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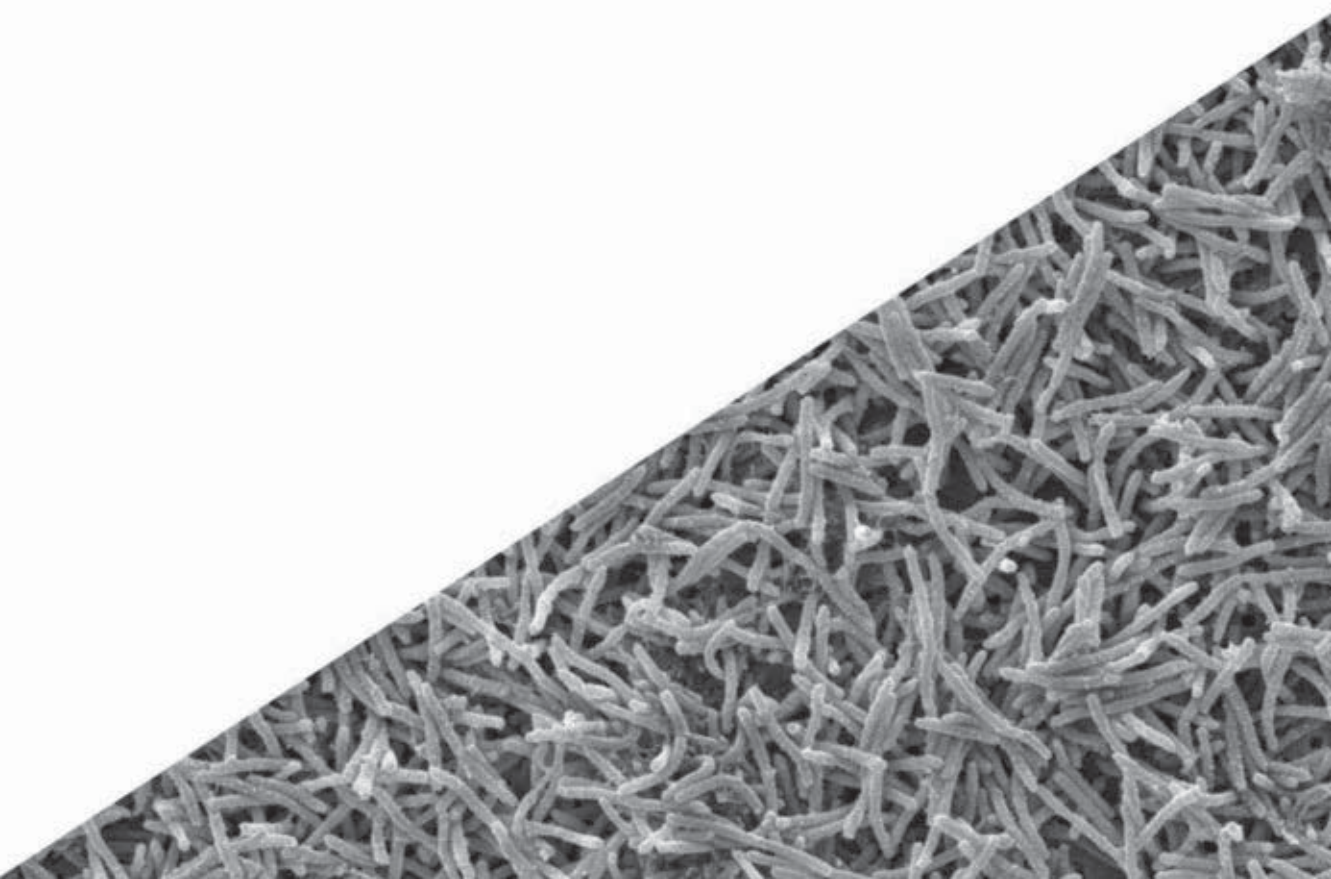
Phase variation in *Flavobacterium psychrophilum*:

Influence on host-pathogen interactions

Eva Högfors-Rönnholm



Phase variation in *Flavobacterium psychrophilum*: Influence on host-pathogen interactions

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To my beautiful, unique and different children
Neo and Bea

"The unlike is joined together, and from differences results the most beautiful harmony."

Heraclitus

ABSTRACT

Phase variation in bacterial cells results in a heterogenic bacterial population consisting of two or more different phenotypes, with slightly different structures and function. Phase variation is, in general, a result of a change in gene expression of one or several cell structures, mostly associated with the surface of the bacterial cell, as a response to changing environmental conditions. Phase variation occurs in pathogenic bacteria as a mechanism to evade the immune system of the host. This complex system is also known to occur in non-pathogenic and commensal bacteria. The cold-water, fish pathogenic bacterium *Flavobacterium psychrophilum*, causing bacterial cold-water disease (BCWD) in farmed fresh water salmonids, is known to exhibit two distinct colony phenotypes when isolated on agar. No detailed characterization of these two phenotypes has, however, been done, nor have the two colony types previously been associated with phase variation in this bacterial pathogen.

The aim of this thesis was to characterize the smooth and rough colony phenotypes of *F. psychrophilum* and further investigate the properties and functions of these phenotypes, in relation to their interaction with the host and with abiotic surfaces. The thesis evaluated *in vitro* interactions of the smooth and rough cells with erythrocytes and macrophages isolated from the rainbow trout (*Oncorhynchus mykiss*) host. The adhesion ability of the smooth and rough cells to polystyrene surfaces was further evaluated. In order to describe bacterial surface structures involved in the interactions with host cells and abiotic surfaces, the bacterial cells and the polystyrene surfaces were treated with different chemicals and enzymes.

Smooth cells of *F. psychrophilum* autoagglutinated, expressed a hydrophobic cell surface and were both hemagglutinating and hemolytic. The smooth cells were also highly adhesive to polystyrene surfaces. These characteristics were associated with proteinaceous structures protruding from extracellular polysaccharides surrounding the bacterial cells. The most dominant proteinaceous structure expressed by the smooth cells was found to be a sialic acid-binding lectin involved in the adhesion to rainbow trout erythrocytes, abiotic surfaces and possibly rainbow trout mucus as well. The rough cells, on the other hand, were shown to be planktonic, expressing a hydrophilic cell surface and to be enzymatically active and highly cytotoxic to

rainbow trout macrophages. These characteristics were, in their turn, associated with the extracellular polysaccharides, possibly a capsule, surrounding the bacterial cells. The smooth cells could further switch phenotype into rough in liquid broth medium. The *in vitro* phase varying structures in *F. psychrophilum* are suggested to be surface proteins, or more precisely lectins, that are possibly protruding from the extracellular polysaccharides surrounding the bacterial cells. Since the lectins were present in both smooth and rough cell types to different extent, lectin expression appears to be differentially regulated than on or off. The different quantity of these proteinaceous structures in the two phenotypes is moreover suggested to determine the surface hydrophobicity of the cells and to determine the organization of the cells in the colonies, which give the colony types their respective smooth and rough appearances on agar medium.

The thesis contributes to an increased knowledge of the virulence of *F. psychrophilum* towards the fish host. Phase variation in *F. psychrophilum* further implies that population heterogeneity is of importance in this fish pathogen, and this could contribute to the persistent and reoccurring disease outbreaks at fish farms.

KEYWORDS: Colony phenotype, phenotypic conversion, hemolysis, hemagglutination, macrophage, cytotoxicity, adhesion, lectin

SAMMANFATTNING

Fasvariation hos bakterier ger i allmänhet upphov till en heterogen bakteriepopulation innehållande två eller flera olika fenotyper med till viss grad olika struktur och funktion. Fasvariationen är generellt sett ett resultat av en förändring i uttryckningen av gener kopplade till en eller flera molekylstrukturer på bakterieytan. Fasvariationen sker oftast på grund av förändringar i bakteriecellernas omgivande miljö och är bland annat ett sätt för patogena bakterier att undvika värdens immunförsvar. Idag är det dock känt att även icke patogena och kommensala bakterier kan uttrycka fasvariation och att mekanismerna bakom fasvariationen kan vara mycket komplexa. Det är känt att den fiskpatogena bakterien *Flavobacterium psychrophilum*, som orsakar bakteriell kallvattensjuka hos odlad laxfisk i sötvatten, kan uppvisa två olika kolonifenotyper på agar. Ingen detaljerad karaktärisering av dessa två fenotyper har dock tidigare utförts. Förekomsten av dessa två kolonifenotyper har inte heller tidigare förknippats med fasvariation hos denna patogen.

Målet med avhandlingen var att karaktärisera cellerna av de släta och grova kolonifenotyperna av *F. psychrophilum* och vidare undersöka olika egenskaper och uppgifter kopplade till värd-patogen interaktioner och interaktioner med abiotiska ytor som dessa två fenotyper uttrycker. Avhandlingen beskriver *in vitro* interaktioner mellan bakteriecellerna och erytrocyter och makrofager isolerade från en av bakteriens huvudvärd, regnbågslax (*Oncorhynchus mykiss*). Avhandlingen beskriver även bakteriecellernas förmåga att fästa sig vid polystyrenytor. Bakteriella ytstrukturer involverade i interaktionen med värdceller och abiotiska ytor undersöktes och beskrevs genom att behandla bakteriecellerna och polystyrenytorna med olika kemikalier och enzymer.

De släta cellerna av *F. psychrophilum* visade sig vara autoagglutinerande, ha en hydrofob cellyta och kunna fästa sig vid både plastytor och erytrocyter. De släta cellerna kunde även lysera erytrocyter. Dessa egenskaper kunde kopplas till strukturer av proteinkaraktär som högst sannolikt är fästa vid eller sticker fram ur extracellulära polysackarider som omger bakteriecellerna. Den mest dominanta proteinstrukturen som uttrycks av de släta cellerna är troligtvis ett lektin som binder till sialinsyra på värdcellerna. Detta lektin var involverat i adhesionen av de släta cellerna till både erytrocyter från regnbågslax, abiotiska ytor och även troligtvis mukus från

regnbågslax. De grova cellerna visade sig å andra sidan vara planktoniska, ha en hydrofil cellyta och vara mer enzymatiskt aktiva och cytotoxiska för makrofager från regnbågslax jämfört med de släta cellerna. Dessa egenskaper kunde i sin tur kopplas till de extracellulära polysackariderna, eller kapseln, som omger bakteriecellerna. De släta cellerna påträffades även ändra fenotyp till grov i flytande tillväxtmedium. De *in vitro* fasvarierande strukturerna hos *F. psychrophilum* antas vara ytproteiner, eller mer exakt lektiner, som på något vis är bundna till eller inbäddade i bakteriecellernas kapsel bestående av extracellulära polysackarider som omger cellerna. Eftersom lektinerna påträffades hos både de släta och de grova celltyperna, om än i olika omfattning, så antas uttryckningen av lektinerna vara reglerad av en upp- och nedregleringsmekanism istället för en av eller på mekanism. Förändringen i ytproteinstrukturerna hos de olika fenotyperna antas även inverka på cellytans hydrofobicitet samt bestämma hur cellerna organiseras i kolonierna, vilket i sin tur ger upphov till koloniernas släta och grova utseende på agarmedium.

Avhandlingen tillför en ökad kunskap om *F. psychrophilum* bakteriens virulens mot fiskvärden. Fasvariation hos *F. psychrophilum* antyder även att populationsheterogenitet är av betydelse hos denna fiskpatogen och detta kan bidra till de ståndaktiga och återkommande sjukdomsutbrotten i fiskodlingar.

NYCKELORD: Kolonifenotyp, fenotypisk förändring, hemolys, hemagglutination, makrofag, cytotoxicitet, adhesion, lektin

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LIST OF ORIGINAL PAPERS

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals (**paper I–IV**). The original articles have been reprinted with the kind permission of the copyright holders; Inter-Research Science Publisher (**paper I**), Elsevier Ltd. (**paper II and III**) and John Wiley & Sons Ltd. (**paper IV**).

I. **Högfors-Rönnholm E.** & Wiklund T. (2010) Phase variation in *Flavobacterium psychrophilum*: characterization of two distinct colony phenotypes. *Diseases of Aquatic Organisms* **90**, 43–53.

II. **Högfors-Rönnholm E.** & Wiklund T. (2010) Hemolytic activity in *Flavobacterium psychrophilum* is a contact-dependent, two-step mechanism and differently expressed in smooth and rough phenotypes. *Microbial Pathogenesis* **49**, 369–375.

III. **Högfors-Rönnholm E.** & Wiklund T. (2012) *In vitro* opsonin-independent interactions between cells of smooth and rough phenotypes of *Flavobacterium psychrophilum* and rainbow trout (*Oncorhynchus mykiss*) head kidney macrophages. *Microbial Pathogenesis* **53**, 214–218.

IV. **Högfors-Rönnholm E.**, Norrgård J. & Wiklund T. (2014) Adhesion of smooth and rough phenotypes of *Flavobacterium psychrophilum* to polystyrene surfaces. *Journal of Fish Diseases*, doi:10.1111/jfd.12250 [Epub ahead of print].

1. INTRODUCTION

Bacteria occupy almost every surface on our planet and without them our planet and our bodies would not function properly. The fact that a single bacterial species can live and thrive in tremendously different habitats, such as the water and the intestines of a host make bacterial cells quite spectacular. However, for this to be possible, the bacterial cells need to be able to respond and adapt to changing environmental conditions (van der Woude 2006). Like all other living organisms, bacteria are in constant interaction with the surrounding environment. However, the only bacterial structure that is in continual contact with the environment is the surface (Brown & Williams 1985). This means that when a change in bacterial function is observed, a change in bacterial surface structures is often noted as well. Depending on the environmental conditions, these functional and structural changes in the bacteria can be very complex and a result of a differential expression of one or several genes coding for surface-exposed proteins or proteins that regulate surface structures (van der Woude & Bäumler 2004). This change in gene expression gives rise to two different subpopulations within the original bacterial population or, in other words, two different phenotypes with slightly different structure and function. This event is called phase variation in bacteria (van der Woude 2006). Phase variation has long been thought to be a random process and occur only in pathogenic bacteria as a way to evade the immune system of the host (van der Woude & Bäumler 2004), but is now thought to occur in most non-pathogenic and commensal bacteria and to be a quite complex system (van der Woude 2006).

My thesis focuses on phase variation of the cold-water, fish pathogenic bacterium *Flavobacterium psychrophilum* and the effect the two known phenotypes have on target cells of the rainbow trout (*Oncorhynchus mykiss*) host. In the subsequent sections of this introduction I will connect the concept of the thesis to overall phase variation in bacteria and to specific interactions between bacterial and host cells.

1.1. Consequence and possible significance of phase variation in bacteria

Phase variation in bacteria often leads to a change in colony morphology, most likely due to a change in surface structures that alters the arrangement of the cells within the colony in relation to each other. Surface structures that

can be affected by phase variation are e.g. proteins and capsular polysaccharides involved in the adhesion to surfaces. For pathogenic bacteria, the capsular polysaccharides can further be involved in the invasion of the host and evasion of host defence mechanisms (van der Woude & Bäumler 2004). Other cell structures affected by phase variation include pili and flagella, which mediate bacterial motility, and lipopolysaccharides, that are known virulence factors in pathogenic bacteria. Phase variation can also involve changes in other proteins, indirectly affecting the surface of the bacterial cell, including metabolism associated proteins and gene regulatory proteins (van der Woude & Bäumler 2004).

Phase variation is a way for pathogenic bacteria to evade the immune system of the host during attachment, colonization and infection (Henderson et al. 1999). However, phase variation also occurs in non-pathogenic bacteria. One hypothesis is that phase variation in commensal and non-pathogenic bacteria results in a heterogeneous bacterial population that enhances the chances for the bacterial population as a whole to survive stressful situations in the environment. However, since many genes affected by phase variation have no role in stress adaptation, phase variation in bacterial populations, as a result of environmental pressure, could further be a means for selecting an optimal population phenotype. Both non-pathogenic and pathogenic bacteria can alternate between a planktonic and sessile cellular state. It is now known that phase variation is involved in this switch between a planktonic and sessile lifestyle (van der Woude 2006), or in the on-off switch of adhesive surface structures (Henderson et al. 1999). Thus, a planktonic phase variant can easily spread in the environment, inside the host, or between hosts, while a sessile phase variant can colonize surfaces, form biofilms and thereby protect the cells inside the population (van der Woude 2006). One of the phenotypes may therefore have a selective advantage and dominate under specific environmental conditions. One phenotype is unlikely to exist alone, since the presence of both phenotypes in a mixed population increases the chances of fitness and survival of the population if the environmental conditions were to change. All of these hypotheses support the importance of heterogeneity in a single species bacterial population (Henderson et al. 1999; van der Woude 2006), whether pathogenic or not.

1.2. Interactions between bacterial and host cells

1.2.1. Adhesion ability of bacterial cells to host cells

In order to interact with a host, both commensal and pathogenic bacteria must adhere to host cells and surfaces. The structures enabling bacterial adhesion are typically adhesive molecules, or adhesins, that are expressed on the surface on the bacterial cells. These adhesins can in turn recognize complementary ligands, e.g. receptors, on the surface of the host cells (Kline et al. 2009). Adhesins may also interact with other surface structures and abiotic materials. The target molecule that an adhesin recognizes is usually very precise for a specific adhesin. This means that the adhesin being expressed at a particular moment will guide the bacterial cell to the right ecological niche, e.g. inside the host. If a bacterial cell only expressed a single type of adhesin it would be relatively straightforward to describe the adhesion mechanisms to a host, or a specific host cell. However, most bacterial cells express a wide variety of adhesins and it is probably the complex cooperation between these adhesins that determine the fate of the interactions with the host cells (Klemm et al. 2010). The reason for this is probably because individual adhesin-ligand interactions can be relatively fragile, while a combination of different adhesins, at different distances from or on the cell surface, can lead to a stronger adhesion (Pieters 2007).

Bacterial pathogen adhesins are often surface expressed lectins, which are a class of proteins that recognizes specific carbohydrates in the form of polysaccharides, glycoproteins and glycolipids on the surface of host cells (Sharon & Lis 1989). It has been found that an individual bacterium can express multiple lectins, either separately or simultaneously, and a single lectin can further be multivalent, i.e. recognize and bind to several different carbohydrates (Sharon & Ofek 2000). The existence and function of a lectin can be suggested by finding the complementary carbohydrate(s) it binds. To date, the best described bacterial lectins are probably the mannose-specific fimbriae (Sharon 2006) and the sialic acid-binding lectins (Mandal & Mandal 1990; Varki 1997). This is probably because many pathogenic bacteria express fimbriae (Kline et al. 2009) and sialic acids are expressed by almost all vertebrate cells (Varki & Gagneux 2012).

Bacterial adhesins such as lectins can be affected by phase variation in order to enhance e.g. the adhesion ability to a host and/or to avoid recognition by the host (Lukáčová et al. 2008). A single species bacterial population may therefore contain both the adhesin expressing and the

adhesin non-expressing phenotype at all times, increasing its overall survival rate. It is also possible for a phase-varying bacterium to shift from a commensal non-adhesive phenotype to a pathogenic and adhesive phenotype. By changing the expression of particular adhesins, the bacterial cell can exploit an ecological niche where it can cause disease, which is the case for *Escherichia coli* in humans (Sharon 2006).

1.2.2. Bacterial cytotoxicity and iron acquisition from host cells

Although adhesion of a bacterium to the host is essential for an infection to proceed, the attachment comes with a cost, since the host is then often able to recognize the bacterium as foreign. To solve this problem, many pathogenic bacteria express fimbrial adhesins that extend out from the cell surface and thereby keep the host cell at a safe distance. Other pathogens produce a protective surface layer in the form of a polysaccharide capsule that may prevent recognition and phagocytosis by host macrophages and neutrophils (Kline et al. 2009). If, however, the bacterial cell is recognized by host phagocytes, it can escape phagocytosis by different strategies. A strategy used by some bacteria is phase variation of surface antigens for avoiding host phagocytes (Celli & Finlay 2002). Some pathogens even see the phagocytes as an opportunity to hide from other immune mechanisms and to spread within the host. Bacteria have consequently evolved ways to survive and replicate inside phagocytic cells (Ernst 2000). Others have developed antiphagocytic mechanisms that involve direct contact with host phagocytes. These antiphagocytic mechanisms can interrupt phagocytic signals or involve bacterial effector proteins that are toxic to the phagocytes (Celli & Finlay 2002). Cytotoxicity can be seen as a pathogen-induced cell death leading to apoptosis (programmed cell death) of the phagocytes after they have encountered the pathogenic bacteria, which results in an induction, or a reduction of the inflammatory reaction. An apoptotic outcome can be a result of the infection strategy of the pathogenic bacteria to stimulate apoptosis but also a way for the phagocytic cells to prevent further spread of the bacteria (Navarre & Zychlinsky 2000). The phagocytes can further die by necrosis after the bacterial cells have damaged the cell membrane. This necrotic outcome may contribute to rapid death of the phagocytes and the spread of the bacterial infection (Stockbauer et al. 2003), but it may also release pro-inflammatory signals that attracts more phagocytes to the infection site (Hornef et al. 2002).

Iron is an essential minor nutrient for the metabolism for practically all living cells, including pathogenic bacteria. However, pathogenic bacteria do not have free availability of iron in the host since all available iron is bound tightly to carrier molecules such as transferrin, lactoferrin and hemoglobin. Some pathogens use iron-chelating agents called siderophores which compete with the host's iron-carrier molecules to obtain iron. Other bacterial pathogens can release hemoglobin from host erythrocytes by lysing the red blood cells with hemolysins or other proteases and then directly use the hemoglobin as sources of iron (Litwin & Calderwood 1993). Bacterial expression of siderophores and hemolysins is often regulated by iron availability, i.e. the expression increases when iron availability is limited and decreases when iron is abundant (Andrews et al. 2003). Therefore, the genes coding for the iron acquisition structure in pathogenic bacteria are often affected by phase variation and this variation in gene expression is probably regulated by the changes in the environment (i.e. low or high iron concentrations) inside the host (van der Woude & Bäumler 2004).

1.3. Microorganism of interest: *Flavobacterium psychrophilum*

A number of *Flavobacterium* species are present in the environment, and most are not pathogenic, but commensal and free-living environmental species, occupying diverse habitats including soil, sediments, microbial mats and water. Some of these *Flavobacterium* species have however been found to be opportunistic pathogens of fish (Bernardet & Bowman 2006; Bernardet 2012; Loch & Faisal 2014), and the species that are thought to affect farmed, and to some extent, wild fish all over the world most severely are *F. branchiophilum, F. columnare* and *F. psychrophilum* (Barnes & Brown 2011; Starliper 2011). These probably have been, or still are, environmental species and a part of the normal bacterial flora on fish, but due to changed environmental conditions, such as the development of aquaculture, these species have evolved into opportunistic pathogens (Bernardet & Bowman 2006).

In Finland and throughout the world, the *Flavobacterium* species having the most negative economic and ecological impact on the cold-water aquaculture today is *F. psychrophilum* (Antaya 2008). This pathogen is widespread in the world and has been detected in fish in most temperate fresh waters (Nicolas et al. 2008). It has been isolated from benthic diatoms (Izumi et al. 2005) and algae (Amita et al. 2000) and from a wide range of

fresh water wild fish species such as carp, eel, perch and roach, often without causing disease. The fish species most affected by *F. psychrophilum* are farmed salmonids, such as rainbow trout and coho salmon (Barnes & Brown 2011). Unpublished studies have discovered that *F. psychrophilum* isolates from the environment and non-salmonid fish shows higher genetic diversity compared with rainbow trout isolates (K. Sundell & T. Wiklund, personal communication). In contrast, most isolates from farmed rainbow trout belong to a genetically less diverse group of genotypes and to a possible epidemic and highly virulent group of isolates (Chen et al. 2008; Siekoula-Nguedia et al. 2012). Mixed infections have, however, been revealed in rainbow trout, involving isolates of distinct genotypes and phenotypes (Sundell et al. 2013). These findings further implicate that the non-virulent isolates found in the environment, in wild fish and possibly even in farmed rainbow trout, are commensal variants of *F. psychrophilum*. The virulent isolates found in farmed rainbow trout have possibly gone through pathoadaptive mutations, due to changed environmental conditions as a result of aquaculture, and thereby gained enhanced virulence (Sokurenko et al. 1999).

1.3.1. Pathogenesis

The disease caused by *F. psychrophilum* is referred to as bacterial cold water disease (BCWD) (Nematollahi et al. 2003b; Barnes & Brown 2011). Although *F. psychrophilum* has been known to cause disease in farmed salmonids since the 1940's, it is still not known for certain how this bacterium enters the fish. Since *F. psychrophilum* is known to survive in fresh waters for several months, horizontal transmission, from the water and environmental surfaces into the fish, is perhaps the most accepted hypothesis of transmission mechanism. However, since *F. psychrophilum* has also been detected in ovarian fluids, milt and on egg surfaces, vertical transmission, from brood fish to offspring, has also been proposed (Starliper 2011; Sundell et al. 2013). The inability to determine how *F. psychrophilum* enters the fish, and the fact that it is rather difficult to infect fish naturally (immersion) with *F. psychrophilum* under laboratory conditions (personal experiences), are additional indications that *F. psychrophilum* could be a commensal bacterium that turns into an opportunistic pathogen under favourable environmental conditions.

However, to cause infection in the fish, *F. psychrophilum* needs to attach to epithelial structures and penetrate the skin, intestine or other surfaces (Kline et al. 2009; Klemm et al. 2010) of the fish. The whole genome of *F. psychrophilum* (JIP02/86) has been delineated and genes coding for several proteins involved in attachment and tissue destruction have been reported (Duchaud et al. 2007). Adhesive capacities to host tissues (Kondo et al. 2002; Nematollahi et al. 2003a) and an adhesin for attachment to host cells (Møller et al. 2003) have further been described. *F. psychrophilum* is also known to form biofilms (Álvarez et al. 2006; Sundell & Wiklund 2011; De la Fuente et al. 2013), another important characteristic for a pathogen causing persistent infections and resisting host immune responses and antimicrobial agents (Costerton et al. 1999). *F. psychrophilum* has also been found to be highly proteolytic and secretes proteases important for degradation of host tissues (Bertolini et al. 1994) and nutrient acquisition (Pérez-Pascual et al. 2011). After attachment to the fish, the bacterial cells must be able to resist or suppress both the innate and adaptive immune system of the fish (Brown & Williams 1985). Regarding the innate immune mechanisms, *F. psychrophilum* has been suggested to resist or manipulate phagocytosis (Decostere et al. 2001; Nematollahi et al. 2005) as well as resist complement activity in rainbow trout (Wiklund & Dalsgaard 2002). Furthermore, specific antibodies towards *F. psychrophilum* have been considered only to partly protect rainbow trout against the pathogen (LaFrentz et al. 2002; 2003), indicating that *F. psychrophilum* can to some extent resist the action of the adaptive immune mechanisms of the host. If the bacterial cells are able to resist the immune system of the host, the next crucial characteristic for the bacterial pathogen to persist the infection is the ability to obtain nutrients from the host and thereby damage the host. As described earlier, iron is probably the most essential minor nutrient for bacterial pathogens (Brown & Williams 1985), including *F. psychrophilum* (Long et al. 2013). It has been reported that *F. psychrophilum* possibly uses siderophores for iron acquisition (Møller et al. 2005) and that the bacterial cells can degrade hemoglobin (Dalsgaard & Madsen 2000). Another indication that *F. psychrophilum* possibly obtains iron from host cells and tissues is that most infected fish suffers from anaemia (Barnes & Brown 2011). In all of these pathogen-host interactions, the bacterial cell surface plays a crucial part (Brown & Williams 1985).

1.3.2. Clinical signs and diagnosis of *F. psychrophilum* infections

Since *F. psychrophilum* is a cold adapted or psychrophilic bacterium (D'Amico et al. 2006), BCWD outbreaks in fish farms usually occur at water temperatures below 16 °C (Starliper 2011), and the outbreaks are most severe at temperatures of 10–15 °C (Barnes & Brown 2011; Starliper 2011). The highest mortality rate from a BCWD outbreak that has been reported to date for farmed rainbow trout fry is 90% (Nilsen et al. 2011). The clinical symptoms differ somewhat between fish of different sizes. Typical external symptoms are lethargy, dark pigmentation, anaemia, exophthalmia and ulcers (Fig. 1 A). Internal symptoms include ascites, an enlarged spleen as well as a pale kidney and liver (Barnes & Brown 2011). However, infected fry often suffer from an acute systemic infection and die without showing any visible symptoms (Starliper 2011). If the infection is severe, *F. psychrophilum* can be detected from several different organs (e.g. spleen and kidney) as well as ulcers from the infected fish by culturing tissue samples on agar medium low in nutrients and at incubation temperatures below 20 °C. The bacterium is very fastidious and slow growing on artificial growth medium, but after a few days of incubation, one or two types of bright yellow colonies (Fig. 1 B) appear on the agar surface (Barnes & Brown 2011). Previously these two types were described as yellow with regular edges and yellow with spreading margins (Pacha 1968; Holt 1987; Bernardet & Kerouault 1989) but I will refer to them as smooth and rough, respectively. These bacteria, forming one or two different colony types, can be further identified as *F. psychrophilum* by polymerase chain reaction (PCR), serology and biochemical tests (Barnes & Brown 2011).

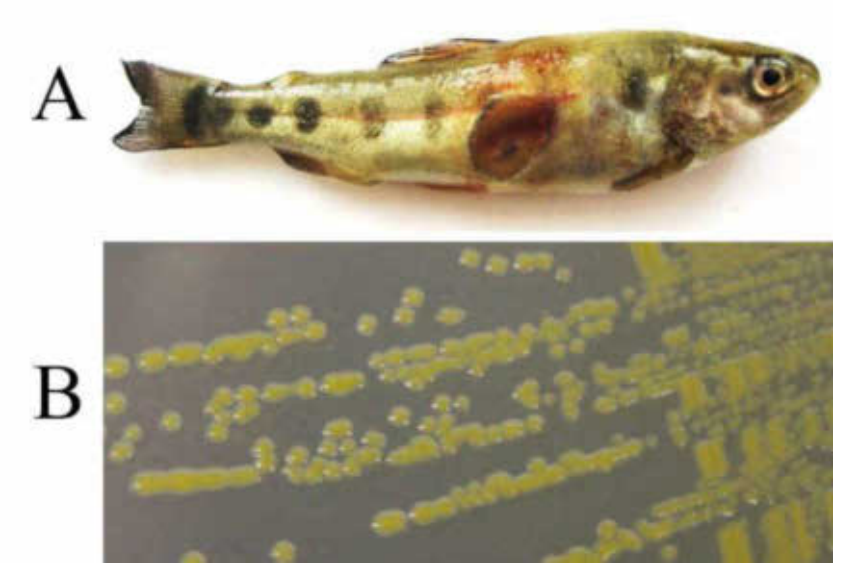

Fig. 1. *(A) A rainbow trout suffering from BCWD showing visible symptoms in form of an ulcer. (B) Bright yellow colonies of F. psychrophilum isolated from internal organs of a BCWD infected rainbow trout and cultured on TYES agar.*

1.3.3. Treatment and prevention of *F. psychrophilum* infections

The treatment method for severe *F. psychrophilum* infections at fish farms is, to this day, antibiotic therapy. However, this is not an effective long-term treatment method since *F. psychrophilum* has been reported to be resistant to some antibiotics (Barnes & Brown 2011). Prevention of infections by disinfecting fish eggs with different chemicals is also regularly done. This method seems to be ineffective regarding the control and spread of *F. psychrophilum* (Starliper 2011). Most research regarding the prevention of *F. psychrophilum* infections has been focused on vaccine development. However, despite extensive effort, there is not yet an effective vaccine against *F. psychrophilum* generally available on the market. This is probably due to the fact that the virulent *F. psychrophilum* strains isolated are serologically and phenotypically different and that even the bacterial population within an outbreak can be very heterogenic (Barnes & Brown 2011; Starliper 2011). Another challenge when it comes to vaccines is that the most severe infections occur in fry populations and these fry have still an undeveloped immune system (Cipriano & Holt 2005) and a vaccine would then probably be useless or even damaging to the fry.

The treatment methods described above all have pros and cons. However a more sustainable choice in preventing *F. psychrophilum* infections in fish farms is likely through management strategies. Stress factors for the fish should be reduced by upholding safe carrying capacities and using fresh and pathogen-free water supplies, using clean tanks and equipment, minimizing the organic material in the tanks from left over feed and last but not least, feeding the fish with high quality food (Barnes & Brown 2011; Starliper 2011). Also reducing the use of pharmaceuticals, such as antibiotics, as much as possible, could be necessary in the future.

2. AIMS OF THE THESIS

Although *F. psychrophilum* has been known as a fish pathogenic bacterium for a long time, its virulence mechanisms are still not well understood. The occurrence of two different colony types of *F. psychrophilum* has long been known, although no further characterization of the two types has ever been done. After initial isolation of *F. psychrophilum* from diseased fish at our laboratory, we observed that the growth mode of the bacterial cells changed in broth culture upon repeated passages. A subsequent change in colony morphology was also observed and the two different colony phenotypes were noted; smooth and rough. I believed that a better understanding of the properties and the importance of these different colony phenotypes of *F. psychrophilum* would help us better understand the infection process of this bacterial pathogen. The overall aim of this thesis was therefore to characterize the two colony phenotypes of *F. psychrophilum* in detail and to further investigate the properties and functions of these phenotypes, in relation to their interaction with the host and with abiotic surfaces.

The specific objectives were to investigate:

1. The characteristics of smooth and rough cells of different *F. psychrophilum* isolates **(paper I)**,

2. The *in vitro* interactions of smooth and rough cells of *F. psychrophilum* and rainbow trout erythrocytes (**paper II**),

3. The *in vitro* interactions of smooth and rough cells of *F. psychrophilum* and rainbow trout macrophages (**paper III**),

4. The *in vitro* interactions of smooth and rough cells of *F. psychrophilum* and inert surfaces (**paper IV**).

Specific research hypotheses and questions are presented in the original articles (**paper I–IV**).

3. MATERIALS AND METHODS

This chapter and Table 1 give a brief overview of the materials and methods and the conditions used in the studies. More detailed descriptions of materials and methods can be found in the original **papers I–IV**.

3.1. Bacterial isolates and culture conditions

The *F. psychrophilum* isolates (P13-4/96, P6-1/07, P6-3/07 and P6-8/07) used were previously isolated from internal organs of diseased farmed rainbow trout in 1996 and 2007. All isolates contained cells of smooth (S) and rough (R) colony variants that were separately subcultivated and named after the corresponding colony type as P13-4S/96, P13-4R/96, P6-1S/07, P6-1R/07, P6-3S/07, P6-3R/07, P6-8S/07 and P6-8R/07. All variants were identified as *F. psychrophilum* by PCR (Wiklund et al. 2000). The *F. psychrophilum* type strain NCIMB 1947^{T} and the wild type (WT) isolate P13-4/96 were also included in **paper I** for comparison. Stock suspensions were maintained in tryptone yeast extract salts (TYES) broth (Holt et al. 1993) with 20% (v/v) glycerol at –70 °C. For experimental use, the bacteria were grown on TYES agar (Holt et al. 1993) at 15 °C and subsequently suspended in media/buffers used in the assays (Table 1).

3.2. Experimental fish

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) were obtained from two freshwater fish farms in Finland. The fish were kept in fiberglass tanks with continuously flowing aerated (12–14 °C) well water and fed with dry commercial pellets (Rehuraisio, Raisio, Finland).

3.3. Characterization of smooth and rough phenotypes (paper I)

3.3.1. Serological and biochemical tests

The smooth and rough bacterial cells were serotyped using a slide agglutination test (Madetoja et al. 2001) with minor modifications. Bacterial colonies, grown on TYES agar were suspended in sodium acetate buffer (0.1 M NaCl, 0.05 M $NaCOOCH_3$, pH 7.5) and heated at 56 °C. Equal volumes of the bacterial suspensions and rabbit antiserum (cross-absorbed anti-Fp^{T}, anti-Fd and anti-Th sera) were mixed on a glass slide and the agglutination

reaction was recorded. Pre-immune serum and 0.9% NaCl, were included in all tests as negative controls.

The bacterial cells were biochemically characterized using tests suitable for *F. psychrophilum* including hydrolysis of starch (Pacha 1968), casein (Bernardet 1989), gelatin, esculin (Madetoja et al. 2001) and elastin, production of catalase (Madetoja et al. 2002), absorption of Congo red (Ishiguro et al. 1985), presence of flexirubin-type pigments (Lorenzen et al. 1997) and cytochrome oxidase activity using the BBL™ Oxidase Reagent Droppers (BD). The enzymatic activity of the bacterial cells were also tested using a semi-quantitative micromethod, API ZYM (bioMérieux Inc.). The gliding motility of the bacterial cells was tested on TYES medium supplemented with 0.5% agar and 0.1% baker's yeast and any spreading of the bacteria around the inoculation site was considered gliding motility.

3.3.2. Growth mode in broth

To investigate the growth mode and possible switching rate in broth, smooth and rough cells, grown on TYES agar, were separately suspended in TYES broth and incubated at 15 °C with 200 rpm agitation. Subsamples, taken daily for 17 days, were diluted in TYES broth and plated onto TYES agar. Following incubation, colony types were identified and counted. The macroscopic growth of the bacterial cells in the tubes was also examined daily and any sign of agglutination recorded.

3.3.3. Cell surface hydrophobicity

The surface hydrophobicity of the smooth and rough cells was determined by the salt aggregation test (SAT) (Lindahl et al. 1981) and by the microbial adhesion to hydrocarbons (MATH) test (Rosenberg et al. 1980). The SAT value was determined to the lowest $(NH_4)_2SO_4$ concentration causing aggregation of the cells. Cells with SAT values under 0.1 were considered highly hydrophobic, between 0.1 and 1.0 hydrophobic and over 1.0 hydrophilic (Møller et al. 2003). In the MATH test the percentage adhesion of the bacterial cells to *n*-hexadecane (Merck KGaA), *n*-octane and *p*-xylene (Sigma-Aldrich) was determined. The cell surface hydrophobicity (%A), presented as the percentage adhesion to the hydrocarbon, was calculated using the following equation:

$$\%A = [OD_{590}(\text{initial bacterial suspension}) - OD_{590}(\text{aqueous phase})] / OD_{590}(\text{initial bacterial suspension})$$

3.3.4. Adhesion to polystyrene

The ability of the smooth and rough cells to adhere to polystyrene surface was tested according to the crystal violet staining method described by Vesterlund et al. (2005) with some modifications, using phosphate buffered saline (PBS) as medium.

3.3.5. Whole cell proteins, cell surface components and extracellular products

Whole cell proteins were extracted from smooth and rough cells, by suspending the cells in PBS and disrupting them by sonication (Branson Sonifier 250 Analog, Branson Ultrasonics Corporation). The lysates were stored at –70 °C. Thawed lysates were centrifuged and the protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific) as described by the manufacturer, and then adjusted to 1000 µg protein ml^{-1}. The samples were subsequently suspended in an equal volume of sample buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8, 40 mM dithiothreitol and 0.01% bromophenol blue), heated at 60 °C for 15 min and centrifuged.

Outer membrane proteins (OMP) were isolated from bacterial cells according to Møller et al. (2005) with minor modifications and stored at –20 °C. The protein concentration was determined in thawed samples as described above and then adjusted to 200 µg protein ml^{-1}. The samples were subsequently suspended in an equal volume of sample buffer and heated at 100 °C for 5 min.

Lipopolysaccharides (LPS) were extracted from bacterial cells according to Valverde et al. (1997) with minor modifications. LPS from *Salmonella enterica* serovar Typhimurium (Sigma-Aldrich) was used as control.

Extracellular products (ECP) produced by the bacterial cells were prepared by the cellophane overlay method according to Sudheesh et al. (2007) with some modifications and stored at –70 °C. The protein concentration was determined in thawed samples as described above and adjusted to 500 µg protein ml^{-1}.

Whole cell lysates (5 µg protein), OMP (2 µg protein), LPS (15 µl sample, 2.5 µg LPS control) and ECP (5 µg protein) were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970) with minor modifications. Whole cell protein, OMP and ECP gels were stained with Bio-Safe Coomassie Stain (Bio-Rad Laboratories), according to the manufacturer's instructions. For visualization of the

carbohydrate portions of the LPS components, LPS gels were stained with a modified silver staining method (Fomsgaard et al. 1990).

3.3.6. Virulence for rainbow trout

The degree of virulence (LD_{50}) was determined for the smooth and rough phenotypic variants according to the method of Reed & Müench (1938) by injecting 10-fold decreasing bacterial dilutions, ranging from approximately 10^7 to 10^3 colony forming units (CFU) ml^{-1}, intramuscularly into five anaesthetized rainbow trout per dilution. Mortalities were examined for re-isolation of cells of smooth or rough colonies of the different isolates by inoculating tissue samples from spleen and kidney onto TYES agar.

3.4. *In vitro* interactions between bacterial cells and rainbow trout erythrocytes (paper II)

3.4.1. Erythrocyte suspension

Blood was collected in an equal volume of Alsever's solution (Sigma-Aldrich) by caudal venipuncture of anesthetized rainbow trout. Erythrocytes were harvested by centrifugation (1000 × g, 5 min, 10 °C) and washed three times with PBS.

3.4.2. Microplate hemolysis assay

Washed erythrocytes were suspended to 5% (v/v) in PBS. An equal amount of erythrocyte and bacterial suspensions in 0.5% NaCl were mixed in a round bottom microtiter plate (Nunc) and incubated for 24 h at 10 °C (5 and 15 °C was additionally tested) and 450 rpm rotation. Following incubation, 0.5% NaCl was added to the wells and the plate was centrifuged (1000 × g, 5 min, 10 °C). The supernatants were transferred to a flat bottom microtiter plate (Nunc) and the absorbance (A) was measured at 540 nm. A negative control ($A^{background}$) and a positive control (total hemolysis, $A^{100\%}$) were included on each plate. The hemolytic activity was calculated according to:

$$\text{Hemolytic activity} = (A - A^{background}) / (A^{100\%} - A^{background})$$

3.4.3. Agarose hemolysis assay

Washed erythrocytes were added to a final concentration of 2% (v/v) to 1% agarose in PBS. Seven ml of the erythrocyte-agarose suspension was poured onto petri dishes containing 20 ml solid, room tempered TYES agar,

forming a double layer, and allowed to solidify. Five µl of the bacterial suspensions in 0.5% NaCl were added in triplicate onto the agarose-agar plates and incubated at 10 °C. The diameter of the hemolysis zone around the inoculates was measured after 24, 48 and 72 h. A negative control (0.5% NaCl) was included on the plates.

For control of hemolysis patterns caused by possible hydrogen peroxide produced by the bacterial cells, catalase from bovine liver (Sigma-Aldrich) was incorporated into the agarose before the addition of the erythrocytes and the plates were inoculated with bacterial suspensions and incubated and analyzed as previously.

3.4.4. Hemagglutination assay

The hemagglutinating ability of smooth and rough cells was tested according to Møller et al. (2003) with minor modifications. Briefly, equal amounts of bacterial dilutions in 0.5% NaCl and erythrocyte suspensions were mixed on microflocculation glass slides (Marienfeld) and incubated for 5 min at 15 °C and 160 rpm rotation. The hemagglutination titer was determined as the reciprocal of the highest dilution displaying an agglutination of the erythrocytes.

3.5. *In vitro* interactions between bacterial cells and rainbow trout macrophages (paper III)

3.5.1. Isolation of rainbow trout monocytes/macrophages

Monocytes/macrophages were isolated from the head kidney of rainbow trout according to the Percoll (GE Healthcare) gradient method described by Secombes (1990) with some modifications.

For the cytotoxicity study, the isolated monocytes/macrophages were suspended in L-15 (Sigma) containing 0.1% fetal bovine serum (FBS, Sigma) and 100 IU ml^{-1} penicillin/100 µg ml^{-1} streptomycin (5000 IU/5 mg ml^{-1}, Sigma). The cell concentration was determined in a Bürker chamber and their viability checked with 0.4% trypan blue dye (Sigma) exclusion and the cell concentration adjusted to 2×10^7 cells ml^{-1}. The monocytes/macrophages for the migration assay were stored overnight at 4 °C in L-15 containing 5% FBS and antibiotics as previously.

The cell population purity was checked using α-naphthyl acetate non-specific esterase staining kit (Sigma) and over 95% of the cells were classified as monocytes/macrophages.

3.5.2. Cytotoxicity assay

The cytotoxic effect of smooth and rough cells on rainbow trout head kidney macrophages was measured as the release of lactate dehydrogenase (LDH) from damaged macrophages using Cytotoxicity Detection Kit (LDH, Roche) according to the manufacturer's instructions. The theoretical multiplicity of infection was approximately 1:100, as the monocyte/macrophage concentration was 2×10^6 cells $well^{-1}$ in L-15+0.1% FBS and the bacterial cell concentration 10^8 cells ml^{-1} in L-15. The incubation time was 24 h at 10 °C (5 and 15 °C was additionally tested). Control wells containing only assay medium (background control), only target cells, only effector cells and target cells lysed with Triton X-100 solution (high control) were included on each plate. After adding a reaction mixture to the supernatants the absorbance was measured at 490 nm using a microplate reader. The percentage cytotoxicity was calculated from absorbance values (A) using the following equation:

$$\text{Cytotoxicity \%} = [(A^{\text{sample}} - A^{\text{background control}}) / (A^{\text{high control}} - A^{\text{background control}})] \times 100$$

3.5.3. Migration assay

Rainbow trout head kidney macrophage migration towards smooth and rough cells was investigated using Transwell® 24-well plates with 5 µm pore polycarbonate membrane inserts (Corning Inc.). Briefly, 100 µl 2×10^5 cells ml^{-1} monocytes/macrophage suspensions in L-15 was added to the upper side of the membrane inserts and 600 µl 10^8 cells ml^{-1} bacterial suspensions in L-15 to the sample wells and incubated 24 h at 10 °C (5 and 15 °C was additionally tested). L-15 without bacteria was used as negative control. The migrated macrophages on the lower side of the membrane inserts were fixed and stained and then counted at 500× magnification under a light microscope.

3.6. *In vitro* interactions between bacterial cells and inert surfaces (paper IV)

3.6.1. Fresh water medium

Fresh lake water, with a natural hardness of 4 °dH (hardness's adjusted to 14 and 28 °dH was additionally tested), was used as medium in the adhesion assay. The pH of the water was set to 7.2, and the water was sterilized and stored at 4 °C until use. The pH of the water was always checked before use.

3.6.2. Adhesion assay

The adhesion ability of smooth and rough cells to 96-well microtiter polystyrene plates with flat bottom (Nunclon Δ Surface, Nunc) was tested according to Álvarez et al. (2006) with some modifications. Briefly, bacterial suspensions in fresh water medium were added to the wells of the microplate and incubated for 1 h at 15 °C (5 and 25 °C was additionally tested). Fresh water medium without bacteria was used as negative control. Following washing, the adhered cells were stained with crystal violet solution and the stain bound to the adhered cells was released with ethanol. The adhesion ability of the bacterial cells was quantified by measuring the absorbance value of the inoculated wells at 595 nm using a microplate reader. The absorbance values were corrected by subtracting the absorbance value of the negative control.

3.7. Treatment of bacterial cells (paper II, III and IV)

To examine if the hemolytic activity of the smooth and rough cells was affected by iron availability or if hemolysins were secreted in ECP, the hemolytic activity of bacterial cells grown on iron-limited media and ECP prepared by the cellophane overlay method was studied (**paper II**).

To investigate if inactivation of bacterial cells affected the hemolytic activity or cytotoxicity, bacterial cells were inactivated by heat (**paper II and III**) and formalin (**paper II**) before use in the hemolysis and cytotoxicity assays described above.

To investigate what kind of surface structures are involved in the hemolysis, the cytotoxicity and adhesion to polystyrene, bacterial cell suspensions were treated with 10 mg ml^{-1} proteinase K, trypsin, sodium (meta)periodate and 25 mM N-acetylneuraminic (sialic) acid (Sigma-Aldrich) (**paper II and III**) or 50 mM D-glucose, D-galactose, D-mannose,

D(-)mannitol (Merck), sodium (meta)periodate and 25 mM sialic acid (**paper IV**). To examine if the structures altered by the sialic acid were involved in the agglutination or the lysis of the erythrocytes, the hemagglutinating ability of sialic acid treated bacterial cells was further tested (**paper II**).

3.8. Treatment of polystyrene surfaces (paper IV)

In order to examine if the adhesion ability of the smooth and rough cells to polystyrene was affected by different carbohydrates and fish mucus, the wells of the plate was pre-treated with 50 mM, D-glucose, D-galactose, D-mannose, D(-)mannitol (Merck) or filter-sterilized rainbow trout skin mucus with a protein concentration of 0.5 mg mL^{-1} in fresh water medium. The adhesion assay was subsequently performed as described above, but using bacterial suspensions and negative control to both carbohydrate or mucus treated and untreated wells for comparison.

3.9. Statistical analysis

All data were analyzed using SPSS 15.0, 17.0 or IBM SPSS Statistics 19 software. The difference in SAT values, %A values and absorbance values was tested between corresponding smooth and rough cells with one-way ANOVA or Mann-Whitney U-test (**paper I**). Differences in hemolytic activity, hemagglutinating ability (**paper II**), cytotoxicity, macrophage migration (**paper III**) and adhesion ability (**paper IV**) between corresponding smooth and rough cells or between untreated and treated bacterial cells were tested with the *T* test or Mann-Whitney U-test. Correlation coefficients between SAT values, %A values and absorbance values (**paper I**), hemolytic activity-bacterial dilutions, hemolytic activity-temperature (**paper II**), cytotoxicity-temperature, cytotoxicity-bacterial dilutions, macrophage migration-temperature (**paper III**), adhesion ability-water hardness and adhesion ability-temperature (**paper IV**) were calculated by using Pearson or Spearman correlation. Differences were considered significant when $P < 0.05$.

Table 1. *Summary of the experimental conditions and analyzes used in the studies. More detailed information can be found in respective* ***paper I–IV****.*

	Paper I	Paper II	Paper III	Paper IV
***F. psychrophilum* isolates**	P13-4S/96 and P13-4R/96 P6-1S/07 and P6-1R/07 P6-3S/07 and P6-3R/07 P6-8S/07 and P6-8R/07 P13-4/96 WT NCIMB 1947^{T}	P13-4S/96 and P13-4R/96 P6-1S/07 and P6-1R/07 P6-3S/07 and P6-3R/07 P6-8S/07 and P6-8R/07	P13-4S/96 and P13-4R/96 P6-1S/07 and P6-1R/07 P6-3S/07 and P6-3R/07	P13-4S/96 and P13-4R/96 P6-1S/07 and P6-1R/07 P6-8S/07 and P6-8R/07
Growth on TYES agar (days)	3–6	5	5	5
Experimental analyzes and assays	Serological test Biochemical tests Growth mode in broth Cell surface hydrophobicity Adhesion to polystyrene Whole cell proteins. cell surface components and extracellular products Degree of virulence	Microplate hemolysis assay Agarose hemolysis assay Hemagglutination assay	Cytotoxicity assay Migration assay	Adhesion assay
Media and buffers for bacterial suspensions	Sodium acetate buffer TYES broth 2 mM NaH_2PO_4 Phosphate urea magnesium sulfate buffer PBS 20 mM Tris-HCl 100 mM EDTA-TEA 0.5% NaCl SDS-PAGE sample buffer	0.5% NaCl	L-15 with or without FBS, heparin and antibiotics	Fresh water medium
Rainbow trout (g)	16	600–1000	200–700	224

4. MAIN FINDINGS OF THE THESIS

The results from the thesis suggest that *F. psychrophilum* is expressing phase variation *in vitro* seen as two different phenotypic cell and colony types, smooth and rough. The cells of the two phenotypes are further suggested to interact with and influence host cells in distinct ways. Smooth cells were characterized as autoagglutinating, hydrophobic, highly adhesive to both inert surfaces and host erythrocytes. Smooth cells also lysed host erythrocytes. In contrast, rough cells were characterized as planktonic, hydrophilic, more enzymatically active and highly cytotoxic to host macrophages. A summary of the distinct characteristics can be seen in Table 2.

Table 2. *Summary of the distinct characteristics of the smooth (S) and rough (R) variants of F. psychrophilum included in the thesis. More detailed results can be found in respective* ***paper I–IV****. NI: not identifiable[a]; ND: not determined.*

Characteristic	*F. psychrophilum* isolates							
	P13-4S/96	P13-4R/96	P6-1S/07	P6-1R/07	P6-3S/07	P6-3R/07	P6-8S/07	P6-8R/07
Phenotype	S	R	S	R	S	R	S	R
Autoagglutination	Yes	No	Yes	No	Yes	No	Yes	No
Phenotypic conversion in TYES broth	Yes	No	Yes	No	ND	ND	ND	ND
Serotype	NI	Th	NI	Th/Fp^T	NI	Fp^T/Th	NI	Th/Fp^T
Acid phosphatase activity	Low	High	Low	High	Low	High	Low	High
Cells surface hydrophobicity	High	Low	High	Low	High	Low	High	Low
Adhesion to polystyrene	High	Low	High	Low	High	Low	High	Low
Hemagglutinating ability	High	None	High	Low	ND	ND	ND	ND
Hemolytic ability	High	Low	High	Low	High	None	High	Low
Cytotoxic activity to macrophages	Low	High	Low	High	Low	High	ND	ND

[a] Not identifiable due to autoagglutination

4.1. Morphology and growth of smooth and rough phenotypes

The typical appearance of the smooth colony type, seen in a dissecting microscope, was shiny and bright yellow with a smooth surface (Fig. 2 A), and when observed at the cell level with scanning electron microscopy (Nation 1983), the cells were tightly lined side by side (Fig. 2 B, unpublished results). When grown in TYES broth, the smooth cells autoagglutinated and

descended to the bottom of the tube (Fig. 2 C, left). The rough colony type had a ruffled appearance and the colour varied from yellow to green (Fig. 2 D) and when seen at the cell level, the cells were irregularly arranged (Fig. 2 E, unpublished results). When grown in TYES broth, the rough cells did not autoagglutinate but were distributed evenly in the tube resulting in a turbid solution (Fig. 2 C, right). In broth, however, the smooth cells had the ability to switch phase to become rough cells. This was seen over time as a more turbid solution instead of clusters at the bottom. Subsamples inoculated on TYES agar subsequently showed both smooth and rough type colonies (**paper I**).

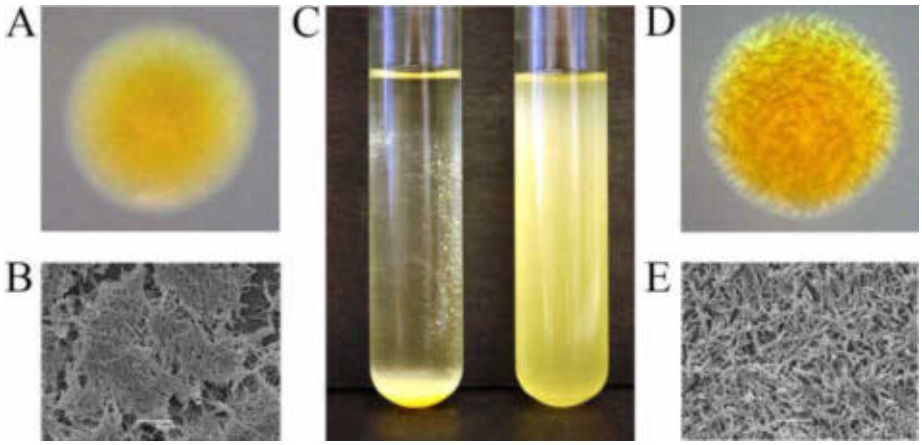

Fig. 2. *The typical appearance of smooth and rough phenotypes of F. psychrophilum when grown on TYES agar and in TYES broth. (A) and (D) show smooth and rough colony types respectively on TYES agar as seen through a preparation microscope and (B) and (E) show smooth and rough cells respectively as seen with a scanning electron microscope. (C) shows the appearance of smooth (left) and rough (right) cells when grown in TYES broth. A, C and D are reprinted with permission from Inter-Research Science Publisher.*

4.2. Phenotypic characteristics of smooth and rough cells

Several phenotypic characteristics were similar between the smooth and rough cells. Biochemically they responded similarly and both types showed gliding motility. Both smooth and rough cells were virulent to rainbow trout when injected intramuscularly, but the LD_{50} value was 3 to 6-fold higher for the smooth cells (**paper I**). No clear differences were found neither in whole

cell protein (Fig. 3 in **paper I**), OMP (Fig. 4 in **paper I**) nor LPS (Fig. 5 in **paper I**) profiles between corresponding smooth and rough cells, the differences were more clear between isolates than between phenotypes. The only variant differing in these characteristics was the rough variant P6-3R/07 that was caseinase negative, non-motile and had a different whole cell protein and OMP profile compared to the other P6 isolates (**paper I**). This variant was also found to be non-virulent for rainbow trout (unpublished results).

The characteristics that differed between the smooth and rough phenotypes were serotype, enzymatic reaction patterns, cell surface hydrophobicity and ECP profiles. Since all smooth cells agglutinated spontaneously, regardless of heating at 56 °C, the slide agglutination test could not be reliably used to determine the serotype of these cells. In contrast, the rough cells were determined to be of serotype Th, or a combination of serotype Th and Fp^T. Enzymatically, the rough cells differed from the smooth cells since the rough cells showed a higher degree of activity for acid phosphatase (**paper I**). Both the SAT and the MATH test with *n*-octane showed that the smooth cells were significantly more hydrophobic compared to corresponding rough cells, that were all considered hydrophilic (Table 1 in **paper I**). The ECP profile (Fig. 6 in **paper I**) differed between the corresponding smooth and rough cells for all isolates. The most noticeable difference was between the smooth and rough cells of isolate P13-4/96.

4.3. Adhesion ability of smooth and rough cells to inert surfaces and rainbow trout erythrocytes

Smooth cells had a significantly higher ability to adhere to polystyrene surfaces compared with the corresponding rough cells, regardless of the medium used (**paper I** and **paper IV**). The adhesion of all smooth cells to polystyrene was increased by pre-treatment of the polystyrene surfaces with D-glucose, D-galactose, D(-)mannitol (Table 1 in **paper IV**) and rainbow trout mucus (Fig. 3 in **paper IV**) and decreased after pre-treatment of the bacterial cells with D-glucose, D-galactose (Table 2 in **paper IV**), sialic acid (Fig. 4 in **paper IV**) and sodium (meta)periodate (Fig. 5 in **paper IV**).

The smooth cells showed further significantly higher adhesion ability to rainbow trout erythrocytes, i.e. hemagglutinating ability, compared with corresponding rough cells and the hemagglutinating ability was completely impaired for both smooth and rough cells after pre-treatment of the bacterial cells with sialic acid (Fig. 2 in **paper II**).

4.4. Hemolytic activity of smooth and rough cells

In the microplate hemolysis assay, the smooth cells showed a significantly higher concentration dependent hemolytic activity, compared with corresponding rough cells (Fig. 1 and Table 1 in **paper II**). Growth on iron-limited medium did not affect the hemolytic activity of the cells and ECP showed a low hemolytic activity (Table 3 in **paper II**). Treatment of the smooth cells with heat, formalin (Table 4 in **paper II**), proteinase K, trypsin (Table 5 in **paper II**) and sialic acid (Table 6 in **paper II**) decreased the hemolytic activity compared with untreated cells. Solely treatment with sialic acid decreased the hemolytic activity of the rough cells (Table 6 in **paper II**).

No differences in hemolytic activity between corresponding smooth and rough cells could be found using the agarose hemolysis assay (**paper II**).

4.5. Interactions between smooth and rough cells and rainbow trout macrophages

The cytotoxic activity to rainbow trout macrophages was found to be temperature dependent (Fig. 2 in **paper III**) and significantly higher for rough cells compared with corresponding smooth cells (Fig. 1 in **paper III**). Treatment of the bacterial cells with heat and proteinase K did not affect the cytotoxicity of the cells. Trypsin treatment (Fig. 3 in **paper III**) of smooth cells decreased their cytotoxic activity compared with untreated cells. Sodium (meta)periodate and sialic acid treatment (Fig. 4 in **paper III**) of the cells of all rough isolates and some of the smooth isolates decreased further the cytotoxic activity of the cells compared with untreated cells.

No differences in migration rate of rainbow trout macrophages towards smooth or rough cells were detected.

5. DISCUSSION

The thesis characterized the smooth and rough colony phenotypes of *F. psychrophilum* and evaluated the influence phase variation had on *in vitro* interactions between host cells and bacterial cells. The results contribute to an increased knowledge of the pathogenicity and complexity of this cold-water fish pathogen.

5.1. Presence and possible consequence of phase variation in *F. psychrophilum*

The results of this thesis demonstrate *in vitro* phase variation in *F. psychrophilum*. The phenotypes are seen on agar plates as smooth and rough colony types, and this difference in colony morphology is likely a result of altered packing of the cells in the colony (van der Woude & Bäumler 2004), as shown by the scanning electron microscopy (Fig. 2 B and E). For *F. psychrophilum*, the growth conditions in liquid broth medium selected for the rough phenotype, since smooth cells could switch into rough cells but not vice versa. Differences in switching rate between isolates were further observed (**paper I**). It is known that many phase variable genes are affected by growth conditions and that, under certain environmental conditions, one phenotype will be more advantageous and it will thereby adapt to environmental changes more successfully than the other phenotype. The switching rate will however depend on the growth conditions (Bayliss 2009). For the Gram-negative, phase-varying human pathogen *Neisseria meningitides*, higher switching rates have been observed among epidemic isolates, compared with non-epidemic isolates. It was also concluded that a high switching rate was more important than the number of genes associated with phase variation, since a rapid increase in heterogeneity increases the epidemic potential of the bacterial population (Richardson et al. 2002). An association between higher switching rate and virulence, seen as mortalities at lower bacterial concentration, was also seen for *F. psychrophilum* (**paper I**). These facts indicate a possible connection between switching rate and pathogenic potential for *F. psychrophilum*. *In vitro* switching rates in steady-state environmental conditions, as in broth medium, cannot be fully compared with switching rates *in vivo* (Bayliss 2009). Phenotypic heterogeneity is known to be a very important adaptive strategy for pathogenic bacterial populations to maximize the survival in an ever

changing environment outside and inside the host and moreover to avoid host defense mechanisms. During an ongoing bacterial epidemic, the level of immunity of the host population is generally increasing. This can however contribute to an even stronger selection for phase variation which could be seen as higher switching rates in epidemic bacterial populations (Casadesús & Low 2013). It can be speculated that the reason why practically no reproducible immersion infection model with *F. psychrophilum* has been reported (Henriksen 2013) could be due to the fact that the important phenotypic heterogeneity is bypassed when *F. psychrophilum* isolates, containing probably only one phenotype, are used and that the bacteria are further grown under steady-state laboratory conditions before the infection challenge.

This thesis primarily investigated the *in vitro* phase variation in *F. psychrophilum*. However, *in vivo* phase variation was tested by injecting smooth and rough cells into rainbow trout. Since no switch between phenotypes was observed when the smooth and rough cells were injected into rainbow trout and subsequently re-isolated from diseased fish (**paper I**), the occurrence of *in vivo* phase variation needs further investigation. There is however new evidence that phase variation of *F. psychrophilum* also could occur *in vivo*. It was recently reported that smooth and rough phenotypes of *F. psychrophilum*, isolated from the same diseased fish individual, shared the same genetic patterns to *in vitro* converted smooth to rough cell variants (Sundell et al. 2013). One would think that genetic changes would be expected as a result of the phase variation, since the phase variation subsequently alters the surface structure of the bacterial cell (Bayliss 2009). It is now known that genetically indistinguishable bacterial populations can contain phenotypically distinct cells or subpopulations with different phenotypes. In these bacteria, the alteration in gene expression does not occur through e.g. mutation or recombination, but through epigenetic mechanisms that result in changes in gene expression without loss or changes to the genome DNA sequence (Casadesús & Low 2013). It is not known in which genes the alteration in expression takes place in *F. psychrophilum*. So the hypothesis that phase variation in *F. psychrophilum* is controlled by epigenetic mechanisms needs to be further investigated. In addition, *in vitro* converted smooth to rough phenotypes always showed identical plasmid profiles with the parent smooth phenotype (Sundell et al. 2013), indicating that the phase-varying genes are unlikely connected to the gain or loss of a plasmid in *F. psychrophilum*.

An advantage for pathogenic bacteria to express phase variation is that the bacterial cell can have two or more different surface proteins displaying the same function. Thereby, by switching the expression of these proteins, the bacterial cell can avoid the immune cells of the host even though the function of the proteins remains the same (Ryall et al. 2012). Since it was found that the smooth cells of *F. psychrophilum* expressed lectins capable of recognizing several different carbohydrates (**paper IV**), it is possible that by switching the expression of these adhesins on and off under certain conditions inside the host, it can facilitate the bacterial cells to avoid the defense mechanisms of the host, but still enable the cells to adhere to host cells. This needs to be further investigated. What is clear though is that the smooth and rough cells of *F. psychrophilum* interact differently with host cells *in vitro*.

5.2. The sticky side of the smooth cells

When smooth cells of *F. psychrophilum* were cultured in TYES broth, the remarkable autoagglutinating or autoaggregating growth mode was immediately apparent. This characteristic made it difficult to serotype the smooth cells with the method used in this study. It was further found that the autoagglutinating smooth cells were highly hydrophobic (**paper I**). The association between autoagglutination and high surface hydrophobicity has previously been shown both for pathogenic (Ljungh et al. 1985) and probiotic bacteria (Del Re et al. 2000; Kos et al. 2003). Autoagglutination and surface hydrophobicity has moreover been reported to decrease after proteolytic treatment of the bacterial cells, suggesting that proteins on the surface of the bacterial cells contribute to these characteristics (Ljungh et al. 1985; Kos et al. 2003). A hydrophobic cell surface has further been suggested to protect the bacteria from host phagocytes and also to help the bacteria in the colonization process of mucosal surfaces (Ljungh et al. 1985). The latter hypothesis is supported as both autoagglutination and high surface hydrophobicity have been linked to the adhesion ability of the bacterial cells to host surfaces (Del Re et al. 2000; Kos et al. 2003). It is not known if the autoagglutination and surface hydrophobicity of smooth cells of *F. psychrophilum* decreases after proteolytic treatment of the bacterial cells. It is quite likely this would indeed be the case, since autoagglutination, high surface hydrophobicity and adhesion ability were also closely connected for cells of *F. psychrophilum* (**paper I**). It has previously been reported that a high surface hydrophobicity is probably a result of the presence of proteinaceous

material on the surface of the bacterial cells (Reifsteck et al. 1987; Cuperus et al. 1993). For the smooth cells of *F. psychrophilum* it was found that the hemolytic ability decreased after proteolytic treatment of the bacterial cells. Since hemagglutination was necessary for the activation of the hemolysis, and since only smooth cells showed high adhesion ability to erythrocytes (**paper II**), autoagglutination and high surface hydrophobicity (**paper I**), it is suggested that proteinaceous structures contribute to all of these characteristics typical for the smooth cells of *F. psychrophilum*.

When it comes to adhering to host cells and surfaces and initiating infection, many bacterial species depend on surface expressed lectins binding to complementary carbohydrates on host cells (Sharon & Lis 1989). These lectins can moreover determine the tissue tropism of the bacterial cells if they are expressed at the cell surface at the right time and at the right place when encountered by the right host cell ligand, i.e. complementary carbohydrate (Korea et al. 2011). Lectins, likely anchored to extracellular polysaccharides of the bacterial cells (**paper IV**) seem to be important for *F. psychrophilum* as well since they have been found to be involved in adhesion to erythrocytes (Møller et al. 2003; **paper II**), fish mucus and inert surfaces (**paper IV**). It has previously been reported for *Flavobacterium columnare*, that a lectin-like structure likely incorporated in the capsule is involved in the bacterial adhesion to gill tissues. The adhesion ability was further reported to correlate with the hemagglutinating ability of *F. columnare* (Decostere et al. 1999). For the smooth cells of *F. psychrophilum*, adhesion to erythrocytes and inert surfaces seemed to be connected (**paper II, IV**), but whether the mechanism behind this adhesion is identical is not clear. It has previously been suggested that, generally, bacterial adhesion to other tissues and cells is mediated by adhesin-ligand mechanisms and the adhesion to abiotic surfaces through nonspecific hydrophobic mechanisms (Carpentier & Cerf 1993). Whether this is the case for *F. psychrophilum* needs to be further investigated since the adhesion to inert surfaces was decreased by treatment of the smooth cells with carbohydrates, indicating that lectin-ligand mechanisms are involved (**paper IV**). Lectin-like structures localized in the capsule were also suggested to be involved in the adhesion of *F. johnsoniae*-like cells to polystyrene surfaces (Chenia & Chadwick 2013). The autoagglutinating and hydrophobic nature of the smooth cells of *F. psychrophilum* and the expression of multiple lectins could also enable the bacterial cells to form biofilms, since these characteristics make it possible for the bacterial cells to adhere to a surface, to

each other and also to other bacterial cells (Dunne 2002). *F. psychrophilum* has been reported to form biofilms (Sundell & Wiklund 2011; De la Fuente et al. 2013), but whether the biofilms are formed solely by smooth cells, rough cells or a combination of both phenotypes needs further investigation.

Although the smooth cells of *F. psychrophilum* appeared to express several lectins able to recognize different complementary carbohydrates, the most dominant lectin was found to adhere to sialic acid (**paper II, IV**). This is not surprising as sialic acid is present on the surface of most cell types, including erythrocytes (Mandal & Mandal 1990; Varki & Gagneux 2012) and in fish mucus (Harris et al. 1973). While the sialic acid-binding lectin seems to be important for the smooth cells of *F. psychrophilum*, it is possible that a combination of this lectin with the other carbohydrate-binding lectins present (**paper IV**) is essential for the tissue tropism. Although an independently expressed lectin is probably important for the bacterial adhesion to certain host cell types, in *E. coli*, serial expression of multiple lectins with different target ligands has been considered critical for directing the bacterial cells to their final location inside the host (Korea et al. 2011). Since sialic acids are so abundant on cell surfaces and in mucus/mucins, another possibility could be that the presence of sialic acids on host cells is of advantage to the host if they are functioning as pathogen decoys. These sialic acid-decoys would then enable the host cells to trap the sialic acid-binding bacterial cells and then subsequently shed off the mucins or surface glycoproteins to prevent the bacterial cells targeting the cell surface. This strategy is however of limited use for the host if the pathogen is able to produce sialidases that can break down sialic acids (Varki & Gagneux 2012). It is not known if cells of *F. psychrophilum* express sialidases. What is known is that infected fish are often anemic (Barnes & Brown 2011) and that the smooth cells can adhere to rainbow trout erythrocytes through a sialic acid-binding lectin (Møller et al. 2003; **paper II**) and subsequently lyse the erythrocytes (**paper II**).

It is known that bacterial lectins often cofunction with lytic enzymes, such as proteases and hemolysins, in so called lectin-lysin pairs. The role of the lectin is thereby to anchor the bacterial cell to the ligand of the host cell. This subsequently gives a signal to the lysin to be activated, which results in the lysis of the host cell or macromolecules in order to protect or provide nutrients to the bacterial cell. If the lectin is inhibited, the lysis will be prevented. The effect of the lectin is thought to be reversible, while the effect of the lysin is always irreversible (Gilboa-Garber & Garber 1989). Based on

the results from this thesis, it is suggested that the hemolytic activity of the smooth cells of *F. psychrophilum* is the result of a cell-bound lectin-lysin cofunction. The lectin involved in the hemolysis recognizes sialic acids on the surface of the host erythrocytes and the lytic enzyme that is subsequently activated is suggested to be a thermolabile proteinaceous structure. The lysis was prevented when the bacterial cells were treated with sialic acid, indicating that hemagglutination is required for the hemolysis to be executed. It is likely that the smooth cells of *F. psychrophilum* lyses host erythrocytes in order to gain iron or other nutrients (**paper II**), since *F. psychrophilum* is known to be able to degrade hemoglobin (Dalsgaard & Madsen 2000). Growing the bacterial cells on iron-limited medium did not, however, increase the hemolytic activity of the smooth cells. The significance of this result is however unclear since the smooth cells already showed high hemolytic activity when grown on medium containing iron (**paper II**).

In addition to being hemolytic, the smooth cells of *F. psychrophilum* also showed some degree (10-30 %) of cytotoxicity to rainbow trout macrophages. Since the cytotoxic activity was impaired after treatment of the smooth cells with sodium (meta)periodate, the cytotoxicity was suggested to be initiated by binding of bacterial surface carbohydrates to lectins on the surface of the phagocytes (**paper III**). This bacterial carbohydrate-lectin binding mechanism to macrophages therefore seems to differ from the bacterial lectin-sialic acid binding mechanism to erythrocytes (**paper II**). For the smooth cells, however, the proteinaceous structures seem to be more important than the structures of carbohydrate nature in determining the hydrophobic and overall adhesive characteristics. For phase-varying *Mycobacterium* species, e.g. *M. abscessus*, it has been reported that the smooth and rough colony morphology is determined by the amount of glycopeptidolipids present in the cell wall of the bacterium (Barrow & Brennan 1982; Howard et al. 2006). It was further concluded that the smooth cells of *M. abscessus*, expressing glycopeptidolipids, were non-virulent but expressed sliding motility and biofilm formation, indicating that the glycopeptidolipids play a role in bacterial adhesion. In contrast, the rough cells, expressing glycopeptidolipids in only small amounts, were reported to be non-motile, lacked the ability to form biofilms, but were highly virulent to mice (Howard et al. 2006). Proteinaceous surface structures are therefore again suggested to be involved in the autoagglutinating, hydrophobic and hemagglutinating characteristics of the smooth cells of *F. psychrophilum*. However, some extracellular polysaccharides seem to be expressed by the

smooth cells, although to a lesser extent compared to proteinaceous structures, due to the ability to be cytotoxic to macrophages (**paper III**) and due to the fact that the adhesion ability to polystyrene was impaired by eliminating carbohydrate material from the surface of the cells (**paper IV**). These extracellular polysaccharides probably belong to the capsule or glycocalyx surrounding the cells of *F. psychrophilum* (LaFrentz et al. 2007). If so, the expression of the genes involved in capsule synthesis are likely affected by the phase variation of *F. psychrophilum*. However, this seems to be a reversible mechanism, since the extracellular polysaccharides are expressed in both smooth and rough phenotypes, although in different amounts. This was also suggested for the expression of glycopeptidolipids in the smooth and rough cells of *M. abscessus* (Howard et al. 2006). Another possibility is that the expression of extracellular polysaccharides by *F. psychrophilum* cells is constant, but that the expression of proteins protruding from the capsule is affected by the phase variation, and is up- and downregulated differently in the two phenotypes.

5.3. Rough cells: drifting but cytotoxic when encountered

In general, planktonic bacterial cells are reported to be more hydrophilic compared to the corresponding hydrophobic biofilm forming cells (Black et al. 2004; George & Kishen 2007). The reason for this could be that surface proteins are downregulated in the planktonic phenotype and upregulated in the corresponding biofilm forming phenotype (Sauer et al. 2002; Black et al. 2004). A hydrophilic surface has moreover been connected with the presence of extracellular polysaccharides surrounding the bacterial cells (Reifsteck et al. 1987; Cuperus et al. 1993). This is logical, since capsules are constructed of hydrophilic and acidic polysaccharides (Wilkinson 1958). The rough cells of *F. psychrophilum* were found to be planktonic when cultured in broth and to be hydrophilic and non-agglutinating, in contrast to the smooth cells (**paper I**). This non-agglutinating type of growth mode was demonstrated via scanning electron microscopy (Fig. 2 E).

Sodium (meta)periodate treatment of the rough cells decreased their cytotoxic activity to rainbow trout macrophages, just as it did for the smooth cells (**paper III**). It is therefore suggested that the extracellular polysaccharides, possibly in form of a capsule or glycocalyx (LaFrentz et al. 2007), surrounding the cells of *F. psychrophilum* are connected to the cytotoxic activity. Capsular polysaccharides have long been thought to be

involved in bacterial dispersal and protection against host opsonophagocytosis (Wilkinson 1958), as the surface polysaccharides can prevent host complement components from attaching to the bacterial surface (Celli & Finlay 2002). However, in **paper III** it is suggested that the macrophages recognizes the bacterial cells through an opsonin-independent mechanism called lectinophagocytosis (Ofek & Sharon 1988), since the cytotoxicity of both the smooth and the rough cells depended on the presence of carbohydrates on the surface of the bacterial cells (**paper III**). Lectinophagocytosis is the interaction between carbohydrates on the surface of bacterial cells and complementary lectins on the surface of host phagocytes, or vice versa (Ofek & Sharon 1988). Lectinophagocytosis is moreover thought to be important in immunodeficient hosts whose opsonin activity is poor, or as a defence against opsonophagocytosis-avoiding bacteria (Sharon & Lis 1989). The fact that the rough cells of *F. psychrophilum* was found to be highly cytotoxic to macrophages compared with the smooth cells (**paper III**) indicates, however, that although the macrophages can recognize and interact with the bacterial cells through lectinophagocytosis, the rough cells can escape the phagocytosis through an antiphagocytic and cytotoxic mechanism also present to some extent in the smooth cells. The cytotoxic mechanism, or the lysis of the macrophages, was found to be different in the smooth and the rough cells (**paper III**). Therefore, since both smooth and rough cells seems to express extracellular polysaccharides but execute the lysis of the macrophages differently, it is likely, as discussed above, that the expression of the extracellular polysaccharides is constant in the two phenotypes but the expression of proteins protruding the polysaccharides is under phase variation, at least *in vitro*.

The rough cells of *F. psychrophilum* were found to be more enzymatically active compared with the corresponding smooth cells **(paper I)**. The group of enzymes that was expressed to a higher degree in rough cells compared with corresponding smooth cells was acid phosphatases, which are known to break down a wide variety of structurally diverse substrates in acidic to neutral environments (Rossolini et al. 1998). Acid phosphatases were previously reported to be involved in antiphagocytic strategies for intracellular bacteria by inhibiting the respiratory burst of phagocytes (Saha et al. 1985; Baca et al. 1993; Mohapatra et al. 2007). Whether acid phosphatases are virulence factors of *F. psychrophilum* and further, if they are involved in the antiphagocytic strategy of the rough cells need additional investigations. The

association between the high acid phosphatase activity and cytotoxic activity in rough cells of *F. psychrophilum* (**paper I, III**) is nonetheless interesting.

Some of the rough cells showed some hemolytic activity, hemagglutinating and adhesion ability (**paper I, II, IV**). The complete reduction of the hemagglutinating ability after treatment of the rough cells with sialic acid indicates that the same sialic acid-binding lectin is involved in the adhesion of rough cells to rainbow trout erythrocytes as for the smooth cells (**paper II**). The results further suggest that the expression of the lectin is not under an on-off switch (Henderson et al. 1999) but instead downregulated in the rough phenotype. Similar to the smooth cells, the rough cells were also found to express other lectins than the sialic acid-binding lectin and the most apparent was probably a D-galactose-binding lectin (**paper IV**). Compared with the smooth phenotype, however, the expression of both the sialic acid- and the D-galactose-binding lectins seems to be downregulated in the rough phenotype (**paper II, IV**). It is however likely that both lectins, though upregulated in the smooth and downregulated in the rough phenotype, are important for the tissue tropism (Korea et al. 2011) of *F. psychrophilum*. Further, when smooth cells of *F. psychrophilum* were grown in broth they were able to shift phase into rough cells (**paper I**). It is therefore very likely that it is the up- and downregulated expression of surface proteins, such as the lectins involved in adhesion, and the subsequent change in cell surface hydrophobicity, that determines the cell organization inside the colonies and that gives the colony types their smooth and rough appearances.

6. CONCLUDING REMARKS

In this thesis I have shown that *in vitro* phase variation occurs in the fish pathogenic bacterium *Flavobacterium psychrophilum* seen as two different phenotypic cell and colony types, smooth and rough. The results contribute to an increased knowledge of the virulence of *F. psychrophilum* towards the fish host.

Smooth cells were characterized as autoagglutinating, hydrophobic, highly adhesive to both inert surfaces and rainbow trout erythrocytes. Smooth cells were also highly hemolytic and somewhat cytotoxic to rainbow trout macrophages. These characteristics were all suggested to be linked to proteinaceous structures possibly protruding from extracellular polysaccharides surrounding the bacterial cells. The most dominating proteinaceous structure found was a sialic acid-binding lectin involved in the adhesion of smooth cells to polystyrene surfaces, rainbow trout erythrocytes and likely rainbow trout mucus.

Rough cells were characterized as planktonic, hydrophilic, enzymatically active and highly cytotoxic to rainbow trout macrophages. These characteristics were suggested to be linked to extracellular polysaccharides surrounding the bacterial cells.

The phase-varying structures in *F. psychrophilum* are suggested to be surface proteins, or more exactly lectins involved in the adhesion to host cells and abiotic surfaces, that are possibly protruding from the extracellular polysaccharides surrounding the bacterial cells. Lectin expression is moreover suggested to be differentially regulated than on or off. The different cell organization inside the colonies, which give the colony types their smooth and rough appearances, is also suggested to be associated with the altered lectin expression in the two phenotypes.

What are the possible consequences of phase variation in *F. psychrophilum in vivo*? Phase variation implies that population heterogeneity is of importance in this cold-water fish pathogen. Phase variation could also be a part of the pathoadaptive evolution of *F. psychrophilum*. More specifically, since the smooth cells of *F. psychrophilum* express all characteristics necessary for biofilm formation, and since *F. psychrophilum* is known to form biofilms, it is likely that this is contributing to the pathogen's resistance towards antimicrobial agents and also to the persistent and reoccurring BCWD outbreaks at fish farms. The phenotype switching, from smooth to rough to

possibly smooth again, could also make a future vaccine inefficient if the vaccine is targeting phase-varying surface proteins of the *F. psychrophilum* cells. One novel treatment method for BCWD outbreaks in the future could, however, be prevention of the adhesion of *F. psychrophilum* to the fish by inhibiting the sialic acid-binding lectin or other lectins expressed by the bacterial cells. This approach would target the adhesion molecules on the surface of the bacterial cells, by e.g. coating the surfaces of the fish or the fish tanks with carbohydrates that subsequently would interfere with bacterial lectins, and thereby prevent the attachment and colonization process. An analogous anti-adhesion strategy has been proposed for the prevention of *F. columnare* infections in channel catfish (*Ictalurus punctatus*) (Beck et al. 2012). The advantages of such a method are that it is not likely to cause harm to the fish, or to cause resistance problems in the bacterial pathogen. The cons are that most bacterial pathogens, as showed for *F. psychrophilum* in this thesis, express several different lectins. It is probably the combination of these lectins, and not one in particular, that are important for the infection process. Further, since it is these lectins that are affected by the phase variation, an anti-adhesion approach for prevention of BCWD outbreaks could be even more complicated.

Since the results of this thesis demonstrates diverse characteristics for the smooth and rough cells of *F. psychrophilum*, it could be necessary to include both phenotypes in future studies and study them separately if possible. Future investigations regarding the phenotypic characterization of the two phenotypes should include detailed examinations of the extracellular polysaccharides, or capsule surrounding the cells and the lectins probably protruding the capsule. Future investigations should moreover examine the *in vivo* phase variation of *F. psychrophilum* and the interaction between the two phenotypes and the role of the phenotypes in biofilm formation and disease process.

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Phase variation in *Flavobacterium psychrophilum*: Influence on host-pathogen interactions

This thesis is a characterization of smooth and rough colony phenotypes of the fish pathogenic bacterium *Flavobacterium psychrophilum*. The thesis further describes the *in vitro* interaction of smooth and rough cells with abiotic surfaces, erythrocytes and macrophages isolated from rainbow trout (*Oncorhynchus mykiss*). The results suggest that phase variation occurs in this pathogen and that smooth and rough cells interact differently with host cells *in vitro*. The phase varying structures are suggested to be adhesive surface proteins possibly protruding from the extracellular polysaccharide capsule surrounding the bacterial cells.

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