In vitro markers for virulence in *Yersinia ruckeri*

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RUNNING TITLE:

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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 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 6h 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.

Keywords: *Yersinia ruckeri*, adherence, invasion, intracellular survival, serum resistance

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, Rodgers & Munn

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, Decostere, Hermans, Ryckaert, Duchateau, Haesebrouck & Chiers 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, Lelivelt & Orndorff 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, Willingham & Pastan 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.

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, Salminen, Bylund & Ouwehand 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 l^{-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, Mano & Hirose (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 Fig. 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.

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 Fig. 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.

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 Fig. 3.

In all the cell lines, *Y. ruckeri* 17.00(2-1) showed a significantly higher adherence capacity, than all the other strains. Fig. 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.

Adherence inhibition assay to CHSE-214

Results are represented in Fig. 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.

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 (Fig. 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 at a significantly ($P < 0.05$) higher rate 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 rate 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 1.5×10^4 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 invasion rates in CHSE-214. Overall, a significantly ($P < 0.05$) higher invasion rate was observed in cell line R1 for all strains compared to the other cell lines.

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 in comparison with 1 h after infection (Fig. 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 rates 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 rate of strain 2198(6) which has the lowest survival.

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 significantly ($P < 0.05$) lower survival rates than strains 17.00(2-1) and 2198(6).

Overall, a significantly ($P < 0.05$) lower survival rate 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 rate.

Invasion inhibition assay in cell lines

The results of the invasion inhibition assays are represented in Fig. 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.

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, Larsen, Madsen & Dalsgaard 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, Chiers, Charlier, Vandenberghe, Van Beeumen, Ducatelle & Haesebrouck 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.

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, Barnes, dos Santos & Ellis 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, van de Westerlo, Jechorek, Haines & Erlandsen

1998; Kawamura, Yoshikawa & Fujiwara 1998; Van Deun, Pasmans, Ducatelle, Flahou, Vissenberg, Martel, Van den Broeck, Van Immerseel & Haesebrouck 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, Esteban & Doménech (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.

REFERENCES

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Analytic Biochemistry* **72**, 248-254.

Davies R.L. (1991) Virulence and serum-resistance in different clonal groups and serotypes of *Yersinia ruckeri*. *Veterinary Microbiology* **29**, 289-297.

Furones M.D., Rodgers C.J. & Munn C.B. (1993) *Yersinia ruckeri*, the causal agent of enteric redmouth disease (ERM) in fish. *Annual Review of Fish Diseases* **3**, 105-125.

García J.A., Esteban M. & Doménech A. (2007) Estimation of the virulence of different strains of *Yersinia ruckeri* on cell culture. *Proceedings of the 13th International Conference of Fish and Shellfish Diseases, Grado, Italy*, 259-259.

Goebel W. & Gross R. (2001) Intracellular survival strategies of mutualistic and parasitic prokaryotes. *Trends in Microbiology* **9**, 267-273.

Kawamura S., Yoshikawa Y. & Fujiwara K. (1998) Enhanced invasion of Tyzzer's organism into cultured mouse hepatocytes by cytochalasin D. *FEMS Microbiology Letters* **160**, 97-100.

Kawula T.H., Lelivelt M.J. & Orndorff P.E. (1996) Using a new inbred fish model and cultured fish tissue cells to study *Aeromonas hydrophila* and *Yersinia ruckeri* pathogenesis. *Microbial Pathogenesis* **20**, 119-125.

Levitzki A., Willingham M. & Pastan I. (1980) Evidence for participation of transglutaminase in receptor-mediated endocytosis. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 2706-2710.

López-Dóriga M.V., Barnes A.C., dos Santos N.M.S. & Ellis A.E. (2000) Invasion of fish epithelial cells by *Photobacterium damselae* subsp. *Piscicida*: evidence for receptor specificity, and effect of capsule and serum. *Microbiology* **146**, 21-30.

Mills S.D. & Finlay B.B. (1994) Comparison of *Salmonella typhi* and *Salmonella typhimurium* invasion, intracellular growth and localization in cultured human epithelial cells. *Microbial Pathogenesis* **17**, 409-423.

Møller J.D., Larsen J.L., Madsen L. & Dalsgaard I. (2003) Involvement of a sialic acid-binding lectin with hemagglutination and hydrophobicity of *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology* **69**, 5275-5280.

Namba A., Mano N. & Hirose H. (2007) Phylogenetic analysis of intestinal bacteria and their adhesive capability in relation to the intestinal mucus of carp. *Journal of Applied Microbiology* **102**, 1307-1317.

Nikoskelainen S., Salminen S., Bylund G. & Ouwehand A.C. (2001) Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. *Applied and Environmental Microbiology* **67**, 2430-2435.

Romalde J.L. & Toranzo A.E. (1993) Pathological activities of *Yersinia ruckeri*, the enteric redmouth (ERM) bacterium. *FEMS Microbiology Letters* **112**, 291-300.

Schliwa M. (1982) Action of cytochalasin D on cytoskeletal networks. *The Journal of Cell Biology* **92**, 79-91.

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.

Van Deun K., Pasmans F., Ducatelle R., Flahou B., Vissenberg K., Martel A., Van den Broeck W., Van Immerseel F. & Haesebrouck F. (2008) Colonization strategy of *Campylobacter jejuni* results in persistent infection of the chicken gut. *Veterinary Microbiology* **130**, 285-297.

Van Overbeke I., Chiers K., Charlier G., Vandenberghe I., Van Beeumen J., Ducatelle R. & Haesebrouck F. (2002) Characterization of the in vitro adhesion of *Actinobacillus pleuropneumoniae* to swine alveolar epithelial cells. *Veterinary Microbiology* **88**, 59-74.

Wang X.H. & Leung K.Y. (2000) Biochemical characterization of different types of adherence of *Vibrio* species to fish epithelial cells. *Microbiology* **146**, 989-998.

Weiss L. (1972) Studies on cellular adhesion in tissue culture XII. Some effects of cytochalasins and colchicine. *Experimental Cell Research* **74**, 21-26.

Wells C. L., van de Westerlo E.M.A., Jechorek R.P., Haines H.M. & Erlandsen S.L. (1998) Cytochalasin-induced actin disruption of polarized enterocytes can augment internalization of bacteria. *Infection and immunity* **66**, 2410-2419.

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.* in press). 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

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 ($P < 0.05$).

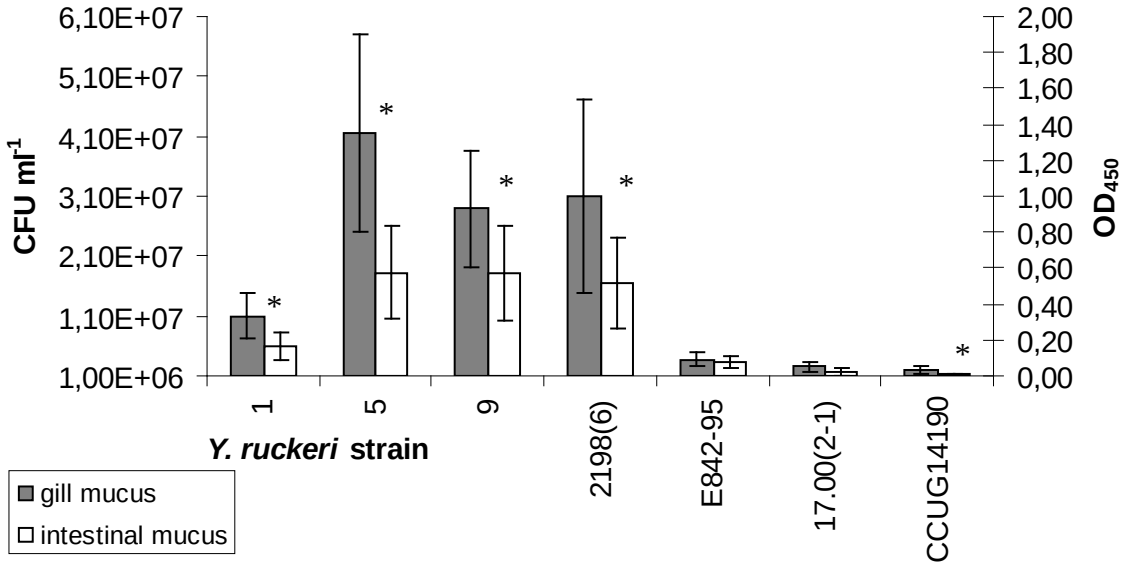


Figure 2 Effect of pre-treatment of the bacterial cells of *Y. ruckeri* strains 5, E842-95 and 17.00(2-1) with enzymes, sodium metaperiodate and carbohydrates on the adhesion to gill and intestinal mucus. The results represent the means ± standard error of three independent experiments conducted in triplicate.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

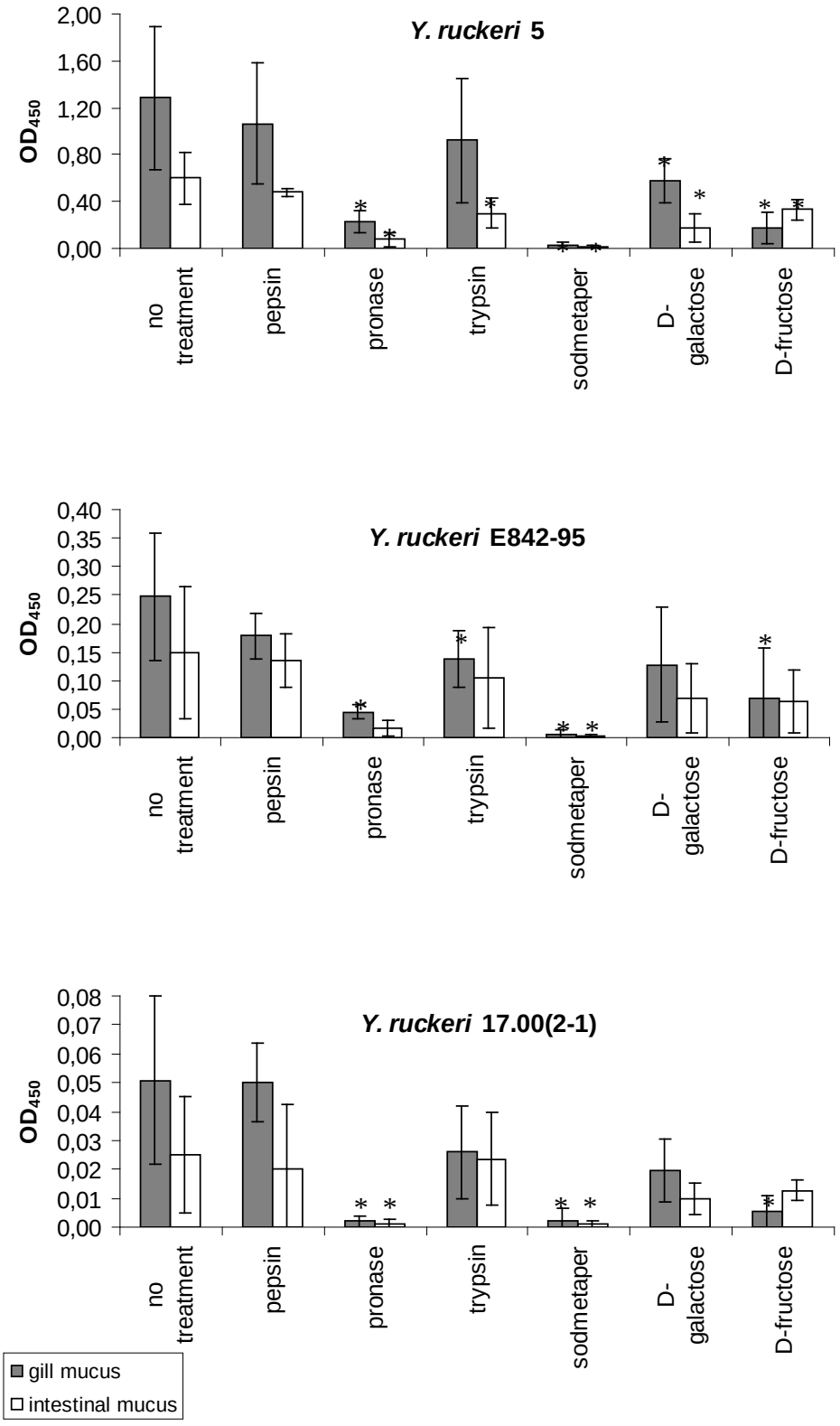

Y. ruckeri 5
OD450
2,00
1,60
1,20
0,80
0,40
0,00
no treatment
pepsin
pronase
trypsin
sodmetaper
D-galactose
D-fructose
Y. ruckeri E842-95
OD450
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
no treatment
pepsin
pronase
trypsin
sodmetaper
D-galactose
D-fructose
Y. ruckeri 17.00(2-1)
OD450
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0,00
no treatment
pepsin
pronase
trypsin
sodmetaper
D-galactose
D-fructose
gill mucus
intestinal mucus

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.

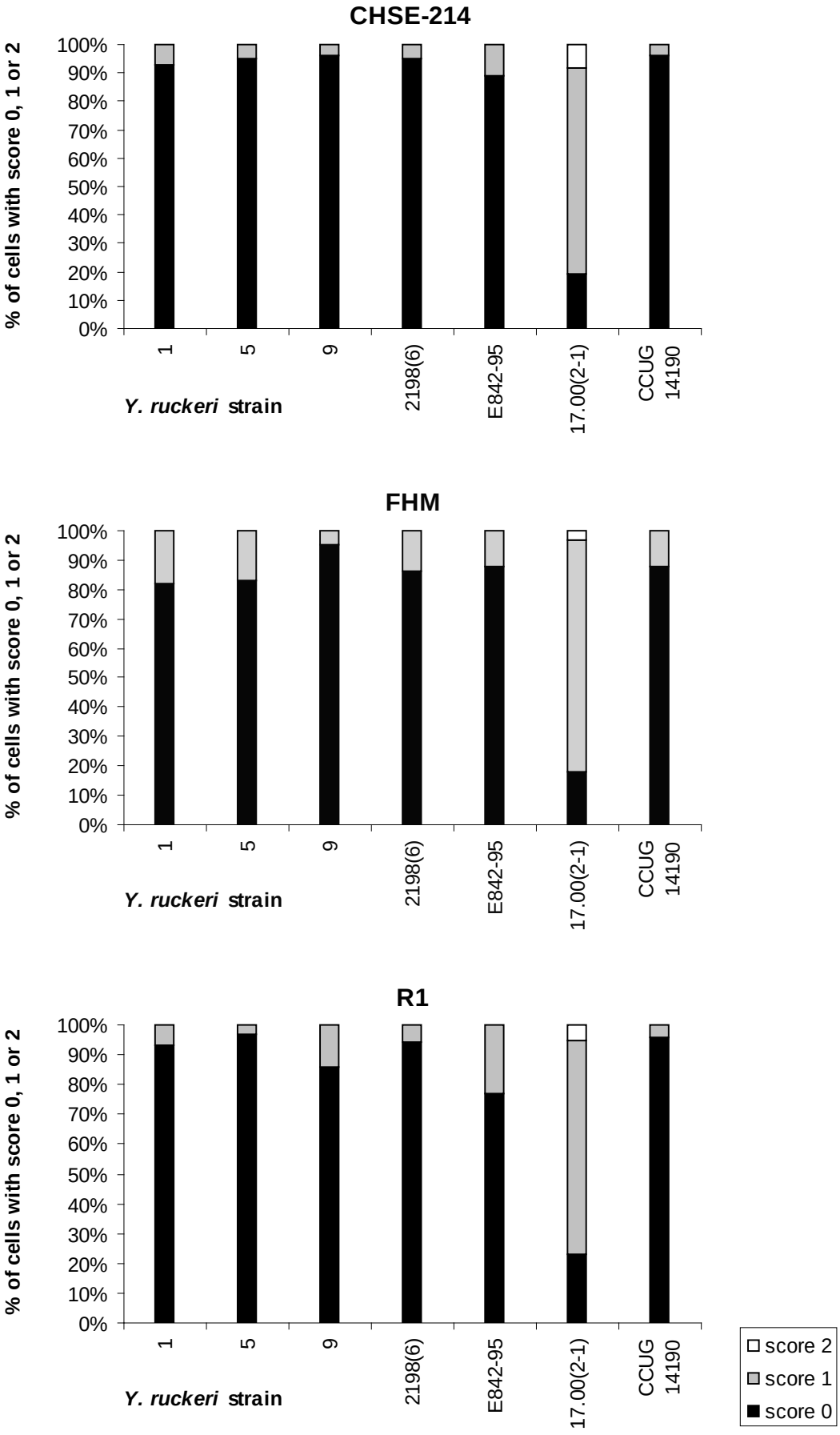

CHSE-214
FHM
R1
% of cells with score 0, 1 or 2
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
5
9
2198(6)
E842-95
17.00(2-1)
CCUG 14190
Y. ruckeri strain
score 2
score 1
score 0

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

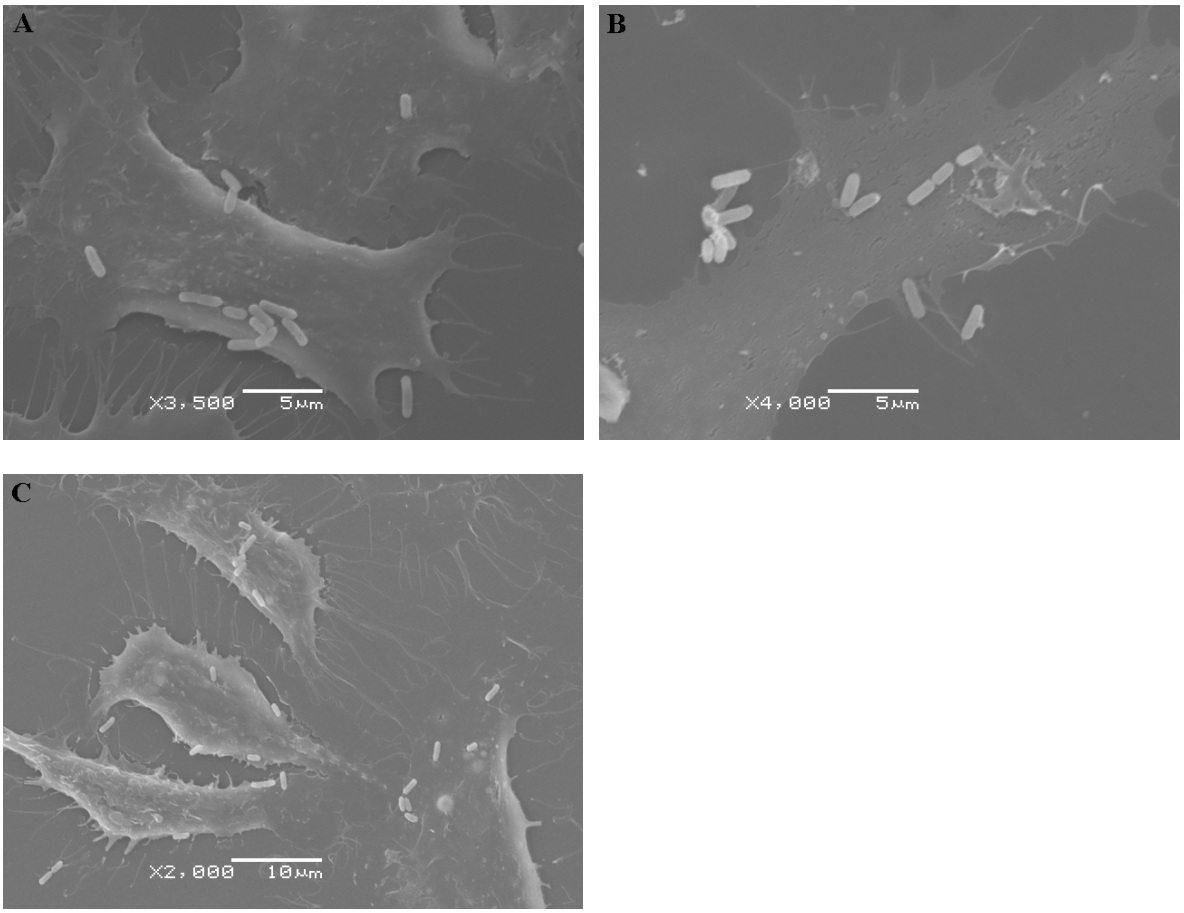


Figure 5 Effect of pre-treatment of *Y. ruckeri* 17.00(2-1) with enzymes, sodium metaperiodate and carbohydrates on the adhesion to CHSE-214 cells. The percentage of cells are shown to which no bacteria (score 0), 1 to 10 (score 1) and 11 to 20 (score 2) bacteria adhered. An asterisk refers to a significantly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

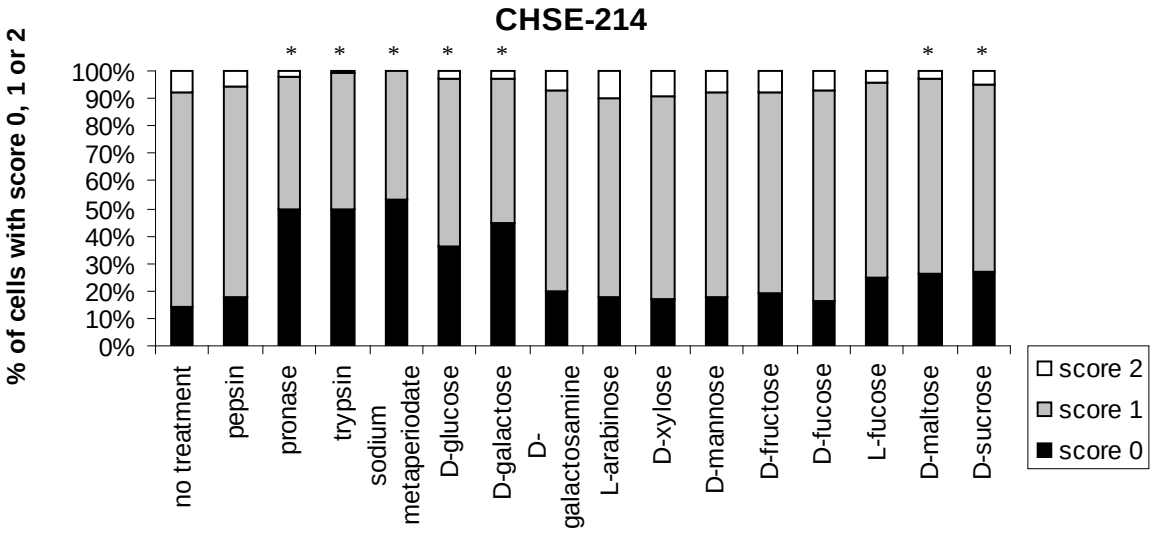


Figure 6 The invasion (1 h) and intracellular survival (6 h) of different *Y. ruckeri* strains in fish cell lines CHSE-214, FHM and R1. The percentages of gentamicin protected bacteria are shown. The results represent the means ± standard error of three or four independent experiments conducted in triplicate.

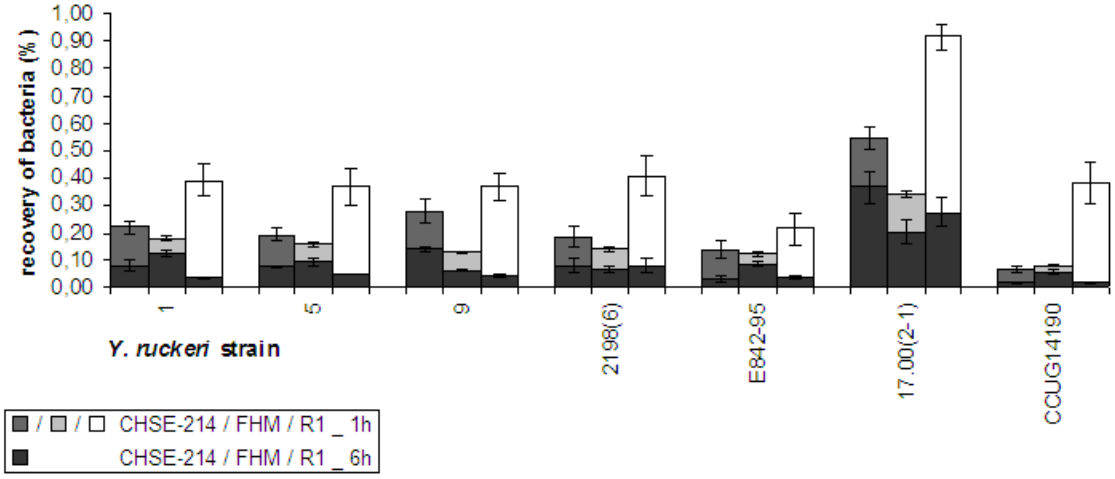
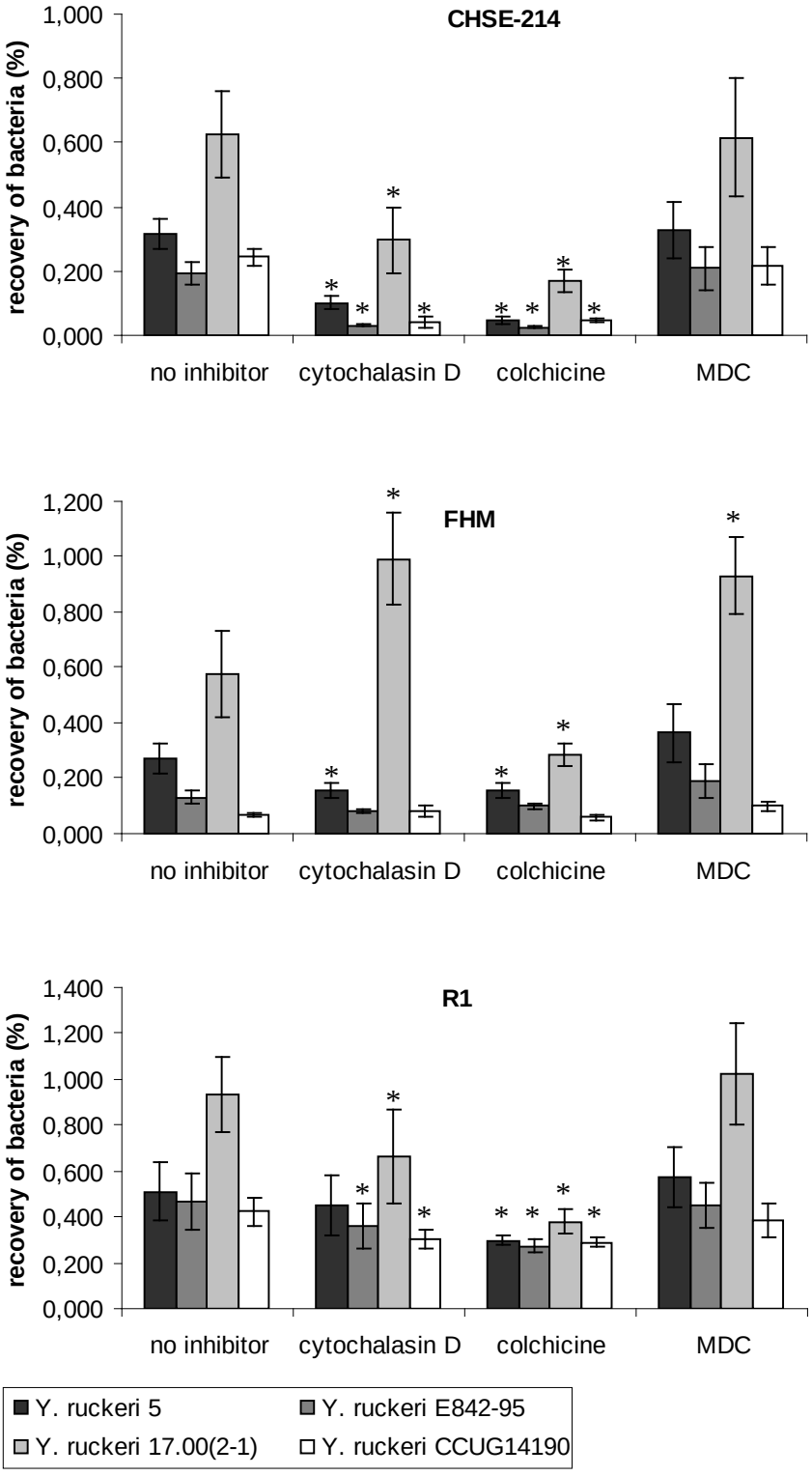


Figure 7 Effect of various inhibitors on *Y. ruckeri* invasion in CHSE-214, FHM and R1 cells. The percentages of gentamicin protected bacteria are shown. The results represent the means ± standard error of three independent experiments conducted in triplicate. An asterisk refers to a significantly different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


CHSE-214
recovery of bacteria (%)
1,000
0,800
0,600
0,400
0,200
0,000
no inhibitor
cytochalasin D
colchicine
MDC
FHM
recovery of bacteria (%)
1,200
1,000
0,800
0,600
0,400
0,200
0,000
no inhibitor
cytochalasin D
colchicine
MDC
R1
recovery of bacteria (%)
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
no inhibitor
cytochalasin D
colchicine
MDC
Y. ruckeri 5
Y. ruckeri E842-95
Y. ruckeri 17.00(2-1)
Y. ruckeri CCUG14190

ACKNOWLEDGEMENTS

The technical assistance of B. De Pauw is gratefully appreciated. The Institute of Aquaculture (Stirling, Scotland) and the Institut National de la Recherche Agronomique (Paris, France) are acknowledged for providing *Y. ruckeri* strains 1, 5, 9 and 2198(6), E842-95, 17.00(2-1) respectively, used in this study. The authors thank J.L. Romalde for serotyping these strains.

This study was supported by The Research Fund of Ghent University, Belgium, grant n° 01110505.