Functional analysis of antibacterial defence mechanisms in homozygous clonal lines of rainbow trout with different resistance to *Flavobacterium psychrophilum* infection

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Abstract

In teleost fish as in other vertebrates two basic functions representing respectively the humoral and cellular aspects of immunity are mainly involved in innate defences opposed to bacterial infections. Bacteriostatic or bactericidal activity of normal serum may result from the action of different effectors among which the complement system usually plays the major part. Phagocytic activity is a characteristic of specialized lymphoid cells which have developed strong capacities in motility, chemosensitivity, capture and killing of bacteria. It occurs at some degree in the humoral compartment and more generally at tissular level, in peripheral tissues or lymphoid organs.

In salmonids infected with the cold-water flavobacteriosis agent, *Flavobacterium psychrophilum*, few researchers have considered these functions yet and a lack of concordance among their observations results from the diversity of the fish species, developmental stages and experimental methods used in these studies. This represents a serious difficulty for drawing a global representation of the fish and bacterium interrelationships in the course of flavobacteriosis.

We have established a collection of isogenic homozygous lines of rainbow trout (RT) using a combination of mitotic and meiotic steps of gynogenesis [1]. These fish were repeatedly tested with virulent strains of *F. psychrophilum*, and lines consistently exhibiting a range of susceptibility or resistance to the bacterium were identified [2]. Such lines represent a convenient material for comparative study of the effectiveness of innate or specific antibacterial defences in susceptible and resistant fish. Here are reported recent observations about normal serum bactericidal effect (NSB) and phagocytosis obtained using several of these lines.

Fish: The homozygous clonal RT lines used in our study (Table 1) had been tested for flavobacteriosis resistance to JIP 02-86 isolate in previous studies [3]. Fish from six different lines were used in the present experiment. Breeders were maintained at the INRA experimental fish farm of Monts d'Arrée (PEIMA, Brittany). Fish used for phagocytosis tests (100-150 g mean body weight) were reared in the farm in a specific pathogen-free (SPF) compartment supplied with spring water. Fish used for bactericidal activity tests (15-20 g)

were introduced in the experimental unit of Jouy-en-Josas (UEP) as disinfected eggs and reared in recirculated tap water units.

Phagocytosis tests: Tests were performed at the ANSES Ploufragan Plouzané Laboratory (formerly LNPAA Brest). Circulating cells were preferred after a preliminary comparison test had shown that anterior kidney cell treatment did not result in better extraction efficiency. Blood (about 2-2.5 ml) was collected from 6 to 7 fish per line and diluted 1/10 in Glasgow minimum essential medium (GMEM) containing antibiotics (penicillin 100 IU and streptomycin 100 µg/ml), fetal calf serum (10 %) and heparin (450 IU).

Table 1. Homozygous trout lines used in the experiments ranked according to susceptibility to *F. psychrophilum* (R= resistant, S= susceptible).

Clonal lines	Phagocytosis assay	Bactericidy
R1	+	+
R2		+
R3	+	+
S1	+	+
S2	+	+
S3		+

For cell extraction, cell suspensions were deposited on 8 ml of cold ficoll 1.077. After centrifugation (150 g) the leucocyte ring was gently collected and rinsed 3 times in L15 medium. An aliquot of the final cell suspension was stained with Trypan blue and observed in a Thoma counting chamber to estimate the viability and numbers of cells which were then adjusted to 10^7 cells/ml. In case of low numbers of cells, samples from different fish could be pooled for further use.

Phagocytosis tests were conducted in flat-bottom 96 well microplates using the nitroblue tetrazolium (NBT) method. Leucocytes (90 µl) were put in contact with l0 µl of a *F. psychrophilum* suspension which had been opsonised with homologous serum 1/10 (30 min at 20 °C) and incubated 24 h at 37 °C for inactivation. After 30 min, 100 µl of NBT 0.2 % were added and incubated 30 min more. Eventually, adherent cells were fixed with methanol 4 % and treated subsequently with KOH 2M and DMSO before reading absorbance at 630 nm.

Bactericidal activity tests: Blood was aseptically collected from 15 fish per line by caudal puncture (on both heparinized and non-heparinized tubes held on ice) and kept at 4 °C until plasmas and sera could be processed and stored at -80 °C. For bactericidal activity study, the rezazurin reduction test (Uptiblue®, Uptima Lab.) was used in 96 well microplates.

The bacterial isolates JIP 02-86, cultured in standard conditions to ensure optimal viability, was suspended in veronal buffer saline (VBS) or VBS supplemented with Ca^{++} and Mg^{++} salts (VBS^+) containing 1% liquid Anacker and Ordal medium. Comparisons were made between pooled sera and plasmas (3 samples per pool), and between fresh and heated (45°C, 30 min) sera which were concurrently incubated with *F. psychrophilum* in VBS or in VBS^+, in order to detect possible involvement of the complement system. The microplate wells received successively the bacterial suspension at 3 x 10^7 CFU/ml in the appropriate buffer (80 µl), the sera or plasma (20 µl) and the Uptiblue® reagent (10 µl), before being incubated 18 h at 18 °C. Reading was performed on a SPECTRA Fluor plus TECAN both in visible and fluorescent light.

Phagocytosis: 4 trout clonal lines (6 fish /clone) were tested with the JIP 02-86 bacterial isolates. Two of them had been characterized as resistant (R1 and R3) and the two other ones susceptible (S1 and S2) to *F. psychrophilum* infection in former studies, In some cases, the number of leucocytes collected was low and the cells of several fish had to be pooled.

Table 2. Activation of phagocytic cells by *F. psychrophilum* JIP 02-86 in disease resistant (R1 and R3) or susceptible (S1 and S2) lines of homozygous trout.

	R1	R3	S1	S2
Basal activity	0,039	0,12	0,006	0,048
Specific activation (JIP 02-86)	0,000 0,048 0,029 0,073	0,030 0,108 0,080 0,014 0,061 0,016	0,023 0,051 0,000 0,000 0,024 0,007	0,098 0,094 0,023 0,054
mean	0,038	0,052	0,018	0,067
standard-error	0,027	0,035	0,018	0,031
activation rate (mean/ basal act.)	0,96	0,43	2,93	1,40

Table 2 shows that the cell extraction method was not properly optimized. Many cells seem to have been lost during the extraction procedure, due to detachment and/or stressful conditions, resulting in very weak reactions. The table also shows a large variability in the basal metabolic activity in the different clone cells. Two clear cases of cell activation (S1 and S2) may be observed, however, and it is interesting to note that in both cases, the fish repeatedly appeared susceptible to experimental infection.

Bactericidal activity of serum and plasma: The results obtained in Uptiblue® tests (some of them are displayed in Fig. 1) show little difference between plasma and serum activity although the plasmas sometimes produced more distinct differences among the fish lines. The growth of *F. psychrophilum* in trout sera displayed large variations among lines and was especially marked in certain clones or when heated serum was combined with Mg^{++} limitation.

Serum antibacterial activity to *F. psychrophilum* was observed, but not constantly. While the presence of divalent cations seemed to favour this activity, the role of complement factors is more questionable and probably not exclusive. Namely, heated sera almost always showed an adverse effect on *F. psychrophilum* suspensions. The homozygous clones tested indeed displayed differences (Fig. 1) but in this case again this was not in the expected way. Altogether, the most pronounced antibacterial activity was found in disease susceptible families.

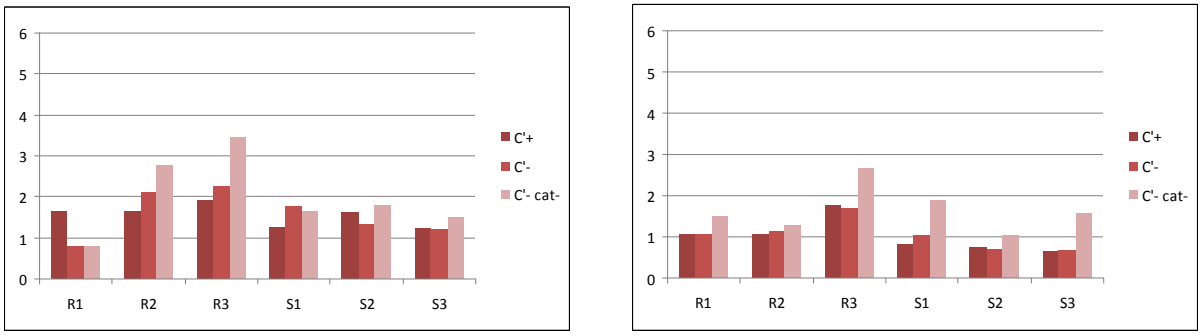

Figure 1. Serum and plasma antimicrobial effect in homozygous resistant (R) and susceptible (S) trout lines tested with JIP 02-86 isolate. C'+: fresh serum; C'-: heated serum; C'- cat-: heated serum with Ca^{++} and Mg^{++}. Activity is expressed as the ratio to the activity of bacteria in VBS^{+}.

In summary, these observations were not expected. They do not suggest a prominent role of classical antimicrobial defence systems in resistance to coldwater disease. They partially confirm, however, some data of Wiklund and Dalsgaard [4], who reported a bactericidal action of both fresh and heated sera and the ability of certain isolates to multiply in serum after exposure for 24 h.

References

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