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Moritella viscosa bypasses Atlantic salmon epidermal keratocyte clearing activity and might use skin surfaces as a port of infection

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ABSTRACT

Moritella viscosa is considered the main causative agent of winter ulcer disease in salmonid fish. In order to obtain more details on route of infection, we challenged Atlantic salmon (*Salmo salar*) epidermal keratocytes with *M. viscosa* and performed an Atlantic salmon immersion challenge. Although keratocytes were able to remove *M. viscosa* from surfaces, their engulfment capability appeared inefficient with reduced ability to reepithelialise superficial wounds (scale less skin surfaces) challenged with the bacterium. The immersion challenge revealed a significant connection between exposure area and mortality. Enhanced invasion ability and mortality was observed by *M. viscosa* exposure of the head and gill region compared to exposure of: the right side of the body; the left side of the body; or the body from pectoral to caudal fin ($p = 0.04$). Ulcer development corresponded to area exposed ($p = 0.002$), suggesting skin ulcer formation to result primarily from direct skin surface colonization. Ulceration of surfaces exposed to *M. viscosa* in parallel with occurrence of septicaemia suggests that both skin and gills may act as possible initiation sites for *M. viscosa* infections.

1. Introduction

Winter ulcer disease continues to challenge the salmonid fish farming industry as long as implemented measures are only semi-preventive and may fail to prevent outbreaks (Bornø et al., 2010; Johansen et al., 2009). The disease, which is reported in association with marine waters at temperatures below 8 °C (Lunder et al., 1995), is characterized by extensive and chronic ulceration of the skin and septicaemia, and results in animal welfare problems and economic loss (Bruno et al., 1998). Mucous membranes of the gastrointestinal tract, skin and gills are often considered potential entry sites for fish pathogens. *Moritella viscosa* is considered the agent causing winter ulcer disease, and although little is known on route of infection and pathogenesis recent evidence indicates the gill-region as port of entry for the bacterium (Løvoll et al., 2009). Bath or cohabitant-infected fish challenged with *M. viscosa* develop disseminated skin ulcers, in parallel with *M. viscosa* septicaemia (Lunder et al., 1995). Intraperitoneal or intramuscular injection also induces *M. viscosa* septicaemia, with ulceration mostly confined to the injection site (Bjørnsdottir et al., 2004; Gudmundsdottir et al., 2006). Another bacterium, *Aliivibrio wodanis*, is often isolated along with *M. viscosa* from winter ulcer diseased salmonids, but no experimental infection has so far reproduced symptoms of winter ulcer disease, and the significance of *A. wodanis* in the pathogenesis, if any, remains unknown (Benediktsdottir et al., 1998; Lunder et al., 1995).

Epidermis, the outermost layer of fish skin, encompasses a variety of viable cell types, of which the structurally most important is the motile keratocyte

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(Harris and Hunt, 1975; Shephard, 1994; Whitear, 1970). Keratocytes can, by rapid migration from the surrounding wound margins, cover fish skin wound surfaces with a new protective layer of cells within hours after wounding (Bullock et al., 1978; Phromsuthirak, 1977). Mucus cells, another important cellular constituent of epidermis, secrete products with protective roles against opportunistic pathogens (Shephard, 1994). In challenge experiments with bacteria, removal of mucus/epidermal cells increased the cumulative mortality in salmonids, compared to undamaged fish (Madetoja et al., 2000; Svendsen and Bøgwald, 1997). In addition to their migratory activity, Atlantic salmon keratocytes were shown to internalize particular matter such as bacteria and other particles (Åsbakk, 2001). The characteristics combined suggest the keratocyte as an important contributor to the fish nonspecific immune response, serving to protect against microorganisms and other potentially harmful substances from the surrounding water (Åsbakk, 2001; Åsbakk and Dalmo, 1998). Given that *M. viscosa* somehow is capable of interfering with such mechanisms, the fish could be more vulnerable to bacterial invasion through the skin. The aim of the present study was to assess: (I) do *M. viscosa* evade the particulate matter-clearing and impact the wound healing capability of Atlantic salmon keratocytes and (II) if by doing so, can *M. viscosa* utilise skin surfaces as a port of entry to the fish body and cause winter ulcer disease.

2. Materials and methods

2.1. Keratocyte cultivation

Atlantic salmon (*Salmo salar*) ($n = 8$, unvaccinated, weight 2–2.5 kg, killed by a blow on the head and exsanguination), were transported from the rearing site (Tromsø Aquaculture Research Station, Kårvik) to the laboratory (≤40 min) in rearing tank seawater. Keratocytes were cultured from whole scale explants as described elsewhere (Åsbakk and Dalmo, 1998). Briefly, after rinsing the skin surface with filtered (0.2 μm, NALGENE® MF75™ SFCA) rearing tank seawater (FT-water), single scales were picked (forceps) and placed in 24 well tissue culture plates (TC-24) (Falcon Multiwell™ Becton Dickinson, NJ, U.S.A., 3 scales/well), or on standard glass microscope slides (5–6 scales/slide) in Petri-dishes containing Hanks' balanced salt solution (HBSS; Gibco 14065, Invitrogen) supplemented with 1 ml/100 ml of Sigma A5955; 25 μg amphotericin B, 10 μg streptomycin and 10,000 IU penicillin/ml (Sigma–Aldrich Co., St. Louis, MO, USA). Volumes were adjusted to liquid column heights of ~3 mm for fluid coverage of scales. In some TC-24 wells, scales were placed on circular microscope cover glasses for subsequent examination of cells by electron microscopy. After two days at room temperature with no disturbance, debris and unattached scales were removed by replenishment of the supplemented HBSS medium. Experiments involving cultured cells were performed no later than five days after seeding of scales. Cells were migrating and without apparent loss of vitality (visual inspection) after 7 days.

2.2. Challenging keratocytes with bacteria

Single colony-forming units (cfu) of *M. viscosa* 06/09/139 and *A. wodanis* 06/09/139 from blood agar (BA) plates (agar base no. 2, Oxoid CM271, supplemented with 7% human whole blood and 2.5%, w/v, NaCl) were subcultured in 10 ml Marine Broth (Difco™, 2216) for two days (7 °C, 200 rpm continuous shaking). Cultured keratocytes (TC-24 wells) received new HBSS (1.0 ml/well) without antibiotics/antimycotics. After acclimatization to 7 °C, 20 μl of 1×10^9 cfu ml^{-1} *M. viscosa* or 1.5×10^9 cfu ml^{-1} *A. wodanis* culture (estimated by colony counting serial diluted aliquots plated on BA plates) was added per well. A Nikon TMS-F inverted microscope, equipped with digital camera (Nikon DS-Fi1) with automatic image capture every 15 s was used for inspection of subsequent development in wells for up to 2 h at room temperature. Images were sequential ordered into time-lapse video using Microsoft® Windows® Movie Maker v5.1.

2.3. Experimental wounding model

After picking of scales for culture, the killed exsanguinated fish (kept in FT-water) were utilised in experimental wounding studies. All parts of wounding study and procedures were at 7 °C. Teleost epidermis is avascular (Bullock and Roberts, 1974), and viable Atlantic salmon epidermal cells can be isolated up to at least 18 h after killing of the fish (Åsbakk and Dalmo, 1998). Rows of six TC-24 plate-wells with bottom removed were fixed onto the skin surface of fish submerged into FT-water (Fig. 4d, fish were mechanically restrained in plastic containers, with top of rows above water surface). Strips of tape fixing rows exerted downward pressure ensuring no fluid leakage between in- and outside of the resulting experimental chambers. Prior to onset of study, skin surface mucus scrapings and FT-water samples were examined for bacteria by plate inoculation and colony counting. Epidermal scales (~0.5 cm^2 area) from inside each chamber were removed (forceps), and 1.0 ml/well of 6.6×10^3 cfu ml^{-1} *M. viscosa* or 4.2×10^3 cfu ml^{-1} *A. wodanis* in FT-water (numbers estimated by colony counting serial diluted aliquots (100 μl) plated on BA) was added. After 8, 12, 24 or 48 h, the bacterial suspension was removed, and after washing with FT-water, 0.5 ml of 1% methylene blue in HBSS was added per well. After 5 min, unbound stain was washed away (FT-water) and a Nikon SMZ-2 T binocular magnifier with a Nikon DS-Fi1 digital camera was used for inspection. Methylene blue gives deep blue staining to non-viable cells and tissue while viable cells and tissue, including regenerated epidermis, remain unstained (Quilhac and Sire, 1999). Also, skin surface samples of an Atlantic salmon smolt (~50 g) not experimentally wounded, but bath challenged for 1 h with *M. viscosa* 06/09/139 (1×10^6 cfu/ml, grown as described above), was after 2 days in 8 °C seawater killed and skin sample specimens were prepared for electron microscopy.

2.4. Electron microscopy

Cultured keratocytes on cover glasses and excised skin specimens were fixed with half-strength fixative for