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Genetic Bases of Resistance *versus* Susceptibility to *Flavobacterium psychrophilum* in Rainbow Trout

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ABSTRACT: *Flavobacterium psychrophilum* is one of the most significant bacterial pathogen in salmonids worldwide. Selective breeding for resistance seems a promising method to control the disease. We searched for resistance QTL in a doubled haploid F2 progeny from two clonal lines chosen for opposite resistance. About 280 fish (6.3g mean body weight) were infected through intramuscular injection (4.2 10^5 cfu/fish) and mortality was recorded daily during one month. Using a genome-scan with 217 microsatellite and 101 SNP markers, QTL for survival were detected on several linkage groups, three of them having a strong effect on survival. To get further insight into the defense mechanisms involved, the location of a set of immune candidate genes was determined and the effect of the QTL on bacterial load in the spleen is being investigated.

Keywords: Bacterial Cold Water Disease; QTL; immune genes

Introduction

The bacterium *Flavobacterium psychrophilum* is one of the most significant pathogen affecting salmonids around the world. This psychrotrophic agent is believed to have been introduced from North America into Western Europe at the beginning of the years 1980 (Bernardet and Bowman (2006)). Since, it was identified in most cold and temperate waters all around the world. In rainbow trout (*Oncorhynchus mykiss*), it occurs as a septicaemic form (the rainbow trout fry syndrome or RTSF) which severely affects the fry. In larger fish, the disease is chronic and known as the bacterial cold-water disease (BCWD). Besides mortalities, common clinical signs are dorsal swelling and muscular lesions, all resulting in heavy economical losses.

Methods to control the disease are highly needed. No effective vaccine is available and the use of veterinary drugs is presently the major way to fight outbreaks. In rainbow trout, the existence of a genetic control of resistance to the disease has been evidenced in a number of studies. Moderate estimates of heritability were obtained (Henryon et al. (2005); Silverstein et al. (2009)) and genetic gain was obtained though selection (Wiens et al. (2013a)). However, as in other livestock species, selection for disease resistance in fish is hampered by practical and economical limitations and the discovery of QTL and markers associated to resistance are expected to substantially help to design efficient selective breeding schemes. Vallejo et al. (2010) have concluded that the variability of resistance to the disease is likely explained by a limited number of loci, and QTL for survival after experimental infection have recently been identified (Wiens et al. (2013b)). QTL detection also paves the way for the discovery of genes responsible for the differences of susceptibility and brings further insight in the defense mechanisms and host-pathogen interactions. Little is known about the antibacterial mechanisms opposed by the host to the infection. The spleen index of healthy fish seems to be genetically associated to resistance (Hadidi et al. (2008); Wiens et al. (2013b)). Johnson et al. (2008) found suggestive evidence for association between MH genes and resistance. A comparative analysis of transcriptomic response to infection in two trout clonal lines with opposite susceptibility to infection revealed similar overall response profiles in the two lines, though the stronger induction of several genes in the resistant line (e.g. complement C3) pointed them as potential candidate genes to explain the variability of resistance (Langevin et al. (2012)).

A collection of gynogenetic clonal lines was previously established as described in Quillet al. (2007) and exhibited a wide range of resistance/susceptibility to several viral diseases (Verrier et al. (2013)). The lines were screened for resistance to *F. psychrophilum* and two lines with opposite resistance were selected as F0 breeders for the present study. The first objective was to search for QTL associated to survival after infection using rainbow trout F2 juveniles issued from the F0 grand-parents. To get further insight into the defense mechanisms associated to QTL, the location of a set of immune candidate genes was determined and their positions were compared to the positions of identified QTL. In addition, the relation between the main QTL and bacterial load in the spleen is being investigated.

Materials and Methods

Experimental design. The two F0 grand-parents were all homozygous individuals from a resistant (R) and a susceptible (S) gynogenetic clonal respectively. F1 females (S female x R male) were reproduced by gynogenesis to produce doubled haploid F2 progeny (fertilization with UV irradiated sperm followed by heat treatment after fertilization). Fertilized eggs were placed in the INRA experimental facilities dedicated to infectiology (IERP, Jouy-en-Josas) and normally reared (tap water, 10°C constant) until infectious challenge. During the whole experiment, animal were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals and by the local Ethics committee. Animal work was approved by the Direction of the Veterinary Services of Versailles (authorization number 78-39).

Infectious challenge and tissue sampling. When they reached about 6g, around 280 fish were infected with *F. psychrophilum* using the JIP 02/86 bacterial isolate, initially isolated in France. Bacterial suspension was produced according to standard procedures (Garcia et al. (2000)) and administered to anaesthetized fish by dorsal intramuscular injection at 4.2 10^5 colony forming units (cfu) per fish. Dead animals were recorded twice a day during 35 days. The fish that survived at that time were sacrificed (lethal dose of phenoxyethanol). All individuals were sampled (fin clip) for subsequent DNA extraction and genotyping.

To investigate the possible effect of QTL on the course of infection, some F2 progeny were reared until about 90g. Fish were infected by intramuscular injection as previously described, except that the dose was 47.5 10^5 cfu/fish, and were sacrificed at successive time points post-infection (8h, 22h, 46h, 76h; 75 fish per date). Thirty seven fish were kept as control. Spleen was sampled for subsequent measurement of bacterial DNA through qPCR detection. A piece of fin was taken on every individual in order to establish the status at QTL from genotype at the closest markers.

Genome scan and QTL detection. The genome scan was performed with 318 markers (217 microsatellites and 101 SNPs). The linkage map was rebuilt for the family using CARTHAGENE software (de Givry et al. (2005)). The mean overall spacing for genome scan was around 9 cM. Resistance was analysed using the whole post-challenge life time dataset where individual data were the surviving status (dead or alive at the end of the period of survey) and the time to death (in days post-infection) of each fish died. Surviving fish corresponded to 'censored' observations.

QTL detection was performed with QTLMAP software (Filangi et al. (2010)), based on an interval mapping method described by Elsen et al. (1999) and performing the Cox model-based survival analysis implemented in QTLMap for non-normal distribution and presence of censored data (Moreno et al. (2005)). The presence of QTL at one location (H1: one QTL) *vs* the null hypothesis (H0: no QTL) was tested with an approximate likelihood ratio test (LRT). To take into account the uniparental origin of the F2 gynogenetic family, it was considered as a sib family where each fish was assigned a virtual unknown parent different for every individual. The empirical distribution of LRT was obtained from 10,000 simulations under the null hypothesis with a trait heritability fixed to 0.5.

Mapping of functional candidate genes. The list of candidate genes tested for a possible association with QTL included interleukins (IL6, IL10), MH genes (MH1-A or UBA, MH1-B or UAA, MH1-C or MH-II and TAP1) and Toll-like receptors (TLR3, TLR22). For each gene, we searched markers that were polymorphic in the QTL family, either using microsatellite markers known to locate near the gene or by designing specific primers from sequence information available in NCBI or Sigenae databases.

Results and Discussion

Overall survival of the F2 progeny at the end of infectious challenge was around 40%. The survival curve was typical of JIP 02/86 isolate, with mortality starting after a period of 5-7 days and progressing regularly for 3 weeks (Figure 1).

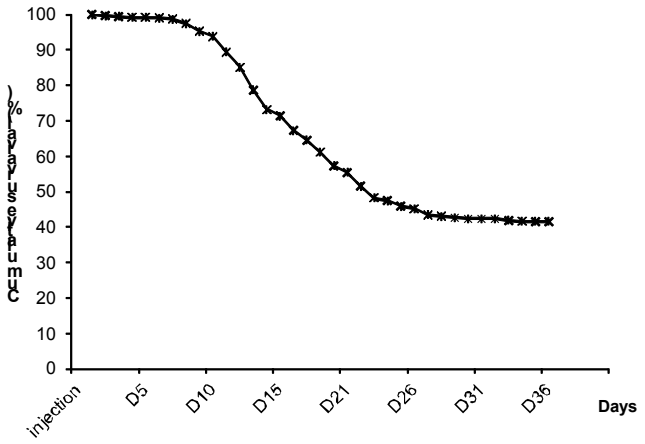

Figure 1. Cumulative survival of the F2 doubled haploid progeny after the infectious challenge (dorsal intramuscular injection of JIP 02/86 *F. psychrophilum* isolate).

QTL for survival were detected on 6 different chromosomes. The 3 most significant ones (P<0.05 at the genome wide level) were located on chromosomes Omy7 (*i.e.* linkage group RT12), Omy17 (RT19) and Omy3

(RT31) (Table 1). Those QTL are different from the ones identified by Wiens et al. (2013b) on chromosomes Omy19 (RT14), Omy16 (RT22) and Omy5 (RT08) in another domestic population of trout. At each of the three QTL, the ultimate survival of fish homozygous for alternative allele at the closest marker ranged from 20-30% to 50-60% respectively. Thus, cumulative effects of QTL resulted in a substantial difference in survival. However, because progeny were all homozygous, no indication of the relative dominance of resistance *vs* susceptibility at the QTL is available.

Table 1. Chromosome location and effect of the main survival QTL detected in the F2 doubled haploid progeny.

Chromosome (linkage group)	Significance*	Relative risk†	Origin‡
Omy7 (RT12)	P<0.001	0.46	R
Omy17 (RT29)	P<0.001	0.34	R
Omy3 (RT31)	P<0.001	0.35	R

* at the chromosome wide level

† with the risk of R allele carrying individuals taken as reference (risk = 1)

‡ F0 grand-parent from which allele resistance is inherited

Position of the different candidate genes is indicated in Table 2. Except TAP1, they were located on linkage groups different from those carrying survival QTL or at a distant position from the QTL. Thus, the potential role of MH genes on Omy14 (RT03) observed by Johnson et al. (2008) was not confirmed in our experimental family. The location of TAP1 on Omy2 (RT27) was confirmed and the associated marker (OMM1080) was at the end of the QTL confidence interval calculated according to Li (2011) which suggested a possible role of TAP1, an antigen peptide transporter, in resistance.

Table 2. Chromosome location of the different candidate genes.

Gene name*	other name†	Chromosome (linkage group)
MH1-A	UBA	Omy18 (RT16)
MH1-B	UAA	Omy24 (RT26)
MH1-C	UCA	Omy14 (RT03)
MH2- DB1	DAB	Omy17 (RT29)
TAP1		Omy2 (RT27)
IL6		Omy14 (RT03)
IL10		Omy7 (RT12)
TLR3		Omy10 (RT20)
TLR22		Omy18 (RT16)

* IMGT MH gene name

†other designation from the literature

Conclusion

Several QTL with substantial effects on survival after infection with *F. psychrophilum* were detected in rainbow trout genome, confirming that resistance is controlled by genetic factors. However, the QTL identified in our experimental cross were different from the main QTL identified by Wiens et al. (2013b), suggesting a complex genetic determinism varying according to families and populations. This may complicate the choice of efficient markers to be used in selection. Suggestive evidence of a contribution of TAP1 to the variability of resistance was found. Other genes, including complement C3, are being tested and the analysis of the spleen bacterial load in the early steps of infection is underway. These results should provide additional clues to better understand the mechanisms underlying the variability of resistance to *F. psychrophilum* in rainbow trout.

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