Hafnia alvei の RTG-2細胞への付着、侵入および生存性
Study of Adherence, Invasion and Survival of *Hafnia alvei* in RTG-2

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ABSTRACT—*Hafnia alvei* is a Gram-negative bacillus that belongs to the family Enterobacteriaceae and has been isolated from different fish species. *H. alvei* causes a wide variety of diseases, including hemorrhagic septicemia, kidney lesions and mortality in different fish species, as rainbow trout *Onchorhyncus mykiss*, cherry salmon *O. masou*, and brown trout *Salmo trutta*. The aim of this study is to investigate the interaction of different *H. alvei* strains with RTG-2 by gentamicin protection and immunofluorescence assays. Our results demonstrated a considerable potential of *H. alvei* to attach to RTG-2 cells, and that the internalized strains remained viable in the cells for 48 h.

Key words: *Hafnia alvei*, adherence, invasion, RTG-2

*Hafnia alvei* is a Gram negative, rod-shaped, facultative anaerobic bacterium of the *Enterobacteriaceae* family that has been isolated from various fish species. In freshwater fish, *H. alvei* has been reported with epizootic hemorrhagic septicemia in rainbow trout, *Onchorhyncus mykiss*, kidney pathology in charr salmon, *O. masou*, and mortality in brown trout *Salmo trutta*, after intraperitoneal inoculation. In marine fish, the susceptibility is apparently very low after intraperitoneal injection in gilthead sea bream, *Sparus aurata*, being the only histopathological manifestations small localized areas of granulomatous inflammation in the peritoneum, liver and kidney, and focal hepatitis without causing the death of fish.

Few data exist on the interaction of *H. alvei* with animal tissues, and the potential for this pathogen to adhere to and to invade fish epithelial and/or fibroblastic cells has not been explored. The precise nature of the mechanism responsible of adherence, and how it may interact with animal cell receptors to facilitate bacterial entry into the host cells may be of importance to understand the pathogenicity of *H. alvei* in fish.

In previous reports, we demonstrated the association of this bacterium with fish epithelial cells in vivo using an immunohistochemical method. Also, different authors has been demonstrated that this bacterium have the capacity to remain viable in marine and freshwater fish tissues for up to 3 months. Recently, our group has demonstrated the capability of *H. alvei* to invade and survive in human epithelial cells. All together, these results prompted us to investigate the in vitro interaction of *H. alvei* isolates with the fibroblastic-like cell line obtained from rainbow trout gonad (RTG-2).

Materials and Methods

Cell line

Rainbow trout gonad (RTG-2) fish cell line was described elsewhere. Cells were grown in 75 cm² flasks containing minimal essential medium (MEM) with 10% foetal bovine serum (FBS, Sigma). In all the experiments, RTG-2 cells were grown to a confluence of 85–95%, at 21°C without CO₂.

Bacterial strains

The *H. alvei* strains used in this study were, 187–95 (septicemia in laying hens), C-34 (rainbow trout), 19–68 (lizard), 4256–83 (human blood) and 1967–82 (human faeces), described previously by Rodríguez *et al.*. Bacteria were grown routinely on Trypticase Soy Agar (TSA) at 25°C.

Adherence and Invasion experiments

Invasion experiments were based on a modification of the gentamicin protection assay described elsewhere. RTG-2 cells were plated at 85–95% confluence into 24-well tissue culture plates (Corning). Bacteria were grown overnight at 25°C in LB broth. Five microliters of bacterial cultures were added to each well containing RTG-2 cells, and the mixtures centrifuged for 4 min at 400 rpm previous to the incubation without CO₂ at 21°C. In the adherence assays, 2 h post-infection, external non adhered bacteria were removed by washing the wells three times with PBS, and cells were lysed with 100 μL of 1% Triton-X100 (Sigma-Aldrich), an additional of 400 μL of PBS per well was added, mixed and serially diluted in PBS and determined by plate counting on TSA. In the invasion assays, two h post-infection the cells were incubated 1 h with MEM containing gentamicin (100 μg/mL). The cells were washed again and then disrupted by addition of Triton X-100, and serial dilutions of the disrupted mixture were plated on TSA and incubated for 24–48 h at 25°C. Control wells without RTG-2 cells were also
included to establish that the antibiotic treatment was effective in killing the extracellular bacteria of all strains used in this study.

For long-term intracellular survival experiments, after extracellular killing of bacteria by gentamicin, culture medium was replaced by MEM containing 10 μg/mL of gentamicin, incubated for further 21 or 45 h, and lysed as described before. For infections, standard conditions (5 μL) accounted for a multiplicity of infection (MOI) of ~40:1 (bacterium:fish cell ratio). In all experiments, adherence and invasion efficiency were calculated as the average of the total number of colony-forming units (CFUs)/total initial inoculum and expressed as a percentage. The integrity of the H. alvei-infected monolayers was examined by light microscopy throughout the 48 h period and the cell viability was assessed using trypan blue exclusion. For invasion experiments, non-invasive Escherichia coli DH5-α and Photobacterium damselae subsp. piscicida strain 94–99 were used as negative and positive controls respectively. In MOCK cells (wells inoculated with 5 μL of LB), cell viability was assessed in the same way in all experiments as described above.

Immunofluorescence

For immunofluorescence experiments we use polyclonal antibodies against the strain C-34 (a fish isolate) using the previously described protocol5. Secondary antibodies Alexa Fluor 594 and 488 goat anti-rabbit IgG were purchased from Invitrogen. Cells were seeded on 12-mm-diameter coverslips in 24-well plates. Five microliters of bacterial cultures were used for infection. Following the incubation periods, cells were carefully washed three times and fixed with cold paraformaldehyde (3.2% in PBS) for 20 min at RT. Primary and secondary antibodies were diluted 1:500 in bovine serum albumin (BSA, Sigma) (1% in PBS). Coverslips were then carefully washed two times with PBS and incubated for 20 min with the polyclonal rabbit anti-H. alvei C-34. Coverslips were carefully washed again and incubated for 20 min with a second anti-rabbit antibody Alexa-594. Following this initial staining, cells were permeabilized with Triton X-100 (0.1% in PBS) for 4 min at RT and carefully washed four times with PBS. To stain intracellular bacteria, coverslips where incubated with primary and secondary antibodies as before, but using an anti-rabbit antibody Alexa-488. After washing, coverslips were mounted on glass slides with ProLong Gold containing DAPI (Invitrogen). All preparations were examined by epifluorescence microscopy using an Olympus BX51 microscope with 60x or 100x oil immersion objectives. Digital images were acquired using an Olympus DP-70 digital camera and merged using Photoshop CS3 (Adobe) software.

To investigate if invasiveness was dependent on the viability of H. alvei and/or a heat-labile receptor, 400 μL of a bacterial culture of the strain 187–95 was heated at 80°C for 20 min in a thermoblock. Three plates of TSA were inoculated with 100 μL each to confirm the non-viability of the bacteria. After inactivation, 5 μL of the culture were used for infections. Cultures infected with heat-killed bacteria were processed for immunofluorescence as described before.

Results

Different adherence assays were performed with the five H. alvei strains, which can adhere to RTG-2 before internalization. Results are expressed as a percentage of the initial inoculum. Surprisingly, of five bacterial strains studied, the human isolate (strain 1967–82) adhered to the cells most efficiently whereas a fish isolate (strain C-34) exhibited the lowest adherence to the fish fibroblastic cell (Fig. 1A). The fish pathogen P.
**Hafnia alvei in RTG-2**

**Fig. 2.** *H. alvei* C-34 in RTG-2 cells. In fluorescent images, extracellular bacteria were detected with anti-*H. alvei* rabbit and alexa fluor 594 antibodies on non-permeabilized cells (A). Total bacteria were detected with alexas 488 and 594 on permeabilized cells (B). In merged images, extracellular bacteria are shown in orange-pink, intracellular bacteria in green, and the DAPI-stained DNA in blue (C). Scale bar, 10 μm.

damselae subsp. picicida* strain 94–99 used as control attaches more efficiently to the cells than the *H. alvei* strains, and the *E. coli* DH5-α used as negative control attaches similar to some *H. alvei* strains, probably reflecting common surface structures (Fig. 1A). Phase-contrast examination of RTG-2 cells confirmed extracellular bacteria in close association with eukaryotic cells (not shown). After gentamicin treatment, no extracellular *H. alvei* was located adherent to the fish cell line. Internalisation of *H. alvei* in RTG-2 was investigated using both gentamicin protection assays and immunofluorescence microscopy. Levels of invasion after 2 h of contact between bacteria and cells varied depending on the strain used (Fig. 1B). Maximal and minimal invasion efficiency was observed also for the strains more and less adherent respectively (1967-82 and C-34). We were unable to detect intracellular *E. coli* by gentamicin assays after 2 h of infection but the positive control (*P. damselae* subsp. *picicida* strain 94–99) were found inside a high percent of RTG-2 cells (80–90%) by immunofluorescence (not shown). To determine whether internalized strains replicate and/or survive in RTG-2 cells, infections were carried out for 24 and 48 h. After this time, numbers of viable bacteria were counted and results were expressed as a percentage of the initial inoculum that remains intracellular (Fig. 1B). Also, no changes in the morphology of the cell monolayer were noted during the course of the infection experiments with the *H. alvei* strains.

Throughout gentamicin protection assays, host cell viability was assessed using trypan blue exclusion, and in comparison to mock-infected RTG-2 cells, no difference in host cell viability was noted when RTG-2 cells were incubated in the presence of *H. alvei*, *E. coli* or *P. damselae* subsp. *picicida* over a 3 h period (infection and gentamicin treatment). To more fully characterize *H. alvei* adherence, we used immunofluorescence microscopy. In immunofluorescence assays, adherent bacteria were labelled red by incubation with the anti-*H. alvei* antibodies, and subsequent incubation with mouse anti-rabbit IgG conjugated to Alexa-549 (Fig. 2A). After cell membrane permeabilisation, both extracellular and intracellular bacteria were labelled green by incubation with anti *H. alvei* and Alexa-488 antibodies (Fig. 2B). Total bacteria were stained also with DAPI and the different channels merged (Fig. 2C).

Immunofluorescent microscopy using anti-*H. alvei* antibodies confirmed the intracellular location of bacteria. Immunofluorescence also demonstrated that heat-killed bacteria were unable to enter the cell line.
Discussion

Our results obtained by fluorescent microscopy and by adherence assays demonstrated a considerable potential of H. alvei to adhere to fish fibroblastic cells. These data showed that two of the five H. alvei strains used were able to attach at very high ratio to the RTG-2 cell line, in a similar way of P. damselae subsp. piscicida. Some authors reported that H. alvei is able to adhere to human epithelial cells, and the possibility existed, therefore, that the adherence capabilities of H. alvei is the result of an adhesive activity perhaps common to many other H. alvei strains in a different host range. Invasion is an important virulence mechanism of several pathogenic bacteria such as Salmonella and E. coli, two species closely related with H. alvei.

Our data showed that H. alvei strains were able to adhere to and to invade fish fibroblastic cells, and that the number of invasive bacteria is highly correlated to the number of bacteria adhered, which indicates that the invasion of fish cells is a postadherence event. Also, we have observed a high relationship between adherence and invasion ratio, with the susceptibility of brown trout to H. alvei by intraperitoneal injection. The more adherent and invasive strain, H. alvei 1967-82, showed a high virulence degree in brown trout (LD₃₀ 1.05 x 10⁶ cfu/fish), while that the less adherent and invasive strains, C-34 and 187-95, were avirulent in brown trout, with LD₃₀ values of 7.4 x 10⁶ cfu/fish and 2.5 x 10⁵ cfu/fish, respectively. The possibility existed, therefore, that the attachment and invasion of H. alvei to RTG-2 cells was the result of an adhesive activity. Further studies will help elucidate the mechanism of this invasion capacity. We reported previously that H. alvei strains may survive for long time inside a host, using a fish model of infection. These data correlates well with our present in vitro observations in the fish cell line, and highlight the possibility that H. alvei may exploit fibroblastic-like cells to survive inside the host.

References