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Antagonistic effect of indigenous skin bacteria of brook charr (*Salvelinus fontinalis*) against *Flavobacterium columnare* and *F. psychrophilum*

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ABSTRACT

Industrial fish production exposes fish to potentially stressful conditions, which in turn may induce infections by opportunistic pathogens. Probiotics appear to be a promising way to prevent opportunistic infections in aquaculture. In this study, we tested the inhibitory potential of endogenous bacterial communities found in the mucus of brook charr (*Salvelinus fontinalis*) against two major pathogens *Flavobacterium columnare* and *Flavobacterium psychrophilum*. Nine bacterial strains were isolated from brook charr skin mucus and tested for potential antagonistic activity. Results from both agar diffusion assays and broth co-culture assays showed the presence of antagonism. We identified seven bacterial strains, collected from unstressed fish, which exerted strong antagonism against *F. psychrophilum* and/or *F. columnare*. These strains were mixed and used to treat columnaris disease in an *in vivo* experiment in which four distinct fish families were tested. This treatment resulted in a decrease of mortality (54–86%) across fish families indicating that candidates from the host microbiota are potentially suitable for probiotic development. This would allow for the efficient (ability to adhere and colonize the host mucus) and durable management (antagonistic effect against pathogens which would be harmless for the host and safe for its environment) of opportunistic diseases in aquaculture.

1. Introduction

Over the last decade, the aquaculture industry has greatly intensified productivity and is now a major economic activity in many countries (FAO, 2007). Massive production in fish farms may expose fish to stressful conditions, which in turn has the potential to induce infection by opportunistic pathogens (Wakabayashi, 1991). Infections are usually prevented and controlled by intrusive veterinary medicines and chemical substances (Rangdale et al., 1997). However, the beneficial effects of antimicrobial agents are counterbalanced by selection for resistant pathogens (Nematollahi et al., 2003). Therefore, alternative strategies to prevent opportunistic infections in aquaculture are strongly needed. The development of probiotics appears to be one of the most promising ways to reach this goal (Merrifield et al., 2010).

The present study focused on two important opportunistic pathogens in Brook charr (*Salvelinus fontinalis*), namely *Flavobacterium columnare* and *Flavobacterium psychrophilum* (Bernardet and Bowman, 2006). Skin microflora was targeted as a potential source of probiotics because isolates from other parts of the body (e.g., gut) are known to be inefficient at inhibiting the growth of skin pathogens (Spanggaard et al., 2001). Microbial communities are sensitive to various stressful environmental conditions (Schimel et al., 2007). In this respect, we hypothesized that stress may imbalance the bacterial community structure of brook charr skin mucus and

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trigger opportunistic infections. Therefore, our general aim was to test whether natural isolates of non-stressed skin mucus bacterial community exhibit an antagonistic effect against two pathogens *F. columnare* and *F. psychrophilum*.

The specific objectives were: (i) to test the presence of inhibitory compounds in brook charr skin mucus; (ii) to evaluate the competitiveness of eight microbial isolates from skin mucus against *F. columnare* and *F. psychrophilum* for nutrients, (iii) to determine which defensive mechanism was exerted, i.e., competitive exclusion or synthesis of antimicrobial substances, (iv) to confirm the *in vitro* effectiveness of our candidates on an *in vivo* experiment for aquaculture application.

2. Experimental procedure

2.1. Sampling of brook charr bacterial community

Sixteen fish families derived from crosses between different parents were raised at the Laboratoire Régional des Sciences Aquatiques (LARSA) at Université Laval, in Quebec City, QC. Among them, two families were collected from a stress experiment. A total of two individuals per family were sampled. One of them was previously exposed to stressful physiological conditions (hypoxia and handling). Skin mucus was sampled using a sterile razor blade and homogenized after addition of 9 mL sterile water.

2.2. Identification of the bacterial isolates

Fish mucus was diluted in sterile water from a 10 fold to a 10^{-7} dilution. Dilutions were spread on TSA and R2A media (Difco). Bacterial colonies were isolated and individualized by cross streaking and incubated at 20 °C for 48 h. A total of nine isolates were identified by 16S rDNA sequencing. Colonies were used as template DNA for PCR amplification of the 16S rRNA gene using the universal bacterial primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) (Marchesi et al., 1998) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Lane et al., 1985). PCRs were carried out in a volume of 25 μL containing 0.2 mM dNTP (Promega), 0.3 μM each primer, 6.4 μg of BSA, 1.25 mM of $MgCl_2$, 1× of Buffer and 0.4 U of Taq DNA polymerase (Promega) and performed in a Biometra T1 Thermocycler. The following amplification conditions were applied: a first step of initial denaturation at 94 °C for 5 min followed by 28 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 90 s and a final extension step at 72 °C for 10 min. PCR products were visualized by agarose gel electrophoresis [0.8% (w/v) agarose, 100 V]. Fragments were sequenced using the big Dye terminator V3 chemistry on an ABI 3130XL sequencer (Applied Biosystem, Foster City) at the Plateforme d'analyse Biomoleculaire (IBIS, Laval University).

2.3. Media used for culture and competition assay

Three general growth media, R2A (Difco) and TSA (Difco) were used for isolation, identification, and culture of brook charr bacterial community isolates. Both pathogens were grown on their respective general growth media, i.e., Anacker and Ordal (AO) (Anacker and Ordal, 1959). This media was used for competition assays with pathogens, *F. columnare* (ATCC 49418) and *F. psychrophilum* (ATCC 49512) isolated from *Oncorhynchus kisutch* and *Salmo trutta* (Bernardet and Grimont, 1989).

2.4. Screening for antagonistic effect in agar diffusion assay

All host bacterial isolate strains were tested for antagonistic effects using well diffusion assays against *F. columnare* and *F. Psychrophilum*. Melted AO cooled to 45 °C was inoculated with each pathogen to a final density of 10^6 cells mL^{-1} agar and poured into Petri dishes (Gram and Melchiorsen, 1996). A volume of 10 μL of probiotic candidate culture was added into a 3 mm well punched in the solidified agar plates. Plates were incubated at 20 °C and observed for zones of growth around the wells during 48 h (Spanggaard et al., 2001). Individual strains exhibiting competition capabilities were then mixed in three co-cultures: two co-cultures containing the same quantity of all strains with specific antagonistic effect against each pathogen *F. columnare* and *F. psychrophilum* (culture C and culture P) and one co-culture with all strains with antagonistic effect against the two pathogens (culture U). These three co-cultures (30 μL) were added into 3 mm wells punched in the solidified agar plates, and observed for zones of growth around the wells during 48 h to check potential synergistic co-culture effects (Timmerman et al., 2004).

2.5. Screening for antagonistic effect in broth co-culture assay

All antagonistic effects observed in the diffusion agar assay were validated by a broth co-culture assay. Candidates (10^4 cells) were added to 1 mL of AO media in competition with 10^4 cells of the pathogen. These co-cultures were made in triplicates and incubated 48 h at 20 °C. Two mono-cultures of 10^4 cells of each pathogen acted as controls. Growth of both candidates and pathogens was observed by spreading the co-culture on AO agar plate along with morphological identification and counting.

2.6. Screening for antagonistic effect of mucus proteins

Forty-three fish were sampled and all mucus samples were mixed and sterilized with UV light for 25 min to minimize degradation of proteins (Williams and Kraus, 1963). Forty-five microliter of mucus were added to 5 μL (500 cells) of pathogen culture in AO, spread on AO agar plate and incubated at 20 °C for 48 h. After incubation, culture was spread on AO agar to observe the growth of pathogen. A volume of 45 μL of PBS mixed with 5 μL of pathogen culture acted as control.

2.7. Probiotic treatment of fish infection

Seven strains selected for their antagonistic activity during the *in vitro* experiment were tested together in a co-culture for logistical reasons. Four different fish families (i.e., resulting from different parental crosses), all selected on the basis of zootechnical traits of interest (growth and