Short Communication

ENHANCEMENT OF THE VIRULENCE FOR TROUT OF *AEROMONAS HYDROPHILA* BY SERIAL ANIMAL PASSAGE

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Aeromonas hydrophila is an opportunistic pathogen associated with human and fish diseases. Toxins and enzymes together with some structural features are considered to be important virulence factors and have been widely investigated (1, 12, 16, 17). The bacteria display a remarkable ability to respond to changes in their surroundings and it is known that the expression of most of the virulence factors is regulated by environmental conditions. It has been demonstrated that a capsule-like structure is synthesized when *A. hydrophila* grows in vivo (10) and that temperature greatly influences the rate of growth of some serotypes as well as the production of enzymes and toxins (9) and the cell surface composition (11).

The increase of bacterial virulence following animal passage as well as attenuation of strains after prolonged cultivation in vitro occurs frequently among microorganisms (7, 8, 19). We have frequently observed a reduction of the virulence for trout of *A. hydrophila* strains, isolated from natural sources, after a long term in vitro cultivation.

The purpose of this study was to determine whether the virulence for trout of *A. hydrophila* L57 maintained for a long time in artificial conditions could be enhanced by serial passage through host tissues. Changes in the expression of surface virulence factors during the enhancement were also studied.

A. hydrophila L57 (Serotype O:34) is a strain of intermediate virulence for fish, lacking the cell surface protein array (S-layer). It was originally isolated from a muscular lesion of a trout and biotyped and serotyped following the protocols described elsewhere (13, 18). The strain was subjected to repeated serial passage

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through rainbow trout (*Oncorhynchus mykiss*). Trout weighing 25 g were intramuscularly injected with 10^7 cfu. Between 24 and 48 h postinoculation, the purulent content of the lesion formed at the site of injection was aseptically removed and streaked on TSA plates. After incubation for 12 h, cells were suspended in saline (0.85%, w/v, NaCl), and 10^7 cfu were injected again as above. Four passages through trout tissues were carried out. Daughter strains were labelled L571, L572, L573, L574, corresponding to passages 1, 2, 3, and 4. The 50% lethal dose was calculated by the Reed and Muench method (14). The susceptibility to bactericidal activity in fresh trout serum was measured by the method described by Davies (4). OMP were obtained following the method described by Chart and Trust (3), and analyzed by SDS-PAGE. LPS was prepared by the method of Hitchcock and Brown (6). Following electrophoresis, the LPS was transferred to nitrocellulose membranes that were blocked, washed and incubated overnight at room temperature in O:34 rabbit antiserum. Finally, they were incubated again with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate and developed by adding the substrate solution that was prepared following the manufacturer's protocol (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Cells of the original strain L57 and the daughter strain L574 grown on TSA were labelled with cationized ferritin using the method of Weiss et al. (20). The negatively stained whole mounts were observed by TEM in a Phillips EM 300 transmission electron microscope, operating at 60 kV under standard conditions.

The virulence of *A. hydrophila* strain L57 was enhanced by repeated serial passages in trout, using intramuscular injections. After four passages, the virulence for trout increased as evidenced by a reduction of the LD_{50} from 3×10^7 to 1×10^6 cells. The enhanced virulence of the strain was also noticed by the increased severity of the lesions and a reduction of the time to develop those lesions.

A progressive increase of the resistance to the bactericidal activity of trout serum after each animal passage was obtained. Serum resistance values expressed as a percentage of survivors and obtained as an average of three determinations were as follows for the original and daughter strains: 17.7% (L57), 20.5% (L571), 22.8% (L572), 25.0% (L573), 55.5% (L574). A positive correlation between the enhancement of the virulence and the increase of the resistance to the fresh serum bactericidal activity have also been described in other microorganisms (2,15).

Electrophoretic patterns of lipopolysaccharide in polyacrylamide gels of the original and daughter strains were studied but, no difference in the O side chain could be observed. Similarly, immunoblots did not show any antigenic variation. No major differences on the electrophoretic patterns of SDS-PAGE of OMPs were detected. Only a band of 38 kDa appears to be decreased in passaged strains (Fig. 1).

In order to examine the correlation between the increase of virulence and structural changes in the bacterial surface, the original (L57) and the most virulent daughter strain (L574) were compared by electron microscopy. To facilitate the detection of additional external capsule-like layers, a specific staining method for

Fig. 1. SDS-PAGE of the outer membrane proteins (OMPs) from *Aeromonas hydrophila* O:34 serotype.

Daughter strain L574 (lane 1) obtained after 4 passages through trout and original strain L57 (lane 2). Molecular mass markers are indicated with arrows from top to bottom; phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soy bean trypsin inhibitor (20 kDa), and alpha-lactalbumin (14.4 kDa).

Fig. 2. Micrograph of whole mount of ferritin-labeled cells of *Aeromonas hydrophila* (L574), showing cells positively stained.

Bar=0.5 μ m.

acidic polysaccharides (ferritin polycationized) was used. Both strains were found to be a mixture of positive (Fig. 2) and negative (Fig. 3) ferritin-binding cells although they clearly differed in the relative proportion of the two phenotypes. The intensity of ferritin-binding to the cellular surface was higher in L574 which showed up to 15% of the cells with a loose but complete coat of ferritin together

Fig. 3. Micrograph of whole mount of ferritin-labeled cells of *Aeromonas hydrophila* (L574), showing a negatively stained cell.
Bar = 0.5 μ m.

with many others uncoated or partially covered by the label. Conversely, most of the cells of L57 had no surrounding label, although, cells with ferritin label on the surface were also occasionally observed. Bacterial populations consisting of a mixture of cells with different properties have also been observed in other bacteria (5, 21). The presence of a higher number of cells coated by a polysaccharide layer, in the most virulent daughter strain L574, may account for the increased resistance to the bactericidal activity of the fresh serum as well as the enhancement of the virulence for trout. It can also indicate that environmental factors represented by in vivo conditions may have a selective effect on the relative cellular composition of the bacterial population.

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