ling *et al.* 2001). Indeed for respiration, gills are highly vascularized with a large number of blood capillaries and therefore, they may provide good entry sites for bacteria to become easily disseminated through the entire body of the fish.

The gut has been proposed to be a portal of entry for *Y. ruckeri* (Busch & Lingg 1975; Valtonen *et al.* 1992). Colonization of this organ may occur through the uptake of contaminated food or water. Therefore, the microorganisms must overcome the acidic secretions and digestive enzymes found in the stomach before reaching the intestinal tract. Both adhesion to mucus and chemotaxis followed by mucus penetration have been shown to promote attachment of pathogenic bacteria to the intestine (Olsson *et al.* 1996). Epithelial penetration or endocytosis of the pathogen may increase the invasion of the host blood and the lymphatic system, resulting in a spread of the pathogen through the body of the fish. Experimental intestinal intubation of pathogens has also shown to give efficient infection and mortality (Olsson *et al.* 1996; Smith *et al.* 1999; Smith *et al.* 2004). Whether bacteria can also enter the intestine of the fish through the anal opening under natural conditions remains unclear, since this condition would require infection against the natural flow of the intestinal contents.

Structurally, the skin is a thicker and more compact natural barrier compared to the gills and the intestine. Some pathogens are able to enter intact skin, but injuries may facilitate the transmission (Svendsen *et al.* 1999; Spanggaard *et al.* 2000; Smith *et al.* 2004). Cultured fish may sometimes suffer mechanical skin damage, whereas also ectoparasites may cause wounds. Following adhesion, a progressive penetration of pathogenic bacteria in deeper tissues and in the muscles has been shown (Smith *et al.* 1999).

Bacteria causing systemic disease possess several features which allows them to exert their pathogenic effects in the host. They have developed several mechanisms to adhere to host surfaces, invade tissues, combat the host defence mechanisms and eventually damage internal organs. It is generally believed that adhesion to the epithelium followed by invasion of the portal of entry are a prerequisite and consequently of major importance in the early pathogenesis of diseases.

Bacterial **adhesion** may be dependent on both hydrophobic interactions as well as the specific binding of a ligand to its receptor. Bacterial pathogens and eukaryotic cells are negatively charged under physiological conditions. Hydrophobic interactions may overcome the repulsion between the negatively charged bacteria and their host cells and may lead to an initial, weak association between both cell types. Strong adhesion is generally mediated by the specific binding of bacterial adhesins to complementary receptors on host surfaces (Figure 1). The largest group of adhesins described among bacterial pathogens are lectins which have often been described as parts of fimbriae or outer membrane components in Gram-negative bacteria. These lectins bind specific carbohydrate structures and are usually classified by their sugar specificity. This is generally demonstrated by inhibiting the adhesion with carbohydrates that compete with the binding of the adhesins to host cells. Besides lectins, other bacterial components have been identified to act as adhesins such as polysaccharides including lipopolysaccharide (LPS) and proteins. Protein-protein interactions may involve bacterial proteins binding to components of the extracellular matrix (ECM) (Ofek *et al.* 2003).

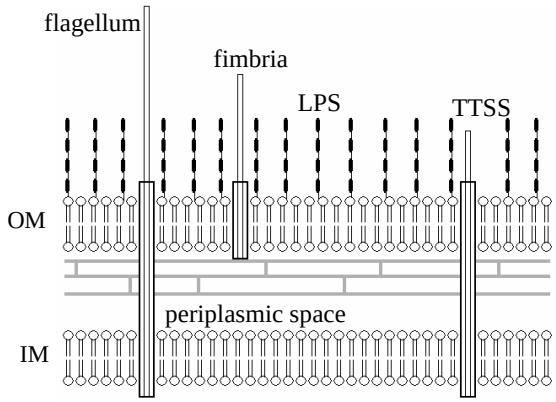

Figure 1 Illustration of the cell surface of a Gram-negative bacterium with its major potential adhesins, including subunits of flagella, fimbriae and outer membrane proteins. Adhesins are generally integral components of the outer membrane (OM), but can also be secreted components that are associated with integral membrane components. (IM: inner membrane; LPS: lipopolysaccharide; TTSS: type three secretion system).

Following adhesion, bacteria may **invade** the host tissue and therefore, they often have to overcome the epithelial barrier and/or the ECM. Interaction between an invasive microbial pathogen and its host cell induce a signal transduction cascade leading to cytoskeletal rearrangements which promote internalization. Three major invasion mechanisms have been recorded among facultative intracellular pathogens: the receptor-mediated or zipper

mechanism, the type three secretion-dependent ruffling of host cell membranes or trigger mechanism and the invasome mechanism (Figure 2).

Pathogens having the zipper mechanism use a single bacterial surface molecule that mediates both the adhesion and invasion processes. High affinity binding of the bacterial adhesin to a complementary receptor on the host cell results in the accumulation of actin filaments and the formation of modest membrane extensions around the entering bacteria. Finally, the bacteria are endocytosed in a clathrin-coated vesicle (Alonso & Portillo 2004).

The trigger mechanism involves the injection of effector proteins in the host cell which interact with the actin cytoskeleton using a type three secretion system (TTSS). Dramatic alterations in the host cell membrane are induced and the formation of membrane ruffles leads to bacterial internalization (Alonso & Portillo 2004).

The invasome mechanism has not yet been thoroughly defined. A bacterial aggregate is formed and engulfed leading to internalization via a unique host cellular structure, termed the invasome. The formation of a large aggregate of bacteria is required for triggering internalization and therefore, it is thought to be quorum sensing-dependent (Ofek *et al.* 2003).

Invasion of the ECM is often mediated by secretion of proteases with subsequent degradation.

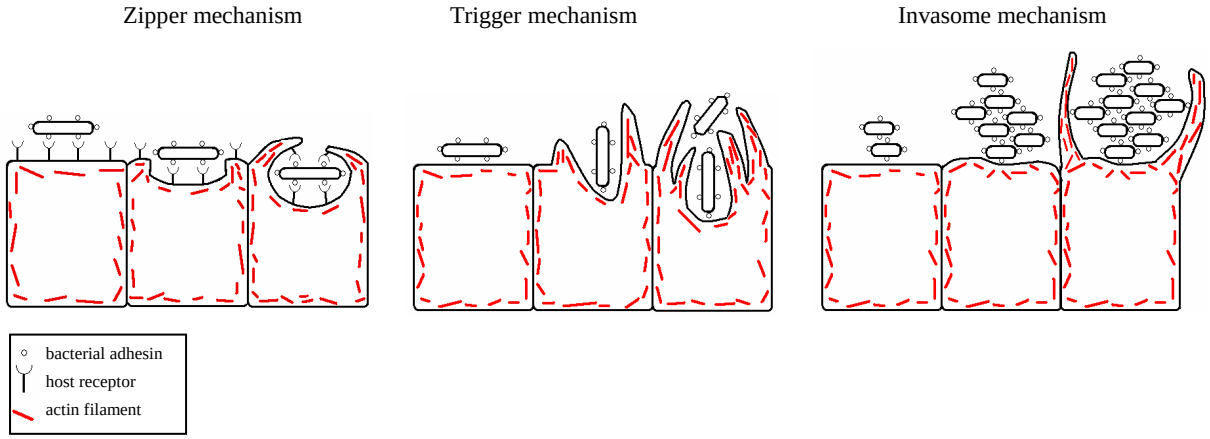

Figure 2 Illustration of the three major invasion mechanisms of facultative intracellular microbial pathogens: the zipper, the trigger and the invasome mechanism.

Y. ruckeri was recently shown to adhere to different glycoproteins in carp intestinal mucus (Schroers *et al.* 2008). The ability of *Y. ruckeri* to adhere to and effectively invade fish cell lines cultured *in vitro* has also been proven, but the adherence capacity was dependent on the cell line employed (Romalde & Toranzo 1993; Kawula *et al.* 1996). Some cell surface associated properties such as hydrophobicity and agglutinating capacities could be responsible for bacterial adhesion, however, these properties were not generally found in *Y. ruckeri* and

could not be related to its pathogenicity (Romalde *et al.* 1990). How *Y. ruckeri* invades host cells has hitherto not been studied, but an extracellular protease called Yrp1 has been found to be important in *Y. ruckeri* invasion. Yrp1 digests a wide range of ECM proteins, particularly laminin which is a major component of basement membranes. Nevertheless, the molecular mechanisms involved in adhesion and invasion remain unknown.

2.1.3. Virulence factors

2.1.3.1. Extracellular toxins: Yrp1 and YhlA

Romalde & Toranzo (1993) showed that extracellular products (ECPs) of *Y. ruckeri*, including lipases, proteases and haemolysins, reproduce some characteristic signs of ERM, such as haemorrhage in the mouth and the intestine, when injected into fish. Therefore, these ECPs seem to play a role in the pathogenesis of *Y. ruckeri* infection.

A molecule, of which the involvement in virulence has been proven is the 47-kDa metalloprotease, designated Yrp1, which is produced at the end of the exponential growth phase (Secades & Guijarro 1999). The protease is secreted by a type I Gram-negative bacterial ABC exporter protein secretion system composed of three genes, *yrpD*, *yrpE* and *yrpF*, and a protease inhibitor *inh* (Fernández *et al.* 2002). The presence of Yrp1 is not a general feature for *Y. ruckeri* and has no relationship with the serovar. Indeed, Secades & Guijarro (1999) found that some strains of serovar I, the most virulent and common serovar, showed protease activity and other strains of the same serovar did not. Two groups of strains were defined, i.e. Azo^+ and Azo^-, according to the presence or absence of the Yrp1 proteolytic activity with azocasein as substrate, respectively (Secades & Guijarro 1999). Analysis of both groups showed that all strains contained the *yrp1* operon, but further research revealed that the operon is regulated at the transcriptional level. The Azo^- phenotype is due to a transcriptionally inactive *yrp1* operon or a very low transcriptional level of the operon, which is not enough for the detection of the proteolytic activity (Fernández *et al.* 2003).

Yrp1 protease contributes to the virulence of the bacterium and is allegedly involved in the colonization and invasion of different tissues. Indeed, Yrp1 protease digests a wide variety of extracellular matrix and muscle proteins, and may lead to membrane alterations and pores in the capillary vessels. This may result in the leaking of blood from these vessels and hence cause the typical haemorrhages especially around the mouth and intestine (Fernández *et al*. 2003).

The expression of the protease is under the control of environmental conditions. Production of Yrp1 has been shown to be sensitive to repression by carbon and nitrogen sources. Glucose and fructose were found to be the greatest production inhibitors, whereas glycerol, mannitol and maltose also had a potent repressive effect. Protease production was also decreased when ammonium was added to the culture medium (Secades & Guijarro 1999). *Yrp1* expression is also regulated by osmolarity and temperature, without the influence of pH. Production of the protease is decreased by increasing the osmotic pressure of the medium (Fernández *et al*. 2003). Expression of *yrp1* operon was found to be high at 18°C and was repressed at 28°C, the optimal growth temperature of *Y. ruckeri* (Fernández *et al*. 2003). Thus, Yrp1 protease is highly expressed at temperatures found in the host and may be an adaptation to the optimal temperature conditions for efficient infection and colonization. Characterization of the protease also showed that it requires Mg^{2+} and Ca^{2+} cations for maximal activity (Secades & Guijarro 1999). Sequence alignment revealed that Yrp 1 shows a high degree of homology with metalloproteases from *Erwinia chrysanthemi* (Fernández *et al.* 2002).

A haemolysin/cytolysin, named YhlA, has been found to play an important role in the pathogenicity of *Y. ruckeri* (Fernández *et al*. 2007b). Two genes are thought to be necessary for the production of YhlA. The upstream gene *yhlB* is involved in the secretion and activation of the haemolysin, encoded by *yhlA*. Both genes showed a high homology to genes encoding haemolysins of the *Serratia*-type pore-forming toxins which are secreted by a two-partner secretion system, also called type V secretion system. Genomic analysis revealed the presence of similar haemolysins in human pathogenic yersiniae, however, their function has not yet been determined (Fernández *et al*. 2007b).

The haemolysin YhlA is able to lyse erythrocytes as well as cultured fish cells and may be related to invasive properties as has been shown for other *Serratia*-type toxins. Fifty percent lethal dose (LD_{50}) experiments using *yhlB* and *yhlA* insertional mutant strains, demonstrated the role of the toxin in the virulence of *Y. ruckeri* (Fernández *et al*. 2007b).

Similar to the *yrp1* operon, the expression of *yhlA* was remarkably higher at 18°C, the infection temperature, than at 28°C, the optimal growth temperature of *Y. ruckeri*. Haemolysin production also increased under iron-starvation conditions and was suggested to be important in the acquisition of iron from the host cells (Fernández *et al*. 2007b).

2.1.3.2. Adhesins and invasins

Y. enterocolitica and *Y. pseudotuberculosis* produce at least three invasion proteins: invasin and Ail are encoded on the chromosome and the YadA protein on a 70-kb virulence plasmid.

Invasin and YadA are two outer membrane adhesins that both bind β1 integrin receptors. Attachment of the bacteria via these adhesins seems to be important for promoting internalization into macrophages as well as into neutrophils and dendritic cells (Hudson *et al.* 2005). Ail is another outer membrane protein that plays a role in cell invasion, although its binding receptor is unknown. Kawula *et al.* (1996) examined the presence of the genes *inv* and *ail* in *Y. ruckeri* by using southern blot analysis. They found no evidence for *inv* or *ail* homologues in *Y. ruckeri* although it should be noted that only one strain was tested (Kawula *et al*. 1996). Using PCR and sequencing analysis, however, Fernández *et al.* (2007a) suggested that *Y. ruckeri* carries an *inv* homologous gene that could be involved in bacterial adherence and invasion into host cells.

Y. pestis does not express invasin, Ail or YadA as the genes encoding these proteins are disrupted by frameshift mutation or transposon insertion (Cowan *et al.* 2000). Nevertheless, *Y. pestis* has been found to be at least as invasive for epithelial cells as the enteropathogenic *Yersinia* species (Cowan *et al*. 2000). Virulence factors that affect invasiveness include the outer membrane serine protease Pla (plasminogen activator protease). This protease, encoded by the *pla* gene located on a 9.6-kb plasmid pPCP1, can activate plasminogen and promote adherence to and invasion in epithelial cells and macrophages. Cowan *et al.* (2000) showed that Pla is an important, but not the sole significant adhesin in *Y. pestis*. However, little is known about additional adhesin(s). No research has hitherto been carried out on the possible presence of Pla in *Y. ruckeri*.

Although it is clear that the majority of the bacterial replication occurs in an extracellular phase, there is also evidence that all three human pathogenic yersiniae survive and multiply in macrophages (Pujol & Bliska 2005). Intracellular survival and replication in macrophages may occur throughout the *Yersinia* infection, but is very important during the initial stages of colonization. There is also evidence that *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* can subvert the normal functions of macrophages while replicating in phagosomes. *Y. pseudotuberculosis* prevents acidification of their phagosomes to low levels that are important in the destruction of intracellular pathogens as well as in phagosome maturation (Tsukano *et al.* 1999). *Y. pestis* and *Y. pseudotuberculosis* share a pigmentation segment, a 102-kb pathogenicity island that is required for replication in activated macrophages when exposed to interferon-γ (IFN-γ). This segment is absent in *Y. enterocolitica* and indeed, phagocytosed *Y. enterocolitica* were efficiently killed in murine macrophages when IFN-γ was added, while *Y. pestis* and *Y. pseudotuberculosis* continued to replicate (Pujol & Bliska 2005). The pigmentation segment also plays an important role in the reduced production of nitric oxide in

macrophages infected with *Y. pestis* or *Y. pseudotuberculosis* (Pujol & Bliska 2005). Inducible nitric oxide synthase is a key protein that is expressed in murine macrophages exposed to LPS and IFN-γ and functions to kill intracellular pathogens via the production of nitric oxide. Intracellular *Y. pestis* and *Y. pseudotuberculosis* are able to interfere with macrophage activation and the production of nitric oxide. No thorough research has been performed on the ability of *Y. ruckeri* to survive in macrophages. Interestingly, a small number of intracellular bacteria were observed in kidney, spleen and peripheral blood phagocytes of rainbow trout after immersion and intraperitoneal infection with a green fluorescent protein (GFP) expressing strain of *Y. ruckeri* (Welch & Wiens 2005).

2.1.3.3. Ruckerbactin

Another molecule of *Y. ruckeri* with a proven involvement in virulence is the iron uptake system ruckerbactin (Fernández *et al.* 2004). Iron acquisition is essential for successful colonization and invasion by many microbial pathogens and therefore they developed high-affinity iron transport systems (Faraldo-Gomez & Sanson 2003). Siderophores are low-molecular-mass Fe^{3+}-chelating compounds which may be regarded as virulence factors, because the availability of iron endows the bacteria with the ability to multiply in the host. Siderophores can be divided into three major classes, catecholates, hydroxamates and heterocyclic compounds. During infection, siderophores can bind host iron because of their high affinity for the metal and transport it back to the bacteria. The complex siderophore-Fe^{3+} recognizes a specific bacterial outer membrane receptor and is translocated into the cytosol where the iron is discharged from its siderophore and utilized for different metabolic pathways (Faraldo-Gomez & Sanson 2003). Fernández *et al*. (2004) showed that genes involved in the siderophore pathway of *Y. ruckeri* are upregulated during the infection of fish. This induction was temperature-dependent, being higher at 18°C, the infection temperature, than at 28°C, the optimal growth temperature. The chemical structure and biosynthetic route of this catechol siderophore, called ruckerbactin, are not yet determined. Sequencing analysis revealed that the ruckerbactin receptor had the highest homology with the ferrichrysobactin receptor from the plant pathogen *Erwinia chrysanthemi* and shows more similarity with hydroxamate receptors than with other catecholate receptors (Fernández *et al.* 2004). The human pathogenic yersiniae only produce and utilize a heterocyclic compound, yersiniabactin, which has been related to pathogenicity and they do not produce either catecholate nor hydroxamate siderophores (Fernández *et al*. 2004). Based on their findings, Fernández *et al.*

(2004) suggest that *Y. ruckeri* is closer related to *E. chrysanthemi* than to other yersiniae in terms of pathogenic mechanisms.

2.1.3.4. Plasmids

The presence of plasmids in *Y. ruckeri* strains has been described by different authors. Although one or more of these plasmids may be involved in the virulence of the pathogen, their function is not clear. Garcia *et al.* (1998) studied the plasmid profile of 183 isolates from a wide variety of sources and detected eight different profiles. The observation of a large plasmid of approximately 75 MDa in most of the *Y. ruckeri* strains agrees with reports from other authors (Guilvout *et al.* 1988; Romalde *et al*. 1993). The existence of this large plasmid was independent of the geographical origin of the isolates and common to all clinical strains. Large plasmids have only been found in strains of serovar O1 and not in other serogroups. Small plasmids have been found in different serovars as well as in serovar O1. However, the small plasmids have received less attention than the large one, probably because of the attempts to correlate the latter with the virulence plasmid of the human pathogenic *Yersinia* species. These latter species harbour a 70-kb virulence plasmid which encodes a TTSS, required for counteracting the immune response of the host and to ensure survival. A TTSS allows direct communication between bacteria and host cells by injecting effector proteins into the cytosol and in this way the bacterium modulates the cell functions to its advantage. A TTSS or 'injectisome' consists of two pairs of rings spanning the two bacterial membranes, linked by a rod and a needle protruding outside the bacterial body. Injection of effectors requires the presence of translocator proteins which form a pore into the target cell (Troisfontaines & Cornelis 2005). This model of a continuous conduit between the bacterial cytosol and the plasma membrane of the host explains how effectors can be efficiently targeted into the host cytosol without 'leaking' and how the integrity of the plasma membrane can be maintained. The Ysc (Yop secretion) injectisome in human pathogenic *Yersinia* species, encoded by the 70-kb virulence plasmid, secretes Yop (*Yersinia* outer protein) effectors under low calcium conditions. Bacterial attachment causes a local decrease in extracellular calcium that in turn activates the Ysc TTSS (Allen 2003). The translocators form the needle to insert into the host plasma membrane and function to transport six effectors (YpkA/YopO, YopH, YopM, YopT, YopP/J and YopE) into the cytosol of the host cell. Yops have a protein kinase or phosphatase activity and can interfere in signalling pathways by phosphorylation or dephosphorylation of involved proteins. Several reviews focus on the biochemical function of these Yops, the signalling pathways they modulate and their role in

Yersinia pathogenesis (Cornelis 1998; Fallman & Gustavsson 2005; Viboud & Bliska 2005). In general, the major functions assigned to Yops in counteracting the immunity of the host include inhibition of bacterial uptake and killing by phagocytes, suppression of proinflammatory cytokine production and induction of macrophage apoptosis.

Guilvout *et al*. (1988) compared the plasmid profile in one American and 18 French *Y. ruckeri* strains with the patterns of *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. The large plasmid of *Y. ruckeri* appears to be significantly different from the virulence plasmid associated with human pathogenic *Yersinia* species.

2.1.3.5. Type three secretion system

A TTSS, different from that shared by human pathogenic *Yersinia* species, has been found in *Y. ruckeri* (Gunasena *et al.* 2003). Sequencing analysis of the gene encoding the most highly conserved proteins of TTSS in fish pathogens, the type three ATPase, and adjacent genes revealed a significant homology with the chromosomally encoded Ysa (*Yersinia* secretion apparatus) TTSS genes of *Y. enterocolitica* biovar 1B (Gunasena *et al*. 2003). This suggests the presence of a Ysa-like TTSS in *Y. ruckeri*. The Ysa TTSS of *Y. enterocolitica* biovar 1B contributes to gastrointestinal stages of infection and the delivery of effector proteins, called Ysps (*Yersinia* secreted proteins), into host cells affecting the outcome of an infection (Venecia & Young 2005). Several of the Ysps seem to be homologous to other virulence factors (Matsumoto & Young 2006), but more research into the precise function is required to understand how *Y. enterocolitica* biovar 1B interacts with host cells. Additionally, more research is needed to reveal the presence and the function of the Ysa TTSS in *Y. ruckeri*.

2.1.3.6. Type four secretion system

A chromosomally encoded cluster of eight genes, *traHIJKCLMN* in short the *tra* operon or *tra* cluster, has been found in *Y. ruckeri* to form part of a virulence-related type four secretion system (TFSS) (Méndez *et al.* 2009). This operon was present in *Y. ruckeri* strains with different backgrounds, indicating that these genes are important in the pathogenesis of this bacterium. Similar TFSS of intracellular pathogens have been identified to participate in the transfer of different effector molecules into target cells to play a role in their survival within macrophages or red blood cells. Therefore, the *tra* operon found in *Y. ruckeri* may be important during the intracellular stage (Méndez *et al.* 2009). Expression of the operon was temperature-dependent as has been seen for other virulence factors, being upregulated at 18°C

compared to 28°C and was also higher under nutrient-limiting conditions (Méndez *et al.* 2009).

The *tra* operon appeared to be similar to the virulence plasmid pADAP from *Serratia entomophila* both in sequence and genetic organization, whereas no similar *tra* operon has been found yet in human pathogenic yersiniae (Méndez *et al.* 2009).

2.1.4. Importance of environmental factors

Environmental factors may have a direct influence on the pathogenicity of microorganisms. This is particularly the case in aquatic environments where both the host and the bacterium are greatly influenced by the fluctuations of ambient water conditions. Factors such as temperature and salinity can influence the establishment and the severity of *Y. ruckeri* infections (Altinok & Grizzle 2001; Altinok 2004).

Temperature regulation is especially important in fish pathogenic bacteria because the production of a specific protein may stop at the temperature corresponding to the upper limit of pathogenicity of the bacteria, which is below the optimal growth temperature. This corresponds with the observation of the repressed activation of the *yrp1* promotor at 28°C, the optimal growth temperature of *Y. ruckeri* (Fernández *et al*. 2003). Similar results were obtained for the expression of the haemolysin YhlA, the siderophore ruckerbactin and the *tra* operon that were higher at infection temperature of 18°C than at 28°C as stated above (Fernández *et al*. 2004).

The effect of salinity has been studied to a limited extent. The transcription of the *yrp1* operon significantly decreased with increasing osmolarity of the growth medium (Fernández *et al.* 2003). This finding is consistent with the observation that outbreaks of ERM are especially found in freshwater fish. Moreover, *Y. ruckeri* has been found to survive for at least four months in unsupplemented water, whereas its survival time notably decreased in aquatic environments with high salinity (Thorsen *et al.* 1992).

2.2. Clinical signs

ERM can affect fish of all ages, but is most acute in small fish up to fingerling size. In larger fish, the disease appears as a more chronic condition.

Changes in fish behaviour may be observed, including swimming near the surface and moving slowly. Affected fish are lethargic, are found in areas of low flow and often lose their appetite. Haemorrhages on the body surface are common, with reddening at the base of the

fins and along the lateral line, as well as in the head region. The characteristic haemorrhages in and around the oral cavity have led to the name ‘redmouth’ disease, although these reddened areas are not apparent in some affected fish and thus absence of classic ‘redmouth’ does not rule out infection with *Y. ruckeri*. Petechial haemorrhage on the surface of the liver, pancreas, pyloric caeca, swim bladder and in the lateral musculature may be obvious. The spleen is often enlarged and may be almost black in colour (Figure 3a). The intestine is inflamed and filled with a thick, opaque and purulent fluid. The abdomen is distended as a result of fluid accumulation. Exopthalmia occurs and is commonly accompanied by orbital haemorrhages (Figure 3b), sometimes seen as haemorrhagic rings around the eyes (Rucker 1966; Horne & Barnes 1999; Avci & Birincioğlu 2005).

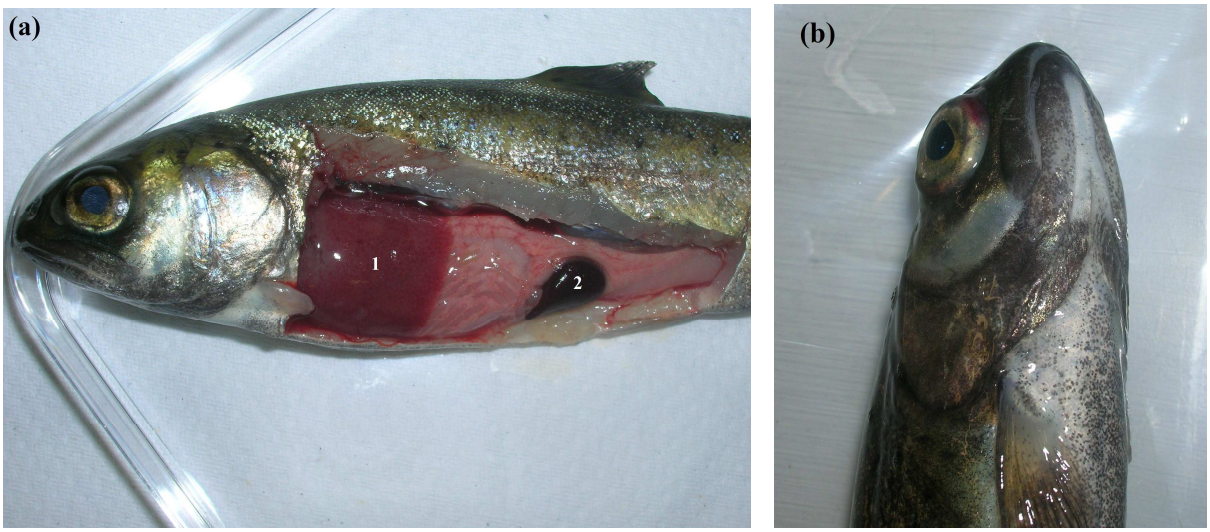

Figure 3 *Oncorhynchus mykiss* with (a) enlarged (1) liver and (2) spleen and (b) exopthalmia with orbital haemorrhages, both typical for yersiniosis.

Histological examination of tissues from infected rainbow trout with acute ERM shows general septicaemia with an inflammatory response in virtually all tissues. Bacterial colonization especially occurs in well-vascularized tissues such as kidney, spleen, heart, liver and gills and in areas of petechial haemorrhage (Rucker 1966). Pathological changes in the gills, including hyperemia, oedema and desquamation of the epithelial cells in the secondary lamellae, have been described in experimentally infected fish. Focal necrosis was observed in the liver, kidney and spleen of some fish after i.p. injection with *Y. ruckeri* (Berc *et al.* 1999; Avci & Birincioğlu 2005).

Fish with chronic yersiniosis may show dark pigmentation or depigmentation of the skin (Avci & Birincioğlu 2005). Prominent peritonitis probably caused by long-lasting

inflammation in the peritoneal cavity and enteritis have been described in carp that survived i.p. injection with *Y. ruckeri* (Berc *et al.* 1999).

Outbreaks of ERM usually begin with low mortality which slowly escalate and may result in high losses. The problem may become large-scale if chronically infected fish are exposed to stressful conditions such as high stocking densities and poor water quality (Horne & Barnes 1999). Severity of yersiniosis is dependent mainly on the virulence of the strain and the degree of environmental stress.

2.3. Immune response

Knowledge about the immune defence mechanisms of fish against bacteria is important in terms of control and prevention. Many non-specific and specific, humoral and cellular mechanisms of fish to resist bacterial disease have been studied (Ellis 1999; Ellis 2001; Claire *et al.* 2002).

One of these important antibacterial defence mechanisms is phagocytosis and killing of invading pathogens. The principal phagocytic cells - neutrophils and macrophages - internalize bacteria and kill them by production of reactive oxygen species (ROS) during the so-called respiratory burst. I.p. injection of 5×10^7 live or formol-killed *Y. ruckeri* in rainbow trout resulted in a rapid influx of large numbers of neutrophils, which were attracted from the blood and haematopoietic organs and phagocytosed the bacteria (Afonso *et al.* 1998a). Neutrophils are present in tissues in significant numbers only when inflammation occurs and as long as the inflammation process persists. In contrast, macrophages are present in all body compartments and are the first phagocytes to encounter the invading pathogens (Afonso *et al*. 1998a). Resident macrophages also phagocytose the bacteria and have also been observed to phagocytose neutrophils containing bacteria. Furthermore, by coming into close contact with macrophages, neutrophils appear to transfer peroxidase-containing granules to the macrophages which play a role in killing bacteria. In the presence of halide ions and H_2O_2, peroxidase halogenates the bacterial cell walls and produces bactericidal hypohalite ions (Afonso *et al.* 1998b). A weak ROS response consistently elicited by serotype I strains was observed in striped bass, *Morone saxatilis* (Walbaum), macrophages, in contrast to serovar II strains which generally elicited increased phagocyte ROS responses (Stave *et al.* 1987). Although its involvement in pathogenicity is still to be determined, a correlation was made with the presence of the large 75 MDa plasmid in all serotype I strains and in general not in serovar II strains. However, one serovar II strain, which did not carry the large plasmid, also

showed a decreased presence of ROS (Stave *et al.* 1987). Whether *Y. ruckeri* can survive inside macrophages and neutrophils has not yet been determined.

Leukocyte phagocytosis is an important reaction activated in *Y. ruckeri*-vaccinated fish as well as antibody production (Cossarini-Dunier 1986) and lymphocyte proliferation (Siwicki & Dunier 1993, Siwicki *et al.* 2001). Enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of many fish pathogens. Cossarini-Dunier (1985) developed an ELISA to detect and quantify specific antibodies in rainbow trout to *Y. ruckeri*. Antibodies against this pathogen have been shown to be produced following vaccination and probably play a role in protection.

Recently, some studies have measured the expression of immune relevant genes in rainbow trout in response to *Y. ruckeri* infection or immunization.

In mammals, Toll-like receptor 3 (TLR3) is involved in double-stranded RNA recognition and host immune response activation when viral infection occurs. To investigate whether TLR3 in trout is involved in antibacterial immunity, Rodriguez *et al.* (2005) examined its transcriptional regulation *in vivo* after *Y. ruckeri* treatment. In healthy rainbow trout, TLR3 is highly expressed in the liver, pyloric caeca, intestine, spleen and anterior and trunk kidney tissues. There was a slightly although non-significant up-regulation of TLR3 mRNA levels in the spleen and a modest down-regulation in the anterior kidney after bath challenge with *Y. ruckeri*. The authors suggest that a larger sample size would be required to determine if the small upregulation in the spleen was statistically and biologically significant.

Wiens *et al.* (2006) described novel CXC chemokines, designated CXCd proteins, in rainbow trout. Chemokines attract lymphocytes to sites of injury and then activate them. *CXCd* was constitutively expressed in skin, gill, visceral fat and posterior kidney tissues, while low mRNA levels were present in the anterior kidney and spleen. Transcript abundance in the spleen and anterior kidney was increased during both *Y. ruckeri* bath vaccination and challenge. The number of viable *Y. ruckeri* was significantly correlated with *CXCd* gene transcript abundance and fish with the highest bacterial loads had the highest *CXCd* expression. In contrast, pro-inflammatory cytokine IL-1β transcript levels were increased in fish infected with low numbers of *Y. ruckeri*, while diminishing in heavily infected fish. Infection with a high injection challenge dose of the rhabdovirus infectious haemopoietic necrosis virus (IHNV), did not lead to changes in *CXCd* expression, suggesting that upregulation may be pathogen-specific. However, Raida & Buchmann (2008a) did not observe increased *CXCd* expression after bath vaccination of rainbow trout with *Y. ruckeri* and induction may not clearly be regulated following vaccination.

Raida & Buchmann (2008a) studied the gene expression of pro-inflammatory and anti-inflammatory cytokines, chemokines, immunoglobulins and cellular receptors after bath vaccination of rainbow trout. They found the upregulation of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-6. These are all produced by T-cells and macrophages and induce local inflammation and the production of acute phase proteins respectively. An increased expression of the anti-inflammatory cytokines IL-10 and TGF-β was also observed, however, their specific role in trout is still unclear. The chemokine IL-8 is considered to have a chemo-attractive effect on neutrophils in trout, but expression was not increased due to vaccination. The teleost specific antibody IgT from which the specific function is unknown as well as cell receptors TcR, CD8α, CD4, C5aR were clearly induced in rainbow trout after bath vaccination. Passive immunisation conducted by transfer of plasma from vaccinated to naïve fish did not provide protection. Therefore, the authors suggest that humoral factors are less important in the protection induced by bath vaccination. Cellular factors such as CD8α were upregulated, suggesting that cellular factors including cytotoxic T-cells could play a role in immunity against *Y. ruckeri* (Raida & Buchmann 2008a).

Raida & Buchmann (2007) also investigated the gene expression of immune-relevant genes in rainbow trout following i.p. injection of a *Y. ruckeri* bacterin. The pro-inflammatory cytokines IL-1β and IFN-γ and the anti-inflammatory cytokine IL-10 were significantly up-regulated after immunization. Moreover, this expression was higher in the spleen compared to the head kidney. Although the head kidney of teleost fish is considered to be of major importance in the clearance of bacteria by macrophages, recruitment and activation of lymphocytes as response to infection has been thought to occur in the spleen (Raida & Buchmann 2008b). IgT was very weakly expressed compared to IgM, whereas C5aR expression was increased following i.p. vaccination (Raida & Buchmann 2007).

Different genes encoding cytokines were also significantly upregulated in the spleen after primary infection with a *Y. ruckeri* O1 strain (Raida & Buchmann 2008b). At the same time, the spleen was enlarged, probably due to the influx or proliferation of cells recruited by inflammatory cytokines. Cytokines are known to activate lymphocytes to initiate an adaptive immune response that could lead to long-lasting protective immunity. Indeed, a weak expression of the cytokine-encoding genes was observed in surviving rainbow trout after re-infection with *Y. ruckeri*, whereas a rapid elimination of the bacteria took place. Moreover, re-challenged fish showed a recovered normal spleen and a highly increased survival compared to the naïve fish receiving a primary infection. This result was attributed to the development of adaptive immunity in the rainbow trout against *Y. ruckeri* (Raida & Buchmann 2008b).

It is important that the immune defence mechanisms of fish against *Y. ruckeri* and the possibility that these bacteria can overcome some of these mechanisms are investigated in more detail to be able to develop new approaches in the protection of fish.

2.4. Diagnosis

Different diagnostic methods have been developed for *Y. ruckeri*, including culturing, serological tests and molecular biological techniques. The isolation of *Y. ruckeri* by using the classic agar media is useful as the bacterium grows fairly rapidly. Tryptic Soy Agar (Austin *et al.* 2003; Sousa *et al.* 2001) is commonly used, although *Y. ruckeri* also grows on Columbia blood agar plates (Bomo *et al.* 2003) and MacConkey agar (Gibello *et al.* 1999).

Y. ruckeri has an optimum growth temperature of 28°C, although it can grow in a wide temperature range (Stevenson *et al.* 1993). After incubation for 48 h at 25°C on blood agar, off-white, opaque colonies of approximately 2-3 mm in diameter appear. The pathogen may be identified using biochemical characteristics as described above.

Based on serological characteristics, other methods may be used in detecting this pathogen such as ELISA, agglutination test and immunofluorescence antibody technique (IFAT) (Smith *et al.* 1987).

Molecular techniques including restriction fragment-length polymorphism (RFLP) (Garcia *et al*. 1998) and polymerase chain reaction (PCR) (Gibello *et al.* 1999; Altinok *et al.* 2001) are often used. Gibello *et al*. (1999) developed a PCR assay, based on the selective amplification of the 16S rRNA gene, for demonstration of *Y. ruckeri* in infected trout tissues. This PCR method has the advantage of being able to detect low levels of *Y. ruckeri* and provides the possibility to detect asymptomatic carriers, which is very important in order to prevent the transmission and spread of ERM. PCR amplification of *yruR/yruI* genes, responsible for the quorum sensing system of *Y. ruckeri*, has also proved to be highly specific (Temprano *et al.* 2001). Altinok *et al*. (2001) described a PCR method for detecting *Y. ruckeri* in the blood of rainbow trout. The use of blood samples does not require necropsy and allows repeated sampling of individual fish. Other nonlethal methods for diagnosis of *Y. ruckeri* infections are culture of faeces from the posterior intestine using inoculating loop stabs (Busch & Lingg 1975; Rodgers 1992) and biopsy of head kidney (Noga *et al.* 1988).

A practical alternative for rapid and sensitive detection of pathogens is the loop-mediated isothermal amplification (LAMP) assay. This nucleic acid amplification method synthesises large amounts of DNA in a short time period with high specificity and requires only simple

laboratory equipment since the amplification procedure takes place under isothermal conditions (Notomi *et al.* 2000). A LAMP assay for the detection of *Y. ruckeri* based on the amplification of the quorum sensing genes *yruR/yruI* has recently been developed and suggested to be a promising tool for molecular detection of ERM in fish farms (Saleh *et al.* 2008).

2.5. Control and prevention

2.5.1. Antimicrobial compounds

Antimicrobial compounds are often used in the treatment of *Y. ruckeri* infections in fish. Rucker (1966) described treatment with sulphamethazine for five days, followed by three days of chloramphenicol or oxytetracycline administration. Potentiated sulphonamide therapy has been of great value in the treatment of both experimental and natural infections (Bullock *et al.* 1983). Rodgers & Austin (1983) used oxolinic acid for prophylaxis and therapy of ERM in rainbow trout. Although *Y. ruckeri* is sensitive to many antibiotics, acquired resistance of *Y. ruckeri* strains to various antimicrobial agents has been reported. Post (1987) highlighted the complete resistance of some isolates in the USA to therapeutic levels of both sulphamerazine and oxytetracycline. Resistance to both tetracyclines and sulphonamides has also been demonstrated by other authors (De Grandis & Stevenson 1985).

2.5.2. Vaccines

Vaccines for the prevention of the ERM disease were the first fish vaccines to be commercialised in 1976 and are composed of formalin-killed whole bacterial cells. The water temperature plays an important role in the elicitation of an efficient immune response. Raida & Buchmann (2008a) found that bath vaccination of rainbow trout with a *Y. ruckeri* bacterin did not offer protection at 5°C or 25°C, in contrast to vaccination at moderate temperature. However, the highest cytokine and antibody response after i.p. injection of this bacterin in rainbow trout was found at higher water temperatures, with major expression at 25°C (Raida & Buchmann 2007). Further on, also administered by spray or oral routes, *Y. ruckeri* bacterins provide good levels of protection against ERM disease. In spite of vaccination, disease outbreaks do occur, however, from time to time under severe stress conditions due to the spreading through the water of bacteria from the faeces of carrier and ill fish (Stevenson 1997). Moreover, some non-motile strains that were isolated in the last years seem to be

unaffected by commercial vaccines (Austin *et al.* 2003; Fouz *et al.* 2006; Arias *et al.* 2007). Thus, new approaches based on subunit or DNA vaccines might be an additional way to eliminate or minimize these outbreaks. Fernández *et al.* (2003) tested the protection against yersiniosis by using a toxoid of the Yrp1 protease as immunogen, because as stated above this extracellular protease is involved in the virulence of *Y. ruckeri*. The efficacy of protection was found to be high by using active immunization with the Yrp1 toxoid through intramuscular injections. It would be interesting to study the application of Yrp1 toxoid in more detail.

Recently, interest in the use of live attenuated vaccines against bacterial pathogens in fish has increased, because they can provide a better protection. In general, they elicit a stronger cell-mediated response than bacterins, although the greater immunity provided by attenuated bacteria in comparison with that provided by killed organisms is possibly due to the induced expression of stress proteins (Temprano *et al.* 2005). Dysfunction of the *aroA* gene, present in bacteria, is finding widespread applications in the development of live vaccines for various fish diseases. This gene encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase which plays a role in the biosynthesis of aromatic amino acids. Dysfunction of the *aroA* gene by the introduction of certain mutations leads to auxotrophy of the bacterium for different metabolites. The bacterium is not able to grow in fish tissues where these metabolites are not available and in this way becomes less virulent. The vaccination of rainbow trout with a *Y. ruckeri aroA* live vaccine has been documented as providing greater protection than the currently used bacterin. Therefore, the highly attenuated *Y. ruckeri* mutant, constructed by the insertion of a DNA fragment containing a kanamycin resistance determinant into the *aroA* gene, has been considered an effective vaccine to protect fish against ERM (Temprano *et al.* 2005). However, care is necessary in the use of live vaccines. The use of *aroA* mutant strains in fish vaccinations may facilitate the spread of these bacteria into aquatic environments (Vivas *et al.* 2004). Therefore, the safe use of live vaccines or genetically modified organisms requires precise studies of potential hazards related to release, spread and survival in natural environments.

2.5.3. Immunostimulants

Resistance to bacterial pathogens can be increased by administration of immunostimulants to improve the effectiveness of vaccines. β-hydroxy-β-methylbutyrate (HMB) is a breakdown product of the amino acid leucine which is an essential building block of proteins in all tissues and has been shown to provide a positive immunostimulatory effect after *in vivo* immunization of rainbow trout with anti-*Y. ruckeri* vaccine (Siwicki *et al.* 2001). Indeed,

HMB, applied in the diet, activated cellular and humoral defence mechanisms and provided protection against ERM in intensive rainbow trout culture. Immunostimulating effects against *Y. ruckeri* have also been demonstrated in rainbow trout using levamisole bathing (Ispir & Yonar 2007). Levamisole has been extensively used in both human and veterinary medicine as an anthelmintic agent and its effect on the immune system of different fish species has already been shown (Ispir & Yonar 2007).

More research is needed on the timing, scheduling and regimens of administering immunomodulators to induce effective protection against yersiniosis.

2.5.4. Probiotics

Control of diseases as ERM has mainly focused on the use of antimicrobial compounds and on vaccination. Recent work, however, has concerned the application of probiotics (Raida *et al.* 2003; Kim & Austin 2006; Capkin & Altinok 2009). Administration of certain probiotics to cultured trout has been shown to enhance their survival when exposed to *Y. ruckeri*. These live microbial feed supplements are defined as beneficially affecting the host by the production of inhibitory compounds, competition for chemicals and adhesion sites, immune modulation and stimulation, and improving the microbial balance. Administration of feed supplemented with spores of *Bacillus subtilis* and *B. licheniformis* (1:1 mixture with total dose: 4×10^4 spores g^{-1} feed) improved resistance in rainbow trout against infection with *Y. ruckeri* (Raida *et al.* 2003). Similarly, feed supplemented with *Carnobacterium maltaromaticum* or *C. divergens* dosed at $> 10^7$ cells g^{-1} feed also conferred protection against challenge with *Y. ruckeri* because these cultures enhanced cellular and humoral immune responses (Kim & Austin 2006). Feeding rainbow trout with 10^8 cells *Enterobacter cloacae* and *Bacillus mojavensis* g^{-1} feed for 60 days, resulted in a notable increased survival of the fish following bath challenge with *Y. ruckeri* (Capkin & Altinok 2009). Such organisms may have an important role in aquaculture for controlling bacterial disease.

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SCIENTIFIC AIMS

Yersiniosis or enteric redmouth disease (ERM) is a worldwide problem in salmonid aquaculture. The etiological agent, *Yersinia ruckeri*, causes a chronic or acute septicaemia in mainly salmonids and especially rainbow trout are most susceptible to infection. Despite the importance of ERM, little information is available on the pathogenesis. The first steps in microbial pathogenesis are considered to be adhesion and invasion of bacterial pathogens to host surfaces. Neither the portal of entry of *Y. ruckeri* nor its adhesion and invasion mechanisms have been studied. However, improved understanding of the first stages of the pathogen-host interaction may lead to the development of innovative and efficient therapeutic and immunoprophylactic strategies.

The present work deals with the study of the early pathogenesis of *Y. ruckeri* infections. The specific aims were:

- To reveal the portal of entry of *Y. ruckeri* in rainbow trout and to investigate its tissue distribution in time using an immersion infection model.
- To study the adhesion and invasion of *Y. ruckeri* to rainbow trout gills and gut using a gill and gut perfusion model.
- To investigate different traits *in vitro* for *Y. ruckeri* that have been associated with bacterial virulence.

EXPERIMENTAL STUDIES

1. **Route of entry and tissue distribution of *Yersinia ruckeri* in experimentally infected rainbow trout (*Oncorhynchus mykiss*, Walbaum)**

2. **Interactions of virulent and avirulent *Yersinia ruckeri* strains with isolated gill arches and intestinal explants of rainbow trout (*Oncorhynchus mykiss*, Walbaum)**

3. ***In vitro* markers for virulence in *Yersinia ruckeri***

1. Route of entry and tissue distribution of *Yersinia ruckeri* in experimentally infected rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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Experimenal study 1

ABSTRACT

Yersinia ruckeri is the causative agent of enteric redmouth disease, which leads to significant losses in salmonid aquaculture worldwide. Despite the significance of the disease, little information is available on the pathogenesis. In this study, the portal of entry was investigated using a contact exposure infection method in rainbow trout (*Oncorhynchus mykiss*, Walbaum) with four different *Y. ruckeri* strains. Bacteriological and histological examination revealed the presence of high numbers of bacteria in the gills immediately after infection resulting in a rapid spread of *Y. ruckeri* in the internal organs. However, only a virulent strain was able to survive and multiply in the host, causing septicaemia and death several days after infection. These findings indicate that gills may be an important site of entry and that virulence of *Y. ruckeri* is related with immune evasion.

INTRODUCTION

Yersinia ruckeri is the causative agent of yersiniosis or enteric redmouth disease (ERM) and causes significant losses in salmonid aquaculture worldwide. Although infection with this agent has been reported in other fish species, salmonids and especially rainbow trout (*Oncorhynchus mykiss*, Walbaum) are most susceptible to ERM (Furones *et al.* 1993). Infection may result in the development of a chronic or acute septicaemia with haemorrhages on the body surface and in the internal organs.

Despite the importance of ERM, little is known about the precise pathogenic mechanisms by which *Y. ruckeri* is able to defeat the host defences and cause disease. Differences in virulence between *Y. ruckeri* strains after experimental infection studies in rainbow trout have been documented by using intraperitoneal and intramuscular injection as well as contact exposure (Davies 1991; Romalde & Toranzo 1993; Austin *et al.* 2003; Fouz *et al.* 2006). This last method, however, seems a more reliable and efficacious infection model which mimics the conditions of natural infection as accurately as possible and ensures that immune mechanisms located at the body surface play their role. Nevertheless, it has not been determined yet how and when rainbow trout are infected by *Y. ruckeri* and where the bacterium resides in the infected fish.

The purpose of this study was to reveal the route of entry of *Y. ruckeri* in rainbow trout (experiment I) and to investigate its tissue distribution at different time intervals (experiment II) after experimental infections with four different *Y. ruckeri* isolates. Additional experimental infections (experiments IIIa and IIIb) were carried out to determine whether a different tissue distribution is related to differences in virulence. These virulence studies were performed at two different temperatures, since temperature is known to have an impact on rainbow trout immune-related functions as well as on *Y. ruckeri* growth and the production of toxins (Nikoskelainen *et al.* 2004; Fernández *et al.* 2007).

MATERIALS AND METHODS

Bacterial strains

Four different *Y. ruckeri* strains were used in the experiments. Strain 5 was originally isolated in 2001 from rainbow trout farmed in the UK with clinical signs of ERM and was obtained from the Institute of Aquaculture (Stirling, Scotland). Strains 17.00(2-1) and E842-95 were provided by the INRA (Paris, France). Strain 17.00(2-1) was isolated from a rainbow trout without clinical manifestation. No background information was available about strain E842-95. Type strain CCUG 14190 was obtained from the culture collection of the University of

Göteborg (Sweden) and was isolated from a rainbow trout with ERM. All the strains belong to serotype O1a except for *Y. ruckeri* E842-95 which is a serotype O1b strain as has been characterized by J.L. Romalde (Universidad de Santiago de Compostela, Spain).

Stock suspensions of the strains were stored at -70°C. After thawing, the bacteria were grown overnight at 20°C on Columbia agar (Oxoid, Drongen, Belgium) with 5% sheep blood (blood agar). Colonies were picked up from the agar plates and grown in nutrient broth (NB; VWR, Haasrode, Belgium) for 24 h at 20°C. The number of colony forming units (CFU) per ml was determined by plating ten-fold serial dilutions on blood agar plates.

Animals

Four different batches (experiment I, II, IIIa and IIIb) of 30, 81, 85 and 120 rainbow trout, weighing 6-45g, 22-55g, 3-20g and 8-60g respectively, were obtained from a fish farm (Gérouville, Belgium) with no history of ERM disease. The fish were acclimatized for ten days and were maintained in 1000 l tanks in filtered, recirculated tap water (pH 8.0-8.2; NH_4^+ < 0.1 mg l^{-1}; NO2 < 0.1 mg l^{-1}). They were fed daily with a commercial diet (Vijver Visvoeder, Merelbeke, Belgium). To ensure that animals were free from *Y. ruckeri*, ten percent of the trout of each newly introduced batch was sacrificed and samples from gills, gut, liver, kidney and spleen were taken for bacteriological examination. Furthermore, before the start of each experiment, swabs were taken from the skin, fins and gills of two randomly chosen fish for microscopic evaluation of external parasites. *Y. ruckeri* was not isolated on blood agar from any sample and all fish were free of external parasitic infestations.

Experimental infections

All the experimental infections were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2005/73, EC 2007/094, EC 2008/026).

In experiment I, four groups of 6 fish were bath challenged with *Y. ruckeri* strains 5, 17.00(2-1), CCUG 14190 and E842-95, respectively at a final concentration of approximately 2 x 10^8 CFU ml^{-1}. After 1 h of contact exposure in aerated aquaria at 18°C, the fish were transferred to their tanks again. At time points 0, 1.5 and 2.5 h post inoculation (p.i.), two fish from each infection group were euthanized using an overdose of a stock solution of benzocaine (1 g ethyl aminobenzoate (Federa, Brussels, Belgium) in 10 ml of acetone). After euthanasia, fish were rinsed with tank water to remove non-adherent bacteria from skin and gills. Six control fish were not infected and were kept under the same conditions, from which two were sampled at every time point in the same way as the infected fish.

In experiment II, groups of 18 trout were contact exposed to a final concentration of approximately 2 x 10^7 CFU ml^{-1} of the four *Y. ruckeri* strains for 1 h at 18°C in the same way as in experiment I. Two fish of each infection group were sampled at 1, 2, 4, 6, 9, 12, 24, 48 and 72 h p.i. Nine control fish were kept under the same conditions as the infected fish from which one was sampled at every time point.

In experiment IIIa, four groups of 20 fish were contact exposed for 1 h at 23°C in the same way as in experiment I. The final concentrations were approximately 2 to 3 x 10^7 CFU ml^{-1} of the four *Y. ruckeri* strains. Five control fish were kept under the same conditions as the infected fish. After inoculation, fish were observed for clinical signs several times a day and mortality was recorded. Dead and moribund fish were necropsied. The remaining fish were euthanized between 14 and 18 days p.i.

In experiment IIIb, four groups of 25 fish were contact exposed for 1h at 16°C in the same way as in experiment I. The final concentrations were approximately 10^7 CFU ml^{-1} of the four *Y. ruckeri* strains. Twenty control fish were kept under the same conditions as the infected fish. Similar as in experiment IIIa, dead and moribund fish were necropsied and the remaining fish were euthanized between 20 and 34 days p.i.

In all the experiments, samples of gills, proximal and distal gut, liver, kidney and spleen were collected for bacteriological, histological and immunohistochemical examination. In experiment I and II, additional samples of middle gut were taken and 1 cm^2 skin tissue along the left lateral line below the dorsal fin was collected from all fish in experiment I.

Bacteriological examination

The collected tissues were homogenized in phosphate buffered saline (PBS) as 10% (w/v) suspensions. The number of CFU *Y. ruckeri* g^{-1} liver, kidney and spleen was determined by plating tenfold dilutions of the suspensions on blood agar plates. Dilutions of gills, gut and skin suspensions were inoculated on MacConkey agar plates (Oxoid). *Y. ruckeri* was identified based on the typical colony shape and negative oxidase reaction. Systematically, identification of one colony of a suspected *Y. ruckeri* isolate of each plate was also confirmed using polymerase chain reaction (PCR).

PCR analysis

DNA was extracted from a loopful of cells using an alkaline lysis method (Baele *et al.* 2000). PCR was carried out using the primers YER8 (5'-GCGAGGAGGAAGGGTTAAGTG-3') and YER10 (5'-GAAGGCACCAAGGCATCTCTG-3') which amplify a 575 bp fragment of

the 16S rRNA gene of *Y. ruckeri*, as previously described by Gibello *et al.* (1991). For each PCR reaction, 1 µl of the prepared DNA was added to 9 µl of the PCR mixture, containing 0.05 U/µl Hotmaster® *Taq* polymerase (Eppendorf, Hamburg, Germany), 10x PCR Buffer (Invitrogen Life Technologies, Merelbeke, Belgium), 25 mM $MgCl_2$ (Invitrogen), 200 µl of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech, Puurs, Belgium), 0.5 µM of each primer (Operon, Cologne, Germany) and sterile distilled water. The used conditions for the amplifications were the following: an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min. A final extension was performed at 72°C for 8 min. PCR products were run in agarose gels containing 1.5% Multi Purpose agarose (Boehringer, Mannheim, Germany) in 1x Tris-borate-EDTA (TBE) buffer, pH 8 and stained with ethidium bromide. The gels were visualised using the Image Master® VDS (Amersham Pharmacia Biotech).

Histological and immunohistochemical analysis

In experiment I, tissue samples were fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid) for at least 2 h, dehydrated in an alcohol-xylene series and embedded in paraffin wax. The organs in experiment II, IIIa and IIIb were fixed in 10% phosphate buffered formaldehyde for at least 24 h before being dehydrated and embedded. Five-µm sections, mounted on glass slides, were stained with haematoxylin and eosin (H&E) and Giemsa.

Additionally, immunohistochemistry was performed, using polyclonal rabbit anti-*Y. ruckeri* antibodies. Hyperimmune serum was obtained by immunizing rabbits (EC 2005/921) with *Y. ruckeri* strain 7 as described by Decostere *et al.* (1999). Five-µm sections of paraffin-embedded tissues were placed on SuperFrost slides (Sigma-Aldrich, Bornem, Belgium), deparaffinized, rehydrated and pretreated by the antigen retrieval microwave technique. Thereafter, slides were incubated with 3% H_2O_2 in methanol to block endogenous peroxidase and were washed once with PBS. Slides then underwent sequential application of 30% goat serum, primary rabbit anti-*Y. ruckeri 7* 1/800 antibody, biotinylated goat anti-rabbit antibody 1/500 (DakoCytomation, Heverlee, Belgium), StreptABComplex/horseradish peroxidase (DakoCytomation), and finally 3,3'-diamino benzidine tetrahydrochloride (Sigma-Aldrich) with each time a wash step. Sections were counterstained by use of an aqueous-based haematoxylin staining and mounted.

Statistical analysis

In experiment I and II, a fixed effects model (SAS Version 9.1.3) was used to compare CFU *Y. ruckeri* g^{-1} tissue using first time and strain as fixed effects and the organ as stratification factor, then time and organ as fixed effects and the strain as stratification factor. The analysis was done at the 5% global significance level and the reported P-values for pairwise comparisons are adjusted using Tukey's multiple comparisons method.

In experiments IIIa and IIIb, the analysis of the time to death outcome was based on the Wilcoxon rank sum test with all surviving fish given the same and highest rank. First, the time to death outcome was compared between the different contact exposed groups within each experiment. Second, the time to death outcome was examined between the two experiments for each strain. As the normal distribution assumption did not hold, the Wilcoxon signed rank test was used to compare CFU *Y. ruckeri* g^{-1} tissue between the different organs of the fish using only animals from which *Y. ruckeri* was isolated from at least one organ.

RESULTS

Experiment I

Bacteriological examination

For experiment I, the results of the reisolation of *Y. ruckeri* from gills, gut and skin at 0, 1.5 and 2.5 h after contact exposure with strains 5, 17.00(2-1), CCUG 14190 and E842-95 are shown in Figure 1.

The highest bacterial numbers were reisolated from the gills. These numbers were significantly higher than these reisolated from the gut between 0 and 2.5 h p.i. for strains 5 ($P = 0.0005$) and 17.00(2-1) ($P = 0.0016$).

For *Y. ruckeri* strains 5, 17.00(2-1) and CCUG 14190, the bacterial numbers reisolated from the gills, skin and gut declined between 0 and 1.5 h p.i. and remained the same or increased slightly between 1.5 and 2.5 h p.i. Higher bacterial numbers were detected in the skin than in the gut at 0, 1.5 and 2.5 h p.i. in fish inoculated with these strains, however, only for strain 5 the number of bacteria in the skin was significantly ($P = 0.0035$) higher than in the gut over this time period.

For *Y. ruckeri* E842-95, the bacterial population in the gills declined gradually between 0 and 2.5 h p.i. The number of bacteria reisolated from the gut also showed a gradual decrease in this time period, however, the bacterial population in the skin increased slightly between 0 and 1.5 h p.i., after which a sharp decrease was observed. Differences in bacterial numbers between these organs were not statistically significant between 0 and 2.5 h p.i.

Bacteria were also found in liver, kidney and spleen (between 10^1 and 10^5 CFU g^{-1}) of all fish immediately after infection with *Y. ruckeri* strains 5, 17.00(2-1), CCUG 14190 and E842-95 and fluctuated between 0 h and 2.5 h p.i (data not shown).

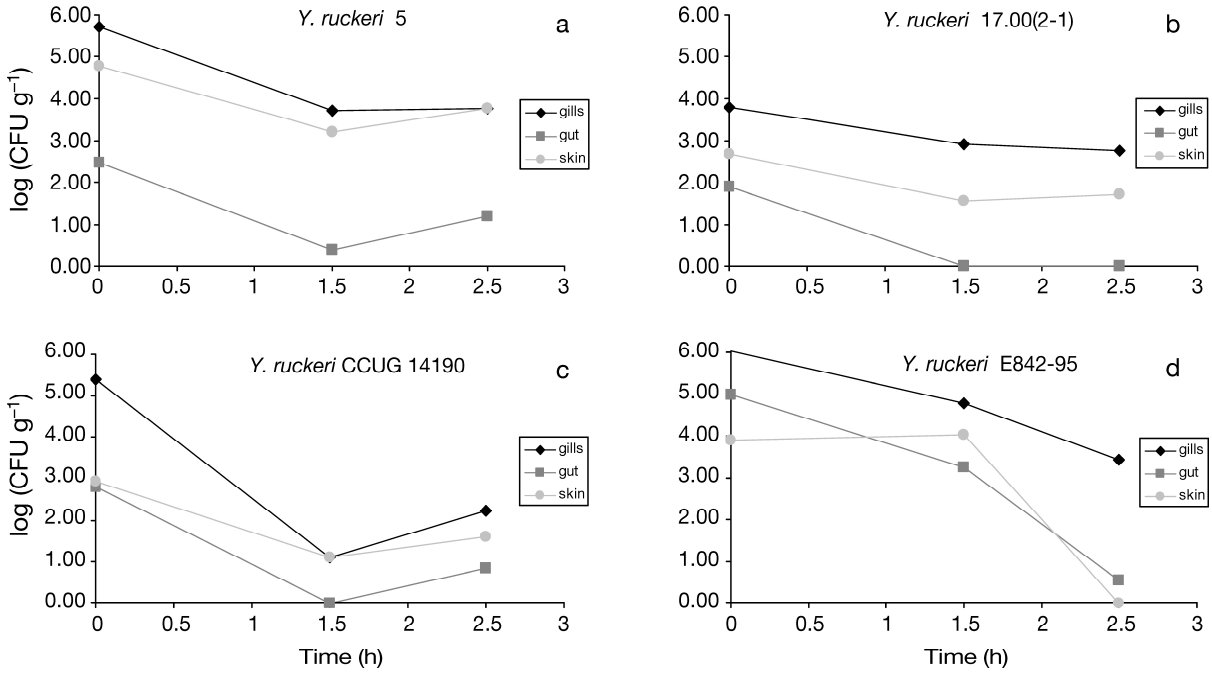

Figure 1 *Oncorhynchus mykiss*. Detection of *Y. ruckeri* in the gills, gut (posterior, middle and anterior gut were pooled) and skin after contact challenge with *Y. ruckeri* (a) 5, (b) 17.00(2-1), (c) CCUG 14190 and (d) E842-95 at 0, 1.5 and 2.5 h p.i. Each time point represents the means of two fish. The number of bacteria in each sample is expressed as log (CFU g^{-1} tissue).

Histological and immunohistochemical analysis

Histopathological changes were not observed in any of the tissues of fish at 0, 1.5 and 2.5 h after contact exposure with *Y. ruckeri* strains 5, 17.00(2-1), CCUG 14190 and E842-95, which were compared with those of negative control fish. Giemsa and immunohistochemical staining revealed the presence of *Y. ruckeri* bacteria in the gills of several fish inoculated with strain 5. At time 0 h p.i., numerous bacteria were observed in the mucus of the primary and secondary lamellae, whereas later on, a lower number of bacteria was found. At time 0 and 2.5 h p.i., two fish showed the presence of a few bacteria in the capillaries of the secondary lamellae (Figure 2a). In the gut of trout inoculated with *Y. ruckeri* 5, few bacteria were noticed in the crypts, attached to the villi and within the mucosa at different time points (Figure 2b).

A notably lower number *Y. ruckeri* bacteria (as low as none) were detected in the gills and gut of fish inoculated with strains 17.00(2-1), CCUG 14190 and E842-95. Bacteria were observed in the mucus of the gills and in the crypts and the mucosa of the gut sections. *Y. ruckeri* was not detectable in the skin, liver, kidney and spleen of infected fish by histology or immunohistochemistry.

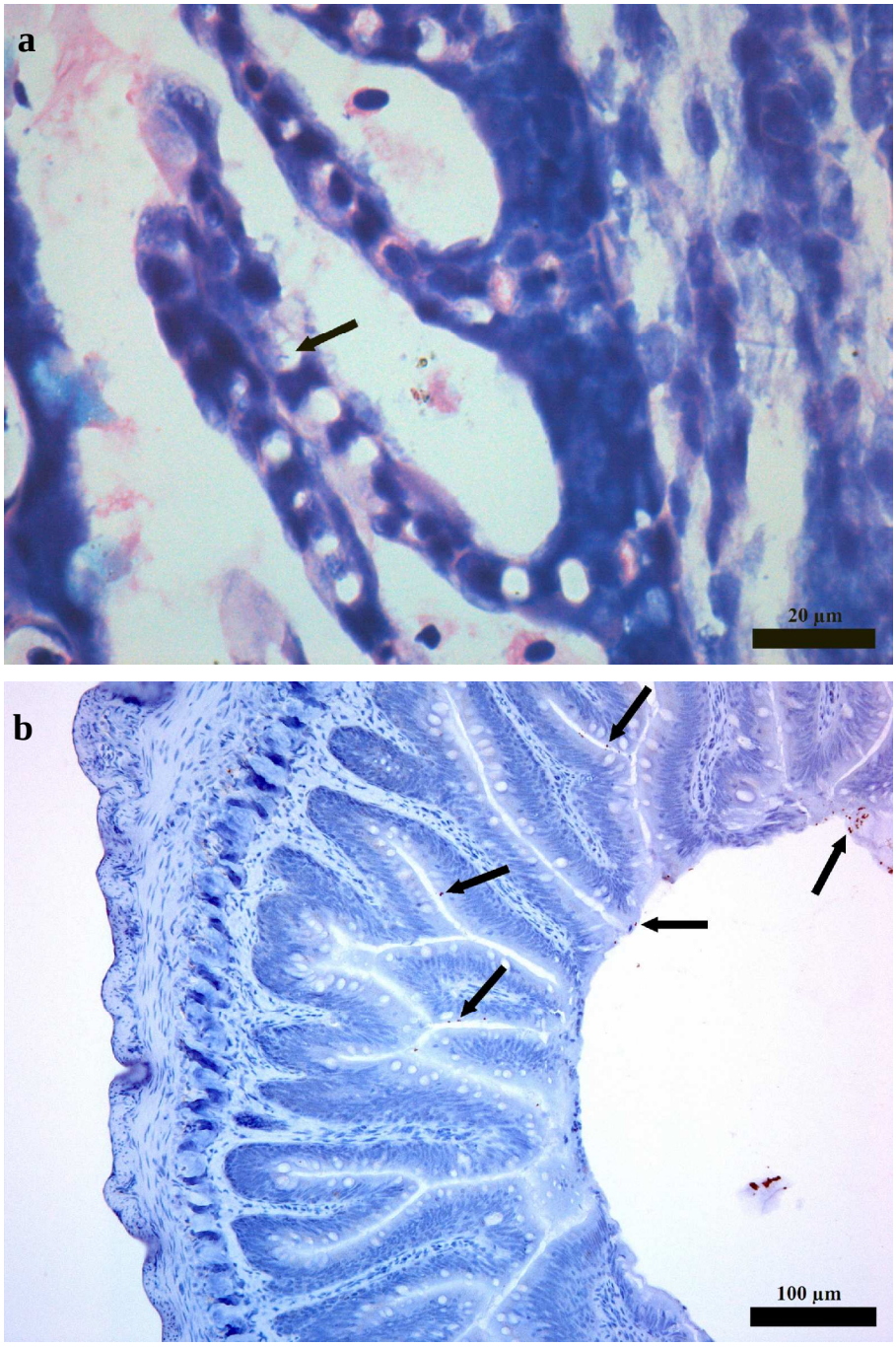

Figure 2 *Oncorhynchus mykiss.* Localisation of *Y. ruckeri* in the gills and the gut after contact challenge with strain 5. *Y. ruckeri* bacteria at 0 h p.i. (a) in a capillary of the gill filament (Giemsa staining) and (b) in the intestinal lumen between the villi and in the mucosa (immunohistochemical staining).

Experiment II

Bacteriological examination

For experiment II, the results of the reisolation of *Y. ruckeri* from gills, gut, liver, kidney and spleen at 1, 2, 4, 6, 9, 12, 24, 48 and 72 h after contact exposure with strains 5, 17.00(2-1), CCUG 14190 and E842-95 are shown in Figure 3 and 4.

The highest numbers of bacteria were reisolated from the gills of trout contact exposed with *Y. ruckeri* strains 5, 17.00(2-1), CCUG 14190 and E842-95 during the first 2 h p.i. After a period of fluctuations in the bacterial population, bacteria were no longer detectable in the gill tissue at 24, 48, 12 and 48 h p.i. for strains 5, 17.00(2-1), CCUG 14190 and E842-95, respectively. However, in the gills of fish inoculated with *Y. ruckeri* 5, the number of bacteria sharply increased between 48 and 72 h p.i., whereas bacteria were not isolated from the gills of fish from the other infection groups.

The number of bacteria reisolated from the gut of fish inoculated with *Y. ruckeri* strains 17.00(2-1) and E842-95 was low during the first 12 h p.i. Bacterial numbers in the gut of these fish were slightly higher at 24 to 48 h p.i. and exceeded the amounts reisolated from the gills at this time. By comparison, the number of bacteria in the gut of fish inoculated with *Y. ruckeri* 5 was higher within the first 12 h p.i. and sharply increased between 48 and 72 h p.i. In the gut of fish inoculated with strain CCUG 14190, none or only small amounts of bacteria were reisolated between 1 and 72 h p.i.

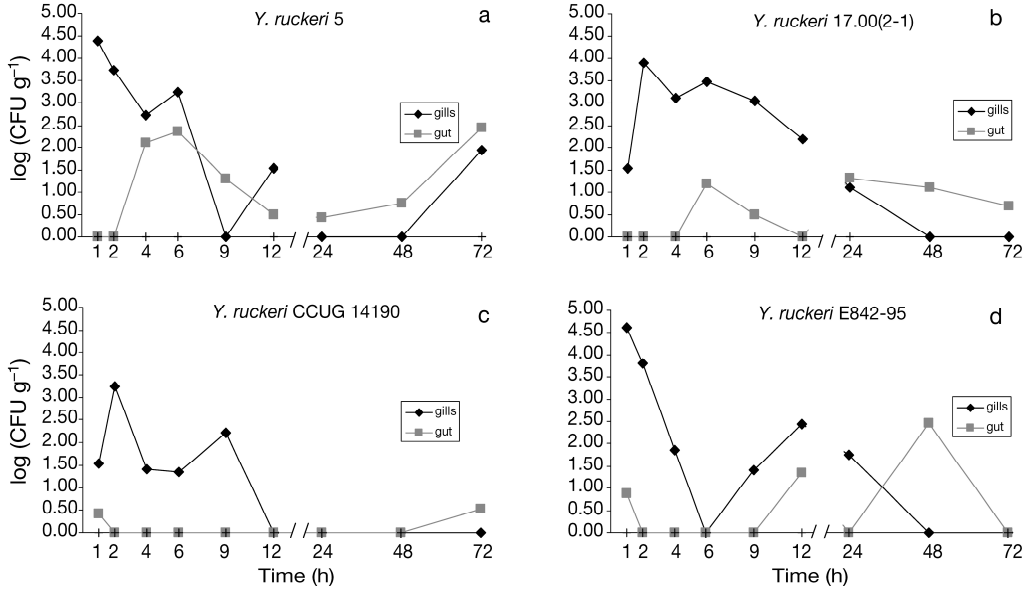

Figure 3 *Oncorhynchus mykiss.* Detection of *Y. ruckeri* in the gills and the gut (posterior, middle and anterior gut were pooled) after contact challenge with *Y. ruckeri* (a) 5, (b) 17.00(2-1), (c) CCUG 14190 and (d) E842-95 at 1, 2, 4, 6, 9, 12, 24, 48 and 72 h p.i. Each time point represents the means of two fish. The number of bacteria in each sample is expressed as log (CFU g^{-1} tissue).

Bacteria were found in the liver, kidney and spleen of fish contact exposed with *Y. ruckeri* 5 between 1 and 72 h p.i. The number of bacteria fluctuated with a peak on 6 h p.i. for the three tissues (between 1.2 x 10^4 and 2.4 x 10^4 CFU g^{-1}) and a sharp increase in bacterial population between 48 and 72 h p.i. was noticed. By comparison, lower bacterial numbers (≤ 4.0 x 10^1 CFU g^{-1}) were reisolated from liver, kidney and spleen of fish challenged with *Y. ruckeri* strains 17.00(2-1) and CCUG 14190 before 9 and 24 h p.i., respectively. Thereafter, bacteria were no longer detected in these organs. A similar trend was seen in the liver, kidney and spleen of fish inoculated with strain E842-95, however, slightly higher amounts of bacteria were reisolated from these tissues between 1 and 24 h p.i.

The number of bacteria in the gills was significantly higher than in the gut (P = 0.0006), liver (P < 0.0001), kidney (P = 0.0003) and spleen (P < 0.0001) from fish inoculated with strain 17.00(2-1). Also for strain CCUG 14190, significantly higher numbers were observed in the gills than in the gut (P = 0.0033), liver (P = 0.0051), kidney (P = 0.0058) and spleen (P = 0.0042). Significantly more bacteria were reisolated from the liver of fish inoculated with strain 5 than from the liver of fish inoculated with strains 17.00(2-1) (P = 0.0003), E842-95 (P = 0.0338) and CCUG 14190 (P = 0.0001). The spleen and kidney of fish infected with strain 5 also showed significantly higher numbers of bacteria compared to the spleen and kidney of fish infected with strains 17.00(2-1) (P = 0.0002 and 0.0025, respectively) and CCUG 14190 (P = 0.0002 and 0.0002, respectively).

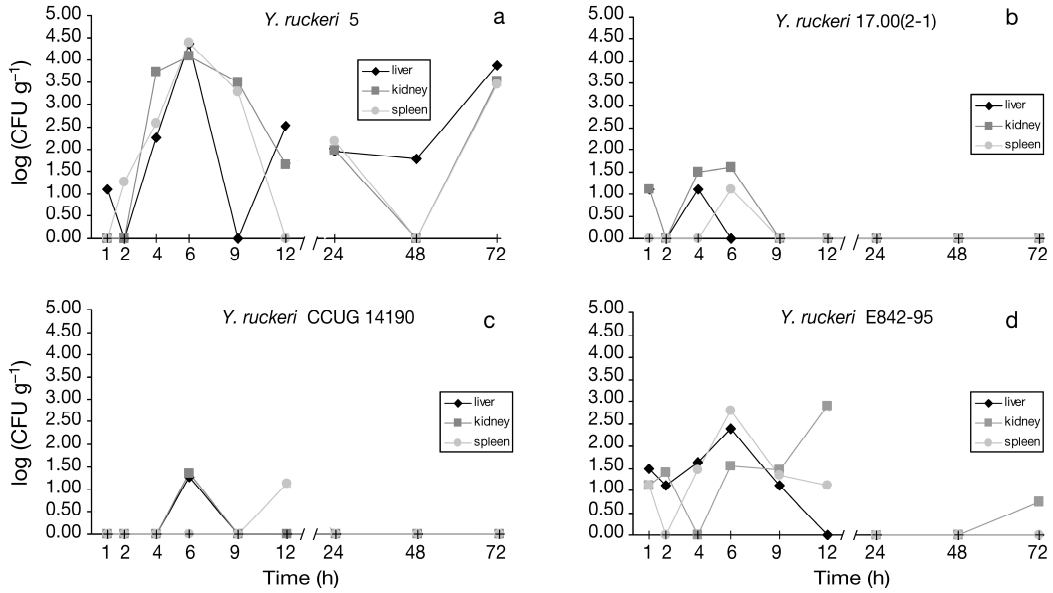

Figure 4 *Oncorhynchus mykiss.* Detection of *Yersinia ruckeri* in the liver, kidney and spleen after contact challenge with *Y. ruckeri* (a) 5, (b) 17.00(2-1), (c) CCUG 14190 and (d) E842-95 at 1, 2, 4, 6, 9, 12, 24, 48 and 72 h p.i. Each time point represents the means of two fish. The number of bacteria in each sample is expressed as log (CFU g^{-1} tissue).

Experimenal study 1

At 72 h p.i., fish contact exposed to *Y. ruckeri* 5 had significantly ($P < 0.0083$) higher bacterial numbers in the liver, kidney and spleen than those infected with the other strains.

Histological and immunohistochemical analysis

Histopathological changes were not observed in any of the tissues of fish between 1 and 72 h after contact exposure with *Y. ruckeri* strains 5, 17.00(2-1), CCUG 14190 and E842-95, which were compared with these of negative control fish. Bacteria were not detected by histological and immunohistochemical analysis.

Experiment IIIa and b

Clinical signs, mortality and necropsy findings

Disease signs or mortality were not noted among the fish belonging to the control groups of experiments IIIa and IIIb. In both experiments, clinical signs and/or mortality were only observed in rainbow trout inoculated with *Y. ruckeri* strain 5 (Table 1).

In experiment IIIa, six of the 20 fish (weighing 7-18g) which were contact exposed to *Y. ruckeri* 5 died within 5 to 11 days p.i., with the highest mortality at day 7 p.i. They did not show any additional clinical signs. The surviving fish neither showed clinical signs or lesions.

In experiment IIIb, four out of 25 fish (weighing 16-37g) contact exposed to *Y. ruckeri* 5 died within 9 days p.i. with a mean of 7.8 days p.i. Two of these fish showed exopthalmia accompanied by orbital haemorrhages and one had an enlarged liver and spleen.

In both immersion experiments, strain 5 showed a significantly higher mortality ($P < 0.0001$) compared to the other strains. For none of the strains, there was a significant difference between the two experiments with respect to time to death.

Table 1 Results of clinical and bacteriological examination of *Oncorhynchus mykiss* experimentally infected with *Y. ruckeri* strain 5, using the immersion challenge method.

Strain	Number of fish			Mean time of death (days p.i.)	No. of *Y. ruckeri* $\log_{10}$ CFU g^{-1} tissue (mean ± SE) isolated from					
	Total	With clinical signs	That died		Gills	Proximal gut	Distal gut	Liver	Spleen	Kidney
EXPERIMENT IIIa										
5	20	0	6	7.2	5.5 ± 1.1	8.6 ± 2.1	7.5 ± 1.1	7.0 ± 0.5	8.2 ± 1.2	7.2 ± 0.5
EXPERIMENT IIIb										
5	25	2	4	7.8	8.0 ± 2.5	7.4 ± 2.5	7.4 ± 2.4	7.8 ± 1.9	7.8 ± 1.9	7.8 ± 1.9

Bacteriological examination

In experiments IIIa and IIIb, *Y. ruckeri* was not isolated from the organs of the control fish. The results of the bacteriological examinations of all the dead fish within each infection group are pooled and summarized in Table 1.

In experiment IIIa, *Y. ruckeri* was reisolated from gills, proximal and distal gut, liver, spleen and kidney of all six fish that died after contact challenge with strain 5 in the range of 10^5 to 10^8 CFU g^{-1} tissue (Table 1). The bacterium was reisolated from one or more organs from four fish which survived after contact exposure with *Y. ruckeri* 5 in the range of 10^2 to10^3 CFU g^{-1} tissue with spleen being the most favoured tissue.

In experiment IIIb, *Y. ruckeri* was reisolated from gills, proximal and distal gut, liver, spleen and kidney of all four fish that died after contact challenge with strain 5 in the range of 10^7 to 10^8 CFU g^{-1} tissue (Table 1). In one surviving fish, euthanized 21 days after contact exposure, 1.7 x 10^2 and 8.3 x 10^2 CFU of *Y. ruckeri* strain 5 g^{-1} tissue was reisolated from kidney and spleen, respectively.

Trout inoculated with *Y. ruckeri* strains 17.00(2-1), CCUG 14190 or E842-95 never showed the presence of the bacterium in their organs at euthanasia.

No differences in CFU *Y. ruckeri* g^{-1} gills, proximal and distal gut, liver, spleen and kidney were observed in animals exposed to strain 5.

Histological and immunohistochemical analysis

Since only *Y. ruckeri* strain 5 caused disease and mortality *in vivo*, the tissue samples of fish infected with this strain were analysed by histology and immunohistochemistry. Tissue samples of some fish that died were susceptible to post-mortem decay and therefore difficult to interpret. Samples that were too deteriorated were not included in the results. To analyse histopathological changes, samples of infected fish were compared with those of negative control fish.

Similar results for experiment IIIa and IIIb were observed. Almost all fish that died after infection showed a moderate to severe oedema in the secondary lamellae whereas the gills of trout that survived exhibited a lower degree or no oedema. Multifocal to coalescing coagulative necrosis (Figure 5a) was observed in the spleen of almost all died animals with the presence of numerous intralesional bacteria. Only one fish that survived contact exposure with *Y. ruckeri* 5 showed a very mild focal necrosis in its spleen. In the kidneys of several surviving fish, contact exposed to *Y. ruckeri* strain 5, degeneration and/or mild necrosis of proximal tubules (Figure 5b) were observed. These findings were rarely present in kidney of

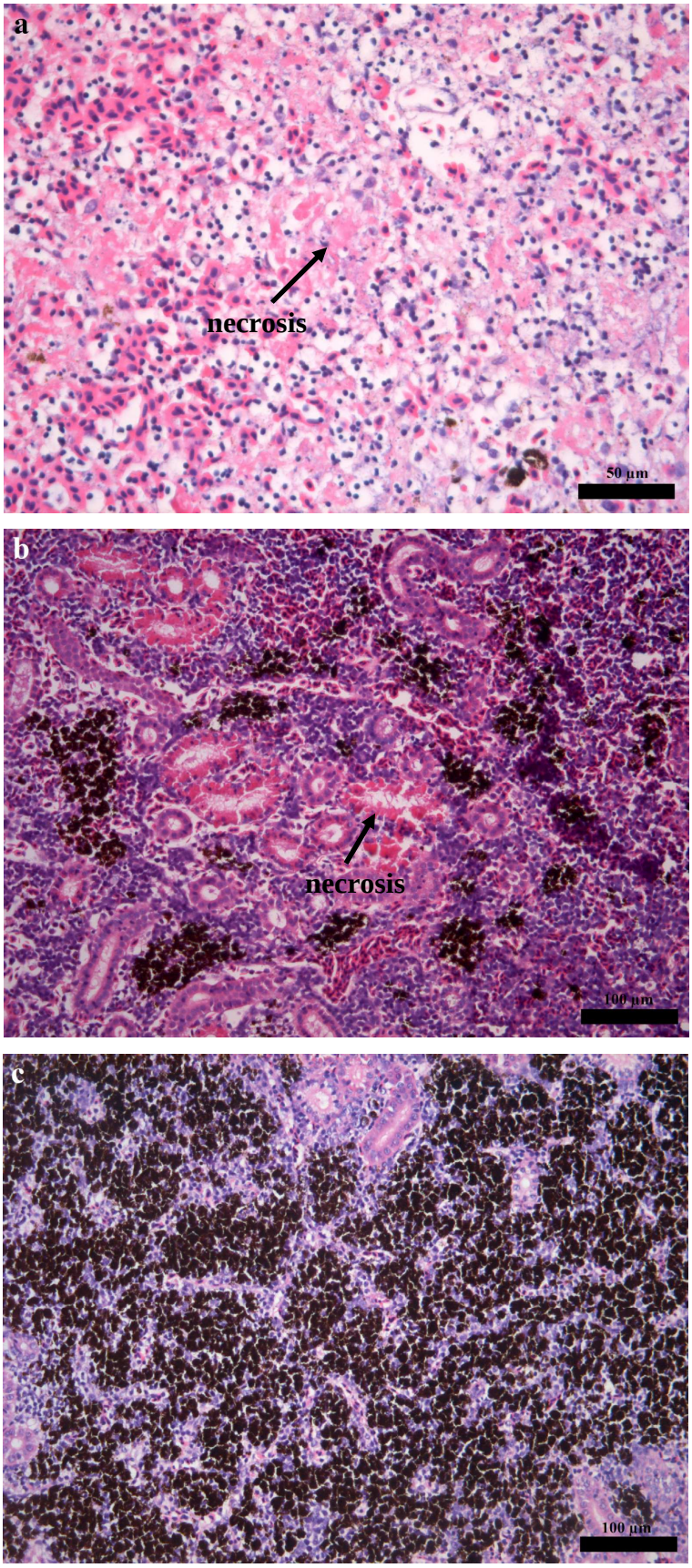

Figure 5 *Oncorhynchus mykiss.* H&E staining of (a) spleen with coagulative necrosis, (b) kidney with degenerated tubules and (c) kidney with a notable increase in melanomacrophages (darkly pigmented cells) of a rainbow trout which died three days p.i. after contact exposure to *Y. ruckeri* strain 5.

fish that died. Increased cellularity of the glomerular tuft was present in a small number of animals that died or survived contact exposure to strain 5. In the kidney, there were slight to moderate increases in the number of macrophages of the majority of fish that survived after infection. A minority of trout that died after infection also showed a slight increase in the number of macrophages. A notable increase in melanomacrophages (Figure 5c) was also observed in about half of the fish that died or survived after infection with *Y. ruckeri* strain 5. No histopathological changes were seen in the gut or the liver.

Immunohistochemistry revealed the presence of bacteria in all organs from which *Y. ruckeri* was isolated. In the spleen, bacteria were mainly found in or surrounding the necrotic areas. In all other organs, bacteria were randomly distributed extracellularly. In the liver, however, bacteria were also found intracytoplasmic.

PCR analysis

The isolates that were identified as *Y. ruckeri* by colony morphology in all experiments were confirmed to be *Y. ruckeri* by PCR.

DISCUSSION

Attachment and entry into the host's body is the first requirement for a bacterial pathogen to establish an infection. To investigate this early interaction between bacteria and its host using histological and immunohistochemical techniques, it is important to maintain the tissue morphology optimally. Carnoy's fixative has been shown to preserve the surface mucous gel layer covering surface mucous cells in different internal organs (Ota & Katsuyama 1992; Shimizu *et al.* 1996; Matsuo *et al.* 1997). Since adhesion of *Y. ruckeri* to mucus may be important during the early stages of infection, tissue samples in experiment I were fixed in Carnoy's solution.

Attachment of *Y. ruckeri* to the gill mucus was obviously seen soon after infection, whereas fewer bacteria were detected in the mucus in the course of time. *Y. ruckeri* was also observed within the gill capillaries at 0 h as well as 2.5 h p.i. This may indicate that bacteria first adhere to gill mucus and thereafter invade the branchial vasculature leading to septicaemia and colonization of the internal organs. Torroba *et al.* (1993) showed that formalin-killed *Y. ruckeri* were taken up by gill epithelial cells during an *in vitro* assay where isolated rainbow trout gills were placed in medium containing the killed bacteria. Zapata *et al.* (1987) demonstrated that killed *Y. ruckeri* bacteria were taken up by the gill cells of Atlantic salmon after bath immunization. However, results obtained with dead bacteria may differ from these

with viable bacteria. The uptake of live *Y. ruckeri* bacteria by the gill epithelium was demonstrated by the use of an isolated perfused rainbow trout head model (McIntosh *et al.* 2000). The results of the present *in vivo* studies demonstrate that gills may indeed be an important portal of entry. Gills have also been reported as a site of entry for other septicaemic fish pathogens, such as *Vibrio anguillarum* and *Edwardsiella tarda* (Baudin-Laurencin & Germon 1987; Ling *et al.* 2001). For respiration, gills are highly vascularized with a large number of blood capillaries; therefore, they may provide good entry sites for bacteria to become easily disseminated to the entire body of the fish (Ling *et al.* 2001).

Although the gills are apparently an important portal of entry for *Y. ruckeri*, the bacterium may also successfully colonize other body surfaces and gain entry to the body by a variety of routes, including the skin and the gut, as has been suggested by Busch & Lingg (1975) and Valtonen *et al.* (1992). In our study, moderate numbers of *Y. ruckeri* were isolated from the skin and gut immediately after infection. At histological examination, some bacteria were noticed in the intestinal crypts, indicating that they evaded the first line of the host defences in the gut mucosa. Further studies are warranted to determine the role of the skin and the gut as portals of entry.

Infection resulted in a rapid spread of *Y. ruckeri* into the internal organs. Other fish pathogens like *E. tarda* and *V. anguillarum* have also been reisolated from different organs within 1 h after contact exposure (Spanggaard *et al.* 2000; Ling *et al.* 2001). In the present study (experiment II), a sharp increase in reisolated *Y. ruckeri* from the internal organs was observed 3 days after infection with strain 5. Only for this strain, mortality appeared from 5 days p.i. onwards with high bacterial numbers present in these organs (experiment IIIa and IIIb). In contrast, no or only few bacteria were detected in the internal organs of fish infected with the other strains at this time and clinical signs and mortality were not observed. Comparable results were seen in blue gourami, *Trichogaster trichopterus*, after contact exposure with *E. tarda.* Infection with a virulent strain resulted in an exponential proliferation of the bacterial population in all examined tissues and organs until day 3 when mortalities in infected fish were observed. After immersion with a non-virulent strain which did not induce mortality, however, the number of bacteria declined over a seven days period within all the tissues (Ling *et al.* 2001). Our findings demonstrate that differences in virulence exist and suggest that the immune system is able to clear non-virulent *Y. ruckeri* bacteria from the organs of the fish. Possibly, virulent isolates are attacked by immune cells resulting in a temporary decrease in the number of bacterial cells within the organs. Survival and proliferation of virulent *Y. ruckeri* in immune cells or in the blood might result in a

subsequent increase of bacteria causing severe septicaemia and death. Therefore, it would be interesting to study the interaction with macrophages as well as the serum resistance of the different *Y. ruckeri* strains.

Different factors may influence the outcome of a bacterial infection, including the temperature. *Y. ruckeri* was found to cause significant losses in the field at water temperatures of 15 to 18°C (Roberts 1983). Danley *et al.* (1999) observed a higher incidence of infection at 18°C than at 22°C in channel catfish, *Ictalurus punctatus* (Rafinesque), intraperitoneally injected with *Y. ruckeri*. Increased severity of infection may be due to upregulation of different *Y. ruckeri* virulence factors at lower temperatures, as has been demonstrated by Fernández *et al*. (2007). However in the present study, contact exposures of trout to *Y. ruckeri* at 16 and 23°C did not result in a significantly different outcome as measured by clinical signs, bacteriological and histological analysis. Possibly, other factors such as the infection dose and fish size may have played a role.

The increase of melanomacrophages in the kidney of some infected fish in our studies has previously not been described. Within melanomacrophage centres, macrophages have been shown to destroy and kill the fungus *Candida albicans* but also to phagocytose rainbow trout erythrocytes in the immediate vicinity (Passantino *et al.* 2002). Consequently, melanomacrophage centers seem to play an important role in the elimination of foreign particulate material and of fish erythrocytes that have ingested microorganisms. Melanomacrophages are believed to be a prominent feature of chronic inflammatory responses (Ferguson 2006). Therefore, our results could indicate a possible role of *Y. ruckeri* in chronic nephritis.

In conclusion, gills may be an important site of entry for *Y. ruckeri* during infection of rainbow trout. Probably, the bacteria first adhere to the gill mucus after which they invade the branchial vasculature and subsequently spread into the internal organs very soon after infection. Virulent strains are probably able to defeat the host immune mechanisms, leading to high bacterial load and necrosis in the organs resulting in death of the fish several days after infection. Exposure to non-virulent strains, however, results in a complete removal of bacteria from the internal organs. This could indicate that immune evasion is a major virulence property of *Y. ruckeri*.

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2. Interactions of virulent and avirulent *Yersinia ruckeri* strains with isolated gill arches and intestinal explants of rainbow trout (*Oncorhynchus mykiss*, Walbaum)

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Experimental study 2

ABSTRACT

Yersinia ruckeri is the causative agent of enteric redmouth disease leading to significant losses in salmonid aquaculture worldwide. Little information is available on the pathogenesis of this disease. Basic steps in the establishment of an infection include attachment to the epithelium followed by invasion at the portal of entry. In this study, the interactions of *Y. ruckeri* with the gills and the gut of rainbow trout (*Oncorhynchus mykiss*, Walbaum) were studied using standardized perfusion models. Virulent and avirulent *Y. ruckeri* isolates appeared to adhere to and invade both tissues without significant differences. For the first time, the gill and gut perfusion models are shown to be suitable to study bacterial invasiveness.

INTRODUCTION

Yersinia ruckeri is the causative agent of enteric redmouth disease (ERM) and causes significant losses in salmonid aquaculture worldwide. Despite the importance of ERM, the precise pathogenic mechanisms of *Y. ruckeri* are not well understood. A comprehensive understanding of the pathobiology of the infection is required for the rational design of vaccines and other preventive measures to combat ERM (Haesebrouck *et al.* 2004). Basic steps in the establishment of an infection include attachment to the epithelium followed by invasion at the portal of entry. Both the gills and intestinal tract have been suggested as possible routes of entry for *Y. ruckeri* (Busch & Lingg 1975; Valtonen *et al.* 1992; Tobback *et al.* 2009). Therefore, the interactions of *Y. ruckeri* with the gills and the gut of rainbow trout (*Oncorhynchus mykiss,* Walbaum) were studied using standardized perfusion models. Virulent and avirulent strains were included to determine whether differences in virulence are related to differences in adherence and invasive capacity.

MATERIALS AND METHODS

Bacterial strains

The *Y. ruckeri* isolates used in the present study are listed in Table 1. Stock suspensions of the strains were stored at -70°C. After thawing, the bacteria were grown overnight at 20°C on Columbia agar (Oxoid, Drongen, Belgium) with 5% sheep blood (blood agar). Colonies were picked up from the agar plates and grown in nutrient broth (NB; VWR, Haasrode, Belgium) for 24 h at 20°C. A non-invasive *Escherichia coli* strain (DH5α) was included as a negative control in adhesion and invasion experiments (Parthasarathy & Mansfield 2009). This strain was cultured in NB for 24 h at 37°C. The number of colony forming units (CFU) per ml was determined by plating tenfold serial dilutions on blood agar plates.

Fish

Twenty-two rainbow trout of 600 g average weight were obtained from a fish farm (Gérouville, Belgium) with no history of ERM disease. The fish were maintained in 1000 l tanks in filtered, recirculated tap water. They were fed daily with a commercial diet (Vijver Visvoeder, Merelbeke, Belgium) until two days before included in the experiments. Swabs were taken from the skin, fins and gills of two ad random chosen fish for microscopic evaluation of external parasites upon arrival. The fish were found to be free of external parasitic infestations.

Experimental study 2

Table 1 *Y. ruckeri* strains

Y. ruckeri strain	Origin	Serotype	Virulence [a]	Mortality [a,b]
5	*O. mykiss* with ERM, UK, 2001	O1a	virulent	6/20
9	*O. mykiss* with ERM, UK, 1995	O1a	virulent	4/20
E842-95	NA	O1b	avirulent	0/20
17.00(2-1)	*O. mykiss*	O1a	avirulent	0/20
CCUG 14190	*O. mykiss* with ERM, USA	O1a	avirulent	0/20

NA: no information available

[a] Virulence and capacity to induce mortality were determined in an immersion infection model using juvenile rainbow trout (Tobback *et al.* 2009). If mortality and persistent infection were not observed, strains were categorized as avirulent according to Davies (1991)

[b] Number of fish that died/total number of fish

Adherence and invasion study using a gill perfusion model

Gill perfusion model

The gill perfusion model used in this study has been previously described (Decostere *et al.* 2002). Briefly, the fish were anaesthetized using a solution of benzocaine (1 g ethyl aminobenzoate (Federa, Brussels, Belgium) in 10 ml of ethanol), injected intraperitoneally with heparin (5000 IU kg^{-1}) and allowed to recover. Thirty minutes later, the fish were euthanized using an overdose of the benzocaine solution. The heart and ventral aorta were exposed by ventral dissection. After a haemostat was clamped onto the ventral aorta, the opercula were removed and the first two or three gill arches on both sides were excised and put into Petri dishes filled with Ringer solution at 20°C. The smaller dorsal part of each gill arch was cut off to remove the nod to prevent from jeopardizing an efficient perfusion.

Thereafter, the afferent (ventral) and efferent (dorsal) arteries of the gill arches were cannulated. Each gill arch was suspended separately in an aerated organ bath filled with 400 ml Ringer solution which was surrounded by a water jacket at 20°C. Perfusion flow was started at ≈ 2 ml min^{-1} $arch^{-1}$ kg^{-1} body weight. Cortland solution + dextran 1% (MW 500 000, Sigma, Steinheim, Germany) was used as perfusion fluid and was delivered by means of a drip (Decostere *et al.* 2002). The isolated gill arches were perfused for 30 min to allow recovering from the most acute effects of the procedure before using them in the adherence and invasion study.

Adherence and invasion study

Bacterial suspensions were prepared in Ringer solution and each added to the separate organ baths at a final concentration of approximately 10^8 CFU ml^{-1}. A non-treated perfused gill arch served as negative control. After inoculation, gill arches were incubated for 1 h and subsequently processed for examination of adhesion. The experiment was conducted three times.

After the incubation period, each perfused gill arch was washed in an excess of phosphate buffered saline (PBS) and cut in half. A randomly selected sample was homogenized into a preweighed vial containing 0.4 ml of PBS. The number of CFU g^{-1} gill tissue was determined by plating tenfold serial dilutions of the suspensions on MacConkey agar (Oxoid, Hampshire, UK) plates.

The second sample of the perfused gill arch was fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid) for at least 2 h and further processed for histological and immunohistochemical (IHC) analysis (Tobback *et al.* 2009). The sections were examined by light microscopy.

To study the invasion capacity, the perfusion fluid at the efferent artery was collected during infection. The number of *Y. ruckeri* or *E. coli* present in the collected fluid was determined by plating tenfold serial dilutions on MacConkey agar plates.

Adherence and invasion study using a gut perfusion model

Gut perfusion model

The gut perfusion model was performed as previously described with slight modifications (Nematollahi *et al.* 2005). Briefly, the fish were anaesthetized, injected intraperitoneally with heparin, allowed to recover and euthanized 30 minutes later as described above. The intestine was exposed by ventral dissection and the *aorta intestinalis ventralis* was cannulated after the pyloric caeca. The cranial and caudal ends of the gut were severed and the gut was put into a Petri dish filled with Ringer solution at 20°C. Subsequently, the gut was flushed with 10 ml PBS and a ligature was tied around both the cranial and caudal end. The gut was suspended in an aerated organ bath filled with 100 ml Ringer solution at 20°C and perfusion was started at ≈ 2 ml min^{-1} $arch^{-1}$ kg^{-1} body weight. Cortland solution + dextran 1% was used as perfusion fluid (Nematollahi *et al.* 2005).

Experimental study 2

Adherence and invasion study

For inoculation, 0.4 ml of bacterial suspension in PBS containing approximately 10^8 CFU was injected inside the lumen of the ligated gut. A gut injected with 0.4 ml PBS served as negative control. After inoculation, the intestines were incubated for 1 h and subsequently processed for examination of adhesion. The experiment was conducted three times.

After the incubation period, each perfused gut was washed in an excess of PBS, flushed with 10 ml PBS after removing the ligatures and cut into three equally sized pieces. The proximal, middle and distal gut sections were processed for bacteriological, histological and IHC examination as described above.

To determine the invasion capacity, the organ bath fluid was collected following perfusion. The fluid was centrifuged at 1300 x g for 10 min, the pellet was resuspended in distilled water and the number of *Y. ruckeri* or *E. coli* was determined by plating tenfold serial dilutions on MacConkey agar plates.

Statistical analysis

Differences in adherence and invasion capacity between the five *Y. ruckeri* strains were compared for the gill and gut tissue using the one-way ANOVA test. For each strain, differences in adhesion to and invasion of both tissues were examined using the independent samples t-test. A significance level of 0.05 was accepted ($P < 0.05$).

RESULTS

Adherence and invasion study using a gill perfusion model

The five *Y. ruckeri* strains highly adhered to the gill tissue and significant differences were not observed (Table 2) whereas *E. coli* DH5α showed lower adherence capacity (4.73 $\log_{10}$ CFU g^{-1} gill tissue). The number of $\log_{10}$ CFU ml^{-1} perfusion fluid was significantly ($P < 0.05$) lower for *Y. ruckeri* CCUG 14190 compared to the virulent strains 5 and 9. *E. coli* DH5α was not recovered from the perfusion fluid.

Table 2 *Oncorhynchus mykiss.* Results of the bacteriological examination of the gill arches and intestines (posterior, middle and anterior gut were pooled) after exposure to *Y. ruckeri*. Adhesion capacity of *Y. ruckeri* is expressed as the numbers of bacteria isolated from the gills and the intestine. Invasion capacity is shown as the bacterial titers isolated from the perfusion fluid collected at the efferent gill artery and of the organ bath fluid collected 1 h after infection in the gill and gut perfusion model, respectively. The results represent the means of three independent experiments ± standard error.

	GILL PERFUSION		GUT PERFUSION	
Y. ruckeri	bacterial titer			
strain	$\log_{10}$ CFU g^{-1} gill tissue	$\log_{10}$ CFU ml^{-1} perfusion fluid	$\log_{10}$ CFU g^{-1} gut tissue	$\log_{10}$ CFU ml^{-1} organ bath fluid
5	6.37 ± 0.38	4.37 ± 0.54	6.52 ± 0.55	3.57 ± 0.37
9	6.22 ± 0.64	5.05 ± 1.03	7.58 ± 0.32	3.79 ± 0.15
E842-95	6.99 ± 0.54	3.49 ± 0.60	6.39 ± 0.29	3.78 ± 0.37
17.00(2-1)	6.44 ± 0.65	2.28 ± 2.02	6.69 ± 0.38	0.99 ± 1.71
CCUG 14190	5.81 ± 0.34	0.77 ± 0.68	6.07 ± 0.53	1.89 ± 1.74

Histopathological changes were not observed in the gill arches immediately after immersion with any of the five *Y. ruckeri* strains. Numerous bacteria were found in the mucus of the primary and secondary lamellae after Giemsa and IHC staining, independent of the *Y. ruckeri* strain used for infection. Moreover, sections often showed bacteria closely associated with the gill tissue (Figure 1a) and sometimes invaded in the lamina propria (Figure 1b).

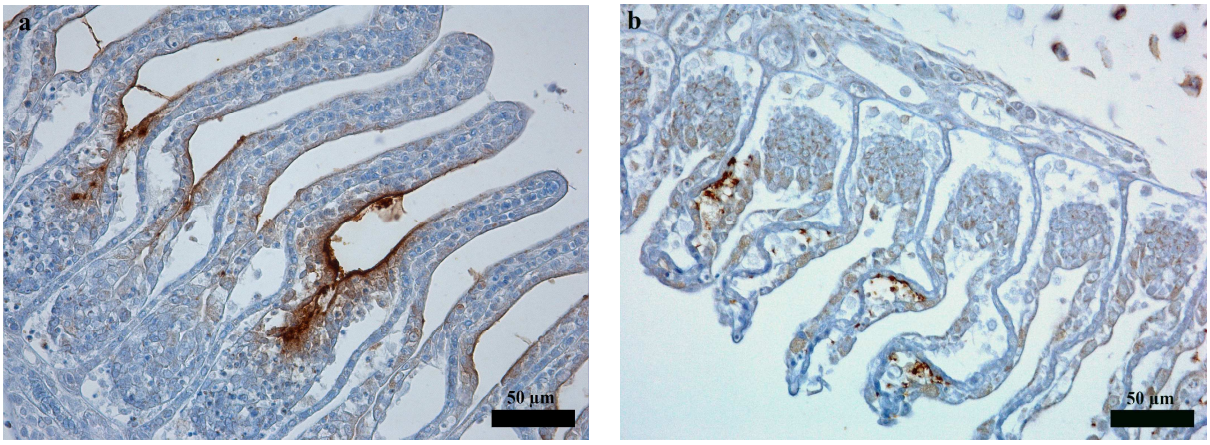

Figure 1 *Oncorhynchus mykiss*. Gill arch 1 h post challenge with *Y. ruckeri* (a) strain 17.00(2-1). Bacteria closely associated with the secondary lamellae (immunohistochemical staining), (b) strain E842-95. Bacterial invasion into the lamina propria (IHC staining).

Experimental study 2

Adherence and invasion study using a gut perfusion model

The five *Y. ruckeri* strains highly adhered to the gut tissue and significant differences were not observed (Table 2) whereas *E. coli* DH5α showed lower adhesion (4.06 $\log_{10}$ CFU g^{-1} gut tissue). The number of $\log_{10}$ CFU ml^{-1} organ bath fluid was lower for strains 17.00(2-1) and CCUG 14190 compared to the other strains, however, this was not significant ($P > 0.05$). *E. coli* DH5α was not recovered from the organ bath fluid.

Histopathological changes were not observed in the gut segments immediately after infection with any of the five *Y. ruckeri* strains. IHC staining revealed the presence of *Y. ruckeri* bacteria in the crypts, attached to the villi and within the epithelial layer of gut segments, independent of the *Y. ruckeri* strain used for infection (Figure 2). Few bacteria were also observed in the lamina propria mucosae.

Adhesion to and invasion of gill tissue compared to gut was not significantly different for any *Y. ruckeri* strain ($P > 0.05$).

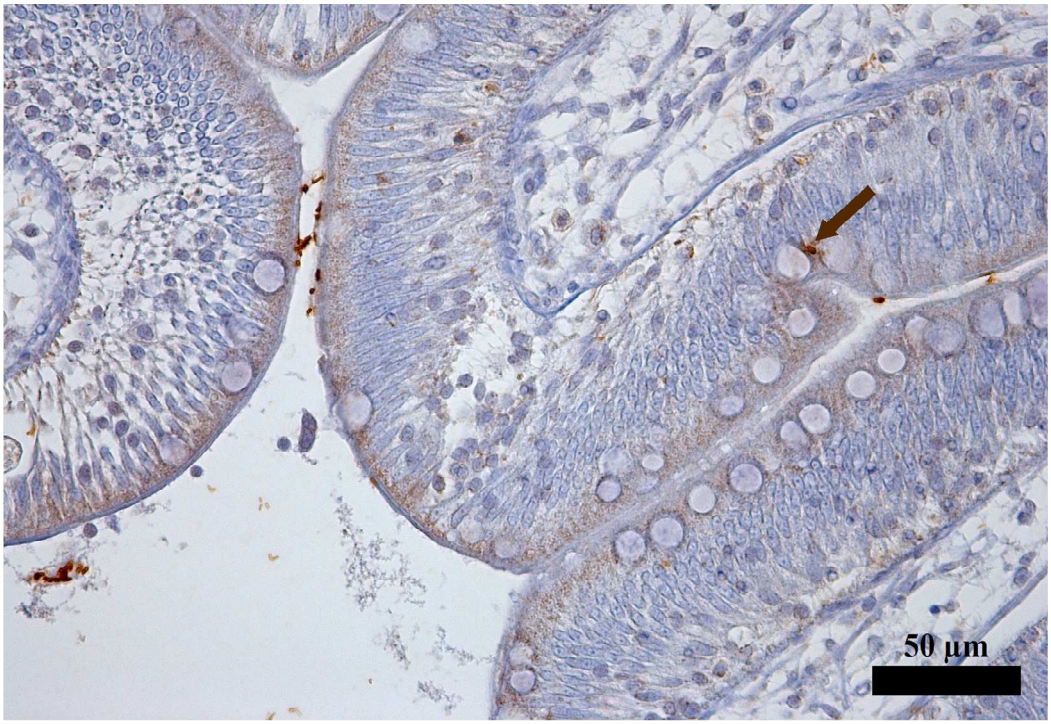

Figure 2 *Oncorhynchus mykiss*. Gut explant 1 h post challenge with *Y. ruckeri* E842-95. Bacteria attached to the mucus, present in the crypts and closely associated with the epithelium and within the mucosa (arrow) (immunohistochemical staining).

DISCUSSION

This is the first time that both perfusion models were used to study bacterial invasiveness. Only *Y. ruckeri* strains and not the non-invasive *E. coli* strain were isolated from the perfusion

fluid and organ bath fluid in the gill and gut perfusion model, respectively. These findings demonstrate that both perfusion models are suitable to study bacterial invasion.

Using the gill perfusion model, IHC examination revealed the presence of numerous *Y. ruckeri* adhered to the gill mucus and epithelium and within the lamina propria. Bacteria were also isolated from the perfusion fluid, indicating that they invaded the gill tissue. This is in agreement with previous *in vivo* studies, in which rainbow trout were experimentally infected (Tobback *et al.* 2009). These findings indicate that the gills are indeed an important portal of entry as has also been reported for other septicaemic fish pathogens such as *Vibrio anguillarum* and *Edwardsiella tarda* (Baudin-Laurencin & Germon 1987; Ling *et al.* 2001). Gills are highly vascularized allowing dissemination of the invaded bacteria to other sites of the body.

Using the gut perfusion model, IHC examination revealed the presence of *Y. ruckeri* adhered to the villi and within the mucosa and the bacteria were isolated from the organ bath fluid, indicating that they invaded the gut mucosa. Bacterial translocation from the gastrointestinal tract has also been shown for various other fish pathogens including *V. anguillarum* and *V. alginolyticus* (Olsson *et al.* 1996; Chen *et al.* 2008).

The ability to adhere to or invade gill and gut tissue was not significantly different between virulent and avirulent *Y. ruckeri* strains. Likely, other virulence factors such as serum resistance or survival in phagocytes may be important to induce disease.

Although different perfusion models were applied, both the gill and gut explants were directly exposed to similar numbers of bacteria. Therefore, the results of the gill perfusion model were statistically compared with those of the gut perfusion model. Analysis did not reveal significant differences in adhesion and invasion capacity of any of the *Y. ruckeri* strains between gill and gut tissue. This indicates that at least under our experimental conditions both organs are of equal importance in the initial interaction of *Y. ruckeri* with its host. This is in contrast to our previous *in vivo* study where lower numbers of bacteria were isolated from the intestine than from the gills after bath challenge of rainbow trout with *Y. ruckeri* (Tobback *et al.* 2009). This difference may be due to a different exposure to bacterial cells. Water taken into the mouth of fish is directly forced over the gills, bringing this organ continuously in close contact with the environment. Freshwater fish are reported to drink less than their saltwater counterparts (Bucking & Wood 2006) and therefore, the gut may possibly be exposed to a lower bacterial number during immersion infection.

In conclusion, *Y. ruckeri* was shown to colonize and invade gills and gut tissue of rainbow trout. Significant differences in adherence and invasive capacity were not observed between virulent and avirulent strains.

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Experimental study 2

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3. *In vitro* markers for virulence in *Yersinia ruckeri*

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Adapted from: *Journal of Fish Diseases*, in press.

Experimental study 3

ABSTRACT

In this study, different traits that have been associated with bacterial virulence were studied in *Yersinia ruckeri*. Two isolates that had been shown to cause disease and mortality in experimentally infected rainbow trout (*Oncorhynchus mykiss*, Walbaum) were compared with five avirulent isolates. Both virulent isolates showed high adhesion to gill and intestinal mucus of rainbow trout, whereas the majority of non-virulent strains demonstrated significantly lower adhesion. A decrease in adherence capability following bacterial treatment with sodium metaperiodate and proteolytic enzymes suggested the involvement of carbohydrates and proteins. All strains were able to adhere to and invade CHSE-214, FHM and R1 cells. One non-virulent strain was highly adhesive and invasive in the three cell lines, whereas the virulent strains showed moderate adhesive and invasive capacity. The internalization of several isolates was inhibited by colchicine and cytochalasin-D, suggesting that microtubules and microfilaments play a role. For all strains, intracellular survival assays showed a decrease of viable bacteria in the cells 6 h after inoculation, suggesting that *Y. ruckeri* is not able to multiply or survive inside cultured cells. Analysis of the susceptibility to the bactericidal effect of rainbow trout serum demonstrated that virulent *Y. ruckeri* strains were serum resistant, whereas non-virulent strains were generally serum sensitive.

INTRODUCTION

Yersinia ruckeri is the causative agent of yersiniosis or enteric redmouth disease (ERM) and causes significant losses in salmonid aquaculture worldwide. Although infection with this agent has been reported in other fish species, salmonids and especially rainbow trout (*Oncorhynchus mykiss*, Walbaum) are most susceptible to ERM (Furones *et al.* 1993). Infection may result in the development of a chronic or acute septicaemia with haemorrhages on the body surface and in the internal organs.

In vivo and *in vitro* studies indicated that the adherence and invasive capacities of *Y. ruckeri* are important in the early pathogenesis (Romalde & Toranzo 1993; Tobback *et al.* 2009). High numbers of bacteria were attached to the gill mucus soon after immersion of rainbow trout. *Y. ruckeri* was also observed within the gill capillaries, suggesting that the pathogen first adheres to the gill mucus and thereafter invades the branchial vasculature leading to septicaemia and colonization of the internal organs (Tobback *et al*. 2009).

It has already been demonstrated that *Y. ruckeri* is able to adhere to fish cell lines cultured *in vitro* (Romalde & Toranzo 1993). However, the components involved in the adhesion of *Y. ruckeri* to fish mucus or cells have not been investigated yet. The adherence capacity of pathogens to host tissue is mediated by surface components, known as adhesins. Different adhesins including the capsule, fimbriae, lipopolysaccharides and outer membrane proteins have been identified for several fish pathogens (Wang & Leung 2000).

Y. ruckeri is able to effectively invade fish cell lines *in vitro* (Romalde & Toranzo 1993; Kawula *et al.* 1996). However, efforts towards the identification of possible mechanisms of cell invasion have not been made for this bacterium. Chemical inhibitors of specific host cytoskeletal or surface elements are commonly used in invasion blocking assays. Cytochalasin-D inhibits the polymerization of actin filaments and is used to examine the need of functional microfilaments in host-directed bacterial endocytosis (Schliwa 1982). Besides microfilaments, microtubules are a major component of the cytoskeleton being depolymerised by colchicine (Weiss 1972). Monodansylcadaverine inhibits transglutaminase activity needed for the formation of clathrin-coated pits in receptor-mediated endocytosis (Levitzki *et al.* 1980).

Both an intracellular and an extracellular phase may be important for the survival of bacteria in the host. Bacteria internalized by non-phagocytic cells are well protected from the host's immune system and therefore, an intracellular phase may favour the spread of infection. Many bacteria are able to survive or even multiply intracellularly either in the vacuoles or in the cytosol of host cells (Goebel & Gross 2001). Extracellular bacteria are exposed to humoral as

well as cellular immune responses. An important host immune mechanism is the bactericidal effect of serum due to the complement system. Davies (1991) found that different virulent *Y. ruckeri* strains all showed serum resistance, whereas avirulent isolates were, with some exceptions, serum sensitive.

The aim of this study was to compare different traits that have been associated with bacterial virulence between virulent and avirulent *Y. ruckeri* strains, including adhesion to mucus and cell lines, invasion and intracellular survival in cell lines and serum resistance. The adhesion and invasion mechanisms were further characterized for selected *Y. ruckeri* strains.

MATERIALS AND METHODS

Bacterial strains

The *Y. ruckeri* isolates used in the present study are listed in Table 1. Stock suspensions of the isolates were stored at -70°C. After thawing, the bacteria were grown overnight at 20°C on Columbia agar (Gibco Life Technologies, Paisley, Scotland) with 5% sheep blood (blood agar). Colonies were picked up from the agar plates and grown in nutrient broth (NB; VWR, Haasrode, Belgium) for 24 h at 20°C. The number of colony forming units (CFU) per ml was determined by plating ten-fold serial dilutions on blood agar plates.

Table 1 *Y. ruckeri* strains

Y. ruckeri strain	Origin	Serotype	Virulence [a]	Mortality [a,b]
1	*O. mykiss* with ERM, UK, 1997	O1a	avirulent	0/20
5	*O. mykiss* with ERM, UK, 2001	O1a	virulent	6/20
9	*O. mykiss* with ERM, UK, 1995	O1a	virulent	4/20
2198(6)	*Psetta maxima* (L.) with ERM, France	O1a	avirulent	0/20
E842-95	NA	O1b	avirulent	0/20
17.00(2-1)	*O. mykiss*	O1a	avirulent	0/20
CCUG 14190	*O. mykiss* with ERM, USA	O1a	avirulent	0/20

NA: no information available

[a] Virulence and capacity to induce mortality were determined in an immersion infection model using juvenile rainbow trout (Tobback *et al.* 2009). If mortality and persistent infection were not observed, strains were categorized as avirulent according to Davies (1991)

[b] Number of fish that died/total number of fish

Isolation of gill and intestinal mucus

Mucus from the gills and intestine of six clinically healthy rainbow trout weighing approximately 300 g was isolated as described earlier with a slight modification (Nikoskelainen *et al.* 2001). The animals were obtained from a fish farm (Gérouville, Belgium) with no history of ERM and not applying vaccination against this disease. After euthanasia of the fish using an overdose of benzocaine (500 mg ml^{-1}), the gills and the intestine were removed. The mucus was carefully scraped off with a sterile surgical blade. The scrapings were dissolved in a small amount of phosphate buffered saline (PBS; pH 7.6) and centrifuged in order to remove cell debris (15000 x *g* for 10 min at 4°C). The mucus samples were pooled and stored in aliquots at -70°C until use.

Adherence assay to gill and intestinal mucus

The protein concentration in the mucus samples was measured using a commercial assay based on the method of Bradford (1976; Biorad Protein Assay, Biorad Laboratories Inc., Hercules, CA, USA). A concentration of 0.5 mg ml^{-1} in PBS was used in the adherence assays which were performed with a slight modification of the method described by Namba *et al.* (2007). Gill and intestinal mucus were immobilized overnight at 4°C on 96-well microtitre plates (100 µl per well; untreated F96 MaxiSorp plates, product number 442404, Nunc, Roskilde, Denmark). Wells were rinsed twice with PBS to remove unbound mucus. One hundred µl of each *Y. ruckeri* suspension (10^8 CFU ml^{-1} NB) or fresh NB only as a negative control was added per well. To examine for non-specific adhesion, all strains were also added to non-treated wells without mucus. The inoculated plates were centrifuged at 300 x *g* for 10 min at 20°C and incubated for 1 h at 20°C. After incubation, unbound bacteria were removed by washing the wells three times with PBS. One hundred µl NB and 10 µl water-soluble tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) were added per well. After 2 h of incubation at 30°C, the optical density at 450 nm (OD_{450}) for the formazan formed was recorded with a Multiscan MCC ELISA reader (Labsystems, Helsinki, Finland). All the tests were performed in triplicate and repeated independently at least three times. According to Namba *et al.* (2007), the number of adhering bacteria per well was calculated from a standard curve.

Adherence inhibition assay to gill and intestinal mucus

Y. ruckeri strains 5, E842-95, 17.00(2-1) and CCUG 14190 were grown in NB for 24 h at 20°C, centrifuged at 1300 x *g* for 10 min and resuspended in fresh NB at a concentration of

10^8 CFU ml^{-1}. The suspensions were subjected to the following treatments: (1) bacteria were incubated for 30 min at 30°C with pepsin solution (1 mg ml^{-1} pepsin in 50 mM citrate-10 mM phosphate buffer, pH3); (2) bacteria were incubated for 1 h at 30°C with pronase solution (1 mg ml^{-1} pronase in 0.01 M sodium acetate and 0.005 M calcium acetate buffer, pH 7.5); (3) bacteria were incubated for 1 h at 30°C with trypsin solution (1 mg ml^{-1} trypsin in PBS, pH 7.5); (4) bacteria were incubated for 1 h at 30°C in a sodium metaperiodate solution (10 mg ml^{-1} sodium metaperiodate in PBS, pH 7.3); (5) bacteria were incubated for 1 h at 30°C with the following carbohydrates: D-glucose, D-galactose, D-galactosamine, L-arabinose, D-xylose, D-mannose, D-fructose, D-fucose, L-fucose, D-maltose, D-sucrose (all saccharides at a final concentration of 200 mM in PBS). Non-treated bacteria maintained under the same conditions were used as controls.

All bacterial preparations were washed three times, resuspended in NB and the adherence assay was carried out as described above. Additionally, the viability of the treated bacterial cells was determined by plating ten-fold serial dilutions on blood agar plates.

Cell lines

Three different fish cell lines were used in this study: chinook salmon embryo cell line (CHSE-214), fathead minnow epithelial cell line (FHM) and rainbow trout liver cell line (R1). CHSE-214 and FHM cells were grown and maintained in minimum essential medium (MEM; Gibco Life Technologies, Paisley, Scotland) containing 2 mM L-glutamine, 1% non essential amino acids (NEAA), 100 µg ml^{-1} kanamycin, 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 10% fetal calf serum (FCS) at 20°C. CHSE-214 was kept at 5% CO_2. The cells were subcultured every 2-3 days after detachment with 10% trypsin at room temperature. R1 cells were maintained in medium 199 with Earle's salts with L-glutamine (Gibco Life Technologies, Paisley, Scotland) supplemented with 100 µg ml^{-1} kanamycin, 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin and 10% fetal calf serum (FCS) at 20°C. The cells were subcultured every 2 weeks after detachment with TNE (50 mM Tris, 0.1 M NaCl, 5 mM EDTA, pH 7.5) at 4°C for 10 min.

Adherence assay to cell lines

CHSE-214, FHM and R1 cells were plated on Thermanox plastic coverslips (Nunc, Rochester, N.Y., USA) in 24-well plates at a density of approximately 10^5 cells per well and were allowed to attach for at least 2 h. After washing three times with PBS, all different *Y. ruckeri* strains were added to the cells at a multiplicity of infection (MOI) of 10. To examine for non-

specific adhesion, all strains were also added to non-treated coverslips without cultured cells. The inoculated plates were centrifuged at 300 x *g* for 10 min at 20°C to allow contact between bacteria and cells. After 1 h incubation at 20°C, the wells were rinsed three times with PBS and further processed for scanning electron microscopy (SEM). Additionally, cell line CHSE-214 was used in adherence inhibition assays using Hemacolor staining.

Scanning electron microscopy (SEM)

The cells were fixed overnight in 5% paraformaldehyde, 437 mM NaCl, 187 mM HEPES, 12.58 mM $CaCl_2.H_2O$, 5.76% glutaraldehyde, pH 7.2. Samples were postfixed in 1% (wt/vol) osmiumtetroxide in distilled water for 2 h at room temperature. After dehydration through a graded series of alcohol and acetone, samples were critical point dried (Balzers, Liechtenstein) and platina sputter-coated with a JFC–1300 Auto fine coater (Japanese Electronic Optical Laboratories, Tokyo, Japan). Analysis was performed on a JSM-5600LV (Japanese Electronic Optical Laboratories, Japan). The number of adhering bacteria was counted for 100 cells.

Hemacolor staining

The plates were stained with Hemacolor staining reagents (Merck, Darmstadt, Germany) and microscopically examined. The number of adhering bacteria was counted for 100 cells.

Adherence inhibition assay to cell line CHSE-214

Similar to the adherence inhibition assay performed on mucus, *Y. ruckeri* strain 17.00(2-1) was pre-treated with pepsin, pronase, trypsin, sodium metaperiodate, D-glucose, D-galactose, D-galactosamine, L-arabinose, D-xylose, D-mannose, D-fructose, D-fucose, L-fucose, D-maltose and D-sucrose before included in the adherence inhibition assay performed on cell line CHSE-214 as described above. Non-treated bacteria were used as controls.

Invasion and intracellular survival assay in cell lines

CHSE-214, FHM and R1 cells were seeded in 24-well plates at a density of approximately 10^5 cells per well. Similar to the adhesion assay, the cells were inoculated with all different *Y. ruckeri* strains at a MOI of 10, centrifuged at 300 x *g* and incubated at 20°C. After 1 h, the wells were rinsed three times with PBS and fresh medium supplemented with 100 µg ml^{-1} gentamicin (Gibco Life Technologies, Paisley, Scotland) was added.

After an additional 1 h incubation at 20°C, the wells were rinsed again three times with PBS. To assess invasion, the cells were lysed with 100 µl of 1% Triton-X100 (Sigma-Aldrich, Bornem, Belgium) in distilled water by shaking the plates for 10 min. An additional 100 µl PBS per well was added, mixed and serial dilutions of the lysates were plated on blood agar to

determine the number of CFU. The invasiveness was expressed as the percentage of bacteria that were recovered in comparison to the number of bacteria that were inoculated.

To assess intracellular survival, the medium containing 100 µg ml^{-1} gentamicin was replaced after the 1 h incubation time with fresh medium supplemented with 15 µg ml^{-1} gentamicin and the number of viable bacteria was assessed 6 h after inoculation as described above.

All experiments were performed in triplicate and repeated independently at least three times. In each experiment, wells containing only cells and only bacteria were used as controls. To confirm that the conditions used in the invasion and intracellular survival assay were sufficient to kill the extracellular bacteria, the susceptibility of *Y. ruckeri* to gentamicin was examined. Therefore, a suspension of 10^6 CFU ml^{-1} of each bacterial strain was prepared. The suspensions and 100 µg ml^{-1} gentamicin were incubated for 1 h at 20°C. In a second test, the gentamicin concentration was lowered to 15 µg ml^{-1} and incubated for an additional 5 h. Following incubation, the viability of the bacteria was determined by plating serial dilutions on blood agar.

Invasion inhibition assay in cell lines

Cytochalasin-D (Sigma-Aldrich), colchicine (Sigma-Aldrich) and monodansylcadaverine (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 1 mM, 20 mM and 20 mM, respectively, divided into aliquots and stored at -20°C. Prior to use, the inhibitors were thawed and diluted in culture medium without antibiotic to 2 µM cytochalasin-D, 20 µM colchicine and 100 µM monodansylcadaverine. In a preliminary experiment, the concentrations mentioned above were found to have no cytotoxic effect on the cultured cells or the bacteria, as measured using trypan blue exclusion and CFU ml^{-1} counts, respectively.

In the inhibition assay, cell cultures (10^5 cells per well) were pre-treated for 1 h with the inhibitors at 20°C and washed three times with PBS before invasion with *Y. ruckeri* strains 5, E842-95, 17.00(2-1) and CCUG 14190 at a MOI of 10 was studied as described above.

Serum resistance assay

Blood was collected by caudal venipuncture from seven rainbow trout weighing approximately 300 g and obtained from the same fish farm as mentioned above. After overnight separation at room temperature, sera were pooled and stored at -70°C. For all *Y. ruckeri* strains, a bacterial suspension in PBS was prepared and used in the serum resistance assay at a final concentration of approximately 10^7 CFU ml^{-1}. The assay was carried out in

triplicate at 20°C with native serum and serum previously heated for 20 min at 46°C to destroy complement activity as described by Davies (1991). The percentage of survival was calculated after 3 h of incubation by dividing the number of viable bacteria after the serum treatment by the initial population before treatment. Serum resistant isolates were defined as those which increased in number after 3 h (Davies 1991).

Statistics

The adherence, invasion and survival data were analysed by one-way analysis of variance. All analyses were done at the 5% significance level and Bonferroni's technique was used to adjust for multiple comparisons. The results of the adherence inhibition assays were analysed by the Kruskal-Wallis one-way non-parametric analysis of variance test using a significance level of 5%.

RESULTS

Adherence assay to gill and intestinal mucus

The *Y. ruckeri* strains did not adhere to non-treated wells without mucus. Results of *Y. ruckeri* adhesion to gill and intestinal mucus are presented in Figure 1. The number of adhering bacteria per well was calculated from the standard curve: CFU ml^{-1} = 3.0 x 10^7 x OD_{450} + 1.0 x 10^6 (R^2 = 0.98).

To gill mucus, *Y. ruckeri* strains 5, 2198(6) and 9 showed the highest adhesion. *Y. ruckeri* 1 exhibited moderate adhesive capability. The other strains showed low adhesion, close to the detection limit (10^6 CFU ml^{-1}). The adhesion of strains E842-95, 17.00(2-1) and CCUG 14190 was significantly ($P < 0.05$) lower than the adhesion of strains 5, 2198(6) and 9. Strain 1 showed significantly ($P < 0.05$) lower adhesive capability than strains 5 and 2198(6).

To intestinal mucus, *Y. ruckeri* strains 5, 9 and 2198(6) exhibited significantly ($P < 0.05$) higher adhesion than the other strains. Strain 1 showed moderate adhesion and the other strains were low adhesive with values close to the detection limit.

In general, adhesion to gill mucus was higher than to intestinal mucus for all strains. This difference was significant ($P < 0.05$) for strains 1, 5, 9, 2198(6) and CCUG 14190.

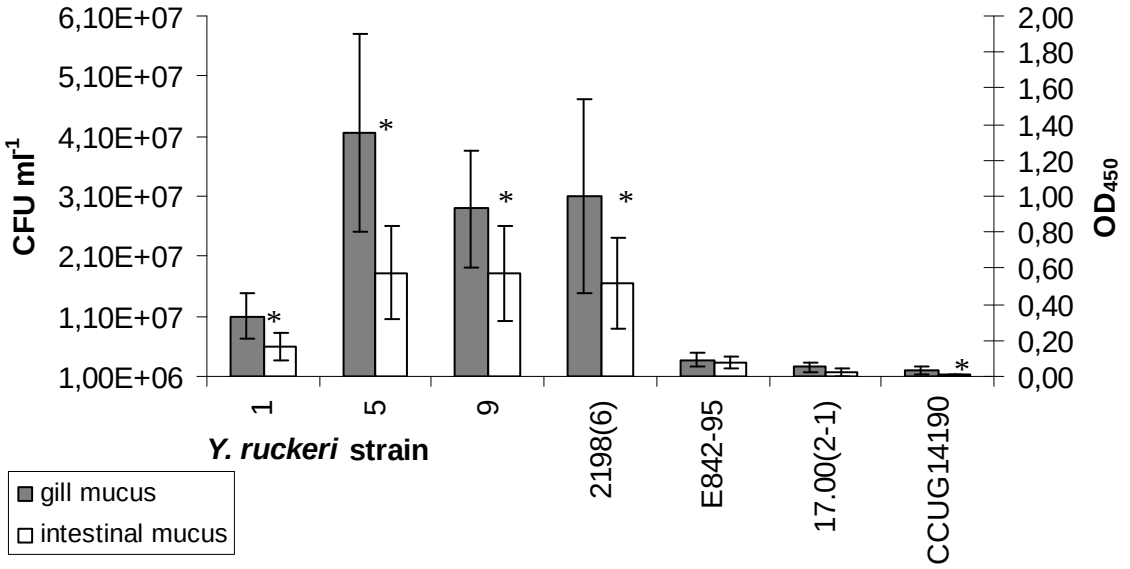

Figure 1 The adhesion of different *Y. ruckeri* strains to gill and intestinal mucus. OD_{450}-values are shown as a measure for the number of adhered bacteria. The results represent the means ± standard error of three or four independent experiments conducted in triplicate. An asterisk refers to a significantly higher adhesion to the gill mucus compared to the intestinal mucus ($P < 0.05$).

Adherence inhibition assay to gill and intestinal mucus

Results of the adherence inhibition assay to gill and intestinal mucus of *Y. ruckeri* strains 5, E842-95 and 17.00(2-1) are presented in Figure 2. The adherence of strain CCUG 14190 was too low to get reliable results in the adherence inhibition assay using WST-1.

Similar results were obtained for the three *Y. ruckeri* strains. Pepsin treatment did not affect the adherence ability, whereas pronase significantly ($P < 0.05$) reduced the level of adhesion. Treatment with trypsin resulted in a lower adhesion, however, this was not always significant. Sodium metaperiodate treatment strongly decreased the adhesion of all strains to gill and intestinal mucus ($P < 0.05$). D-galactose and D-fructose caused a lower adhesion to both gill and intestinal mucus; however, D-fructose inhibited the adherence to gill mucus more than to intestinal mucus. Treatment with the other saccharides did not inhibit the adherence of the *Y. ruckeri* strains.

Pronase and trypsin treatment of the bacterial cells did not have any significant effect on the viability. Pepsin significantly ($P < 0.05$) reduced the viability. Treatment with 10 mg ml^{-1} sodium metaperiodate slightly reduced the viability of *Y. ruckeri*, however, this was not significant.

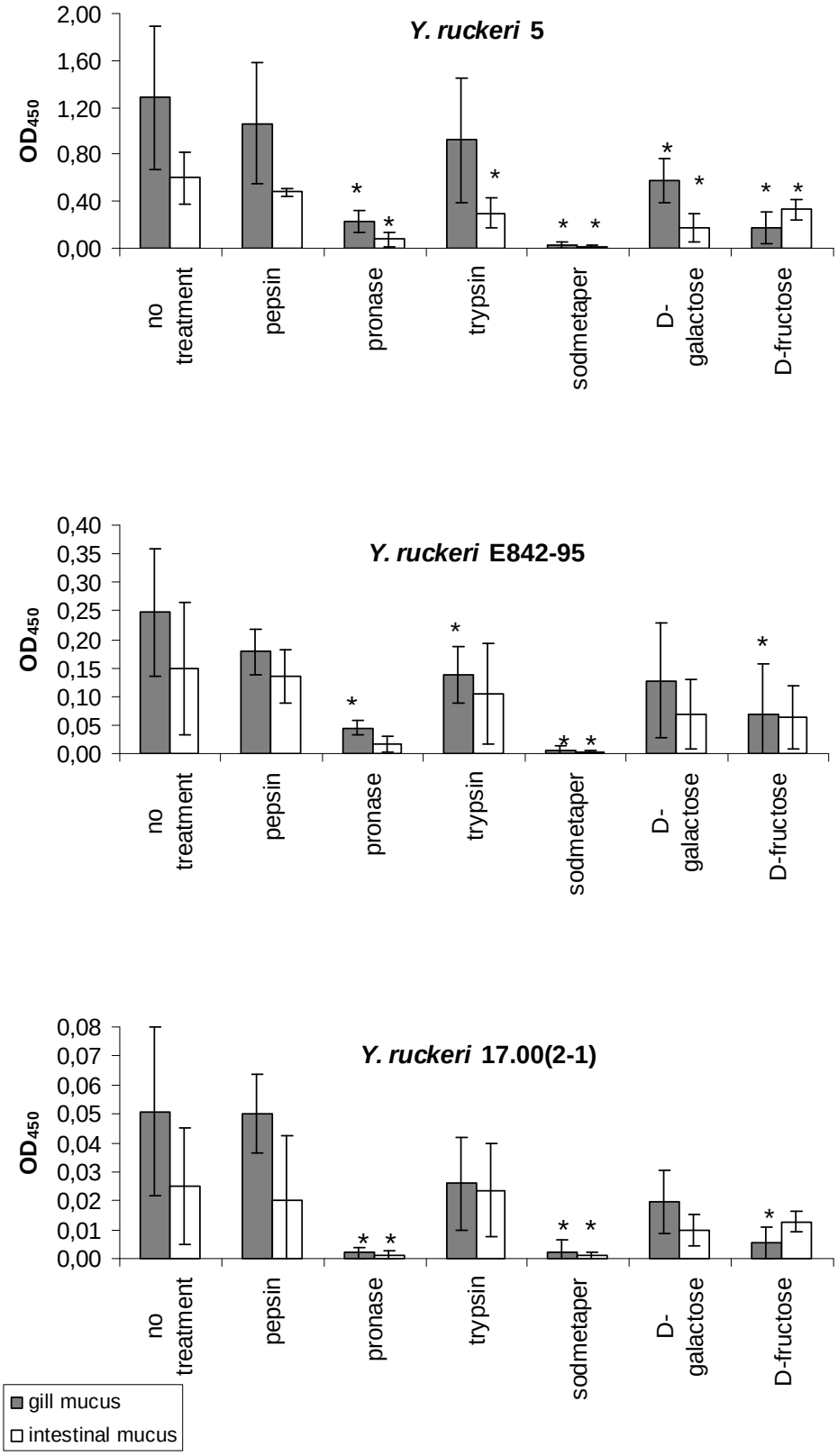

Figure 2 Effect of pre-treatment of the bacterial cells of *Y. ruckeri* strains 5, E842-95 and 17.00(2-1) with enzymes, sodium metaperiodate and carbohydrates on the adhesion to gill and intestinal mucus. The results represent the means ± standard error of three independent experiments conducted in triplicate. An asterisk refers to a significantly lower adhesion relative to bacteria that were not pre-treated ($P < 0.05$).

Experimental study 3

Adherence assay to cell lines

The *Y. ruckeri* strains did not adhere to non-treated coverslips without cultured cells. Results of *Y. ruckeri* adherence to CHSE-214, FHM and R1 cells are presented in Figure 3.

In all the cell lines, *Y. ruckeri* 17.00(2-1) showed a significantly higher adherence capacity, than all the other strains. Figure 4 illustrates attached *Y. ruckeri* 17.00(2-1) bacteria to the three cell lines. The other strains did not significantly differ from each other in adhesion capacity.

In cell line CHSE-214, strain E842-95 showed a slightly higher adhesion than strains 1, 5, 2198(6), 9 and CCUG 14190. In cell line FHM, strains 1, 5, 2198(6), E842-95 and CCUG 14190 showed similar adhesion capacity, being slightly higher than strain 9. In cell line R1, strains E842-95 and 9 showed slightly higher adhesion than strains 1, CCUG 14190 and 5.

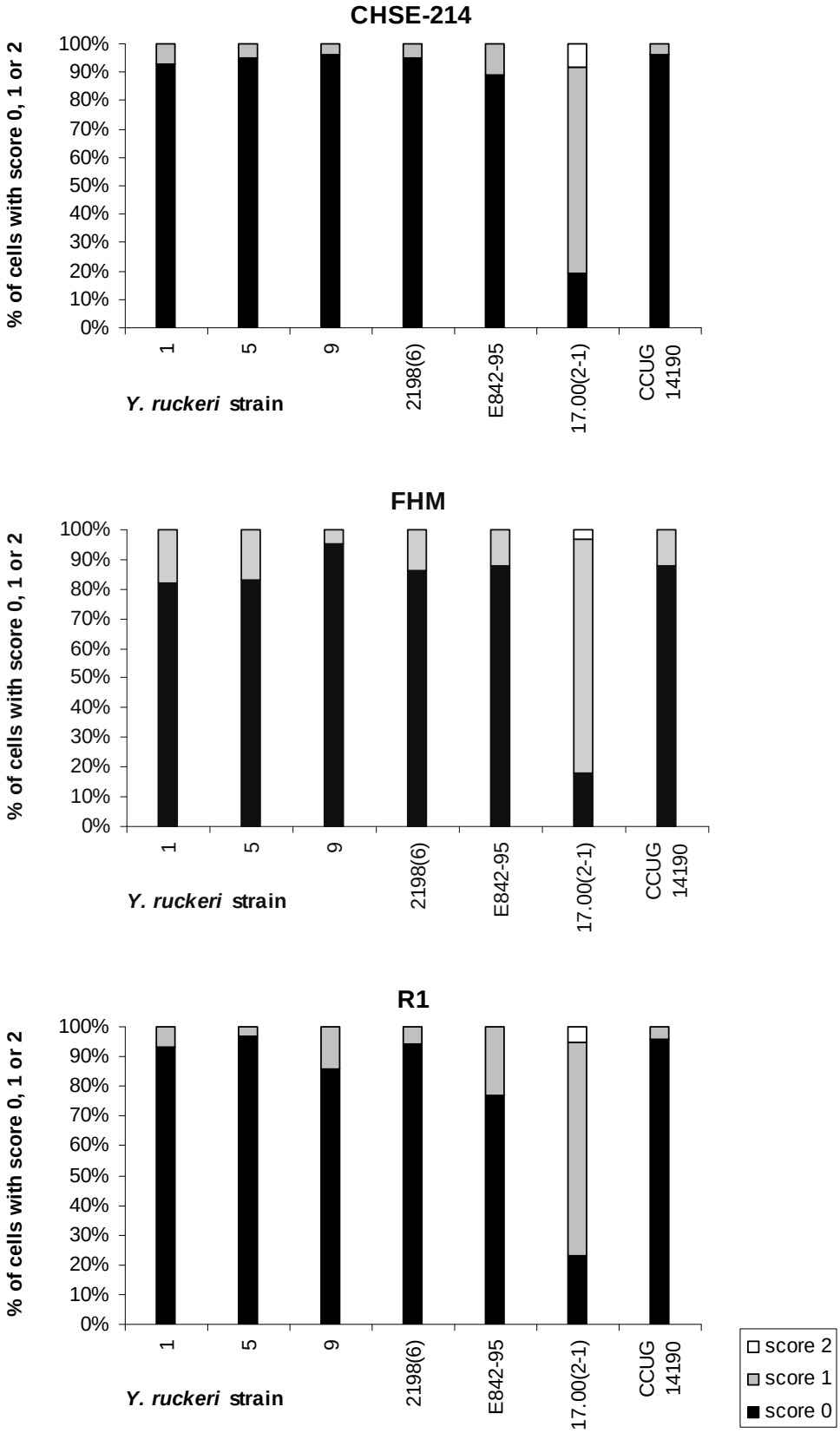

Figure 3 The adhesion of different *Y. ruckeri* strains to CHSE-214, FHM and R1 cells. The percentage of cells are shown to which no bacteria (score 0), 1 to 10 (score 1) and 11 to 20 (score 2) bacteria adhered.

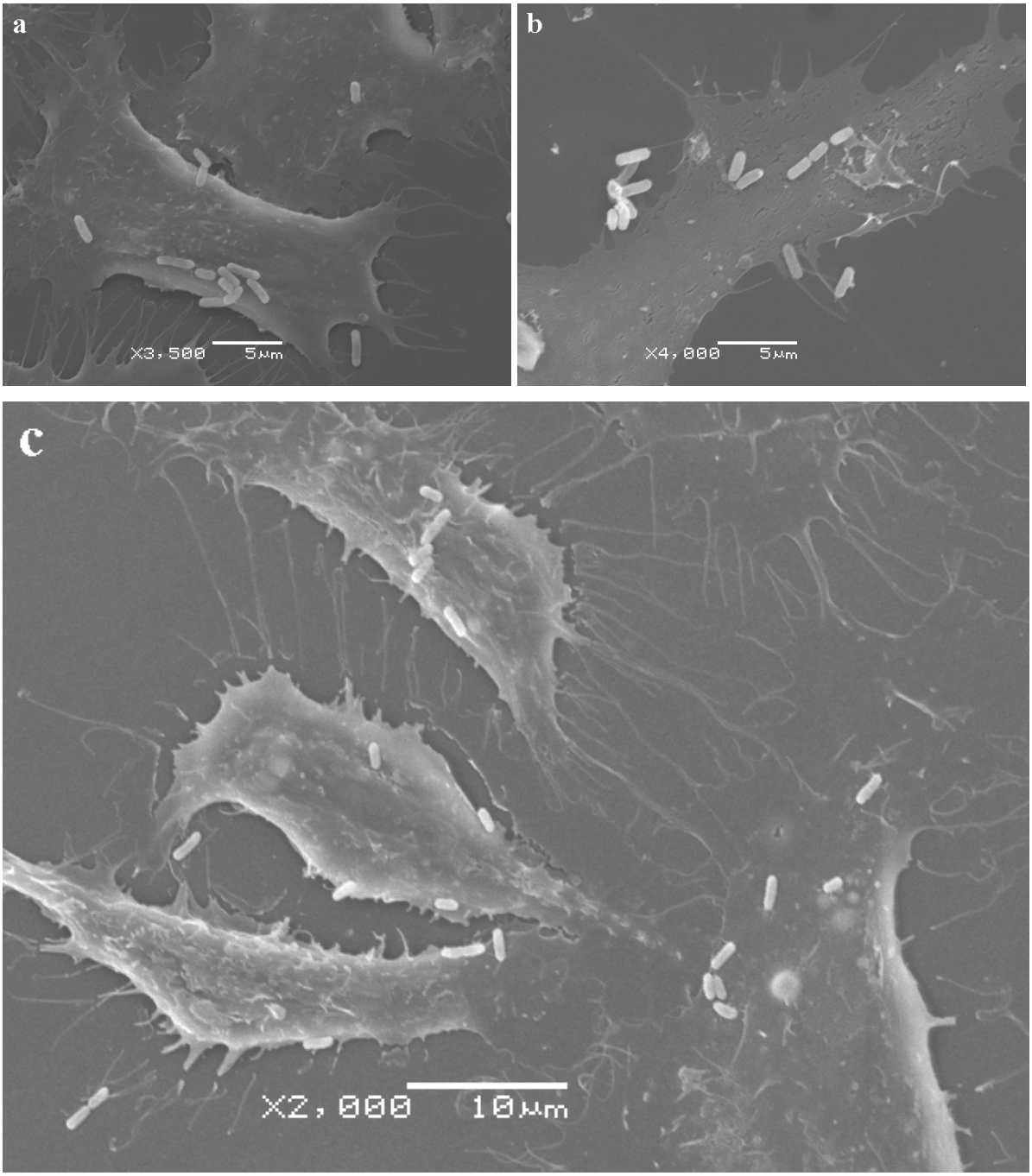

Figure 4 Adhesion of *Y. ruckeri* 17.00(2-1) to (a) CHSE-214, (b) FHM and (c) R1 cells (Scanning electron microscopy).

Adherence inhibition assay to CHSE-214

Results are represented in Figure 5. Treatment of *Y. ruckeri* 17.00(2-1) with sodium metaperiodate, pronase and trypsin significantly ($P < 0.05$) decreased the adhesion to CHSE-214 cells. In descending order, inhibition was observed with D-galactose, D-glucose, D-maltose and D-sucrose ($P < 0.05$). The other saccharides and pepsin did not significantly inhibit adherence of *Y. ruckeri* 17.00(2-1) to CHSE-214 cells.

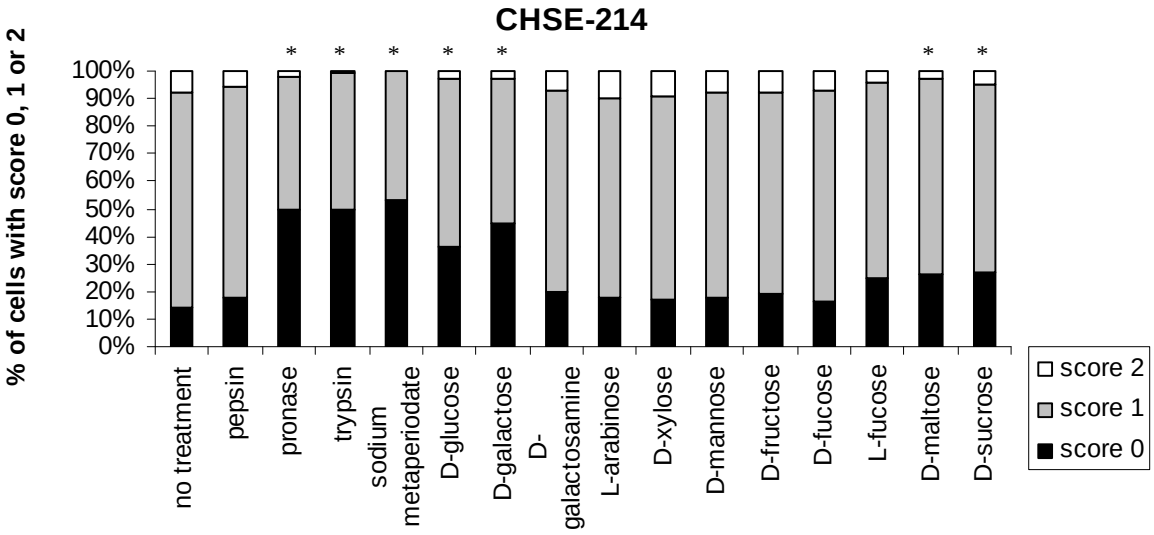

Figure 5 Effect of pre-treatment of *Y. ruckeri* 17.00(2-1) with enzymes, sodium metaperiodate and different sugars on the adhesion to CHSE-214. The percentage of cells are shown to which no bacteria (score 0), 1 to 10 (score 1) and 11 to 20 (score 2) bacteria adhered. An asterisk refers to a significantly lower adhesion relative to bacteria that were not pre-treated ($P < 0.05$).

Invasion assay in cell lines

Similar results were observed in the repetitions of the assays. The positive controls (wells containing only bacteria) and the bactericidal tests confirmed that the gentamicin concentration and incubation time used for the invasion assay were sufficient to kill the extracellular bacteria. All the isolates were invasive in the three cell lines (Figure 6).

In cell line CHSE-214, *Y. ruckeri* strain 17.00(2-1) showed a significantly ($P < 0.05$) higher invasiveness than all the other strains of 0.55% which corresponded with a recovery of bacteria of 5.3×10^3 CFU ml^{-1}. Strain 9 invaded the cells significantly ($P < 0.05$) higher than strains E842-95 and CCUG 14190. Strains 1 and 5 exhibited a similar invasiveness, significantly ($P < 0.05$) higher than strain CCUG 14190. Strains 2198(6), E842-95 and CCUG 14190 showed the lowest invasions in descending order.

In the FHM cells, the invasion of strain 17.00(2-1) was significantly ($P < 0.05$) higher than the invasion of all the other strains corresponding with a recovery of 2.3×10^3 CFU bacteria ml^{-1}. Strain 1 showed a significantly ($P < 0.05$) higher invasion than strains E842-95 and CCUG 14190, followed by strains 5 and 2198(6) which significantly ($P < 0.05$) higher invaded the cells than strain CCUG 14190. Strains 9, E842-95 and CCUG 14190 exhibited the lowest invasions in descending order.

In cell line R1, strain 17.00(2-1) also showed a significantly ($P < 0.05$) higher invasiveness than all the other strains, corresponding with a recovery of bacteria of 1.5 x 10^4 CFU ml^{-1}. Strains 2198(6), 1, CCUG 14190, 5 and 9 showed similar invasiveness values. Strain E842-95 exhibited the lowest invasion.

Generally, the lowest invasiveness values were observed in FHM when invasion was compared between the three different cell lines, however, these were not significantly different from the invasiverness values in CHSE-214. Overall, a significantly ($P < 0.05$) higher invasion was observed in cell line R1 for all strains compared to the other cell lines.

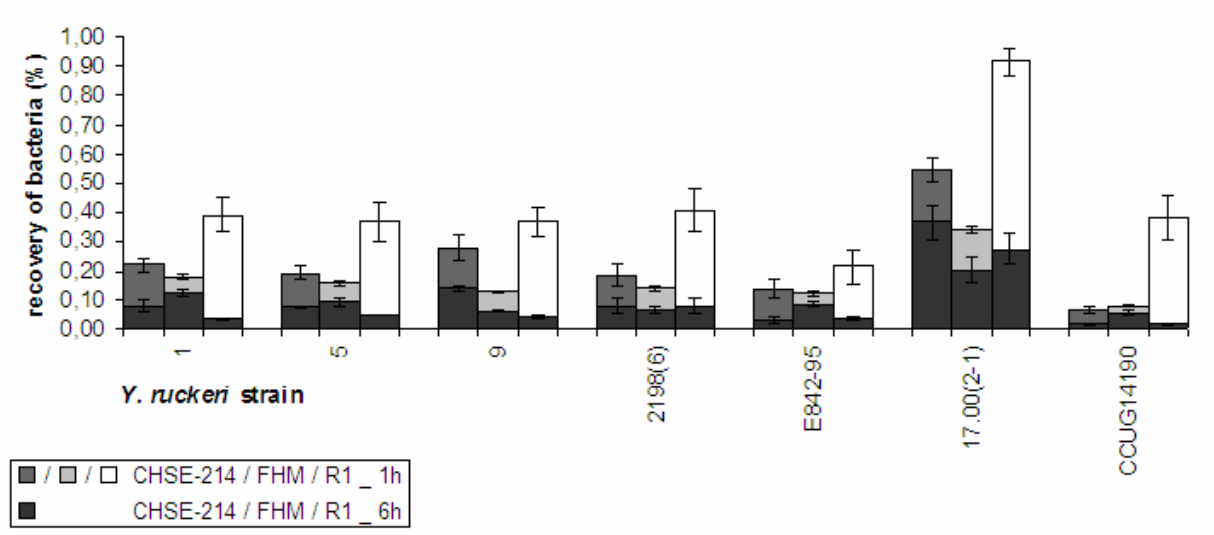

Figure 6 The invasion (1 h) and intracellular survival (6 h) of different *Y. ruckeri* strains in fish cell lines CHSE-214, FHM and R1. The percentages of gentamicin protected bacteria are shown. The results represent the means ± standard error of three or four independent experiments conducted in triplicate.

Intracellular survival in cell lines

Similar results were observed in the repetitions of the assays. The number of viable *Y. ruckeri* in the three cell lines was lower 6 h after infection in comparison with 1 h after infection (Figure 6).

In cell line CHSE-214, *Y. ruckeri* 17.00(2-1) showed the highest intracellular survival 6 h after infection, which is significantly ($P < 0.05$) higher than the survival of strains 1, CCUG 14190 and E842-95. Strains 9, 5 and 2198(6) showed significantly ($P < 0.05$) higher survival values than strains CCUG 14190 and E842-95.

In cell line FHM, *Y. ruckeri* E842-95 showed the highest intracellular survival 6 h after infection, which is significantly ($P < 0.05$) higher than the survival of strain 2198(6) which has the lowest value.

In cell line R1, *Y. ruckeri* 17.00(2-1) had a significantly ($P < 0.05$) higher intracellular survival than all the other strains. Strains 1 and CCUG 14190 showed a significantly ($P < 0.05$) lower survival than strains 17.00(2-1) and 2198(6).

Overall, a significantly ($P < 0.05$) lower survival was observed in cell line R1 for all strains compared to FHM and for most strains compared to CHSE-214. In FHM compared to CHSE-214, only strains E842-95, CCUG 14190 and 1 showed a significantly ($P < 0.05$) higher survival.

Invasion inhibition assay in cell lines

The results of the invasion inhibition assays are represented in Figure 7. Monodansylcadaverine never inhibited invasion.

In cell line CHSE-214, the invasion of the four *Y. ruckeri* strains was significantly ($P < 0.05$) inhibited by cytochalasin-D and colchicine.

In cell line FHM, only strains 5 and 17.00(2-1) were significantly inhibited by colchicine. Treatment with cytochalasin-D significantly ($P < 0.05$) inhibited invasion of strain 5. Cytochalasin-D and monodansylcadaverine significantly ($P < 0.05$) increased invasion of strain 17.00(2-1). The invasion of the other two strains was not influenced by any of the inhibitors.

In the R1 cells, all the strains were inhibited by colchicine, whereas treatment with cytochalasin-D significantly ($P < 0.05$) inhibited invasion of strains 17.00(2-1), E842-95 and CCUG 14190.

Serum resistance assay

Y. ruckeri strains 1, 5 and 9 were serum resistant and showed 345 ± 35%, 528 ± 51% and 143 ± 19% viable bacteria after 3 h incubation, respectively. Strains 2198(6), E842-95, 17.00(2-1) and CCUG 14190 were serum sensitive with 22 ± 7%, 64 ± 7%, 0 ± 0% and 63 ± 4% viable bacteria, respectively.

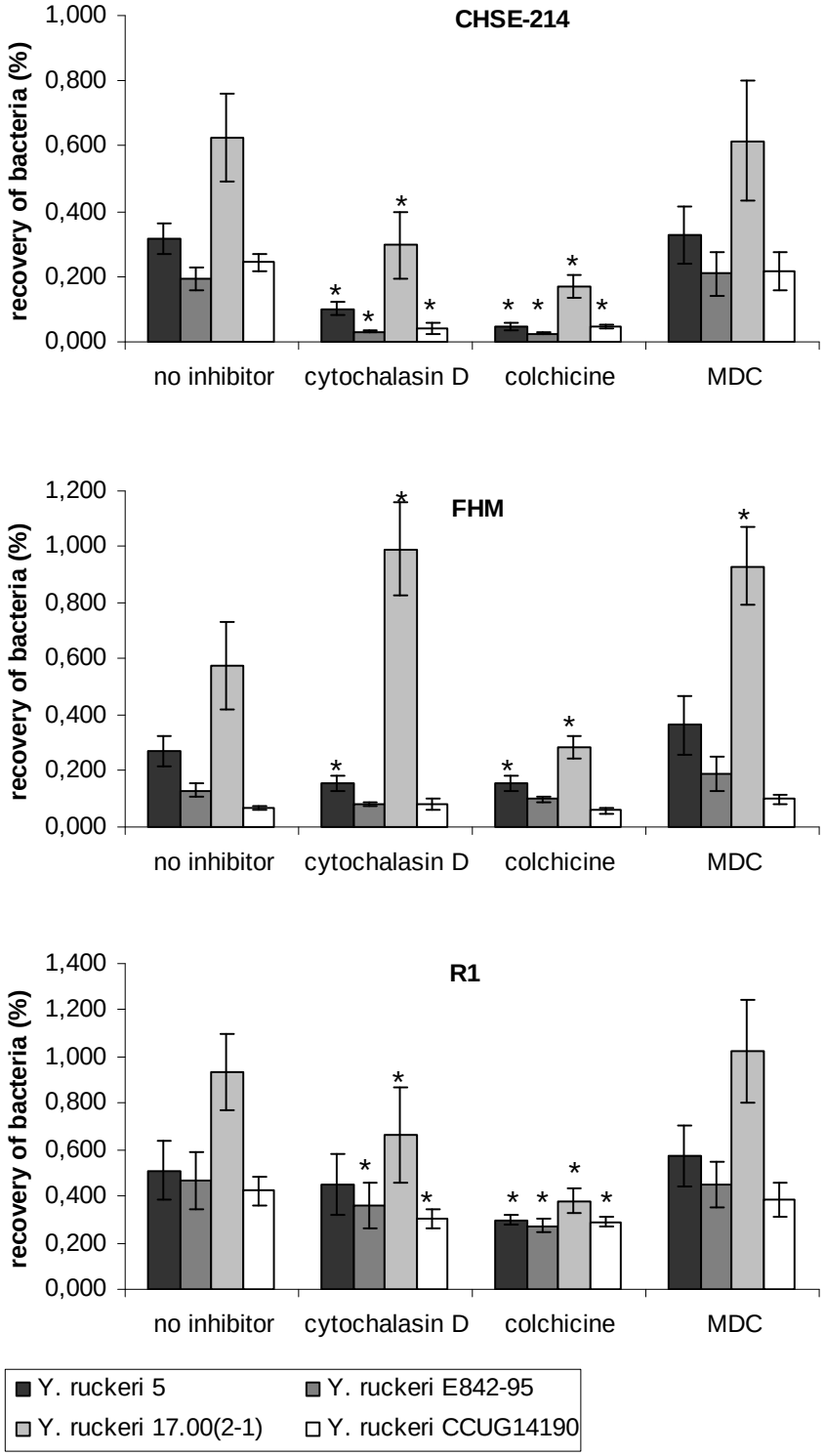

Figure 7 Effect of various inhibitors on *Y. ruckeri* invasion in CHSE-214, FHM and R1 cells. The percentages of gentamicin protected bacteria are shown. The results represent the means ± standard error of three independent experiments conducted in triplicate. An asterisk refers to a significantly different value compared to the values in the absence of the inhibitor ($P < 0.05$).

DISCUSSION

In the present study, *in vitro* adhesion was found to be higher to gill mucus than to intestinal mucus for all strains. Previously, attachment of numerous *Y. ruckeri* bacteria to the gill mucus was clearly seen immediately after infection of rainbow trout using the immersion model (Tobback *et al.* 2009). This indicates that *Y. ruckeri* preferentially adheres to the gill mucosal surface and possibly constitutes an important initial step in the pathogenesis.

Y. ruckeri strains 5, 9 and 2198(6) showed a significantly higher *in vitro* adhesion than the other strains to both types of mucus. Strains 5 and 9 were previously found to cause disease and mortality in rainbow trout after experimental infection, whereas the other strains were found to be avirulent (Tobback *et al.* 2009). These results suggest that adhesion to mucus is associated with virulence in *Y. ruckeri*. Most probably, other factors are important in the pathogenesis of a *Y. ruckeri* infection as well since strain 2198(6) was highly adhesive to mucus but did not induce disease.

To all tested cultured cells, the avirulent strain *Y. ruckeri* 17.00(2-1) adhered to a higher extent compared to the other isolates. Accordingly, the *in vitro* adherence does not appear to correlate with the previously tested virulence. The same discrepancy was also observed in adhesion to erythrocytes of virulent and avirulent *Flavobacterium psychrophilum* strains (Møller *et al.* 2003). In their study, two out of five avirulent strains showed high adhesion, whereas two out of three virulent strains were greatly reduced in adhesive capacity. It is believed that strains with a lower adhesion showed a decreased expression of adhesive factors and therefore, were more resistant to complement-mediated bacteriolysis (Møller *et al.* 2003). Further research is needed to reveal if the pathogenicity of *Y. ruckeri* is influenced in a similar way.

Bacterial attachment to external surfaces involves microbial adhesins. In this study, treatment of *Y. ruckeri* with sodium metaperiodate, pronase and trypsin reduced the adherence to mucus and cultured cells without significantly affecting bacterial viability. These results suggest that carbohydrates as well as proteins play a role and consequently, glycoproteins or lectins may be involved in the adhesion (Wang & Leung 2000; Van Overbeke *et al.* 2002).

Our results suggest that different adhesins are involved in *Y. ruckeri* adhesion to mucus and cultured cells, since adherence to both surfaces was inhibited by different sugars. D-fructose and D-galactose reduced the adherence of *Y. ruckeri* to both types of mucus, whereas D-galactose, D-glucose, D-maltose and D-sucrose inhibited the adherence to CHSE-214 cells in descending order. The fact that both disaccharides inhibited adhesion is not completely surprising, since both contain D-glucose.

Experimental study 3

The ability of *Y. ruckeri* to invade fish cell lines was demonstrated using gentamicin protection assays. The invasive properties of *Y. ruckeri* depended on the bacterial strain and the cell line used, as has also been demonstrated for other pathogens (Mills & Finlay 1994; López-Dóriga *et al.* 2000). Interestingly, *Y. ruckeri* 17.00(2-1), which adhered to all tested cells to a higher extent compared to the other isolates, also showed the highest invasiveness, although this strain did not cause disease *in vivo* as previously determined (Tobback *et al.* 2009). On the other hand, strains 5 and 9 which caused disease signs and mortality *in vivo* appeared to be moderately invasive *in vitro*. Consequently, the virulence of *Y. ruckeri* for rainbow trout does not seem to correlate with *in vitro* invasiveness in cell lines. Similar results were seen in an invasion study with *Aeromonas hydrophila*. *A. hydrophila* strain TF7 did not invade four different cell types, although this strain appeared to be highly virulent for fish *in vivo* (Kawula *et al.* 1996).

Our study toward the identification of possible invasion mechanisms showed that the uptake of *Y. ruckeri* involved the remodelling of cytoskeletal components. The ability to invade cultured cell lines was reduced for almost all strains after colchicine and for many strains after cytochalasin-D treatment. Thus, microtubules and microfilaments seemed to play a role in *in vitro* invasion, but the internalization mechanisms triggered by *Y. ruckeri* may be cell type and strain dependent, since no clear relation with the virulence was found. Surprisingly, cytochalasin-D and monodansylcadaverine stimulated the uptake of *Y. ruckeri* 17.00(2-1) into FHM cells. Enhanced invasion caused by cytochalasin-D has been reported a few times for other bacteria in different cell types (Wells *et al.* 1998; Kawamura *et al.* 1998; Van Deun *et al.* 2008). It has been suggested that microfilament disruption resulted in increased exposure of a submembrane fraction, being the preferred site of entry for some bacterial strains.

The intracellular survival assays in the three cell lines showed a decrease in the number of viable bacteria at 6 h in comparison with 1 h after infection. García *et al.* (2007) demonstrated a drop in viable *Y. ruckeri* bacteria 4 and 24 h after inoculation of epithelial papulosum cyprini carp (EPC) cells. These findings suggest that *Y. ruckeri* is not able to multiply or even survive inside cultured cells. *Y. ruckeri* was previously found to survive in the internal organs of rainbow trout up to 4 weeks after immersion (Tobback *et al.* 2009). At histology, most bacteria were found extracellularly, indicating that intracellular survival is of minor importance in the pathogenesis of *Y. ruckeri*. In liver, however, bacteria were also found intracytoplasmic (Tobback *et al.* 2009). It would be interesting to investigate if this was due to phagocytosis or to specific invasion and survival in monocytes.

Analysis of susceptibility to the bactericidal effect of non-immune rainbow trout serum demonstrated that three *Y. ruckeri* isolates were serum resistant including strains 5 and 9 which were previously determined to be virulent for rainbow trout (Tobback *et al.* 2009). Remarkably, all the serum sensitive strains were previously found to be avirulent. These findings are in agreement with Davies (1991), who also suggested that serum resistance plays a role in the pathogenesis of *Y. ruckeri* infections and probably, this trait is important in the extracellular survival of the pathogen in the host.

In conclusion, both virulent *Y. ruckeri* strains highly adhered to gill and intestinal mucus and were serum resistant, whereas the avirulent strains showed, with some exceptions, significantly lower adhesion to mucus and were serum sensitive. Therefore, adherence to rainbow trout mucus and resistance to the bactericidal effect of serum seems to be correlated with *in vivo* virulence.

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Experimental study 3

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GENERAL DISCUSSION

Identification of the portal of entry of *Y. ruckeri* in rainbow trout

Information on the portal of entry is important in bacterial pathogenesis in order to develop new therapeutic and prophylactic strategies. Despite the importance of ERM and the need to innovative approaches, the initial site of infection has previously not been studied for *Y. ruckeri*. In this research, both an *in vivo* immersion infection model as well as *in vitro* perfusion models were used to reveal the early interactions between *Y. ruckeri* and rainbow trout.

The immersion infection model, also called contact exposure or bath challenge, strongly mimics the natural way of infection compared to the relatively artificial intraperitoneal or intramuscular injection of bacterial cells into the host. These last methods are not sufficient to examine the route of infection since they bypass natural defence mechanisms of the mucus, skin, gills and gut of the fish. Therefore, bath immersion as experimental infection model was preferred in this research. In both virulence studies, only *Y. ruckeri* 5 and 9 caused mortality and clinical signs in rainbow trout and interestingly, both strains were originally isolated from diseased rainbow trout. This indeed indicates that our model is suitable to study infections mimicking the natural route of infection. Two strains, *Y. ruckeri* 1 and CCUG 14190 which were also isolated from diseased rainbow trout, did not cause disease in our experiments. Different factors may influence the outcome of *Y. ruckeri* infections in the field including concurrent infections with other pathogens, the water quality and the temperature.

In vitro studies were performed using a gill and gut perfusion model. These models were previously shown to be reliable to study the early interactions between a pathogen and its host since they closely resemble *in vivo* conditions (Decostere *et al.* 2002; Nematollahi *et al.* 2005). The various components and the global structure of the tissue are maintained in contrast to *in vitro* primary cultures and cell lines. For example, in both perfusion models, the mucus layer overlying the epithelium remains intact, whereas this is absent in cell cultures. Adhesion to and invasion in this mucus layer has been found to be a crucial initial step of infection for different fish pathogens (Larsen *et al.* 2001; Chen *et al.* 2008). The preservation of the tissue structure is indeed a main advantage of the perfusion model in order to extrapolate the results to the *in vivo* situation.

A primary step in the pathogenesis of *Y. ruckeri* disease was demonstrated to be the colonization of the gills. Using the immersion infection model, bacteriological and histological examination revealed the presence of numerous bacteria in the gill mucus

immediately after infection. Adhesion to the gill mucus seemed to give subsequent invasion of the underlying tissue, since *Y. ruckeri* was detected in the branchial capillaries soon after bath infection. Similar results were obtained using the gill perfusion model. Adhesion of *Y. ruckeri* to the gill mucus and epithelium was observed and invasion in the gill tissue was demonstrated after isolation of bacteria from the perfusion fluid at the efferent gill artery. It is not surprising that the gills are an important site of infection for septicaemic pathogens such as *Y. ruckeri* since the gill tissue has a large surface area and a minimal diffusion distance to the capillaries. Indeed, other septicaemic fish pathogens including *Vibrio anguillarum* and *Edwardsiella tarda* have been shown to infect their host via the gills (Baudin-Laurencin & Germon 1987; Ling *et al.* 2001).

The gut is also in constant contact with the external medium and therefore, should be considered as a portal of entry in microbial pathogenesis. Indeed, several investigators, performing immersion infection studies with other fish pathogens, clearly isolated high numbers of bacteria from the intestine immediately after inoculation (Spanggaard *et al.* 2000; Ling *et al.* 2001). Using the immersion infection model, moderate numbers of *Y. ruckeri* were recovered immediately after bath challenge compared to the gills. Nevertheless, bacterial cells were identified within the mucosa and the crypts immediately after infection, indicating invasion in the gut tissue. This hypothesis was confirmed using the gut perfusion model which revealed *Y. ruckeri* attached to the villi and invaded into the deeper tissue layers. Invasion was also demonstrated after isolation of bacteria in the surrounding organ bath fluid, revealing the potential importance of the gut in the initial contact with *Y. ruckeri*. In general, bacteria may be ingested during drinking, making the intestine more vulnerable and accessible to pathogen attack. Indeed, although the gills were shown to serve as an important route of infection for *V. anguillarum*, the pathogen was also detected in the anterior and posterior intestine shortly after bath challenge (Baudin-Laurencin & Germon 1987). Oral and rectal administration of *V. anguillarum* confirmed the spread of this pathogen to the internal organs, indicating the importance of the gut as portal of entry (Olsson *et al.* 1996).

Showing that both the gills and the gut may be important routes for entry, the question raises if *Y. ruckeri* shows a preference in initial tissue colonization. Using the immersion model, a lower number of *Y. ruckeri* was isolated from the gut compared to the gills immediately after infection. This may be due to a different exposure to bacterial cells. Whereas water flows continuously over the gill tissue, the gut may have less contact with the surrounding water

since freshwater fish are reported to drink less than saltwater fish (Bucking & Wood 2006). Freshwater fish are hyperosmotic to their environment and constantly take up water through the gills and to a minor extent through the skin by osmosis. These fish developed different strategies to maintain the internal ion concentration including drinking less water. Comparing the results of the gill and gut perfusion models via statistical analysis, significant differences were however not observed in adhesion and invasion capacity of any of the *Y. ruckeri* strains between both tissues. This indicates that the gills and the gut may be of equal importance in the initial contact of *Y. ruckeri* with its host.

Moderate numbers of *Y. ruckeri* were also isolated from the rainbow trout skin immediately after bath challenge, however, bacterial cells were not observed by histological or immunohistochemical examination. Different factors may influence the capacity of bacteria to adhere to host tissues including the temperature, the ion composition of the water and the presence of nitrite or organic matter (Decostere *et al.* 1999a). Therefore, these factors should be taken into account when interpreting results and drawing conclusions.

Using the immersion infection model, the skin could not be excluded as possible site of infection and further studies are needed to reveal if *Y. ruckeri* is able to colonize and invade this tissue. Other models have been described in the literature for these purposes. Patch contact revealed the importance of the skin in the early interaction between *Piscirickettsia salmonis* and juvenile rainbow trout. Mortalities occurred and skin lesions including haemorrhages in the underlying tissues were observed at the site of contact with paper patches, soaked in a bacterial suspension (Smith *et al.* 1999). Similar results were observed in coho salmon, *Oncorhynchus kisutch* (Walbaum), after exposing a skin region to a calibrated bacterial drop (Smith *et al.* 2004). *In vitro* models to further investigate the role of the skin in *Y. ruckeri* infections may be the use of rainbow trout skin mucus, primary cultures or cell lines. Skin explants more closely resemble the *in vivo* condition and have been developed for rainbow trout (Nolan *et al.* 2002). The trout skin explant system was found to actively grow during 8 days evaluation and the levels of proliferation and apoptosis were similar to those reported for fish epithelia *in vivo* (Nolan *et al.* 2002). Therefore, this system may be a promising model to further investigate the interaction with *Y. ruckeri*.

Reddening at the base of the fins is often seen in ERM affected fish and therefore, the fins may also be a possible port of entry. Wounds have been reported to facilitate the entrance of a pathogen to host tissues (Svendsen *et al.* 1999) but these were macroscopically not observed

in our fish. Nevertheless, it would be interesting to examine skin damage as an additional site of infection.

Adherence mechanisms of *Y. ruckeri*

Adhesion of bacterial pathogens to host surfaces is widely recognized as an important step in the initiation of infectious diseases. The process is characterized by the attraction of the pathogen to the mucosal surface and subsequent association within the mucus gel or attachment to the underlying epithelial cells.

Using immersion infection and perfusion models, *Y. ruckeri* was histologically demonstrated in the gill and intestinal mucus of rainbow trout. *In vitro* adherence assays confirmed the efficient adhesion of this pathogen to both types of mucus. However, bacterial adherence to the mucus layer should be interpreted carefully since mucus is continuously sloughed off and replaced to remove trapped bacteria. Mucus also contains many compounds of the innate immune system such as lysozyme, immunoglobulins, complement and proteolytic enzymes (Ellis 2001). The mucus layer is the first line of defence against micro-organisms but conversely, the tendency of bacteria to associate with the mucus and facilitate the colonization of the underlying tissues has been widely reported (Larsen *et al.* 2001; Chen *et al.* 2008; van der Marel *et al.* 2008).

The adherence of bacteria to external surfaces generally occurs by the binding of microbial adhesins to complementary host structures. Performing adherence assays to rainbow trout mucus, this binding was shown to involve carbohydrate-binding proteins or lectins on the *Y. ruckeri* cell. Indeed, all our strains appeared to bind to galactose- and fructose-containing receptors in the gill and gut mucus. Mucus mainly consists of water and mucins composed of a core peptide backbone substituted with a range of O-linked oligosaccharides (Bavington & Page 2005). Carbohydrates are the abundant molecules present in mucus, however, limited information is available on the precise composition. Galactose has been identified as a major monosaccharide present in rainbow trout gill and intestinal mucus and therefore, is probably part of the *Y. ruckeri* adhesin-receptor complex (Lumsden & Ferguson 1994; O'Toole *et al.* 1999). Although fructose has hitherto not been identified as a main component of fish mucus, it has also been found to inhibit adherence of *Vibrio alginolyticus* to skin, gill and intestinal mucus of large yellow croaker *Pseudosciaena crocea* (Chen *et al.* 2008).

Y. ruckeri was recently shown to moderately adhere to different glycoproteins in carp intestinal mucus (Schroers *et al.* 2008). This was examined by allowing fluorescently labelled bacteria to attach to mucus fractions containing molecules with different molecular weight. Although a direct comparison with our findings obtained from rainbow trout intestinal mucus can not be made, the adhesion of *Y. ruckeri* to both types of mucus may differ since the mucus composition has been observed to vary between fish species (Roberts & Powell 2005). The saccharide profile of carp mucus as well as its carbohydrate components to which *Y. ruckeri* adheres has not been studied well, but bacterial adhesion may be higher to mucus derived from its preferable host.

Other variables including the environmental salinity, the age and the health status of fish have been reported to influence the mucus composition (Roberts & Powell 2005). Therefore, differences in sugar ratios caused by these variables may also influence the susceptibility of differently aged fish to ERM.

For successful colonization, a pathogen has to establish itself and move through the mucus gel to the epithelium. To cope with the continuous renewal, it should be advantageous for pathogenic bacteria to replicate and multiply in the host mucus. Indeed, different fish pathogens have been demonstrated to use mucus as a nutrient source for their growth (Larsen *et al.* 2001; van der Marel *et al.* 2008). This has hitherto not been studied for *Y. ruckeri* and it would be interesting to monitor bacterial growth in medium without or supplemented with rainbow trout mucus.

Flagel-mediated motility and chemotaxis can enable fish pathogens to penetrate the mucus layer (Larsen *et al.* 2001; Chen *et al.* 2008). Some bacteria also produce proteolytic enzymes or toxins to facilitate their movement through the mucus by degrading mucins and subsequently reach the epithelial cells (Valiente *et al.* 2008). Mucus may also play an active role in altering the expression of bacterial genes involved in the penetration of the mucus gel layer. For example, biofilm formation and extracellular protease production in virulent *Flavobacterium columnare* was promoted by Atlantic salmon skin mucus (Staroscik & Nelson 2008). How *Y. ruckeri* moves through this protective layer is not known and it is clear that much more research is needed. Taking these different mechanisms into account, it would be interesting to investigate if the presence of flagella, biofilm-forming capacities and protease production enables this pathogen to move through the mucus to reach the underlying epithelium and if this is associated with virulence.

General discussion

Using immersion infection and perfusion models, histological analysis showed *Y. ruckeri* bacteria closely associated with the gill and gut epithelium. *In vitro* adherence assays to cultured fish cell lines also demonstrated the capacity of *Y. ruckeri* to adhere to epithelial cells. Adherence of *Y. ruckeri* 17.00(2-1) to CHSE-214 was inhibited after pre-treatment of the bacteria with the monosaccharides D-galactose and D-glucose and the disaccharides D-maltose and D-sucrose, both containing D-glucose. Although the receptors on epithelial cells have been scarcely studied, D-galactose and D-glucose have been shown to inhibit adhesion of other fish pathogens to cells (Wang & Leung 2000). This suggests that both monosaccharides may be part of fish cell receptors, being important in the adherence of pathogenic bacteria. Cell receptor specificity in *Y. ruckeri* adherence should be further studied including a virulent strain.

Our *in vitro* adherence assay to cultured cell lines showed that *Y. ruckeri* adhesion seemed not to be correlated with *in vivo* virulence. The same discrepancy was also observed in adhesion to erythrocytes of virulent and avirulent *Flavobacterium psychrophilum* strains (Møller *et al.* 2003). The authors believed that strains with a lower adhesion capacity showed a decreased expression of adhesive factors and were more resistant to complement-mediated bacteriolysis. Lectins involved in adherence to host surfaces have often been shown to be associated with surface appendages such as fimbriae and flagella (Proft & Baker 2009). Flagella seemed not to be an absolute requirement and may play a moderate role in the initial contact of *Y. ruckeri* with his host. The ability of a constructed *Y. ruckeri* mutant in the *flaA* flagellin gene and its parental strain to establish infection in rainbow trout was investigated by bath challenge (Kim 2000). Flagella provided a slight advantage to *Y. ruckeri* at early stages of infection as measured by bacterial loads in the gills, liver, kidney and spleen. However, the numbers of the parental strain isolated from these organs were no longer significantly higher than these of the non-flagellar mutant strain, six days after infection. The authors hypothesized that *Y. ruckeri* flagella may be opsonized by host antibodies inside the fish and therefore, flagellated strains may be more sensitive to complement-mediated bacteriolysis (Kim 2000). This may explain why adhesion to cell lines was not correlated with virulence in our study. *Y. ruckeri* 17.00(2-1) adhered to all tested cells to a higher extent compared to the other isolates, but did not cause disease *in vivo*. This strain possibly expresses more adhesins giving a higher initial colonization of the host, but leading to a higher attack of the host immune system.

Nevertheless, the loss of fimbriae or flagellae after invasion in tissues where specific attachment is no longer important has been described. This is probably a bacterial adaptation

to the described detrimental effect to survival caused by the presence of fimbriae or flagellae (Guzmán & Pruzzo 1992).

Adhesion to gill and intestinal mucus as well as to cultured fish cells was inhibited by more than one monosaccharide, suggesting that *Y. ruckeri* possesses more than one lectin involved in the adhesion to host tissues. The co-expression of several adhesins has been described for other pathogens and is thought to be an adaptation for efficient colonization of different surfaces and tissues (Ofek *et al.* 2003). Bacteria must be able to interact with different receptors present on distinct surfaces in order to maintain themselves in different environments (Ofek *et al.* 2003). A combination of sugars could possibly more effectively inhibit *Y. ruckeri* adhesion compared to individual saccharides. This would be interesting to study in the future with regard to the development of innovative therapeutic treatments. The applied gill and gut perfusions could be useful to perform these studies since these models come close to the *in vivo* situation.

Invasion mechanisms of *Y. ruckeri*

Our *in vivo* and *in vitro* studies clearly indicate that virulent as well as non-virulent *Y. ruckeri* strains show invasive capacities. Immediate activation of cytoskeletal responses seems generally to occur during the entry of invasive pathogens. Using chemical blockers, the importance of actin filaments and microtubules during *Y. ruckeri* invasion was demonstrated in cultured fish cells. However, it seems that the mechanism by which *Y. ruckeri* gains entrance inside the host cell could be cell type and strain dependent, rather than universal. Moreover, different factors may influence the invasion of *Y. ruckeri*.

An invasin-homologue has previously been suggested to play a role in *Y. ruckeri* invasion (Fernández *et al.* 2007a). Invasin is an important protein for efficient entry of *Y. enterocolitica* and *Y. pseudotuberculosis* into the host. Fernández *et al.* (2007a) revealed evidence for an *inv* homologue in *Y. ruckeri*, however, Kawula *et al.* (1996) did not find a similar gene. Looking towards the mechanism by which invasin mediates invasion of other *Yersinia* species, cytoskeletal rearrangements as well as receptor-mediated endocytosis play a role. These steps are inhibited by cytochalasin D and monodansylcadaverine (Young *et al.* 1992). High affinity binding of invasin to β1 integrin receptors leads to the clustering of integrins in the host cell. Focal adhesion kinase (FAK) binds to the clustered cytoplasmic domains of integrin, inducing

signal transduction and protein phosphorylation. This leads to cytoskelet rearrangement including the dissociation of actin filaments from the integrin receptors and subsequent accumulation around entering bacteria. Then, the cytoplasmic domain of the integrin receptors interacts with binding factors associated with receptor-mediated endocytosis including clathrin and the bacterium becomes internalized within an endocytic vacuole (Isberg & Tran Van Nhieu 1995).
In our study, monodansylcadaverine never inhibited *Y. ruckeri* invasion in different fish cells and therefore, evidence for receptor-mediated endocytosis via clathrin-coated pits was not provided.

Besides receptor-mediated endocytosis, the type III secretion-dependent ruffling of host cell membranes is another major invasion mechanism involving cytoskeletal rearrangements that has been described for pathogenic bacteria (Meyer *et al.* 1997; Ofek *et al.* 2003). These bacteria use a TTSS to inject effector proteins into the host cell that interact with the actin cytoskeleton. Massive polymerized actin accumulates in the area where the bacterium interacts with the host and projects membrane ruffles outward, enabling bacterial internalization. This invasion mechanism has been exemplified by members of the genera *Salmonella* and *Shigella* and is inhibited by cytochalasin D but not by dansylcadaverine (Meyer *et al.* 1997).
Y. ruckeri also possesses a TTSS, however, its role has hitherto not been revealed (Gunasena *et al.* 2003). In our invasion inhibition study in cultured fish cells, cytochalasin D inhibited the invasion of most strains whereas monodansylcadaverine did not. Therefore, the TTSS identified in *Y. ruckeri* may also be involved in an actin-dependent invasion mechanism in non-phagocytic cells.

The toxins Yrp1 and YhlA have been thought to be involved in *Y. ruckeri* invasion of different tissues, however, their precise role in the internalization process is not known (Fernández *et al.* 2003; Fernández *et al.* 2007b). Possibly, the cytolytic activity of YhlA may enable *Y. ruckeri* to penetrate tissue layers and invade the host in a similar way as has been indicated for the homologue *Serratia marcescens* hemolysin ShlA (Fernández *et al.* 2007b). However, the precise mechanisms by which *S. marcescens* invades host cells are not known and only limited information is available about ShlA activity. Comparing a ShlA-negative isogenic mutant with a wild type strain revealed that ShlA was not involved in adhesion of *S. marcescens* to cultured cells, but played a crucial role in cell invasion and cytotoxicity.

Monodansylcadaverine did not inhibit *S. marcescens* invasion, whereas cytochalasin D and colchicine clearly showed inhibitory capacities. Accordingly, ShlA triggers microfilament- and microtubule-dependent invasion of epithelial cells and subsequent lysis seems to be important to release the bacteria (Hertle & Schwarz 2004).
Because our results suggest that *Y. ruckeri* invasion is dependent on actin filaments and microtubules, it would be interesting to investigate if YhlA plays a similar role in the internalization process using site-directed mutagenesis.

The importance of intracellular and extracellular *Y. ruckeri*

Both an intracellular and an extracellular phase may be important for the survival of a pathogen in the host. Bacteria internalized by non-phagocytic cells are well protected from the host's immune system and therefore, an intracellular phase may contribute to the progression of a disease.

Our results of the intracellular survival assays in three fish cell lines showed a decrease in the number of viable bacteria at 6 h in comparison with 1 h after infection. These findings may suggest that *Y. ruckeri* is not able to multiply or even survive inside cultured epithelial cells.
Another hypothesis is that *Y. ruckeri* remains intracellularly for only a short period. Thereafter, bacteria may leave the host cells by exocytosis or cytolysis as has been shown for *S. marcescens*. Its hemolysin ShlA, counterpart of YhlA in *Y. ruckeri*, mediates invasion in cell lines and causes the release of bacteria after cell lysis (Hertle & Schwarz 2004). Taking a similar mechanism of YhlA into account, a decrease in the number of viable *Y. ruckeri* protected against gentamicin in intracellular survival assays would be attributed to the lysis of host cells and the subsequent exposure of bacteria to gentamicin. Indeed, gentamicin is lethal to bacteria in damaged and disintegrating cells. This hypothesis should be investigated by replacing the cell medium containing 100 µg ml^{-1} gentamicin with fresh medium without antibiotics in our assay after 1 h incubation time. By titrating the collected supernatant for bacterial quantification, the release of bacteria can be studied as has been done for other pathogens (Hertle & Schwarz 2004; Van Deun *et al.* 2008).

The bactericidal effect of non-immune serum plays an important role in host defence against extracellular bacteria. We found a correlation between virulence and serum resistance using non-immune rainbow trout serum. Both virulent *Y. ruckeri* strains were serum resistant,

whereas the avirulent strains were, with one exception, serum sensitive. The same correlation has been found for other fish pathogens such as *Aeromonas hydrophila* (Leung *et al.* 1995) and *E. tarda* (Han *et al*. 2006). Indeed, several bacteria are capable of interfering with the complement system that consists of a number of proteins found in the serum. Some pathogens have developed mechanisms to accelerate the decay of the C3 convertase enzyme, whereas others bind host complement regulators onto their own cell surfaces (Han *et al.* 2006). Serum resistance can allow bacteria to survive within vascular channels leading to organ dissemination. Nevertheless, how *Y. ruckeri* resists the bactericidal properties of rainbow trout serum is not known. Interestingly, Furones *et al.* (1990) revealed the presence of a heat sensitive factor (HSF) in virulent serotype O1a strains that could be involved in the resistance of *Y. ruckeri* to the bactericidal activity of serum or to phagocytic killing. Still, more research is needed to reveal the nature and activity of this factor.

New insights in the prevention and control of *Y. ruckeri* infections

Attachment of a pathogen to host tissues is considered to be the first step in microbial colonization and pathogenesis. This is often mediated by bacterial lectins which target specific carbohydrate structures on host surfaces. Our studies revealed the interaction of *Y. ruckeri* adhesins with sugars and therefore, the development of carbohydrate anti-adhesives and the design of adhesin vaccines may contribute to its control and prevention.

Carbohydrate anti-adhesives

The gills and the gut of rainbow trout appeared to be important sites of entry for *Y. ruckeri*. Because its adhesion to gill and intestinal mucus was inhibited by D-galactose and D-fructose, it may be possible to prevent or control ERM by adding these sugars to the water of the fish. Indeed, carbohydrates have been shown to inhibit adhesion of fish pathogens to host surfaces *in vitro* (Decostere *et al.* 1999b; Chen *et al.* 2008) and to protect different mammals against experimental infection by lectin-carrying bacteria (Sharon 2006). Although the development of carbohydrate anti-adhesives may be a promising tool in the therapeutic and prophylactic control of bacterial diseases, much more research is needed to support this idea.

A big problem in the use of carbohydrates in fisheries is that the addition of large amounts of sugars to the water may induce massive growth of different aquatic organisms, causing a decrease in the water quality. Another issue is that in the case of *Y. ruckeri* the anti-adhesives should continuously and excessive be present on the gill and the gut surface in order to

compete with the host receptors. This may be difficult to achieve because the mucus layer is continuously renewed. Considering the use of these anti-adhesives in practice, it would be interesting to focus on the development of multifunctional receptor analogues which block more than one bacterial binding ligand with high affinity.

Adhesin vaccines

Commercial vaccines against ERM are based on killed whole cells of *Y. ruckeri*, however, outbreaks occur from time to time. Therefore, there is an interest for new approaches such as the development of subunit vaccines. This requires the identification of bacterial components involved in different aspects of virulence including the adhesion. Anti-adhesive vaccines prepared from bacterial adhesins have already been shown to give protection against experimental infection in mammals (Cachia & Hodges 2003). Lectins located on the surface of *Y. ruckeri* may be considered candidate antigens for the development of a subunit vaccine. Taking the portal of entry in rainbow trout into account, the gills and the gut may be the target sites for new *Y. ruckeri* vaccine approaches. Bath immunization or oral administration may be the route of choice in order to stimulate the immune responses at the level of the gill and gut surfaces. Although potential advantages of subunit vaccines include increased safety and low antigenic competition, it is often laborious to purify adhesins causing an increase in the price of such vaccines (Babiuk 1999).

Conclusions

In conclusion, this thesis offers new insights in the early pathogenesis of *Y. ruckeri* infections in rainbow trout (Figure 1). The gill and the gut were identified as possible important portals of entry. Adherence of this pathogen seems to occur by the binding of microbial adhesins to complementary structures on these tissues. Subsequent invasion of the gills and/or the gut lead to a rapid spread of *Y. ruckeri* in the internal organs. Virulence of *Y. ruckeri* appears to be a complex combination of colonization capacity and resistance to killing by the host. Further research is needed to identify the bacterial virulence factors involved in these processes.

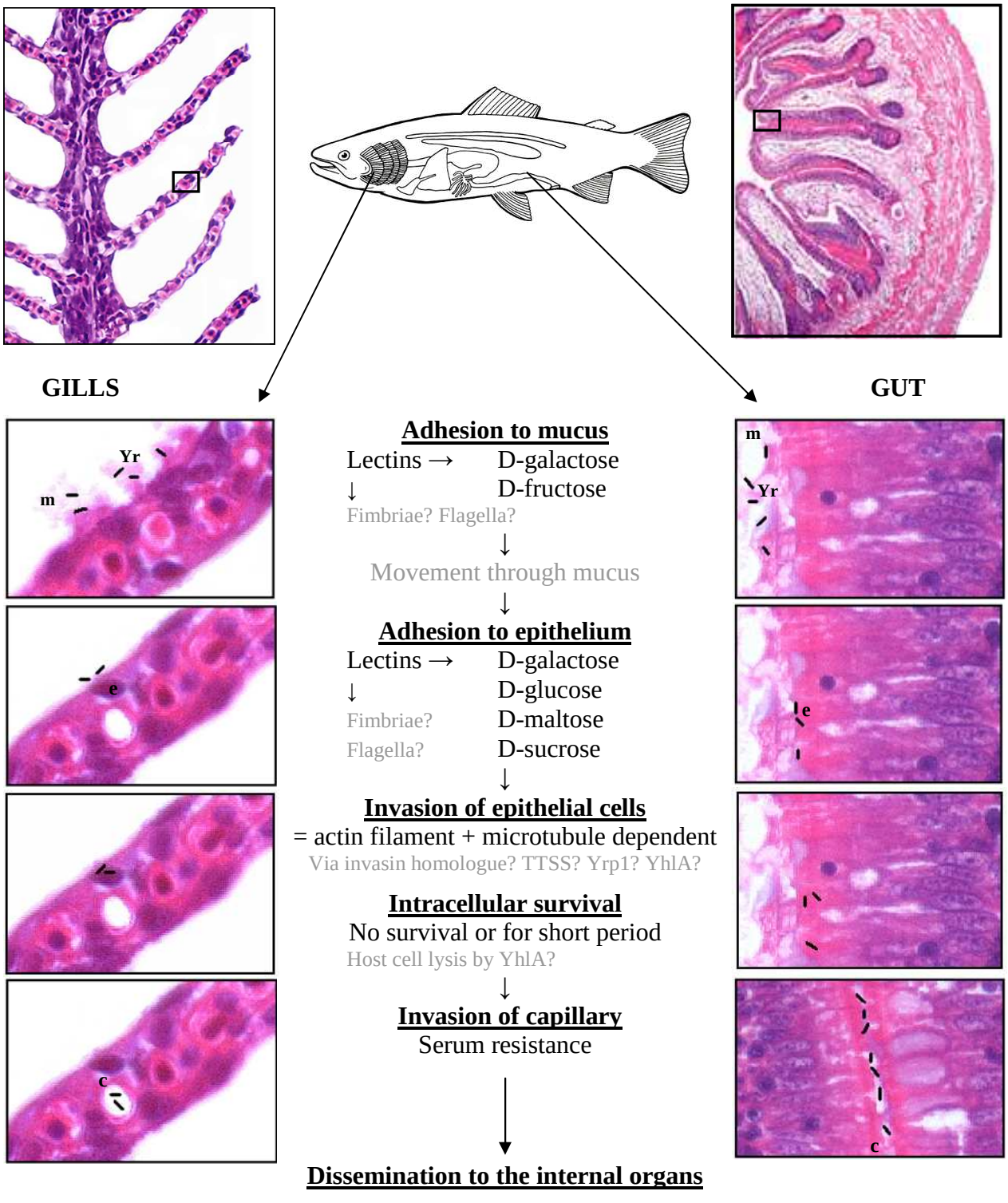

Figure 1 Proposed early pathogenesis of *Y. ruckeri* infections in rainbow trout based on our results: interactions of *Y. ruckeri* (Yr) with the mucus (m), the epithelial cells (e) and the capillaries (c) of the gills and the gut. The pathogenic steps represented in black were identified in the present thesis. The steps in grey still need to be further investigated.

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SUMMARY

Summary

Yersinia ruckeri is the causative agent of yersiniosis or enteric redmouth disease (ERM) and causes significant losses in salmonid aquaculture worldwide. Although infection with this agent has been reported in other fish species, salmonids and especially rainbow trout are most susceptible to ERM. Vaccination of fish with a commercial *Y. ruckeri* bacterin provides good levels of protection, however, disease outbreaks do occur from time to time. The understanding of how this pathogen causes disease is crucial in order to develop new preventive and therapeutic approaches. The aim of this thesis was to study the early pathogenesis of *Y. ruckeri* infections, with emphasis on the identification of the portal of entry and the investigation of different traits *in vitro* that have been associated with bacterial virulence.

The General Introduction gives an overview of the micro-organism, *Y. ruckeri*, and the disease it causes. From this review it is clear that only little information is available about the pathogenesis of *Y. ruckeri*. Only recently, the mechanisms by which the pathogen causes disease have started to be unravelled.

In the first experimental study, the portal of entry of *Y. ruckeri* and its tissue distribution at different time intervals were investigated using an immersion infection model in rainbow trout. This model strongly mimics the natural way of infection and therefore, is suitable to study infectious diseases in fish. Additional experimental infections were carried out to determine whether a different tissue distribution is related to differences in virulence. Bacteriological and histological examination revealed the presence of high numbers of bacteria in the gills immediately after infection. Many *Y. ruckeri* were found to be attached to the gill mucus and some were detected within the gill capillaries between 0 and 2.5 h post infection (p.i.). This indicates that bacteria first adhere to gill mucus and thereafter invade the branchial vasculature leading to colonization of the internal organs. These findings show that the gills are an important portal of entry. Nevertheless, only a virulent strain was able to survive and multiply in the host, causing septicaemia and death several days after infection whereas exposure to avirulent strains resulted in a complete removal of bacteria from the internal organs. This could indicate that immune evasion is a major virulence property of *Y. ruckeri*.

In the second experimental study, the early interactions of virulent and avirulent *Y. ruckeri* strains with the gill and gut tissue of rainbow trout were determined using a standardized gill

and gut perfusion model. For this purpose, perfused gill and gut explants were inoculated for 1 h with *Y. ruckeri* and thereafter, processed for bacteriological, histological and immunohistochemical examination for demonstration of bacterial adherence. For the first time, both perfusion models were also used to study bacterial invasion. Therefore, the perfusion fluid at the efferent gill arch during gill perfusion and the organ bath fluid following gut perfusion were examined bacteriologically. A non-invasive *Escherichia coli* strain was included as negative control for invasion. In both the gill and gut perfusion models, virulent and avirulent *Y. ruckeri* strains highly adhered to gill and gut tissue and significant differences were not observed. Bacteria were seen in the mucus, associated with the epithelium and invaded in the lamina propria of the gills. *Y. ruckeri* was also observed in the crypts, attached to the villi and within the epithelial layer of the gut. Few bacteria were also detected in the intestinal lamina propria mucosae. Our results showed that virulent and avirulent *Y. ruckeri* invaded the gill and gut whereas a non-invasive *E. coli* strain was not isolated from the perfusion fluid and organ bath fluid in the gill and gut perfusion model, respectively. These findings indicate that *Y. ruckeri* is able to colonize and invade the gills and intestine of rainbow trout. Significant differences in adherence and invasive capacity were not observed between virulent and avirulent strains.

In the third experimental study, different traits that have been associated with bacterial virulence were compared between virulent and avirulent *Y. ruckeri* strains, including adhesion to mucus and cell lines, invasion and intracellular survival in cell lines and serum resistance. The adhesion and invasion mechanisms were further characterized for selected *Y. ruckeri* strains. The virulent isolates highly adhered to isolated gill and gut mucus of rainbow trout, whereas the majority of the avirulent strains demonstrated significantly lower adhesion. A decrease in adherence capability following bacterial treatment with sodium metaperiodate and proteolytic enzymes suggested that carbohydrates as well as proteins play a role and consequently, lectins may be involved in the adhesion. All *Y. ruckeri* strains were able to adhere to and invade CHSE-214, FHM and R1 cells. One avirulent strain showed high adherence and invasive capacity in the three cell lines, whereas the virulent strains were moderately adhesive and invasive. The internalization of several isolates was inhibited by colchicine and cytochalasin-D, suggesting the involvement of microtubules and microfilaments. Monodansylcadaverine never inhibited *Y. ruckeri* internalization and therefore, the formation of clathrin-coated pits in receptor-mediated endocytosis may not play a role. For all strains, intracellular survival assays showed a decrease of viable bacteria in the

cells 6 h after inoculation. This suggests that *Y. ruckeri* is not able to multiply or survive inside cultured cells or remains intracellularly for only a short period. Analysis of the susceptibility to the bactericidal effect of rainbow trout serum demonstrated that virulent strains were serum resistant, whereas avirulent strains were generally serum sensitive. Taken together, adherence to mucus and resistance to the bactericidal effect of serum seems to be correlated with *in vivo* virulence.

In the General Discussion, the results of the three experimental studies are discussed and hypothetical virulence mechanisms are proposed in relation to the known virulence factors of *Y. ruckeri*. Finally, perspectives are given on how these findings may contribute to the prevention of ERM.

In conclusion, the gills and gut were identified as possible important portals of entry for *Y. ruckeri* in rainbow trout. This pathogen is shown to be able to adhere to and invade in non-phagocytic cells. The adherence of the pathogen seems to involve bacterial lectins that bind specific carbohydrate structures on host surfaces. Invasion appears to be a microtubule and microfilament dependent process. Virulence of *Y. ruckeri* isolates is probably a complex combination of adhesion and colonization capacity, resistance to the bactericidal effect of serum and other factors which still needs to be determined.

SAMENVATTING

Yersinia ruckeri verzoorzaakt yersiniosis of ‘enteric redmouth disease’ (ERM) wat leidt tot enorme verliezen binnen de aquacultuur van de Salmonidae op wereldschaal. Hoewel infectie door dit agens beschreven is bij andere vissoorten, zijn zalmachtigen en in het bijzonder de regenboogforel meest gevoelig aan ERM. Hoewel de vaccinatie van vissen met een commercieel *Y. ruckeri* bacterin een goede bescherming geeft, komen ziekteuitbraken soms nog voor. Het is van cruciaal belang te weten hoe deze pathogeen ziekte veroorzaakt om nieuwe preventieve en therapeutische maatregelen te ontwikkelen. De algemene doelstelling van deze thesis was de vroege pathogenese van *Y. ruckeri* infecties te bestuderen met nadruk op de identificatie van de toegangspoort en *in vitro* onderzoek naar mogelijke eigenschappen die geassocieerd worden met bacteriële virulentie.

In de algemene inleiding wordt een literatuuroverzicht gegeven van het agens en de ziekte. Hierin wordt duidelijk dat slechts beperkte informatie beschikbaar is over de pathogenese van *Y. ruckeri*. Het is pas recent dat pathogenese-mechanismen van deze belangrijke kiem worden ontrafeld.

In de eerste experimentele studie werd de toegangspoort van *Y. ruckeri* en zijn verspreiding in de weefsels op verschillende tijdsintervallen onderzocht door middel van een immersie infectie model in regenboogforellen. Dit model bootst de natuurlijke manier van infectie na waardoor het zich verleent voor de studie van infectieuze ziekten bij vissen. Bijkomende experimentele infecties werden uitgevoerd om na te gaan of verschillen in weefseldistributie gerelateerd zijn met verschillen in virulentie. Bij bacteriologisch en histologisch onderzoek werd meteen na infectie een hoog aantal bacteriën teruggevonden in de kieuwen. *Y. ruckeri* was vastgehecht aan de kieuwmucus en enkele kiemen waren aanwezig in de capillairen van de kieuwen tussen 0 en 2.5 uur na infectie. Dit wijst erop dat deze bacteriën vermoedelijk eerst adhereren aan kieuwmucus, vervolgens het vasculair systeem invaderen en uiteindelijk de interne organen koloniseren en dat de kieuwen een belangrijke toegangspoort kunnen zijn. Toch was enkel een virulente *Y. ruckeri* stam in staat om te overleven en te vermenigvuldigen in de gastheer, met septicaemie en de dood tot gevolg. Na infectie met avirulente stammen werden de bacteriën na enkele dagen niet meer teruggevonden in de interne organen wat erop wijst dat omzeiling van het immuunsysteem een belangrijk virulentiekenmerk blijkt te van *Y. ruckeri*.

Samenvatting

In de tweede experimentele studie werden de vroegtijdige interacties van virulente en avirulente *Y. ruckeri* stammen met kieuw- en darmweefsel van de regenboogforel bestudeerd door middel van een gestandaardiseerd kieuw- en darmperfusiemodel. In deze modellen werden geperfuseerde kieuw- en darmexplants gedurende één uur geïnoculeerd met *Y. ruckeri* en vervolgens werd de adhesie bacteriologisch, histologisch en immunohistochemisch bestudeerd. Voor de eerste keer werden deze perfusiemodellen gebruikt om bacteriële invasie te bestuderen. In het kieuwperfusiemodel werd de perfusievloeistof aan de efferente kieuwboog opgevangen tijdens perfusie en bacteriologisch getest op aanwezigheid van *Y. ruckeri*. In het darmperfusiemodel werd de vloeistof van het orgaanbad na perfusie getest. Een niet-invasieve *Escherichia coli* stam werd ingesloten als negatieve controle voor invasie. In beide modellen vertoonden zowel virulente als avirulente *Y. ruckeri* stammen een sterke adhesie aan kieuw- en darmweefsel zonder onderlinge significante verschillen. Bacteriën werden waargenomen in de mucus, gehecht aan het epithelium en geïnvadeerd in de lamina propria van het kieuwweefsel. In de darm werd *Y. ruckeri* geobserveerd in de crypten, geadhereerd aan de villi en in de epitheellaag. Enkele kiemen waren ook zichtbaar in de intestinale lamina propria mucosae. Virulente en avirulente *Y. ruckeri* stammen invadeerden de kieuwen en de darm terwijl de niet-invasieve *E. coli* stam niet geïsoleerd werd uit de perfusievloeistof van de kieuw en het orgaanbad in het darmperfusiemodel. Deze bevindingen tonen aan dat *Y. ruckeri* in staat is om kieuwen en darm van de regenboogforel te koloniseren en invaderen. Significante verschillen in adhesie- en invasiecapaciteit werden niet waargenomen tussen virulente en avirulente stammen.

In de derde experimentele studie werden verschillende eigenschappen, geassocieerd met bacteriële virulentie, vergeleken tussen virulente en avirulente *Y. ruckeri* stammen: adhesie aan mucus en cellijnen, invasie en intracellulaire overleving in cellijnen en serumresistentie. De adhesie- en invasiemechanismen werden verder gekarakterisereerd voor geselecteerde *Y. ruckeri* stammen. De virulente isolaten vertoonden een hoge adhesie aan geïsoleerde mucus van kieuwen en darm van regenboogforellen, terwijl de meerderheid van de avirulente stammen een significant lagere adhesiecapaciteit hadden. Adhesie werd geïnhibeerd nadat de kiemen voorbehandeld werden met natrium metaperiodaat en proteolytische enzymen. Dit wijst erop dat vermoedelijk carbohydraten en proteïnen een rol spelen en bijgevolg lectinen betrokken zijn in het adhesieproces. Alle *Y. ruckeri* stammen waren in staat te adheren aan en te invaderen in CHSE-214, FHM en R1 cellen. Eén avirulente stam vertoonde een hoge adhesie- en invasiecapaciteit in de drie cellijnen, terwijl de virulente stammen matig adhesief

en invasief waren. De invasie van verschillende isolaten werd geïnhibeerd door colchicine en cytochalasine-D wat suggereert dat microtubuli en microfilamenten hierbij betrokken zijn. Monodansylcadaverine kon de invasie van geen enkele *Y. ruckeri* stam inhiberen wat doet vermoeden dat instulpingen gecoat met clathrine in het proces van receptor bemiddelde endocytose niet gevormd worden. Alle *Y. ruckeri* stammen vertoonden een afname in leefbare bacteriën na 6 uur inoculatie in de intracellulaire overlevingstesten. Dit suggereert dat *Y. ruckeri* niet vermenigvuldigt of overleeft in cellen of slechts gedurende een korte periode intracellulair blijft. De resultaten van de serumresistentietesten toonden aan dat de virulente stammen resistent waren, terwijl de avirulente *Y. ruckeri* stammen over het algemeen gevoelig waren aan het afdodend effect van het serum. Samengevat kan gesteld worden dat adhesie aan mucus en serum resistentie gecorreleerd blijken te zijn aan *in vivo* virulentie.

In de algemene discussie worden de resultaten van de drie experimentele studies bediscussieerd. Hypothetische virulentie mechanismen worden voorgesteld, rekening houdend met de reeds gekende virulentiefactoren van *Y. ruckeri*.

Als algemeen besluit van dit werk kunnen we stellen dat de kieuwen en de darm kunnen fungeren als belangrijke toegangspoorten voor *Y. ruckeri* bij regenboogforellen. Deze pathogeen is in staat te adhereren aan niet-fagocyterende cellen en deze vervolgens te invaderen. Bacteriële lectinen zijn vermoedelijk belangrijk in de adhesie aan specifieke suikerstructuren op gastheeroppervlakken. Het invasieproces blijkt afhankelijk te zijn van microtubuli en microfilamenten. De virulentie van *Y. ruckeri* isolaten is waarschijnlijk een complexe combinatie van mogelijkheid tot adhesie en kolonisatie, weerstand tegen het afdodend effect van serum en andere factoren die tot op heden nog niet geïdentificeerd zijn.

Els Tobback werd geboren op 31 december 1980 te Bonheiden. Na het beëindigen van haar studies algemeen secundair onderwijs, richting Wetenschappen-Wiskunde, aan de Voorzienigheid te Diest, begon ze in 1998 met de studies Bio-ingenieur aan de Vrije Universiteit Brussel (VUB). In 2003 studeerde ze af in de optie Cel- en Genbiotechnologie met onderscheiding. Een jaar later behaalde ze het diploma Aanvullende studies van Culturen en Ontwikkeling met onderscheiding aan de Katholieke Universiteit Leuven (KULeuven).

In 2005 trad zij in dienst als wetenschappelijk medewerker bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten waar zij vier jaar onderzoek verrichtte naar de vroege pathogenese van *Yersinia ruckeri* infecties bij de regenboogforel dat gefinancierd werd door het Bijzonder Onderzoeks Fonds (BOF).

In 2007 behaalde ze aan de Faculteit Psychologie en Pedagogische Wetenschappen aan de Universiteit Gent (UGent) het diploma van de Academische Initiële Lerarenopleiding (AILO) met grote onderscheiding. In 2008 voltooide ze de doctoraatsopleiding in de Diergeneeskundige Wetenschappen.

Els Tobback is auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften.

Publications in international journals

Tobback E., Decostere A., Hermans K., Haesebrouck F. & Chiers K. (2007) *Yersinia ruckeri* infections in salmonid fish. *Journal of Fish Diseases* **30**, 257-268.

Tobback E., Decostere A., Hermans K., Ryckaert J., Duchateau L., Haesebrouck F. & Chiers K. (2009) Route of entry and tissue distribution of *Yersinia ruckeri* in experimentally infected rainbow trout (*Oncorhynchus mykiss*). *Diseases of Aquatic Organisms* **84**, 219-228.

Tobback E., Decostere A., Hermans K., Van den Broeck W., Haesebrouck F. & Chiers K. (2009) *In vitro* markers for virulence in *Yersinia ruckeri*, in press.

Abstract in international congress

Tobback E., Hermans K., Decostere A., Duchateau L., Haesebrouck F. & Chiers K. (2007) Virulence of *Yersinia ruckeri* (*Oncorhynchus mykiss*) does not seem to correlate with *in vitro* invasiveness in cell lines. Proceedings of the 13th international EAFP conference on fish and shellfish diseases 2007, 63-63.

Na vier jaren ‘ver van huis’ ben ik aan het einde gekomen van dit werk. Rest mij het meest gelezen deel van dit boekje af te werken: het dankwoord. Een doctoraat maak je niet alleen, zoveel is duidelijk, maar hoe bedank je in een paar zinnen iedereen die hieraan een bijdrage heeft geleverd? Ik doe een poging...

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