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Hemolytic activity in *Flavobacterium psychrophilum* is a contact-dependent, two-step mechanism and differently expressed in smooth and rough phenotypes

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ABSTRACT

The hemolytic activity of cells of smooth and rough phenotypic variants of the Gram-negative fish pathogen *Flavobacterium psychrophilum* was investigated in two different assays, a microplate and an agarose hemolysis assay, using rainbow trout erythrocytes. The smooth cells showed a high and the rough cells a negligible, concentration dependent, hemolytic activity in the microplate assay. Both smooth and rough cells showed a rather weak hemolytic activity, with two distinct hemolytic patterns, in the agarose assay. The hemolytic activity of the cells was not regulated by iron availability and cell-free extracellular products did not show any hemolytic activity. The smooth cells, in contrast to the rough cells, showed a high ability to agglutinate erythrocytes and both hemagglutination and hemolytic activity was impaired by treatment of the cells with sialic acid. The hemolytic activity was furthermore reduced after proteolytic and heat treatment of the cells. The results from the present study suggest that the hemolytic activity in *F. psychrophilum* is highly expressed in the smooth phenotype, and that it is a contact-dependent and two-step mechanism that is initiated by the binding of the bacterial cells to the erythrocytes through sialic acid-binding lectins and then executed by thermolabile proteinaceous hemolysins.

1. Introduction

A significant trait of a pathogenic bacterium is the ability to obtain iron from the host's cells. One mechanism for iron acquisition is the ability of the bacteria to lyse host erythrocytes, i.e. have a hemolytic activity and produce hemolysins that release iron bound to heme or hemoglobin [1].

For *Flavobacterium psychrophilum*, a Gram-negative bacterium causing systemic infections in mostly farmed freshwater salmonids known as bacterial cold water disease [2], the hemolytic activity has not been properly investigated. The distinct anemia in fish infected with *F. psychrophilum* does indicate an interaction between the bacterium and the host's erythrocytes. *F. psychrophilum* does not, however, grow on normally used blood agar that is rich in nutrients as the bacterium optimally grows on media with low nutrient content [2]. *F. psychrophilum* has been found to partially lyse rainbow trout erythrocytes incorporated into low nutrient media [3] and to be able to degrade hemoglobin [4]. The fact that some *F. psychrophilum* isolates are able to agglutinate erythrocytes through a sialic acid-binding lectin [5], is an indication of a possible hemolytic activity in *F. psychrophilum* as adhesion to erythrocytes is important in contact-dependent hemolysis [6]. As *F. psychrophilum* is described as highly proteolytic [2], contact-independent hemolysis through secreted extracellular products (ECPs) containing hemolysins, as e.g. in *Aeromonas hydrophila* [7], could be another alternative of iron acquisition for this pathogen.

Flavobacterium species with known hemagglutinating and contact-dependent hemolytic activity includes *Flavobacterium meningosepticum* and *Flavobacterium indologenes*, two opportunistic human pathogens causing infantile meningitis. The hemagglutinating and hemolytic reactions of these *Flavobacterium* species, caused by lipoamino acids, are known to function by initially adhering to and then lysing the erythrocytes by the loss of one molecule of fatty acid from the lipoamino acid [8].

The objective of this study was to investigate the possible hemolytic activity of *F. psychrophilum* and differences in the activity between phenotypic variants. As experimental isolates, four smooth and four rough phenotypic variants of *F. psychrophilum* were used. Phase variation occurs in this pathogen, and these two phenotypes have previously been reported to possess different agglutinating, adhesive and hydrophobic characteristics [9]. Two different hemolysis assays, a microplate and an agarose assay, were examined as the use of low nutrient blood agar in pilot studies was not a sensitive

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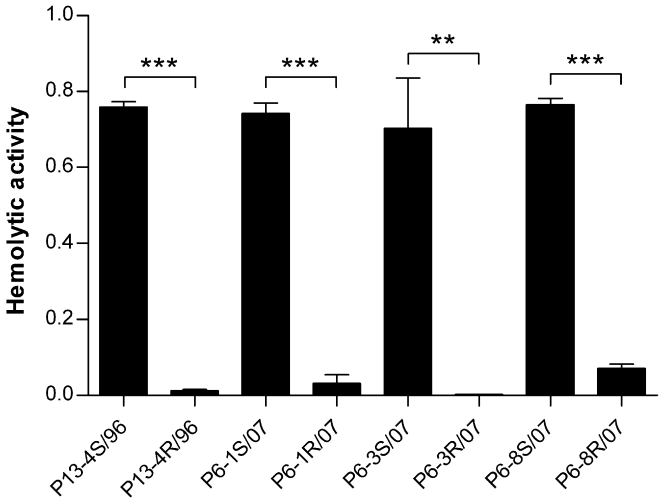

Fig. 1. Microplate assay hemolytic activity (mean ± SD) of cells of *Flavobacterium psychrophilum* smooth (S) and rough (R) phenotypic variants. Statistical differences (*T*-test) between corresponding smooth and rough cells: **, $P < 0.01$; ***, $P < 0.001$.

enough method to detect hemolytic activity in this pathogen. By using two approaches to investigate the hemolytic activity, different hemolytic mechanisms could also possibly be detected.

2. Results

The hemolytic activity of the smooth cells was significantly higher in the microplate assay compared with the hemolytic activity of the corresponding rough cells (Fig. 1). The hemolytic activity of the smooth and rough cells, except for cells of variant P6-3R/07, also significantly decreased with decreasing bacterial cell concentration (Table 1). In the microplate assay, the hemolytic activity of the rough cells showed furthermore a positive correlation with increasing temperature. The hemolytic activity of the smooth cells, on the other hand, did not show any correlation with temperature and was constantly high (Table 2).

No difference in hemolytic activity could be found between corresponding smooth and rough cells with the agarose assay, except that the cells of the P6-3R/07 variant did not show any hemolytic activity at all. Two different hemolysis patterns were, however, noted with the agarose assay. The first hemolysis pattern appeared after 24 h as an incomplete hemolysis zone that was rapidly spreading from the inoculum and was difficult to interpret. After 72 h, a second hemolysis pattern appeared as a narrow, transparent and complete hemolysis zone around the inoculum. Incorporation of catalase in the agarose did not affect these hemolysis patterns in any way.

Table 2
Microplate assay hemolytic activity (mean ± SD) of cells of *Flavobacterium psychrophilum* smooth (S) and rough (R) phenotypic variants at three different temperatures (5, 10 and 15 °C). Pearson correlation was tested between hemolytic activity of variants and temperature. Significant correlations: *, $P < 0.05$; **, $P < 0.01$.

Variant	5 °C	10 °C	15 °C	Pearson correlation
P13-4S/96	0.922 ± 0.029	0.759 ± 0.015	0.875 ± 0.155	$r = -0.501$
P13-4R/96	0.012 ± 0.002	0.012 ± 0.003	0.022 ± 0.001	$r = 0.799$*
P6-1S/07	0.920 ± 0.039	0.742 ± 0.028	0.833 ± 0.086	$r = -0.411$
P6-1R/07	0.023 ± 0.004	0.032 ± 0.022	0.200 ± 0.095[a]	$r = 0.777$*
P6-3S/07	0.919 ± 0.055	0.703 ± 0.132	0.843 ± 0.134	$r = -0.243$
P6-3R/07	0.001 ± 0.001	0.002 ± 0.000	0.010 ± 0.002	$r = 0.851$**
P6-8S/07	0.906 ± 0.025	0.766 ± 0.016	0.949 ± 0.011	$r = 0.218$
P6-8R/07	0.034 ± 0.006	0.070 ± 0.016	0.319 ± 0.242	$r = 0.682$*

[a] $n = 2$.

Smooth and rough cells grown on TYES-DPD showed no difference in hemolytic activity with the microplate assay compared with bacterial cells grown on TYES agar. The exception was cells of variant P13-4R/96 grown on TYES-DPD that showed significantly lower hemolytic activity compared with cells grown on TYES agar. ECPs of smooth variants grown on TYES-DPD showed significantly inferior hemolytic activity compared with cells grown on TYES-DPD and TYES agar. The hemolytic activity of ECPs of rough variants was negligible, but only the activity of ECPs of variant P13-4R/96 was significantly lower compared with cells grown on TYES agar (Table 3). With the agarose assay, no difference in hemolytic activity could be found between bacterial cells grown on TYES-DPD and TYES agar. ECPs of both smooth and rough variants did not show any hemolytic activity at all with the agarose assay.

All heat inactivated smooth cells and cells of the rough variant P13-4R/96 showed significantly inferior hemolytic activity compared with untreated cells in the microplate assay (Table 4). The cells of the rough variant P6-3R/07 showed constantly a low hemolytic activity but the activity was significantly elevated when the cells were heat inactivated. All formalin inactivated smooth cells, except for cells of variant P6-8S/07, showed significantly lower hemolytic activity compared to untreated cells. The hemolytic activity of formalin inactivated rough cells of the variant P13-4R/96 was significantly inferior compared with the untreated cells (Table 4). Neither inactivated smooth nor rough cells showed hemolytic activity with the agarose assay.

In the microplate assay, the hemolytic activity of smooth cells treated with proteinase K and trypsin was significantly inferior compared with the untreated controls. The hemolytic activity of the rough cells of variant P6-1R/07 was, on the other hand, significantly elevated after proteinase K treatment compared with the hemolytic activity of the untreated cells (Table 5). Sodium (meta)periodate treatment of smooth cells did not affect their hemolytic activity. For the rough cells of variant P13-4R/96 the activity was significantly

Table 1
Microplate assay hemolytic activity (mean ± SD) of cells of *Flavobacterium psychrophilum* smooth (S) and rough (R) phenotypic variants of diluted bacterial suspensions. The 1:1 suspension was adjusted with 0.5% NaCl to an OD of 0.45 ± 0.02 at 520 nm. Spearman correlation was tested between hemolytic activity of variants and dilutions. Significant correlations: **, $P < 0.01$.

Variant	Bacterial dilutions in 0.5% NaCl							Spearman correlation
	1:1	1:2	1:5	1:10	1:50	1:100	1:500	
P13-4S/96	0.844 ± 0.011	0.636 ± 0.074	0.192 ± 0.008	0.148 ± 0.016	0.030 ± 0.011	0.019 ± 0.004	0.004 ± 0.002	$r_s = 0.983$**
P13-4R/96	0.021 ± 0.004	0.012 ± 0.003	0.012 ± 0.002	0.006 ± 0.010	0.009 ± 0.005	0.001 ± 0.001	0.000 ± 0.000	$r_s = 0.788$**
P6-1S/07	0.638 ± 0.147	0.325 ± 0.273	0.085 ± 0.030	0.036 ± 0.018	0.004 ± 0.004	0.002 ± 0.003	0.000 ± 0.000	$r_s = 0.956$**
P6-1R/07	0.023 ± 0.004	0.039 ± 0.022	0.038 ± 0.015	0.039 ± 0.008	0.007 ± 0.004	0.000 ± 0.001	0.001 ± 0.002	$r_s = 0.695$**
P6-3S/07	0.798 ± 0.059	0.615 ± 0.120	0.169 ± 0.071	0.127 ± 0.011	0.027 ± 0.003	0.004 ± 0.003	0.000 ± 0.000	$r_s = 0.977$**
P6-3R/07	0.000 ± 0.000	0.000 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.001	$r_s = -0.023$
P6-8S/07	0.764 ± 0.138	0.386 ± 0.197	0.128 ± 0.054	0.108 ± 0.008	0.034 ± 0.002	0.014 ± 0.005	0.000 ± 0.000	$r_s = 0.967$**
P6-8R/07	0.095 ± 0.082	0.029 ± 0.006	0.112 ± 0.113	0.116 ± 0.032	0.019 ± 0.002	0.006 ± 0.004	0.000 ± 0.001	$r_s = 0.750$**