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Effect of *Flavobacterium psychrophilum* strains and their metabolites on the oxidative activity of rainbow trout *Oncorhynchus mykiss* phagocytes

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ABSTRACT: The oxidative activity of rainbow trout phagocytes was studied using a chemiluminescence technique using 12 different *Flavobacterium psychrophilum* strains and their metabolites. Phagocytes were obtained from the head kidney of rainbow trout *Oncorhynchus mykiss*. The addition of viable *F. psychrophilum* or their metabolites to the phagocytes resulted in an immediate chemiluminescence response. The stimulating effects of both the *F. psychrophilum* and their metabolites on the phagocytes were found to be heat stable. No significant differences in stimulation capacity were found between the strains tested. To investigate the nature of the stimulating agent, both the bacteria and the supernatant were treated with either sodium metaperiodate or polymyxin B. Adding polymyxin B to the bacterial cells and supernatant did not change the chemiluminescence pattern, suggesting that the capacity of *F. psychrophilum* to stimulate the phagocytes probably is not due to lipopolysaccharides (LPS). However, following incubation of the bacteria and their metabolites with sodium metaperiodate, the capacity to stimulate phagocytes was significantly impaired. This suggests that a carbohydrate component most likely plays an important role in the ability of *F. psychrophilum* to stimulate phagocytes. Opsonisation of the bacteria with native trout serum or with rabbit anti-*F. psychrophilum* serum resulted in an additional chemiluminescence peak which was significantly higher than the first peak. This extra peak disappeared following heat treatment of the trout serum and the rabbit anti-*F. psychrophilum* serum, pointing towards the involvement of heat labile complement in opsonisation.

KEY WORDS: *Flavobacterium psychrophilum* · Chemiluminescence · Oxidative burst · Rainbow trout · Phagocytes

INTRODUCTION

Rainbow trout fry syndrome (RTFS) is currently recognised as a serious bacterial disease affecting hatchery reared rainbow trout *Oncorhynchus mykiss* fry and fingerlings in many parts of Europe (Lorenzen & Olesen 1997, Madsen & Dalsgaard 1999). The aetiological agent is *Flavobacterium psychrophilum* (Bernardet et al. 1996), a Gram-negative filamentous chromogenic rod (Wood & Yasutake 1956, Holt et al. 1993). In outbreaks of RTFS, mortalities of 10 to 30% are involved, and are quite often as high as up to 70% (Chua 1991, Bruno 1992, Santos et al. 1992).

Despite the importance of the disease, there is a significant lack of knowledge on the way the pathogen interacts with its host and causes disease (Rangdale 1995). For example, little is known about the role of phagocytes as a host defense mechanism. Rangdale (1995) demonstrated phagocytic activity of a granulocyte-like cell in which *Flavobacterium psychrophilum* was seen enclosed within phagocytic vesicles surrounded by refractile granules. The interaction between *F. psychrophilum* and phagocytes is of interest, as fish phagocytes play a vital role in both the specific and non-specific defence against micro-organisms (Secombes & Fletcher 1992). One of the key elements of the bactericidal action of phagocytes is termed the respiratory burst. This process of phagocytosis and subsequent killing of ingested pathogens in the phago-

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lysosomes is associated with the production and release of highly reactive oxygen metabolites (Rossi et al. 1985). Reversion of these unstable oxygen metabolites to their basic state is responsible for the emission of photons, which is termed chemiluminescence (CL). Luminol, a cyclic hydrazide, enhances the magnitude of the light produced during CL (Van Dyke et al. 1977).

The main purpose of the present study was to investigate the early interaction of *Flavobacterium psychrophilum* and their metabolites with rainbow trout head kidney phagocytes and to preliminary characterize the agent responsible for this interaction. This was done using luminol-enhanced chemiluminescence.

MATERIAL AND METHODS

Phagocytes. Forty-five rainbow trout *Oncorhynchus mykiss*, weighing 300 to 500 g, were obtained from a trout farm in Dilbeek, Belgium. Fish were acclimatized for at least 2 wk in 150 l aquaria containing recirculated tap water of 12 to 14°C. Fish were daily fed dry commercial pellets ad libitum (Extruvet, Trouw, The Netherlands). All trout used in the studies appeared to be healthy. Five individuals were sacrified for bacteriological and parasitological examination. *Flavobacterium psychrophilum* was not isolated on Shieh agar (Shieh 1980) from samples of skin, gills, kidney, spleen and brain. Parasite infestations were not noted upon examination of wet mount preparations made from the skin and gills.

From the other trout, head kidney phagocytes were collected according to the procedure of Sørensen et al. (1997) with some modifications. Briefly, fish were euthanized using an overdose of a solution of benzocaine (1 g benzocaine [ethyl aminobenzoate] [Federa, Brussels, Belgium] in 10 ml acetone). The cranial part of the kidneys (±3 g) were aseptically removed and pushed through a 150 µm nylon mesh (Solana NV, Schoten, Belgium) with Leibovitz L-15 medium (Gibco, Merelbeke, Belgium) containing 2% fetal calf serum (FCS; Gibco), 100 U ml^{-1} penicillin/streptomycin (P/S; Gibco) and 10 U ml^{-1} heparin (Leo Pharmaceutical products, Vilvoorde, Belgium). The resultant cell suspension was layered on top of a discontinuous 34%/51% Percoll gradient (Pharmacia Biotech, Roosendael, The Netherlands). Following centrifugation (400 × *g*, 25 min, 4°C) in a conical centrifuge tube, the band of cells lying at the 34/51% interface was collected and washed once with sterile Hanks' balanced salt solution (HBSS) without Ca/Mg (Gibco). The cells were resuspended in L-15 medium supplemented with 0.1% FCS and 100 U ml^{-1} P/S and adjusted to 10^7 phagocytes ml^{-1}. A Hemacolour stain of the phagocytes was performed in order to detect the percentage of macrophages and neutrophils. Their viability was determined by exclusion of trypan blue. The phagocytes were used in CL assays immediately after collection, except for the assays in which the viability of the phagocytes was assessed until Day 2.

Bacterial strains, supernatant collection and opsonisation. Twelve different *Flavobacterium psychrophilum* strains were used in these studies. The strains and their sources are given in Table 1. Stock suspensions were stored at –70°C. After thawing, bacteria were grown for 4 d in 4 ml Shieh broth (Shieh 1980) at 17°C. Subsequently, 0.5 ml was transferred to 10 ml Shieh broth and grown for 3 d at 17°C. The cultured broth was centrifuged (10 000 × *g*, 10 min, 4°C) and the resulting pellet and supernatant were separated.

The pellet was resuspended in phosphate buffered saline (PBS). The suspension was checked for purity and the number of colony forming units (CFU) was determined by plating 10-fold serial dilutions on Shieh plates. The bacterial suspensions were immediately used in the CL assays.

The supernatant was filtered through a 0.2 µm filter (Cornins Laboratory Sciences Company, New York, USA) and used undiluted, 1/10 or 1/20 diluted in PBS in the CL assays.

In order to evaluate their heat sensitivity, both bacteria and supernatant were heated for 20 min at 100°C before testing.

Table 1. *Flavobacterium psychrophilum* strains used in the chemiluminescence assays. NK: serotype not known

Strain	Origin	Serotype
32/97	Rainbow trout *Oncorhynchus mykiss*, Chile, 1997	NK
JIP 30/98	Eel *Anguilla anguilla*, France, 1998	NK
B97034 E4	Rainbow trout, Scotland, 1997	NK
B97026 P1	Rainbow trout, Scotland, 1997	NK
Dubois	Rainbow trout, Belgium, 1998	NK
OSU 84-254	Coho salmon *Oncorhynchus kisutch*, British Colombia, 1984	NK
UCD 004-95	Rainbow trout, Idaho, USA, 1995	NK
SVS 910611-1	Rainbow trout, Denmark, 1991	Fp^T
FPC 840	Ayu *Plecoglossus altivelis*, Japan, 1987	O_2
LVDI 5/I	Carp *Cyprinus carpio*, France, 1992	Fp^T
950106-1/1	Rainbow trout, Denmark, 1995	Fd
99/10A	Rainbow trout, Denmark, 1995	Fp^T

Strain sources: 32/97, JIP 30/98, B97034 E4 and B97026 P1: strains provided by K. Thompson, University of Stirling, Stirling, Scotland. Dubois: strain provided by F. Lieffrig, Centre d'Economie Rurale, Marloie, Belgium OSU 84-254, UCD 004-95, SVS 910611-1, FPC 840 and LVDI 5/I: strains provided by J.-F. Bernardet, Unité de Virologie et Immunologie Moléculaires, Institut National de la Recherche Agronomique, Centre de Recherches de Jouy-en-Josas, France. 950106-1/1 and 99/10A: strains provided by I. Dalsgaard, Danish Institute for Fisheries Research, Denmark

For opsonisation, an equal volume of a viable and heat-inactivated bacterial suspension of each *Flavobacterium psychrophilum* strain in PBS was incubated with heat treated (30 min, 60°C) or non-treated (viable) *F. psychrophilum* antiserum against the Belgian Dubois strain.

The antiserum was prepared in rabbits according to the procedure of Decostere et al. (1999a). The titer of agglutinating antibodies of the antiserum against the 12 *Flavobacterium psychrophilum* strains varied between 1:64 and 1:128.

Additionally, an equal volume of a viable and heat-inactivated bacterial suspension of each *Flavobacterium psychrophilum* strain in PBS was incubated with either native rainbow trout serum or heat treated (30 min at 56°C) native trout serum.

All incubations of the bacterial cells with the sera occurred for 15 min at 17°C. Following, each suspension was centrifuged (10 000 × *g*, 10 min, 4°C), the supernatant was discarded, the pellet resuspended in PBS and used in the CL assays immediately.

Chemiluminescence assay. The CL assays were performed in a 6-channel luminometer (Lumicon, Hamilton, Bonaduz, Switzerland), using polystyrene biolum cuvettes (Lumac, Landgraaf, The Netherlands). Luminol (Sigma, St. Louis, MO, USA) was used as chemiluminigenic probe (CLP). Luminol was dissolved in dimethyl sulfoxide to give a concentration of 104 mM and stored in small aliquots at −70°C. Prior to use, the luminol stock was thawed and diluted (1/50) in HBSS to a final concentration of 400 µmol l^{-1}. The cuvettes were not stirred during the assay. The protocol of the CL assays was as follows. The cuvettes were filled with 500 µl of purified phagocytes (10^7 ml^{-1}) suspended in L-15 medium supplemented with FCS and P/S. After 3 h of incubation at 17°C allowing adherence of the cells, the medium was discarded and replaced by 400 µl HBSS and 100 µl of the diluted luminol solution in HBSS. The cuvettes were placed in the luminometer and the CL background value was recorded for at least 15 min. The data were collected at 10 s intervals and presented as counts per minute (cpm). After stabilisation of the background value, 20 µl of one of the products to be tested for triggering activity (see below) was added per cuvette. The CL response was recorded for 45 min and expressed as cpm. The peak value (PV) and time to peak (TP) was determined. Each CL assay was repeated 4 times at 15°C.

Influences of different *Flavobacterium psychrophilum* strains, their supernatant or other triggering agents on the respiratory activity of phagocytes. In each CL assay, Zymosan A was used as a positive control. For that purpose, a suspension of 25 mg Zymosan A (Sigma) in 10 ml HBSS was heated for 30 min in a water bath at 90°C. After cooling, the suspension was aliquoted and stored at −70°C. The final assay concentration of Zymosan A was 9.6×10^{-3} mg ml^{-1}.

Additionally, in each CL assay, a negative control sample was included, consisting of macrophage-free cuvettes supplemented with the agent to be tested for triggering activity.

An initial experiment to investigate the effect of the viable and heat treated bacterial cells of the different *Flavobacterium psychrophilum* strains on the oxidative activity of phagocytes was carried out. As stated above, following the recording of the background value, 20 µl of PBS containing different numbers of CFU (10^5, 10^6, 10^7 or 10^8) of each viable strain was added to the cuvettes. Additionally, 10^7 CFU of heat-treated bacteria were added to evaluate heat sensitivity. An additional negative control sample was included, consisting of 400 µl HBSS, 100 µl diluted luminol suspension in HBSS and 20 µl PBS without bacteria.

To determine whether the bacteria remain viable following contact with the phagocytes, at the end of the run in which 10^7 CFU of each viable strain was used as the triggering agent and 2, 6 and 16 h later, the number of CFU $cuvette^{-1}$ was determined by plating 10-fold serial dilutions on Shieh plates. After 4 d of incubation at 17°C, the number of CFU was compared at each sampling time between the macrophage supplemented and the macrophage free cuvettes.

In the second experiment, the capacity of the native and heat-treated native supernatant of each *Flavobacterium psychrophilum* strain to stimulate the phagocytes was investigated. For that purpose, the native supernatant (undiluted and 1/10 or 1/20 diluted in PBS) or heat-treated native supernatant (undiluted) was added to the cuvettes. In this case, as an additional negative control, a cuvette supplemented with uncultured Shieh broth was included.

A third assay was performed to determine the effect of opsonisation of *Flavobacterium psychrophilum* on their capacity to stimulate phagocytes. Bacterial cells opsonised with rabbit antiserum, native trout serum, heat-treated rabbit antiserum or heat-treated native trout serum were added to the cuvette. As a negative control, native rainbow trout serum or rabbit antiserum without *F. psychrophilum* were used in the CL assay.

A final assay was carried out to determine the nature of the stimulating agent in the CL assays. For that purpose, polymyxin B (PMB; Prodivet Pharmaceutical, Eynatten, Belgium) (100, 250 or 500 U ml^{-1}) or sodium metaperiodate (SMP; Sigma) (10 mg l^{-1}) were added to the bacterial suspensions and the supernatant. After 15 min of incubation at 17°C, the suspension was centrifuged (10 000 × *g*, 10 min, 4°C). The supernatant or the bacteria, washed and resuspended in PBS, were consequently used in CL assays.

At the end of each of the 4 CL assays, 2 h, 6 h, 1 d and 2 d later, in order to check the viability of the phagocytes, the stimulating agent Zymosan A was added and the CL response measured.

Statistical analysis. For the evaluation of the oxygenation activity, in each assay, PV and TP of the 12 different strains were transformed to natural logs and compared with the 1-way analysis of variance (ANOVA) (computer program STATISTIX 4.1, Analytical software) and Scheffé's pairwise comparison of means. A significance level of 0.05 was used.

RESULTS

Collection of phagocytes

Approximately 10^8 phagocytes were harvested from each rainbow trout head kidney. The phagocytes contained 80% of macrophage-like cells, together with 20% of neutrophils. The viability exceeded 98%, however, after 1 d viability decreased significantly. There was no more activity recorded after 2 d. Following freezing, storage in liquid nitrogen and thawing, the viability of the phagocytes was reduced to 10%. For that reason, only phagocytes that were collected the same day were used in CL assays.

Table 2. Luminol enhanced chemiluminescence from fish phagocytes stimulated with 10^7 CFU of different *Flavobacterium psychrophilum* strains

Strain	Peak value (cpm × 10^3)	Time to peak (min)
32/97	226.1 ± 12.9	3
JIP 30/98	234.3 ± 13.6	3
B97034 E4	226.3 ± 17.2	3
B97026 P1	246.7 ± 11.9	3
Dubois	254.4 ± 10.3	3
OSU 84-254	238.3 ± 11.3	3
UCD 004-95	250.3 ± 16.2	3
SVS 910611-1	227.2 ± 18.3	3
FPC 840	228.3 ± 12	3
LVDI 5/I	218.1 ± 18.1	3
950106-1/1	230.5 ± 16.3	3
99/10A	217.6 ± 12.6	3

Chemiluminescence assays

The effects of the addition of the various triggering agents on the CL response of the fish phagocytes are given in Tables 2 & 3 and depicted in Fig. 1.

Table 3. Luminol enhanced chemiluminescence from fish phagocytes stimulated with different triggering agents. NS: not significant; I: first peak value; II: second peak value

Triggering agent	Final concentration	Treatment	Peak value (cpm × 10^3)[a]	Time to peak (min)[b]
Zymosan A	9.6 mg 100 ml^{-1}	–	690.3 ± 31.2	12
Flavobacterium psychrophilum	10^5 CFU	–	141.5 ± 10.5	3
F. psychrophilum	10^6 CFU	–	185.1 ± 12.6	3
F. psychrophilum	10^7 CFU	–	233.2 ± 14.2	3
F. psychrophilum	10^8 CFU	–	231.9 ± 13.2	3
F. psychrophilum	10^7 CFU	Heat	229.4 ± 12.6	3
Supernatant	Undiluted	–	540.0 ± 21.1	3
Supernatant	1/10	–	380.4 ± 20.0	3
Supernatant	1/20	–	NS	–
Supernatant	Undiluted	Heat	529.3 ± 20.3	3
F. psychrophilum	10^7 CFU	Opsonisation with native trout serum	I: 230.2 ± 13.2 II: 390.1 ± 17.2	3 25
F. psychrophilum	10^7 CFU	Opsonisation with heat-inactivated native trout serum	230.3 ± 13.5	3
F. psychrophilum	10^7 CFU	Opsonisation with rabbit Dubois antiserum	I: 224.3 ± 12.3 II: 350.6 ± 16.2	3 22
F. psychrophilum	10^7 CFU	Opsonisation with heat-inactivated rabbit Dubois antiserum	233.2 ± 14.1	3
F. psychrophilum	10^7 CFU	Sodium metaperiodate	NS	–
F. psychrophilum	10^7 CFU	Polymyxin B	234.8 ± 14.3	3
Supernatant	Undiluted	Sodium metaperiodate	NS	–
Supernatant	Undiluted	Polymyxin B	536.2 ± 19.9	3

[a]Each value represents the mean ± SD of the 12 strains tested in 4 independent experiments
[b]Each value represents the mean of the 12 strains tested in 4 independent experiments

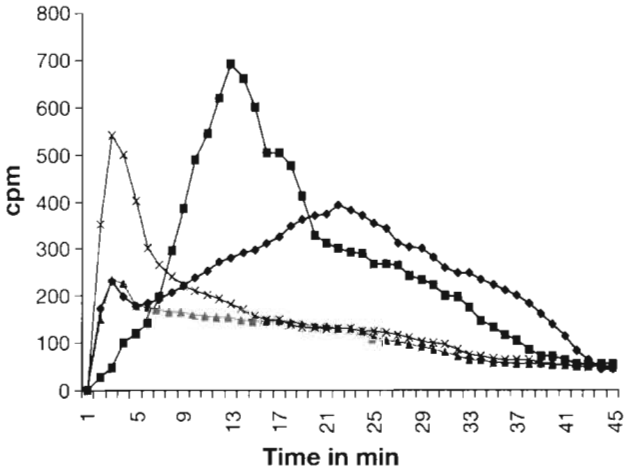

Fig. 1. Chemiluminescence pattern of head kidney phagocytes induced by 10^7 CFU viable *Flavobacterium psychrophilum* (—▲—), 10^7 CFU viable *F. psychrophilum*, opsonised with native trout serum (—◆—), undiluted supernatant (—×—) and Zymosan A (—■—)

When Zymosan A was added to the phagocytes (positive control), the PV was 690 cpm with a TP of 12 min. All negative controls included in each of the 4 assays gave no PV.

Adding 10^5, 10^6, 10^7 or 10^8 CFU of the viable *Flavobacterium psychrophilum* strains stimulated the oxygenation activity of the phagocytes significantly ($p < 0.05$). Having reached a PV after approximately 3 min, the amount of light emission decreased (Fig. 1). The PV were not significantly different between the 12 strains tested (Table 2). Therefore, the PV were pooled and averaged, resulting in mean PV for each bacterial concentration tested (Table 3). Within each strain, the PV was highest after addition of 10^7 CFU, which was used as the final concentration in all further assays. Lower bacterial titers resulted in lower PV, while a higher titer did not induce a significantly higher PV.

Similarly, no significant differences were found in TP values between the 12 strains tested nor between the different bacterial titers within the strains. Therefore, the TP values of all strains were pooled and averaged (Table 3).

The treatment of the bacterial cells with heat did not significantly impair their ability to stimulate the phagocytes.

Concerning the viability of the bacteria following contact with the phagocytes, it was noted that the number of CFU (10^7) of all *Flavobacterium psychrophilum* strains decreased in the course of time. After 2 and 6 h, respectively 10^4 CFU and 4×10^2 CFU was cultivated on agar. After 16 h, *F. psychrophilum* could no longer be cultivated on agar. This phenomenon was noted in both the macrophage supplemented cuvettes and the cuvettes devoid of phagocytes hence merely containing the bacterial cells.

The addition of the undiluted or 1/10 diluted supernatant to the phagocytes resulted in a PV which was significantly higher compared to the PV caused by the bacterial cells ($p < 0.05$). Again, no significant differences in PV and TP were noted between the supernatant of the 12 *Flavobacterium psychrophilum* strains tested and results were therefore pooled and averaged. The 1/20 diluted supernatant gave no significant PV. As with the bacterial cells, heat treatment of the supernatant did not abrogate its capacity to stimulate phagocytes.

Opsonisation of the bacterial cells with uninactivated rabbit antiserum and native trout serum resulted in a second CL peak, which occurred 20 min later and was significantly higher compared to the first peak. Opsonizing the bacterial cells with heat treated native trout serum and rabbit antiserum however did not result in the additional peak as induced by non treated serum. Again, no significant differences in response to opsonisation was noted between the 12 *Flavobacterium psychrophilum* strains tested.

The incubation of the supernatant and the bacterial cells with polymyxin B did not significantly influence their capacity to stimulate the phagocytes. However, sodium metaperiodate treated bacterial cells and supernatant were no longer able to induce a significant CL response in the phagocytes.

Regarding the viability of the phagocytes after each CL run, the following was noted. At the end of each assay, 2 and 6 h later, the phagocytes could still be stimulated by Zymosan A. The height of the obtained peak (670 cpm ± 30.02) and the TP values (12 min) were similar to the values obtained with immediate stimulation. After 1 d, the PV decreased significantly (450 cpm ± 51.54) and after 2 d, there was no PV recorded.

DISCUSSION

The present study reveals that the CL assay is suitable for studying the influence of *Flavobacterium psychrophilum* and their metabolites on the respiratory burst activity of rainbow trout head kidney phagocytes. To our knowledge, this is the first report of a CL assay for this fish pathogen. This study hence significantly contributes to the elucidation of the interaction between *F. psychrophilum* and the innate host immune system.

We demonstrated that injection of rainbow trout phagocytes with different *Flavobacterium psychrophilum* strains induced an immediate respiratory burst

response. This peak may suggest that 'de novo' synthesis is not required for an increased capacity to release oxidative radicals. The NADPH oxydase can in some instances be activated within seconds, as demonstrated by Adams & Hamilton (1984).

Regarding the viability of the bacteria following contact with the phagocytes, it was found that the number of CFU of *Flavobacterium psychrophilum* in the cuvettes decreased with time. However, there was no significant difference in bacterial survival between the macrophage free and the macrophage supplemented cuvettes. This suggests that the decreasing number of CFU of *F. psychrophilum* is not due to the contact with the phagocytes but rather to the inability of the bacteria to survive in the HBSS and luminol medium. Additionally, attention should be drawn to the fact that, using the above enumeration method, only the extracellular bacteria are enumerated. This ignores the possibility of bacterial internalization or even intracellular survival, as has been noted for fish pathogens such as *Renibacterium salmoninarum* (Gutenberger et al. 1997) or *Aeromonas salmonicida* (Garduno et al. 1993). Further studies to investigate the stages following initial contact between bacteria and phagocytes and to examine the eventual occurrence of bacterial survival and/or multiplication within fish phagocytes are planned.

Although the Danish *Flavobacterium psychrophilum* strains (the virulent elastin-degrading strain 950106-1/1 and the avirulent elastin-non-degrading strain 99/10A) induced different rates of mortality (respectively 71% after injection of 4.5×10^5 CFU ml^{-1} and 7% after injection of 2×10^5 CFU ml^{-1}) in rainbow trout following intraperitoneal injection of the bacterial cells (Madsen & Dalsgaard 1999), we did not observe any significant differences between the strains in their capacity to stimulate the phagocytes. This may be due to different reasons. A first explanation may be that, adapting the CL technique, we only investigated the initial stages of the contact between the phagocytes and the bacteria. Indeed, adherence of a pathogen to the phagocyte membrane or vice versa can be sufficient to cause the release of oxidative radicals. Hence, ingestion does not appear to be necessary for the respiratory burst to occur. Differences between the strains, although not observed in the CL assays, may be present in the stages following initial contact of the bacterium phagocyte interaction. These differences may then be reflected in the inequality in virulence. Another reason may certainly be that other factors are involved in virulence such as the ability to produce proteolytic enzymes and/or toxins, the capacity to spread in the body or the ability to sequester iron (Dalsgaard 1993). These aspects and the way they interrelate merit further investigation.

There was no significant difference in CL values between heat treated and viable *Flavobacterium psychrophilum* bacterial cells, indicating that the stimulating component is heat stable and hence most likely not of protein nature. Incubation of *F. psychrophilum* bacterial cells with sodium metaperiodate, which modifies the structure of surface-borne antigens, impaired their capacity to stimulate the phagocytes. This finding linked to the heat stable trait of the stimulating agent points towards the suggestion that the initial stage of interaction of *F. psychrophilum* with phagocytes most likely involves recognition of bacterial surface sugars by the fish phagocytic cell. Indeed, for several bacterial pathogens the capsular polysaccharides have been shown to mediate adherence to phagocytes (Pruimboom et al. 1996, Keisari et al. 1997, Maganti et al. 1998). Rangdale (1995) suggested that cells of *F. psychrophilum* do possess a surface extracellular polysaccharide layer as a capsule. She speculated that this capsule aids in motility and adhesion to host cells. It is worthwhile to make the comparison with *F. columnare*, the warmwater counterpart of *F. psychrophilum*. This pathogen appears to be surrounded by a polysaccharide capsule which most likely mediates adhesion to the host tissue (Decostere et al. 1999b).

Pacha (1968) noted the actively proteolytic nature of *Flavobacterium psychrophilum*, suggesting this plays an important role in the pathogenicity of the organism. Likewise, Otis (1984) described extracellular enzymes produced by several *F. psychrophilum* strains capable of degrading collagen, fibrinogen, chondroitin sulphate and fish muscle extract (Dalsgaard 1993). We were able to demonstrate that the metabolites and/or bacterial components present in the supernatant of *F. psychrophilum* are able to stimulate head kidney phagocytes. The agent responsible for stimulation was heat stable, excluding the possibility of the involvement of the above mentioned exotoxins, which are known to be heat lable. In an attempt to investigate the possible role of heat stable lipopolysaccharides (LPS) in the ability of the supernatant to stimulate phagocytes, the antibiotic polymyxin B was adopted. This antibiotic is capable of binding to and neutralizing lipid A, the biologically active component of LPS. The addition of polymyxin B did not inhibit the supernatant in its capacity to elicit a significant CL response. It could thus be speculated that the stimulation of the phagocytes is most likely not due to LPS. The abolishing effect of sodium metaperiodate on the ability of the supernatant to stimulate phagocytes and the heat stability of the stimulating component point towards the possible involvement of a carbohydrate component present in the growth medium. Whether this is due to a partial shedding of an eventual polysaccharide layer (see above) in the growth medium or the actual release

of extracellular carbohydrates remains to be elucidated in the future.

In the present study, we investigated the role played in opsonisation by complement of *Flavobacterium psychrophilum*. Opsonisation of *F. psychrophilum* bacteria, with rabbit antiserum or native trout serum resulted in the formation of an additional CL peak, indicating a successful opsonisation of the bacteria. The use of heat-inactivated native rainbow trout serum and rabbit antiserum abrogated the above additional CL peak. These opsonisation data demonstrate that complement receptors take an important part in opsonophagocytosis of *F. psychrophilum* in rainbow trout.

In conclusion, it can be stated that this study demonstrates the capacity of *Flavobacterium psychrophilum* and their metabolites to stimulate rainbow trout phagocytes. The stimulating component appears to be heat stable, insensitive to polymyxin B and sensitive to sodium metaperiodate treatment, pointing towards a carbohydrate nature. Studies are underway to characterize the agent responsible for stimulation and to delineate its precise role in the pathogenesis of the disease.

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