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Isolation and characterization of skin mucus galectin from Atlantic salmon *Salmo salar*

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Abstract

Galectin-3 is a member of a growing animal lectin family that specifically binds to β-galactosides. It is involved in several biological activities such as signal transduction, inflammation, bacterial agglutination, cell-cell and cell-matrix interaction. In this study a novel isoform of galectin-3 (galectin-3C) was isolated from *Salmo salar* skin mucosa by α-lactose agarose based affinity chromatography followed by Sephadex G-15 gel filtration. The full length *Salmo salar* galectin-3 is 29 kDa whereas electrophoretic and mass spectrometric analysis showed that galectin-3C is 15 kDa and do not have the N-terminal domain. The hemagglutination activity of galectin-3C was calcium independent and inhibited by lactose. The *leg3* mRNA showed high expression in skin and gill followed by muscle, hindgut, spleen, stomach, foregut, head kidney and liver. The galectin-3C agglutinated the Gram negative bacteria *Moritella viscosa*. Furthermore, the proteome of *Moritella viscosa* grown in presence of galectin-3C showed an increased level of proteins involved in protein synthesis and drug transport compared to bacteria grown in absence of galectin-3C. Increased expression of protein synthesis proteins, and multidrug transporter protein has been observed in antibiotic treatment of bacteria. qPCR results indicated that galectin-3C has an effect on growth of *Moritella viscosa* or was killing it, and the increased expression of the transporter might be a strategy for the bacteria to avoid the host immune barriers. Over all, this study shows the involvement of galectin-3C in pathogen recognition and immune defense of Atlantic salmon, *Salmo salar*.

1. Introduction

Aquaculture has successfully stridden forward as one of the rapid growing food sectors for decades. It has contributed 66.63 million tonnes of food fish (commodity produced for human consumption) to the world food basket in 2012 (FAO 2012). The consumer demand for fish is increasing day by day due to its alluring nutritional components like the omega- 3 fatty acids EPA (eicosapentanoic acid) and DHA (docosahexanoic acid) (Howe et al. 2006). This increasing demand is further putting the industry in pressure to produce more fish. Technological advancements and sustainable intensification of aquaculture systems is the only solution to meet this increasing demand. Like other farming sectors, also in aquaculture intensification and expansion results in disease (Mohapatra et al. 2013). This problem occurs when an organism's immune system fails to cope with its surrounding environment. The immune system is a complex network of cells, tissues and proteins, which protects the organism against disease (Rauta et al. 2012). Hence, to avoid disease, it's very important to have knowledge about the immune system, its components, and their interaction with disease causing factors.

1.1. Atlantic salmon: a species of interest

Atlantic salmon, *Salmo salar* is a highly prized fish all over the world, with a high content of omega-3 fatty acid (Mozaffarian and Rimm 2006). Culture of Atlantic salmon began long back in 19th century in UK. Use of sea cages for salmon farming started in 1960s in Norway. It achieved success to become an industry in 1980s. The Norwegian Atlantic salmon industry is ranked 1st in the world with a production of 1.23 million tonnes in 2012 (FAO 2012; Marine harvest 2013). It contributes a noticeable part to the national economy. Norway exported salmon worth NOK 3.7 billion in March 2014 that is 27% higher than in March 2013. In March 2014 the average price of fresh, whole Norwegian Atlantic salmon per kilo has increased to NOK 43.88 compared to NOK 37.59 in March 2013 (Norwegian seafood council 2014).

1.1.1. Habitat and biology

Atlantic salmon is distributed to several parts of the world including Europe, United States of America and Canada. It is an anadromous fish. It spends its juvenile phase in freshwater till it achieves the physiological changes in its body to be able to osmoregulate in

sea water, a process called smoltification. Then it migrates towards sea and returns back to the natal river for spawning, known as homing (FAO 2012). In captivity light and temperature is regulated to mimic the wild conditions to produce smolts, which are then transferred to the sea cages to achieve marketable size. The scientific position of Atlantic salmon in animal kingdom is:

Kingdom: Animalia
Phylum: Chordata
Class: Actinopterigii
Order: Salmoniformes
Family: Salmonidae
Genus: *Salmo*
Species: *S. salar*

Figure 1. Atlantic salmon, *Salmo salar*

1.1.2. Diseases in Atlantic salmon

Key for a successful aquaculture has always been associated with good health management practices. Healthy and disease free fish gives good monetary returns to the producer and provides good nutrition to the consumers. On the other hand poor health management leads to diseases and sometimes death of fish and economic losses.

Table.1. Diseases in Atlantic salmon

Disease	**Pathogen**	**Reference**
Bacterial disease		
Winter ulcer/ winter sores	*Moritella viscosa*	(Coyne et al. 2006; Karlsen et al. 2012)
Furunculosis	*Aeromonas salmonicida*	(Jarp et al. 1995; Midtlying et al. 1996)
Bacterial kidney disease (BKD)	*Renibacterium salmoninarum*	(Matejusova et al. 2013)
Enteric red mouth (ERM)	*Yersinia ruckeri*	(Bridle et al. 2012)
Salmon rickettsial disease	*Piscirikettsia salmonis*	(Tobar et al. 2011; Tacchi et al. 2011)

Cold water vibriosis	*Vibrio salmonicida*	(Bjelland et al. 2012; Bjelland et al. 2013)
Vibriosis	*Vibrio anguillarum*	(Tapia-Cammas et al. 2011)
Parasitic disease		
Sea lice	*Lepeophtherius salmonis*	(Ross et al. 2000; Torrissen et al. 2013)
Viral disease		
Infectious salmon anemia (ISA)	Orthomyxovirus	(Marshall et al. 2014)
Viral hemorrhagic septicaemia (VHS)	Rhabdovirus	(Garver et al. 2013)
Infectious pancreatic necrosis (IPN)	Birnavirus	(Jarp et al. 1995)

1.1.2.1. Winter ulcer disease

Winter ulcer is one of the prevalent bacterial diseases caused by the bacterium *Moritella viscosa.* This disease was first reported in farmed Atlantic salmon (*Salmo salar*) in Norway (Lunder et al. 1995). It generally occurs in cold waters. High level of mortalities are found in low temperature at around 4º C compared to high water temperatures (Tunsjø et al. 2007)

Moritella viscosa is a Gram negative, motile, psychrophilic, non spore forming bacteria. Previously it was known as *Vibrio viscosa*, but after 16S rRNA sequencing this bacterium was included in the family Moritellaceae and named *Moritella viscosa*. It has 99.1% sequence similarity in the 16S rRNA with *Moritella marina. M. viscosa* grows well in media supplemented with sodium chloride at temperature between 4-21º C. Temperature and NaCl concentration plays an important role in determining the growth of these bacteria in laboratory conditions (Tunsjø et al. 2007).

M. viscosa is thought to mainly enter the fish body through gills and passes the defense barrier of the host by covering itself by its polysaccharide capsule. Then the bacterium spreads throughout the body to establish clinical signs. The immune responses by host against this infection is found to be appearing very late which suggests that this bacteria is smart enough to escape the initial host defense mechanisms (Løvoll et al. 2009). A study on Chinook salmon embryo cell line showed that *M. viscosa* disrupts the cytoskeleton and affects the cell structure and its rigidity by forming pores and subsequently it lyses the tissue using its secreted products (Tunsjø et al. 2009). However, there is very little information on host-pathogen interaction involving *Moritella viscosa.*

1.2. A general overview of fish immune system

Knowledge of the immune system and its underlying molecules is a fundamental requirement for better understanding of a disease and interaction between host and pathogen. The immune system consists of two arms, innate and adaptive. Being the earliest class of vertebrates, teleosts are equipped with both innate and acquired immunity (Uribe et al. 2011). Even though the immune system is divided into innate and adaptive there is no specific boundary line between these two systems. Increasing research and evidences are showing that innate immunity plays an instructive role to bring adaptive system to an action. So the mechanism and actions of the immune system against an invader is more of combinative than isolative nature (Magnadottir 2006).

Innate immune system is regarded as the ancient immune system found in both invertebrate and vertebrate. It is non-specific and acts as barrier for a wide range of invaders. Classically it is referred as the first line of defense (Whyte 2007). Innate immunity is inheritable and is the only defense mechanism for developing eggs as well as the new born till the adaptive immune system develops. The innate immune system consists of mucosal/epithelial barriers, humoral and cellular factors (Magnadottir 2010).

The adaptive immune system develops after birth. It takes several days to weeks depending on the species of fish (Zapata et al. 2006). Adaptive immunity is very specific for different pathogens and it can also keep the memory for any future encounter from the same pathogen. It consists of humoral and cellular parameters. Immunoglobulins are the key humoral parameter of adaptive immune system (Magnadottir 2010).

Mucosal immune defense has gained increasing focus as an interesting topic of research for fish immunologists since the past few years. The immune system of vertebrates is comprised of both mucosal and systemic factors. Among these two factors, the mucosal immune system is the one which gives protection against the first attack of pathogen. In fish the mucosa associated lymphoid tissue (MALT) has been divided into three groups based on anatomical considerations. These are (i) gut associated lymphoid tissue (GALT) (ii) skin associated lymphoid tissue and (SALT) (iii) gill associated lymphoid tissue (GIALT) (Ángeles Esteban 2012; Xu et al. 2013). The mucosa associated lymphoid tissues in fish shows structural as well as immunologically differences from species to species (Salinas et al. 2011).

1.3. Immunological defenses of fish skin mucus

All vertebrates possess a similar structure of the skin. It consists of two layers (i) epidermis and (ii) dermis. Embryonic origin of skin is the same for all vertebrates, that is the epidermis is originated from ectoderm and the underlying layers are originated from mesoderm. The histological variation in the skin of vertebrates such as presence of hair, feathers, scales, secretion of sweat or mucus depends on the habitat in which the organism is living. The early vertebrate living in aquatic environment like the bony fish possess mucosal skin surfaces, lacks keratinized skin and have living epithelial cells in direct contact with its external milieu. When organisms started to live in on the dry land they lost the mucosal layer and acquired keratinized cells due to evolutionary changes (e.g. hairs in human and feathers in birds) (Schempp et al. 2009; Xu et al. 2013). Fish lives in an environment rich in pathogens. It is the skin which protects the internal body from the external environment. Fish skin is different from mammal because it secretes mucus which plays an important role in immune defense (Salinas et al. 2011). The epidermis of fish skin contains unicellular glands named goblet cells which are involved in mucus secretion. The number of goblet cells and production of mucus varies in different situations. For example, from mucus cells enumeration it has been found that the number of goblet cells increases when the fish is stressed (Vatsos et al. 2010). Also the composition of mucus varies from healthy to diseased fish. Thus, the composition and characteristics of skin mucus is very important to be maintained for a healthy living fish (Cone 2009). The composition of mucus also depends on several factors like species, sex, developmental stage, infection, stress and other environmental factors. Other secretory cells found in skin mucus of fish are malpighian cells, club cells and sacciform cells (Zaccone et al. 2001). Skin mucus in fish acts as the first barrier against pathogenic infections. The malpighian (Asbakk 2001) and goblets cells (Iger and Abraham 1990) are found to be involved in phagocytic activities. Club cells are very specific and they are not common for all fishes. The antimicrobial activity of skin mucus has been demonstrated by several researchers. The mucus can trap the pathogen and remove it in form of shedding the mucus before the pathogen tries to evade the epithelial layers of skin. It can do so as it is impermeable to many pathogens. Thus skin mucus acts as a storehouse of many biologically active factors which are involved in defense mechanisms (Ángeles Esteban 2012). Below some of the important immune parameters found in skin mucus has been described.

Mucin: It is a high molecular weight, densely glycosylated glycoprotein which is highly abundant in fish skin mucus. Protein from the mucin family contains a tandem repeat structure. These structures usually have a high content of proline, threonine and serine that forms the PTS domain. Glycosylation occurs in the PTS domain through the N-acetylgalactosamine O- linkages at threonine and serine residues (Kufe 2009). Mucins are very adhesive and have matrix forming ability, where other antimicrobial components can reside (Ángeles Esteban 2012).

Enzymes: Various enzymes present in fish skin mucus have been reported to be involved in innate immune function. **Lysozyme** is one of the well studied mucosal enzymes. Lysozyme is also known as N- acetylmuramide glucanohydrolase or muramidase. Its bactericidal activity makes it a suitable component of the defense system. It can lyse both Gram positive and Gram negative bacteria. It is involved in opsonization, complement activation and phagocytosis (Saurabh and Sahoo 2008). **Acid and alkaline phosphatases** are important enzymes in lysosomal degradation. These are involved in hydrolysis of phosphate containing compounds. Acid phosphatase is demonstrated as a marker for detection of lysosome in cells, where as alkaline phosphatase serves as a stress indicator (Ross et al. 2000; Mazorra et al. 2002). Presence of **proteases** in fish skin mucus has been reported from several fishes. It can directly kill pathogen by cleaving their proteins or it could also be involved in complement activation, enhancement of production of several immune factors like antimicrobial peptides or immunoglobulin. Proteases have several groups such as serine, aspartic, cysteine and metalloproteases. Some of the proteases identified in fish skin mucus are trypsin, cathepsin D, B and L, and metalloproteases. Trypsin is a serine protease, Cathepsin B and L are cysteine protease, and cathepsin D belongs to aspartic protease group (Hjelmeland et al. 1983; Firth et al. 2000; Ángeles Esteban 2012). Other important enzymes found to be involved in immune functions in skin mucus are superoxide dismutase, easterase, cystine arylamidase, beta glucoronidase, beta galactosidase etc. (Ángeles Esteban 2012).

Antimicrobial peptides: These are small amphipathic and cationic peptides capable of interacting with cell membranes forming pores by specific mechanisms. These are involved in growth inhibition and killing of pathogenic microorganisms (Brogden 2005). Cathelicidin, defensin, piscidin, pleurocidin, paradaxin, are some of the types of antimicrobial peptides isolated from fishes (Cole et al. 1997; Adermann et al. 1998; Chang et al. 2006; Zou et al. 2007; Maier et al. 2008; Ruangsri et al. 2012).

Other proteins involved in immune function: Several additional proteins are also found to be involved in immune defense of fish skin mucus. Some of the important proteins are lectin, lactoferrin, histone, ribosomal proteins etc. (Ángeles Esteban 2012).

1.4. Lectin

Lectins are sugar binding proteins with specific carbohydrate recognition domains (CRD) (Sharon and Lis 1987; Barondes 1988). These are widely distributed among all living organisms from single cell bacteria to multicellular plants and animals. The first report on identification of lectin was documented in a doctoral thesis by Stillmark in 1888. While working on castor beans (*Ricinus communis*) Stillmark observed that a partially purified toxic protein was able to agglutinate erythrocytes. He named this agglutinating agent as ricin (Sharon and Lis 1987). His work on ricin initiated an array of research on lectins.

1.4.1. Classification of fish lectins

Lectins are classified based on their specificity of carbohydrate binding domain and dependence on divalent cations for their activities. The major classes of lectins identified in fish are galectins, C-type lectins, F- type lectin, rhamnose binding lectin, pufflectin, X-type lectin, pentraxins, calnexin and calreticulins (Taylor and Drickamer 2003; Vasta et al. 2011).

1.4.1.1. Galectins

Galectins are family of evolutionarily conserved animal lectins containing one or two CRDs (carbohydrate recognition domain). This lectin family was previously known as S-type or S-Lac lectins. In 1994 the family name changed to galectin with a refined definition which includes two specific criteria for a lectin to be included in this family. One of the criteria is the affinity for β- galactosidases and another one is the significant sequence similarities of CRD (Barondes et al. 1994). Activities of galectins are not metal dependent. Binding of galectin to carbohydrate do not require calcium. Galectin lacks the signal peptide which is necessary for their secretion through the classical secretory understood pathway. Galectins are generally located in cytoplasm and also have been found in some intracellular vesicles (Chen et al. 2012). Till date galectin has been isolated from several biological sources from insects to mammals. It has also been identified in a number of teleost fishes as listed in table. 2.

Table.2. Galectins identified in teleost fish

Organism	Protein name	Galectin type	Reference
Anguilla japonica	AJL-1	Proto	(Tasumi et al. 2004)
Conger myriaster	Congerin I	Proto	(Kamiya et al. 1988)
Conger myriaster	Congerin II	Proto	(Kamiya et al. 1988)
Conger myriaster	Con-P	-	(Watanabe et al. 2012)
Danio rerio	Drgal1-L1	Proto	(Ahmed et al. 2004)
Danio rerio	Drgal1-L2	Proto	(Ahmed et al. 2004)
Danio rerio	Drgal1-L3	Proto	(Ahmed et al. 2004)
Dicentrarchus labrax	Sbgalectin1	Proto	(Poisa-Beiro et al. 2009)
Electrophorus electricus	Electrolectin	Proto	(Levi and Teichberg 1981)
Gadus morhua	Codgal1-1	Proto	(Rajan et al. 2013)
Gadus morhua	Codgal1-2	Proto	(Rajan et al. 2013)
Onchorhyncus mykiss	-	Tandem repeat	(Inagawa et al. 2001)
Rhodeus uyekii	RuGlec9	Tandem repeat	(Kong et al. 2012)
Trachidermus fasciatus	TfGal	-	(Yang et al. 2013)

Based on domain organization (structure and number of CRD) galectins are classified into three types; proto, chimera and tandem repeats (Figure 2) (Hirabayashi and Kasai 1993).

Proto type: The proto type galectins have single CRD (containing approximately 130 amino acids) that forms homodimers (Chen et al. 2014). The member of galectin belongs to the proto type are galectin 1, 2, 5, 7, 10, 11, 13, 14 and 15.

Tandem repeat: Tandem repeat galectins possess two different but homologous CRDs on a single polypeptide joined by a hinge region. This type includes galectin 4, 6, 8, 9, 12.

Chimera type: Galectin-3 is the sole member of chimera type galectin. It contains a single CRD. It has a non lectin domain in its N terminal connected to its CRD in the C terminal end.

Galectins plays several biological roles. It is involved in cell extracellular matrix interaction, cell-cell interaction, cell signaling, complement activation, opsonization, inflammation and

also serves as pathogen recognition receptors through its carbohydrate recognition domain (Rubinstein et al. 2004; Nakahara and Raz 2006; Song et al 2013).

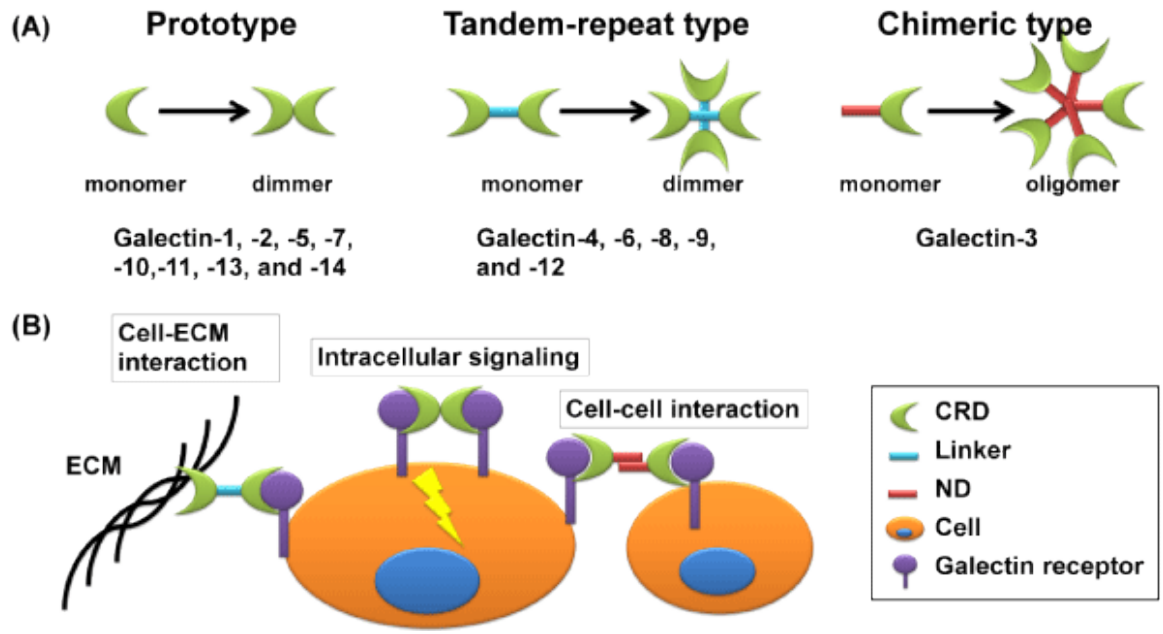

Figure 2. Structure of galectins and their functions (A) Showing structure of galectin CRDs (B) Function of extracellular galectins. Bivalent and multivalent galectin cross linking results incell-cell interaction, cell signal transduction and cell-matrix interaction. CRD- carbohydrate recognition domain, ND-N-terminal domain, ECM- extracellular matrix (Song et al. 2013)

1.4.1.1.1. Studies on teleost galectins

The above mentioned features of galectins make them interesting topics for research. Four galectin-1 like proteins Drgal1-L1, Drgal1-L2, Drgal1-L3, and a splice variant of Drgal1-L2 have been identified and characterized in zebra fish (Ahmed et al. 2004**).** A study conducted on zebra fish showed that knocking down of galectin-1 like protein (Drgal1L-1) in zebra fish embryos resulted in skeletal deformities showing a bent tail and disorganized muscle fibers. The author concluded that galectin-1 in notochord plays an important role in cell differentiation and development (Ahmed et al. 2009). Another study on partial knock down of Drgal1-L2 showed significant reduction of regeneration of rod photoreceptors suggesting that this protein is required for regeneration of rod cells (Craig et al. 2010).

Several studies show agglutination activity of galectins. In Atlantic cod, *Gadus morhua* two galectin-1 proteins showed agglutination activity for both Gram positive and Gram negative bacteria (Rajan et al, 2013). Recombinant galectin from roughskin sculpin (TfGal) could agglutinate both bacteria (both Gram positive and Gram negative) and yeast

(Yang et al. 2013). Proto type galectin AJL-1 from Japanese eel, congerins from Japanese conger showed agglutination against *Streptococcus difficile,* and *Vibrio anguillarum* respectively (Tasumi et al. 2004; Nakamura et al. 2006).

Congerin is found to have opsonic and agglutinating activities in the intestinal lumen of conger eel, *Conger myriaster*, but the actual mechanism is still unknown (Nakamura et al. 2007). Studies from the same author revealed that congerin I could encapsulate nematode named *Cucullanus* in abdominal cavity by enhancing host cell adhesion (Nakamura et al. 2012). Further research on conger eel came up with a new isotype of congerin named Con-P showing 21.9%, 22.6%, 20.4% amino acid sequence similarity of with congerin I, congerin II and human galectin 1 respectively. Recombinant Con-P displayed a unique property by recognizing oligo mannose sugar rather than recognizing lactose and galactose like congerin I and II. Therefore, it could be possible that Con-P is involved in immune functions by binding with mannosides displayed on cell surfaces of pathogens (Watanabe et al. 2012). Galectin-1 isolated from Atlantic cod was found to be present in macrophage like cells from head kidney, and localise to *Francisella noatunensis* granulomas in infected samples (Rajan et al, 2013). AJL-1 was isolated from skin mucus of *Anguilla japonica*, Japanese eel in higher level in fish that survived an infection than those that died. The mechanism could be trapping of bacteria in skin mucus and subsequently sloughing it out, giving resistance to the fish against infection and disease (Tasumi et al. 2004).

There are also reports on galectins showing antiviral activities. Olive flounder, *Paralichthys olivaceus* challenged with poly I:C showed expression of galectin-1 (fglec1) in head kidney. It could neutralize lymphocystis disease virus (LCDV) and was found to be involved in anti inflammatory activity (Liu et al. 2013). Sbgalectin from *Dicentrarchus labrax* showed the same activities as fglec1 against noda virus infection (Poisa-Beiro et al. 2009). A Ca^{+2} dependent α- methyl galactose binding lectin purified from serum of Indian cat fish, *Clarius batrachus* also agglutinated several strains of *Aeromonas* showing their role in the innate immune system. It was also observed that this lectin could induce proliferation of head kidney lymphocytes. This suggests that there might be lectin receptors present on surface of lymphocytes (Dutta et al, 2005). Findings of Fock et al (2001) showed that even very low quantity (nano gram) of N-acetyl-galactosamine-binding lectin was enough to induce phagocytic activity against virulent bacteria, *A. hydrophila* by macrophages of blue gourami, *Trichogaster tricopterus*.

1.4.1.2. Other lectin classes found in fish

C-type lectins are mostly Ca^{+2} dependent lectins. CRDs of these lectins are homologous, but still they are very selective in recognizing sugars. They as a group interact with a variety of sugars through their multiple structural domains. This lectin family has two subgroups playing an important role in innate immunity, they are collectins and mannose macrophage receptors. Collectins recognizes the pathogen and initiates complement activity. Mannose macrophage receptor is directly involved in phagocytosis. Selectins, proteoglycan core protein, endocytic receptors are also subgroups belonging to C-type lectins family. A C-type lectin named nattectin, isolated from venomous fish *Thalassophryne nattereri* has found to be involved in activation and consequent release of chemotactic factors, which helps in immediate recruitment of circulating neutrophils to the site of infection (Lopes-Ferreira et al. 2011). Studies on Atlantic salmon, *Salmo salar* C- type serum lectin demonstrated its role in pathogen recognition, by actively binding to bacteria like *Vibrio anguillarum* and *Aeromonas salmonicida* (Ewart et al, 1999).

F-type lectins are also called fucolectins. The name F-type has come from its specificity towards $_{L}$- fucose sugar. This lectin has been identified in number of teleost species. Few of them are sea bass, *Dicentrarchus labrax* (Salerno et al. 2009), *Sparus aurata* (Cammarata et al. 2007), etc.

Rhamnose binding lectins, as the name suggests, this family of lectin has specificity for $_{L}$- rhamnose sugar. It is also called roe lectin or egg lectin. A tandem repeat rhamnose binding lectin has been isolated from skin mucus of pony fish, *Leiognathus nuchalis* (Okamoto et al. 2005). Isolated rhamnose binding lectin from sea bass plasma showed agglutination and opsonization activities against pathogenic bacteria (Cammarata et al. 2014).

Pufflectin has been found in puffer fish, *Fugu rubripes.* This lectin showed 30% identity to mannose binding lectin of monocotyledonous plant It is a mannose specific lectin widely distributed in inner and outer surface tissues (Suzuki et al. 2003).

X-type lectins are also known as intelectin. In teleost this lectin has been isolated from plasma of rainbow trout, *Onchorynchus mykiss* and named as RTInt (rainbow trout plasma intelectin). The activities of RTInt are calcium dependent. It showed specificity for N-acetylglucosamine (GlcNAc). It showed binding activities against bacteria showing its involvement in defense mechanism (Russell et al. 2008)

Pentraxins are a family of serum proteins. CRP (C- reactive protein) and SAP (serum amyloid protein) are considered as important members of this family. These proteins are

evolutionary conserved and involved in acute phase responses. This group of lectin depends on cations like Ca^{+2} for its various activities (Bottazzi et al. 2010). Pentraxins have been identified in several fish species including Atlantic salmon, rainbow trout, Atlantic halibut etc. (Whyte 2007)

Calnexin (CNX) and **calreticulin** (CALR or CRT) are lectin like chaperons in the endoplasmic reticulum (ER). CNX is a nonglycosylated type I membrane protein and CRT is highly conserved calcium binding protein. Both the proteins have identical lectin specificities. They promote proper folding of misfolded proteins, assemblage of oligomers and quality control in ER. CNX and CRT have been found to be associated with folding of class I major histocompatibilty complex (MHC) molecules (Michalak et al. 1999; Vasta et al. 2011). In fish CNX has been found to be associated with class II MHC molecules of channel catfish (*Ictalurus punctatus*) and CRT has been identified in rainbow trout (*Onchorynchus mykiss*) (Kales et al. 2004; Fuller et al. 2004; Liu et al. 2011)

1.5. Methodological aspects

1.5.1. Isolation and purification of lectins

Lectins have been isolated from numerous biological sources like plants, animal, and microorganisms. The most used method for lectin isolation is carbohydrate affinity chromatography. This method is based on the principle of protein carbohydrate interaction of lectins.

Carbohydrate affinity chromatography

Affinity chromatography is widely used to purify proteins that bind non covalently and reversibly to their ligands. Binding of lectins to its carbohydrate ligand is generally non covalent and reversible including interactions like van der Waals, electrostatic and hydrophobic. These relatively weak bindings allow the lectin to be released from an affinity column through the competitive elution method, where a ligand is added in excess. In competitive elution the column is washed with a solution containing excess ligand, where the ligand competes with the target protein for attachment. As a result the target protein get released to the mobile phase (because of weak and reversible attachment) and can be recovered (Firer 2001). These properties of lectin are suitable for using carbohydrate affinity chromatography. One of the important steps in this technique is preparation of the chromatography column. There is a range of adsorbents used by several studies for lectin purification. It could be a carbohydrate/glycoprotein ligand immobilized in a carrier matrix or

an adsorbent ligand. In a carrier matrix, the matrix is activated and coupled with a ligand whereas in adsorbent matrices; the matrix itself binds to the lectin. Both types of adsorbents have been used for purification of lectins from fish tissues (Table. 3). Extraction of lectin from tissue is an important step of purification. It could be done by mechanical disruption, such as sonication. As lectin is water soluble aqueous solutions are used as the medium for extraction. In the present study the lectin was isolated using carbohydrate affinity chromatography.

Table.3. Different matrix and ligands used in lectin purification

Species	Ligand and matrix	Lectin identified	Reference
Japanese eel *Anguilla japonica*	L-fucose agarose	Eel serum fucolectin	(Honda et al. 2000)
Japanese eel (*Anguilla japonica*)	Lactose sepharose 6B	Skin mucus	(Takayama et al. 2009)
Steelhead trout (*Onchorhyncus mykiss*)	L-rhamnose-Sepharose 6B	Egg lectin Rhamnose binding lectin	(Tateno et al. 1998)
Skipjack tuna (*Katsuwonus pelamis*)	Asialofetuin Sepharose 4B	Fish egg	(Jung et al. 2003)
Bighead carp (*Aristichthys nobilis*)	DEAE Sepharose FF	Fucose binding lectin	(Pan et al. 2010)
Gilthead sea bream (*Sparus aurata*)	L-fucose-agarose	F-type lectin from serum	(Cammarata et al. 2007)
Blue gourami (*Trichogaster tricopterus*)	N-acetyl D galactosamine immobilized on epoxy activated Sepharose 4B	Serum lectin	(Fock et al. 2000)
Indian catfish (*Clarius batrachus*)	Con A Sepharose 4B	Galactose binding serum lectin	(Dutta et al. 2005)
Genypterus blacodes		First lectin isolated from fish	(Oda et al. 1984)
Ayu (*Plecoglossus altevilis*)	L-rhamnose-Sepharose 4B	Rhamnose binding lectin	(Watanabe et al. 2008)
Chum salmon (Onchorhyncus keta)	L-rhamnose-Sepharose 6B	Rhamnose binding lectin from egg	(Shiina et al. 2002)
Atlantic cod (*Gadus morhua*)	Alfa lactose agarose	Galectin	Rajan et al., 2013

1.5.2 Separation of proteins on one dimensional and two dimensional gel electrophoresis

One dimensional SDS-PAGE (sodium dodecyl sulfate – poly acrylamide gel electrophoresis) is a preferred technique for protein study by electrophoresis. Protein separation in SDS-PAGE is mediated by its molecular weight. High molecular weight proteins get trapped in the acrylamide gel mesh and move slowly whereas low molecular weights protein move faster. Therefore, the ratio of acrylamide and bis-acrylamide crosslinker is very important for protein separation (Rath et al. 2013). This is a suitable method for monitoring and checking the purity in protein purification as well as to estimate the molecular weight of the unknown purified proteins (Brunelle and Green 2014). In this thesis glycine based electrophoresis, also called Laemmli SDS-PAGE (Laemmli 1970) has been used to resolve the purified product obtained from the affinity column. This method has been successfully used by several authors for assessing the relative molecular weight and purity of different types of purified lectins such as galectin (Rajan et al. 2013), rhamnose binding lectin (Cammarata et al. 2014), C-type lectin (Uribe et al. 2013) etc.

2D gel electrophoresis is a robust and straight forward method used in biochemistry and molecular biology. In this thesis 2D gel based proteomics is used for resolution of bacterial proteins. Like other biological samples 2D gel based proteomics has successfully been implemented for bacterial proteomics (Curreem et al. 2012). In this method proteins are separated on the basis of their isoelectric point and molecular weight. Some of the advantageous features of this method are (i) its high resolution and separating ability (ii) with narrow pH range of IPG strips 1000s of spot can be resolved at one time (iii) protein spots can be visualize at nanogram levels of sample concentrations (iv) good for expression studies (v) good for separation of protein isoforms. (Rogowska-Wrzesinska et al. 2013). Disadvantages are underrepresentation of hydrophobic and basic proteins and the fact that proteins present in low amount might not be visible.

In both one and two dimensional gel electrophoresis the final result can only be visualized after proper staining of the acrylamide gels. Some of the stains widely used are the Coomassie blue stain, silver stain and commercially available fluorescent stains. Silver stain and SYPRO fluorescent stains are more sensitive than Coomassie blue stain (Sasse and Gallagher 2004). However, Coomassie blue staining is simple and cost effective as compared to the other stains. In the present study one dimensional gels and 2D gels were stained with colloidal Coomassie G-250 and SYPRO Ruby stain respectively.

2. Objectives of the study

Lectins are sugar binding proteins involved in immune defense of teleost fish, involved in among others pathogen recognition, opsonization, complement activation, signal transduction. The aim of the present study is to isolate and characterize lactose binding lectin from the skin mucus of Atlantic salmon. The specific objectives of the study are

- To purify skin mucus lactose binding lectin from Atlantic salmon, *Salmo salar*.
- To identify and characterize the purified protein using mass spectrometry and bioinformatics tools.
- To elucidate the distribution of the identified lectin mRNA in different tissues
- To study the possible hemagglutination and bacterial agglutination activity of the identified lectin.
- To determine possible antimicrobial activities of the identified lectin.

3. Materials and methods

3.1. Fish and their maintenance

Atlantic salmon used for the study were reared in indoor tanks at Mørkvedbukta research station, University of Nordland, Bodø, Norway. The fishes were maintained at temperature 12ºC and raised on commercial diet (Skretting, Norway) till they attain a weight of approximately 1 kg.

3.2. Sampling of skin mucus and tissues

Fishes weighing approximately 1 kg of varying length were used for sampling. Prior to sampling the fishes were anesthetized with MS-222 (70 mg/L). Using a sterile glass slide, mucus from the skin of Atlantic salmon was scraped from the dorsal and ventral body surface, avoiding the anal region to prevent fecal contamination. The collected skin mucus samples were immediately kept on ice and then stored in -80ºC freezer for later use. Tissue samples from skin, muscle, gill, foregut, hindgut, stomach, liver, kidney and spleen were collected and immediately frozen in liquid nitrogen and moved to a -80ºC freezer for storage until further analysis.

3.3. Purification of *Salmo salar* galectin-3 from skin mucus

3.3.1. Preparation of skin mucus sample for purification

Skin mucus samples stored at -80ºC were taken out and thawed on ice. Five ml of mucus sample in a 50 ml tube was mixed with protease inhibitor (10 µl/ml) (GE Healthcare, USA). Next, the mucus sample with protease inhibitor was diluted by adding 3 volumes of binding buffer (20mM Tris-HCl, 10mM $CaCl_2$, 10mM $MgCl_2$, 0.5M NaCl, pH-7.5) and mixed well by pipetting. The diluted mucus sample was sonicated for 3 times 5s using an ultrasonic processor (SONICS Vibracell VCX750, USA). Sonicated mucus sample was centrifuged at 14000 g for 30 min at 4ºC to remove the cell debris. Supernatant of the centrifuged mucus sample was used for purification.

3.3.2. Binding of skin mucus to α- Lactose agarose

The purification was done by following the protocol described by Rajan et al (2013) with few modifications. Approximately 5 ml of α-Lactose agarose resin (Sigma-Aldrich, USA) in a 50

ml tube was washed twice with 3 volumes of 15Ω analytic grade water milipore water. The washed resin was equilibrated with 3 volumes of binding buffer at 4ºC. To the equilibrated resin 20 ml of previously prepared skin mucus was added and allowed to bind for 2h at 4ºC under rotation. The skin mucus bound resin was washed three times with binding buffer and loaded to a 10 ml Bio-Rad gravity purification column. The bound proteins from skin mucus were eluted manually using elution buffer (20mM Tris-HCl, 0.5M NaCl, 0.5M α-Lactose). Ten fractions, each of 500 µl of eluate were collected and the protein was quantified. The protein quantification was done by using Qubit® Protein Assay Kit in a Qubit fluorometer (Life Technologies, USA). The column was then washed twice with binding buffer and stored at 4ºC for further use. Each fraction containing protein was subjected for desalting to remove the salts and impurities.

3.3.3. Desalting of purified protein using Sephadex G-15

Dry beads of Sephadex G-15 (GE Healthcare, Life Sciences, UK) were washed and soaked for overnight in analytic grade water (15Ω). Two ml of overnight soaked beads were loaded in a 10 ml Bio-Rad gravity purification column and equilibrated with PBS (phosphate buffered saline). Five hundred micro liters of eluted fraction from α-Lactose agarose column was loaded to the equilibrated Sephadex G-15 column. Ten fractions (0.5 ml each) were eluted from the column. Eluted fractions containing protein were pooled and up concentrated using 3K cut off centrifugal filters (VWR, North America). The protein after concentration was again quantified using Qubit® Protein Assay Kit and Qubit fluorometer (Life Technologies, USA) and kept in -20º C for further use.

3.4. SDS-PAGE

The protein profile of the purified product was determined by running it in a 15% poly acrylamide gel (Laemmli 1970). Sample was mixed with protein loading buffer (Laemmli sample buffer mixed with beta- mercaptoethanol, Bio- Rad) and incubated at 95ºC for 5 min before loading to the gel. The gel was run in Bio-Rad Mini PROTEAN® Tetra cell, at 100V. Protein marker (Precision Plus, Kaleidoscope™, Protein™ Standards, Bio-Rad) was used to estimate molecular weight of the protein. Gels were stained in colloidal Coomassie G-250 overnight, destained using analytic grade water, and digitalized (ChemiDoc™ XRS System, Bio-Rad.

3.5. LC/MS-MS analysis

The protein band appeared in gel was excised, reduced, alkylated and trypsinized to obtain a peptide mixture as described elsewhere (Øverbye et al 2007). The peptide mixture was subjected to LC-MS/MS analysis using ESI-Quad-TOF, USA and Q-Exactive, Thermo Scientific. Peak lists (pkl) were obtained by Protein Lynx Global server software (version 2.1). The pkl files were analysed for the protein identification using appropriate bioinformatics tools.

3.6. Bioinformatic analysis of LC-MS/MS data

The peak list file obtained were analyzed using MASCOT MS/MS Ions search (http://www.matrixscience.com) against NCBI non redundant data base. The parameters set for analysis were fixed carbamidomethyl (C) modifications, variable oxidation (M) modifications, peptide mass tolerance of 100 ppm, fragment mass tolerance of 0.1 Da, monoisotopic mass values, maximum 1 mixed cleavage by the enzyme trypsin. Hits having individual ion score >56 were taken into consideration for the protein identification.

3.7. Hemagglutination and inhibition assays

Hemagglutination activity was measured by using horse erythrocytes from Statens Serum Institute, Copenhagen, Denmark. The erythrocytes were treated in 0.05% trypsin EDTA and fixed in 1% gluteraldehyde as described by Nowak et al., 1977. The activity was determined using serial two fold dilutions of the purified protein (200 µg/ml). The assay was performed in an U bottomed microtiter plate (VWR, Norway). Each well contained 25µl of 1% BSA in PBS, 25µl of PBS, 25µl of diluted protein sample and 25µl of 4% erythrocyte suspension. For control the protein sample was replaced by 25µl of PBS and for hemagglutination inhibition assay PBS was replaced with 0.5M lactose in PBS. Mixing was done for 30s at slow speed in a microplate vortex (Genie®, Scientific industries, INC, Long islands, USA). The plate was incubated for an hour at room temperature and hemagglutination activity was determined.

3.8. Tissue distribution

3.8. 1. RNA extraction and cDNA synthesis

RNA from gill, skin, muscle, stomach, foregut, hindgut, spleen, head kidney and liver of healthy Atlantic salmon were extracted using EZNA total RNA kit (Omega Bio-Tek, Norcross, GA, USA) following manufacturer's manual. The extracted RNA was quantified

using Qubit® RNA BR Assay kit and Qubit® fluorometer (Life technologies, USA). RNA quality was evaluated by running it in a 1.2% agarose gel. The cDNA was synthesized using QuantiTect Reverse Transcription kit with integrated removal of genomic DNA contamination (Qiagen, USA) following the manufacturer's protocol.

3.8.2. Primer designing

The mRNA sequence of Atlantic salmon (*Salmo salar*) galectin-3, (Accession No: NM_001140833 GI: 213514683) was retrieved from NCBI (http://www.ncbi.nlm.nih.gov). The retrieved mRNA sequence was blasted against NCBI Primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to pick primers specific for galectin-3 (gene symbol: *leg3*). Primers obtained from NCBI Primer Blast were analyzed for melting temperature and secondary structures (hairpins, self dimers, cross dimers, palindromes, repeats and runs) using Net Primer (http://www.premierbiosoft.com/netprimer) software. Two set of primers having minimum secondary structures and suitable thermal conditions were chosen for the study (Table. 4). The designed primers were used for tissue distribution analysis.

Table.4 Galectin-3 primers used in the study

Gene name	**Gene symbol**	**Sequence (5'→3')**
galectin-3	*leg3*	Reverse: GGCTGAAACCAACCCTGCTA
		Forward: GAGTTCAAACACCGCATCCG

3.8.3. Real time PCR for tissue distribution analysis

Real time quantitative PCR was performed to localize the distribution of *leg3* mRNA in different tissues of healthy Atlantic salmon. The primer used for real time PCR is shown in Table: 1. StepOneplus real time PCR system (Applied Biosystems, CA, USA) was used to perform all the PCR reactions. The thermocycling condition used was: holding stage of 20s at 95ºC for activation of Taq DNA polymerase, followed by 45 cycles of denaturation at 95ºC for 3s, annealing at 60ºC for 30s, a data acquisition step of 15s at 60ºC during the annealing stage in each cycle. Each PCR reaction mixture was of a volume of 10 µl containing 5 µl of SYBR Green master mix (Applied Biosystems, USA), 4 µl of 25x diluted DNA, 0.5 µl of each primer. The reaction mixture was dispended in 96 wells plate and covered with an

optical adhesive (Applied Biosystems, USA). The prepared plate was centrifuged for a minute and loaded into the machine. Each reaction was run in duplicates with appropriate standards and negative control (no template reaction). Standard was run by using a fivefold dilution series of pooled cDNA. The same reaction set up and PCR conditions were used for the reference gene *elongation factor 1 alfa* (Olsvik et al. 2005; Ingerslev et al. 2006)

3.9. Effect of *Salmo salar* galectin-3C on proteome of *Moritella viscosa*

3.9.1. Bacterial strain and experimental set up

Stock culture of *M. viscosa* (Glomfjord isolate) (Sirriyappagouder 2013) stored at -80ºC was used in the study. A loop full of bacteria from -80ºC stock was streaked on agar plate supplemented with 3% TSB and 1.5% NaCl and incubated at 15ºC. A colony from the plate was picked and inoculated in media containing 3% TSB (Tryptic soy broth, Fluka, Sigma-Aldrich) supplemented with 1.5% NaCl at 15ºC.

The experimental media included 3% TSB, 1.5% NaCl supplemented with 13 µg of purified galectin-3C (change of name is explained in section 4.2) per ml of media. To observe the inhibitory effect of lactose in one set up 0.5M lactose was added to the media along with galectin-3C (13 µg/ml of medium). One more set up was made by adding only 0.5M lactose but no galectin-3C. In control (3%TSB + 1.5% NaCl) samples PBS was added in place of galectin-3C to make the volume equal in all set ups. A bacterial culture of 0.6 OD was used as inoculum for the experiment. The experiment was done in duplicates at 15ºC. Cell growth of bacteria was measured at 590 nm at interval of every 4 h. Samples for 2D analysis and qPCR were taken at each time point. For 2D the samples were harvested by centrifugation (4000 g, 10 min, 4ºC) and subsequently the pellets were washed 2 times in a low salt buffer (3mM KCl, 1.5mM KH_2PO_4, 20mM NaCl and 9mM NaH_2PO_4) (Raeder et al. 2007). Washed pellets were stored at -80º C for further analysis. For qPCR analysis the DNA was extracted using DNeasy Blood and tissue kit (Qiagen, USA) following the manufacturer's instructions.

3.9.2. Sample preparation for 2D analysis

Sample for 2D was prepared by following the protocol of Raeder et al. (2007) with few modifications. Briefly, bacterial cell pellet obtained from the experiment was thawed in lysis buffer (9 M urea, 4% CHAPS, 1% DTT and 0.8% ampholyte (Bio-Lyte 3/10 ampholyte, USA)). The lysis buffer containing cell pellet was sonicated (2 x 5s) using an ultrasonic processor (SONICS Vibracell VCX750, USA). One µl of benzonase (Benzonase® Nuclease,

Sigma-Aldrich, USA) was added to reduce the viscosity of the sample. The solubilized sample in lysis buffer was dialyzed in 3kD cut off Nanosep spin columns (VWR, Norway) following the manufacturer's protocol. Protein content of the dialyzed sample was estimated using Qubit® Protein Assay Kit and Qubit™ fluorometer (Life Technologies, USA). A volume of the obtained sample was mixed with rehydration buffer (7 M urea, 2M thiourea, 4% CHAPS, 15mM DTT, 0.5% Biolyte 3-10, 10% glycerol and 0.001% bromophenol blue) to achieve a final protein concentration of 100 µg. Prepared sample (300 µl) was applied to a dry IPG strip (pH 3-10, Bio-Rad, USA) of 17 cm and subjected for rehydration.

Isoelectric focusing of the rehydrated IPG strips were carried out in Bio-Rad Protean IEF cell to a total volt hours of 60,000 at a maximum of 10,000 V using three steps of slow ramping at a constant temperature of 20ºC (Rajan et al. 2011). Next, the electro focused IPG strips were reduced and alkylated in equilibration buffer (6M urea, 0.375 M Tris- HCl (pH 8.8), 2% SDS, 20% glycerol) containing 2% w/v DDT or 3% w/v iodoacetamide each for 15 min respectively. The equilibrated strips were subjected to a second dimension run in 12.5% polyacrylamide gels using Bio-Rad Protean IIxii system (USA). After completion of the run gels were stained (Sypro® Ruby Protein gel stain, Life technologies, USA) and images were documented using ChemiDoc™ XRS system (Bio-Rad). The documented gels were analysed using PDQuest™ Advanced 2D analysis software (Bio-Rad). Next, the selected spots from the analysis were excised manually on a blue light transilluminator (Safe Imager™ 2.0 Blue-Light Transilluminator, Life technologies, USA) and subjected for LC-MS/MS analysis. The LC-MS/MS and MASCOT analysis for protein identification was performed as described earlier (section 5 and 6).

3.9.3. qPCR for Moritella viscosa

The primers and probes used for qPCR were designed by Grove et al. (2008). The assay was performed on the StepOnePlus real time PCR system (Applied Biosystems, CA, USA). Each 12 µl PCR reaction contained TaqMan 2X master mix (Applied Biosystems), primers (900nM), probes (200nM) and 1 µl of template DNA. Each reaction was run in triplicates with negative (template was replace by molecular grade water) and positive controls. The amplification was achieved with the following condition: initial hold of 95º C for 10 min, followed by 45 cycles at 95º C for 15s and 60º C for 1 min. The Ct values were obtained by arbitrarily selected base line in the log phase.

3.9.4. Bacterial agglutination

Moritella viscosa was grown in media supplemented with purified galectin-3C as mentioned earlier in the experimental setup. In control purified galectin-3C was replaced by PBS. Forty hours old bacterial culture (OD<1) was smeared on a sterile glass slide and observed under microscope (Leica microsystems). To check the inhibition of bacterial agglutination 0.5 M of lactose was supplemented along with galectin-3C and bacteria was grown till 40 h and observed under microscope.

4. Results

4.1. Purification of galectin from Atlantic salmon skin mucus

The purified protein obtained from Atlantic salmon skin mucus after α- Lactose agarose affinity purification and Sephadex G-15 gel filtration showed a single band of approximately 15 kDa in the polyacrylamide gel (Fig. 3).

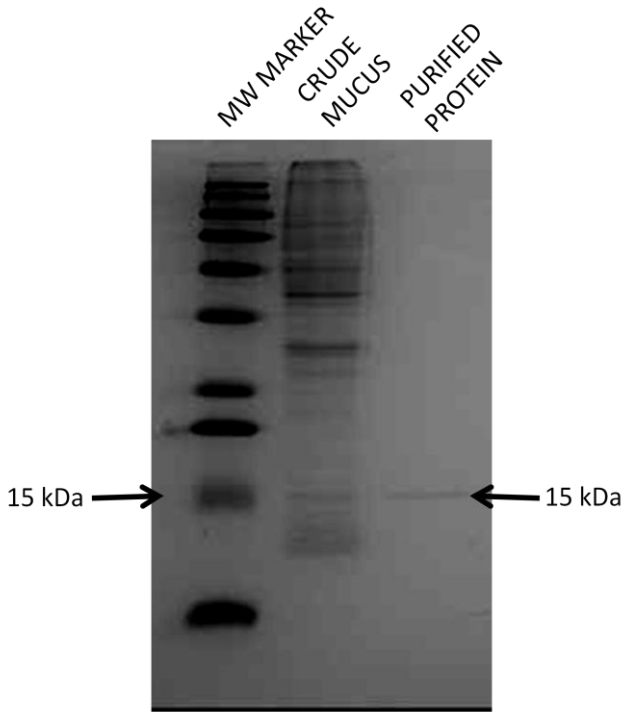

Figure 3. Confirmation of purity of *Salmo salar* galectin. The purified product from gel filteration showed a molecular weight of approximately 15kDa in a 15% SDS polyacrylamide gel.

4.2. Sequence analysis

The band was excised and analysed by MS on the ESI-Q-TOF instrument, the result file obtained was analyzed using MASCOT. The analysis identified the purified protein as *Salmo salar* galectin-3 (Accession: GI: 213514684) with a score of 220 and protein coverage of 19%. The predicted protein has a molecular weight of 29 kDa. Since the SDS PAGE showed a molecular weight of ~15 kDa (Fig. 3) for the isolated galectin-3 from skin mucus, the isolated protein was further analyzed by Q- Exactive quadrupole orbitrap mass spectrometer (Thermo scientific) to get a higher coverage to see which part of the full length protein had been isolated. The Q-Exactive result showed a protein coverage of 38.75% of the full length

protein with a score of 3739.77. Figure 4A shows the identified peptides and that the short form corresponds to the C-terminal part of full length galectin-3.

A

1 MDLSDALGGE PGWPSQNNQQ SSGGVWPGGQ PNQPTWPGQP GGQPTWPGQQ

51 QPGQPAPMWP GQQPNPSQPS WPGQPGGGQP SQPTWPGQPG GGQPSQPTWP

101 GQPGQISQPT GPGWPSPSPG PGPAQPTAPQ QSSLKMPYDL NLPNGCYDKM

151 LITIRGTINQ *NAKMFTINLT KGNDIAMHVN PR*FNDQGKKT IVR*NSQIGNT*

201 *WGKEEREHNH FPFIQGQPFE MKIMCTNSEF KVAVNSSHIL EFK*HRIRDIH

251 SIKHLGIYND VTLTSVEIDK L

B

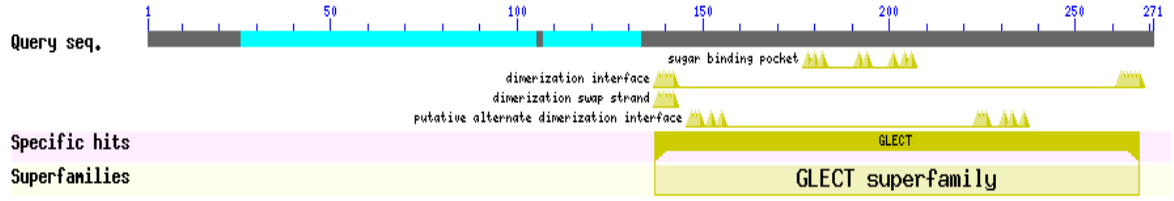

Figure 4. *Salmo salar* galectin-3 amino acid sequence and conserved domains
A. *Salmo salar* galectin-3 amino acid sequence. Underlined, the galectin domain. Amino acids highlighted in red were covered by Q- Exactive, the sequence in italics was found in the ESI-Q-TOF analysis.
B. Conserved domains of full length *Salmo salar* galectin-3. Picture from http://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html. The C-terminal part

A detail of the result from Q-Exactive is shown in Table 1. Analysis for conserved domain in *Salmo salar* galectin-3 showed a conserved galectin domain (binds to beta galactosidases) from amino acid 137- 167 at the C-terminal end (Fig.4B) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The amino acid sequence of the purified galectin-3 has the conserved C-terminal domain, but lacks the N-terminal sequence. From here after we will designate the purified galectin-3 as galectin-3C. The peptides identified are listed in Appendix I.

Table.5 Summary of analysis by Q-Exactive

Protein name	Galectin-3 [*Salmo salar*]
Accession	213514684
Score	3739.77
Coverage	38.75
Proteins	1
Unique Peptides	10
Peptides	11
PSMs	202
Pfam IDs	Pf00337
Gene IDs	100195804
GO Terms	[GO:0030246]; GO:0005488; GO:0003674
AAs	271
MW [kDa]	29.5
calc. pI	8.05

4.3. Tissue distribution of *leg3* gene

Quantitative real time PCR was used to determine the tissue distribution of *leg3* gene in 9 Atlantic salmon tissues; skin, gill, muscle, spleen, liver, head kidney, stomach, foregut and hindgut. The expression of *leg3*, *i.e.* mRNA, was detected in all tissues tested (Fig.5). Expression was high in gill and skin whereas liver showed the lowest level of expression; medium expression was found in the other tissues examined.

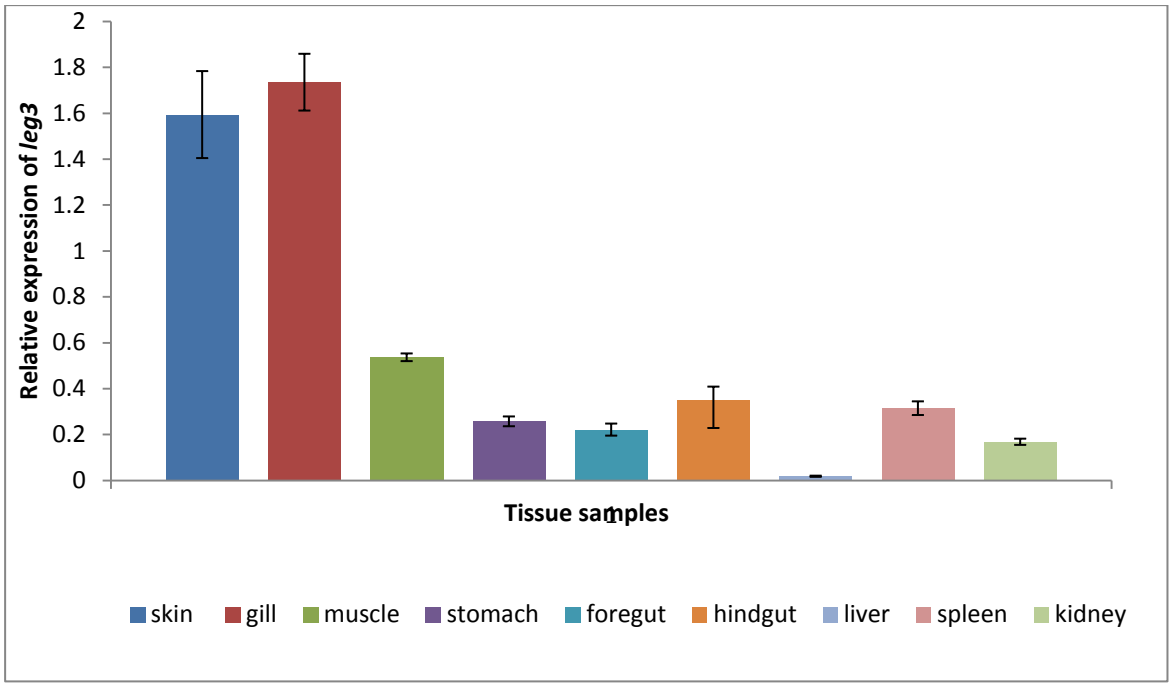

Figure 5. Tissue distribution of *leg3* in healthy Atlantic salmon tissues. Values are mean *leg3* expression relative to *EF1alfa*. Values shown are mean, n=2 Error bars show standard deviation.

4.4. Hemagglutination and inhibition activity

The gel filtration eluate showed hemagglutination activity of galectin-3C at a titre of 4 and this activity was inhibited by 0.5 M lactose (Fig. 6).

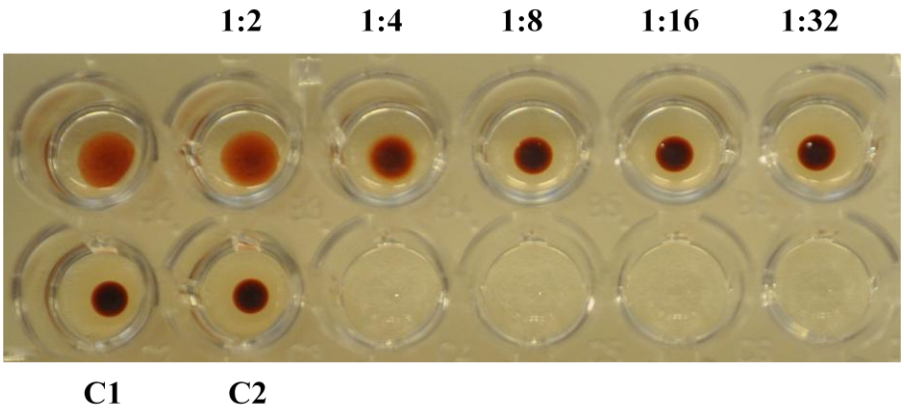

Figure 6. Hemagglutination and inhibition assay; The purified galectin-3C (50 µg protein/ml, final concentration in 1st well) was two fold serially diluted and mixed with 4% horse blood, 1% BSA and PBS, upper row; C1(control with PBS only) no agglutination, C2 (control with 0.5M lactose in the presence of galectin-3C), showing inhibition of hemagglutination activity.

4.5. Growth performance of *Moritella viscosa*

Galectins are known to interact with bacteria by binding to their surface. To investigate whether the isolated 15 kD isoform could have functional activities, *M viscosa* was incubated in the presence of the galectin-3C. Growth of *M. viscosa* was measured in 4 h intervals (Figure 7). Bacteria were grown in medium (3% TSB and 1.5% NaCl) in the absence (control) or presence of purified galectin-3C. In control the galectin-3C volume was replaced by PBS. The bacteria did not show any difference in growth between galectin-3C supplemented medium and control till 36 h, but after that the optical density (OD) continued to increase in galectin-3C added medium whereas in the control it decreased. Two more experimental set ups were also included in the same study. In one, bacteria were grown in medium supplemented with both galectin-3C and 0.5 M lactose, and in the second the medium was supplemented with only 0.5 M lactose. Both these experiment sets showed a different pattern of growth rate from either control or galectin-3C. Both showed lower optical density than control and galectin-3C only supplemented medium. But, after 36h the medium supplemented with both galectin-3C and 0.5 M lactose showed an increase in OD value like that shown in the galectin-3C alone supplemented medium whereas the medium with only 0.5 M lactose showed a decrease in OD value like the control. All OD readings were done at 590 nm.

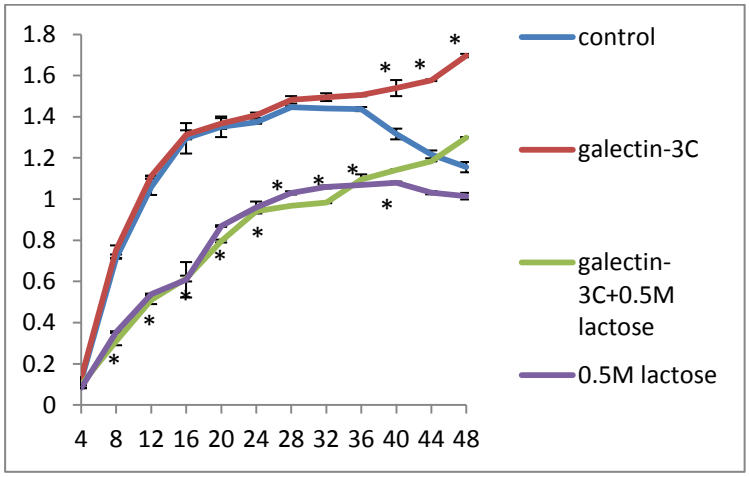

Figure 7. Growth curve of *Moritella viscosa* at 15°C. *M viscosa* was grown in tubes under orbital shaking (200 rpm) at 15°C in medium (3% TSB and 1.5% NaCl) without (PBS, control) or with galectin-3C for the indicated time points. Aliquots (n=3) were removed at each time point and OD at 590 nm was measured. Error bars show SD. Stars indicate significantly different from control, one way annova. The figure is a representative figure from one out of 3 independent experiments with similar results.

4.6. qPCR for *Moritella viscosa*

The DNA was extracted using 1 ml of 40h bacterial culture from control, galectin-3C added medium, 0.5M lactose added medium and medium with both galectin-3C and 0.5 M lactose. qPCR on *tonB* gene was performed to check the DNA concentration based on the Ct values obtained to quantify the relative number of cells in the culture. The Ct values of control (15.62) and culture with both lactose and galectin-3C (15.49) were not different, where as bacteria incubated in the presence of galectin-3C alone (24.45) and 0.5M lactose alone (30.68) showed higher Ct value. This shows that the amount of *M viscosa* DNA was less in the galectin-3C incubated and lactose (without galectin-3C) incubated sample than in control, and indicates that there are fewer bacteria in these two sample.

4.7. Bacterial agglutination assay

The 40h *M viscosa* grown in presence of purified galectin-3C showed agglutination activity where as no agglutination was observed in control (without galectin-3C). The bacteria cultured in presence of both 0.5M lactose and galectin-3C showed very little agglutination.

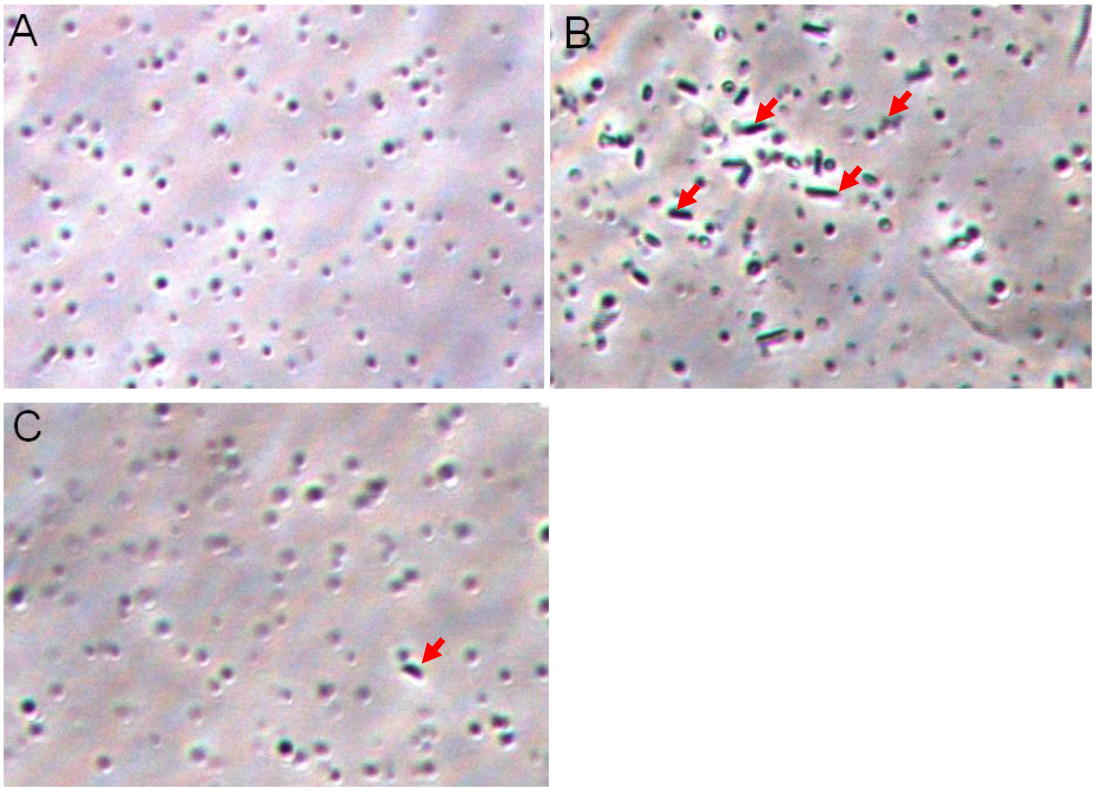

Figure 8. Bacterial agglutination. *Moritella viscosa* was incubated for 40h in the absence (A) or presence of galectin-3C, 13 µg/ml (B*).* galectin-3C led to the formation of rod shaped aggregates of *M. viscosa* (red arrows). These structures (red arrow) were inhibited when 0.5 M lactose was present during the incubation (C).

4.7. 2D analysis

Moritella viscosa 40h old culture were harvested and 2D analysis was performed. The 2D gels were analyzed with the PD-Quest advanced software. Spots in the galectin-3 treated culture showing statistically significant more than 10 fold changes compared with control and were sequenced and analyzed for their protein identity using MASCOT. The MASCOT search was done against NCBInr database for eubacteria. Eight spots were sequenced, out of which two did not show any significant hit. Protein name and parameters obtained from MASCOT search is recorded in table two. The MASCOT summary of results for each spot is mentioned in Appendix II.

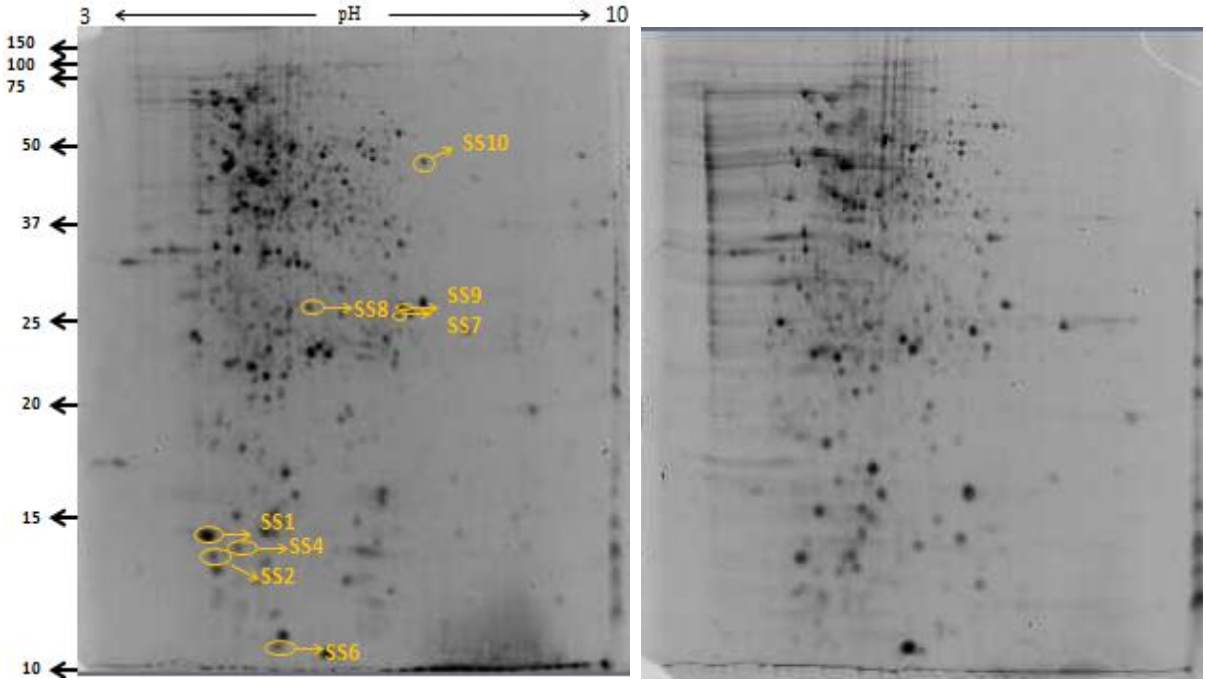

Figure 9. Representative gel images from the experiment. Gel marked with sequenced spots (SS1-10) from galectin-3C treated bacteria (left) and control (right).

Table.6 MASCOT result of identified spots

Spot no.	Accession no.	Protein	Calculated pI/Nominal mass (M_r)	Mascot ion score	Sequence coverage (%)	Major protein domain
SS1	WP_006791261	50S ribosomal protein L7/L12 [*Alteromonadels*]	4.45/12164	518	80	Ribosomal_L7_L12 superfamily
SS2	WP_002962189	50S ribosomal protein L19 [*Pseudoalteromonas haloplanktis*]	10.49/13317	328	50	Ribosomal_L19 superfamily
SS4	YP_341288	30S ribosomal protein S13 [*Pseudoalteromonas haloplanktis* TAC125]	10.95/13162	123	11	Ribosomal_S13 superfamily
SS6	Not identified					
SS7	Not identified					
SS8	WP_016707503	Multidrug transporter [*Pseudoalteromonas haloplanktis*]	5.34/26438	407	40	SapC superfamily
SS9	WP_019442080	30S ribosomal protein S2 [*Moritella marina*]	6.32/26819	194	19	RPS2 superfamily
SS10	WP_016710022	Transcription termination factor Rho [*Pseudoalteromonas haloplanktis*]	6.83/47117	282	19	Rho

5. Discussion

Galectins are beta-galactoside binding proteins. They are found both outside and inside the cells and play a variety of roles in immune defense. Extracellular functions of galectins include cell-matrix and cell-cell interactions. Intracellularly, galectins are involved in apoptosis and migration. Galectins are also a component of spliceosomes and found to be involved in pre-mRNA splicing in nucleus (Chen et al. 2012). Galectins have been identified in many organisms including insects, sponges, nematodes, molluscs, arthropods, reptiles, fungi, fishes, avians and mammals (Stierstorfer et al. 2000; Solis et al. 2000; Lyimo et al. 2011; Wei et al. 2012; Chen et al. 2012; Wang et al. 2013; Wang and Wang 2013; Yang et al. 2013).

5.1. Purification of galectin-3 from Atlantic salmon (*Salmo salar*) skin mucus

In the present study we have isolated and purified a galectin from Atlantic salmon skin mucus. Galectins have been purified from skin mucus of teleosts including *Anguilla japonica* (Japanese eel; Kamiya et al. 1988), *Conger myriaster* (Japanese conger; Tasumi et al. 2004), and *Gadus morhua* (Atlantic cod; Rajan et al. 2013). Carbohydrate affinity chromatography is a widely used technique for lectin purification. Galectins have an affinity towards lactose; its binding with lactose is reversible and the bound galectin in the affinity column can be released easily by a buffer containing the sugar lactose through competitive elution (Pohleven et al. 2012). Lactose in the elution buffer competes with the columns ligands of bound galectins for their attachment and as a result galectin falls out from the column.

In this study purification was done by a two step chromatography procedure where the pooled eluted fractions from the α- Lactose agarose affinity column were loaded onto a gel filtration Sephadex G-15 column for desalting. The second step chromatography fraction showed a single 15 kDa band on SDS gel. LC-MS/MS analysis of the excised protein band and subsequent bioinformatic analysis of the obtained peak list file for similarity search in the NCBInr database identified the purified product as *Salmo salar* galectin-3. Hemagglutination is the classical assay for lectin activity. Inhibition of this activity by different sugars shows the carbohydrate specificity of the lectin. Like other galectins hemagglutination activity of the purified galectin-3 is calcium independent and the activity was inhibited in presence of the sugar α- Lactose. We did not use Ca^{+2} in the hemagglutination assay and the PBS used also did not have Ca^{+2}, which shows that the agglutinating activity of purified galectin-3C is not

dependent on calcium. Similar results were obtained for Atlantic cod galectin-1(Rajan et al. 2013) and *Anguilla japonica* β-galactoside specific lectin (AJL-1) (Suzuki et al. 2003).

Salmo salar galectin-3 is a 29 kDa protein with a full length of 271 amino acids (GenBank: ACN10264.1). This protein is the sole member of animal chimera type galectin and possesses a unique structure. It is a multidomain protein consists of two distinct domain structures in a single polypeptide chain, the N-terminal domain and C-terminal domain. The N-terminal domain is a collagen like domain, rich in proline and glycine (non CRD) and it is responsible for formation of multimeric chimera structure through its small extended tail. The C-terminal domain has the conserved carbohydrate binding domain (CRD) for galectins, and are responsible for the lectin activity of the protein. The N-terminal domain is susceptible to collagenase treatment but not the C-terminal domain (Barboni et al. 2000; Dumic et al. 2006). The purified galectin-3 in the present study showed a molecular weight of approximately 15 kDa. The amino acid sequence of purified galectin-3C has the C-terminal domain and is lacking the N-terminal domain. There are studies showing that the N-terminal domain is vulnerable to selective proteolysis by matrix metalloproteinases (MMP), especially MMP-2 and MMP-9 as reported with recombinant human galectin-3, where the 30 kDa human galectin-3 was cleaved into two fragments of 22kDa and 9 kDa molecular weight. The 22 kDa fragment had intact CRD and the 9 k Da fragment had the N-terminal domain (Ochieng 1998; Nangia-Makker et al. 2010). As the purified galectin-3C from Atlantic salmon skin mucus is lacking its N- terminal domain, one could speculate that it could be due the cleavage of protein by matrix metalloproteinases. However, the cleavage by the human MMPs is at the alanine62-tyrosine63 bond of human galectin-3, in salmon galectin-3 there are no alanine-tyrosine bond in the sequence (Figure 2). MMPs are also reported to cleave leucine-lysine bonds, one is found just N-terminally of the first peptide observed in our study (Figure 4A) (Ochieng et al. 1994). Hence it could be possible that MMPs also have a role in salmon galectin-3 processing, but this needs to be further studied. The possibility exist that the shorter galectin-3C observed in salmon mucus is an artifact, including protease inhibitors in mucus during processing did not result in a longer fragment being isolated, indicating that this was not the case.

Chicken *gal3* has been shown to be alternatively spliced in osteoclast-like cells, giving rise to proteins of different size (Gorski et al. 2002), alternative splicing has not been reported in other species. (Ochieng 1998) reported that the cleavage of galectin-3 alters the lectin CRD which allows it to bind more tightly to the glycoconjugates but at the same time it also

reduces the self-association which affects the biological properties like hemagglutination, aggregation for red blood cells from non-self species. The self association or oligomer formation of galectin-3 molecules occurs by association of N-terminal domains of individual monomers, which forms the basis of lectin hemagglutination. The human 22 kDa fragment lacking the N-terminal (Ochieng 1998) did not show hemagglutination activity, where as in our study the 15 kDa Atlantic salmon galectin-3C showed hemagglution activity. Nonetheless, there are also evidences supporting self association of galectin-3 through its C-terminal CRD. The sugar binding site of one CRD binds to opposite side of another CRD and the N-terminal domain is not needed for this type of self association (Lepur et al. 2012). Another theory for self association without N-terminal domain is formation of dimers, where the sugar binding sites faces each other and form dimers (Yang et al. 1998). Together this suggests that even if the purified galectin-3 is lacking the N-terminal, it can still self associate and show hemagglutination.

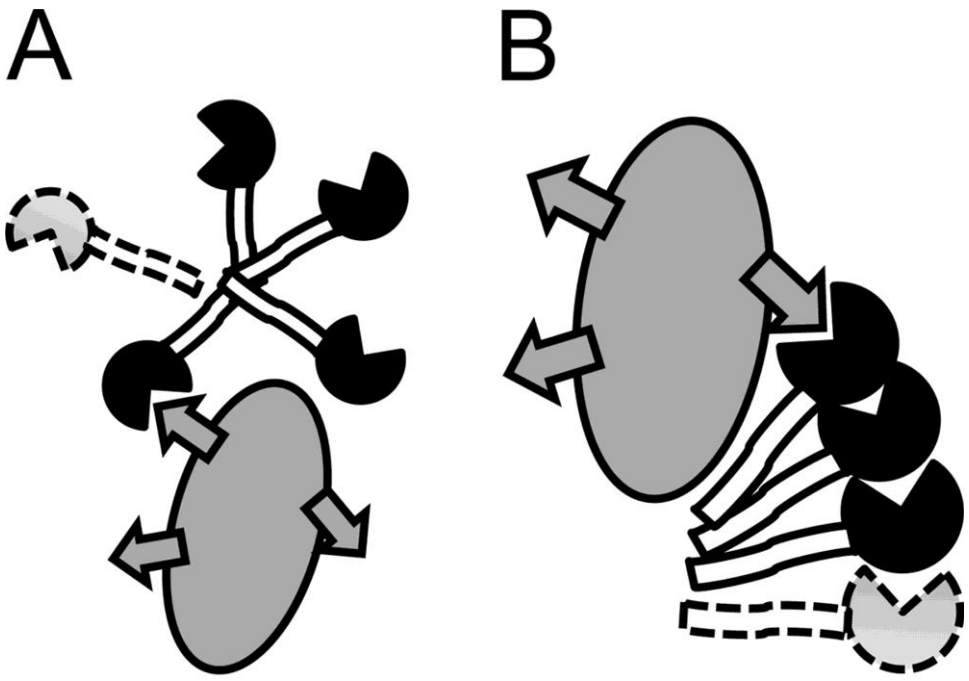

Figure 10. Self association of galectin-3. (A) Showing association through N-terminal end and (B) Association by C-terminal CRD (Lepur et al. 2012)

Absence of the N-terminal domain in mammals, chick, nematodes and mushrooms reduces the affinity towards lactose in affinity columns. Frontal affinity chromatographic analysis showed that the affinity of galectin-3 towards lactose or *N-acetyllactosamine* is reduced by approximately 4 times when the N-termnial domain is removed (Hirabayashi 2002).

5.2. Tissue distribution of *leg3* mRNA

In healthy Atlantic salmon the *leg3* mRNA is mainly expressed in gill and skin with very high level of expression followed by muscle, hindgut, spleen, stomach, foregut, kidney and liver. Being an aquatic animal, fish lives in an environment rich in pathogenic microorganisms. Gills in fish are involved in gas exchange, excretion of nitrogenous wastes through frequent water exchange with the external environment (Hwang and Chou 2013). On the other hand skin covers the largest part of the fish body and it is always exposed to the outer milieu (Ángeles Esteban 2012). Therefore, skin and gills are continuously exposed to the outer environment and serves as an entry point for pathogens. The high expression levels of *leg3* in skin and gills suggest that galectin-3 may be involved in innate immune response against pathogen invasion serving as first line of defence. Similarly, fish lily type lectin CsLTL-1 mRNA was distributed in gill and skin tissues of healthy *Channa striatus* (striped murrel) suggesting their involvement in immune responses (Arasu et al. 2013). The pufflectin mRNA was also expressed in gill, oral cavity, esophagus and skin (Tsutsui et al. 2003). The mucosal protein galectin, congerin in conger eel (*Conger myriaster*) was distributed in epidermal cells of skin, gills, oral cavity and oesophagus, but not in stomach as seen by immunocistochemistry (Nakamura et al. 2001). Two isoforms of galectin-1 from Atlantic cod were found mainly in skin, gills, spleen and head kidney using western blot (Rajan et al. 2013).

The reference gene used for the tissue distribution analysis was *elongation factor1 alfa* (EF1-α). The purpose of using reference genes in real time PCR is to minimize the differences introduced due to sampling and sample processing such as differences in the RNA quality and quantity. In our study we did not evaluate the reference genes for Atlantic salmon. However, EF1-α has been evaluated as a more stable and potential reference gene to be used for Atlantic salmon than the commonly used β- actin gene (Olsvik et al. 2005; Ingerslev et al. 2006).

5.3. Growth performance of *Moritella viscosa*, qPCR and bacterial binding assays

There was no significant change observed in the growth rate of bacteria in control, and in galectin-3C supplemented media till 36h. After that the control showed a declining trend in the growth curve whereas bacteria grown in galectin-3C supplemented media showed an increasing trend.

M. viscosa is a non-pigmented bacterium. Therefore, the spectrophotometer measures the turbidity of the solution rather than the absorbance. This bacterium is also of very slimy nature and the colonies tend to aggregate together. To our knowledge there is no evidence of galectin-3 augmenting bacterial growth. Therefore, we interpret that the increase in OD value as increased growth could be a false interpretation of the data, and that the increase in optical density is due to the combined effect of agglutination activity of galectin-3C and the slimy nature of the bacteria. There are number of factors affecting the measurement of OD for bacterial density such as the type of spectrophotometer, use of light, and nature of the bacteria. To further investigate this we ran qPCR on *tonB* gene (Grove et al. 2008) of *M. viscosa* at 40 h time point that is after the bacteria in the control entered the declining phase. The Ct value in the galectin-3C supplemented incubation was quite high compared to the control and medium supplemented with both lactose and galectin-3C, suggesting the presence of less DNA. Hence fewer bacteria seem to be present after galectin-3C incubation suggesting a suppressed growth rate and/or bactericidal effect in the presence of galectin-3C. To check if aggregation of bacteria could be observed, live bacterial cells from the 40h culture were observed under the microscope, and clear aggregation of bacteria was observed. Hence, the aggregation and qPCR results both are supporting our speculation that the interpretation of high OD measurement in the spectrophotometer as an indication of high bacterial cell density might be false.

If the galectin-3 effect on growth is CRD dependent, lactose should inhibit the galectin-3 activity and allow normal growth of the bacteria. But, in our study, the growth rate of bacteria cultured in medium with both lactose and galectin-3C was low compared to control and only galectin-3C supplemented medium. Slow growth rate was also observed in medium supplemented with only lactose. *M. viscosa* is a lactose negative bacterium and it cannot utilize lactose as an energy source (Xu et al. 2003). This could be the reason of slow growth of bacteria when lactose was added to the media. Microscopical observations and real time PCR showed that in the case of lactose alone (results not shown) and galectin-3C with lactose, single bacteria was observed. For these incubations the OD value observed fits the Ct value observed. This indicates that the OD values for these incubations could be used to enumerate the bacteria, and that turbidity measurement is a valid method as long as one observes the bacteria in microscope to ensure no aggregates are present.

5.4. Spots identified from MASCOT analysis

Spots appearing in the proteome of bacteria grown in galectin-3C supplemented media were analysed with mass spectrometry (MS) and the proteins were identified using MASCOT. Four out of eight spots were identified as ribosomal proteins, two of them did not show any significant hit, one was identified as a multidrug transporter protein and one as transcription termination factor (Zou et al. 2014) The search was done against eubacteria in the NCBI non redundant database. Even though we did not get specific hits for *Moritella viscosa* the results obtained were statistically significant and matching the identified bacterial proteins with a good score and showing unique peptide sequences (see Appendix II). In our study the proteins identified showed similarity with proteins from *Alteromonadels* (SS1), *Moritella marina* (SS9) and *Pseudoalteromonas haloplanktis* (SS2, SS4, SS8, SS10). Both the genera *Moritella* (*Moritellaceae* family) and *Pseudoalteromonas* (*Pseudoalteromonaceae* family) comes under the order *Alteromonadels* (Brenner et al. 2005). 16S rRNA from *Moritella marina* shares 99.1% sequence similarity with *Moritella viscose* (Benediktsdóttir et al. 2000). Giving the close taxonomic relationship between the two species, it is not surprising that significant hits mapping to *Moritella marina* for protein identification was found. However, only the draft uncompleted genome of this species is available (Kautharapu and Jarboe 2012). The full length sequence of the *Moritella viscosa* genome is underway (Zou et al. 2014). Reanalysis of the MS data should be done after these *Moritella* species have been sequenced.

Ribosomal proteins

The ribosome is essential machinery used in protein synthesis for decoding the genetic information from mRNA to make proteins. The prokaryotic ribosome is a 70S ribonucleoprotein particle composed of two subunits, one large (50S) and one small (30S) subunit (Lecompte 2002). In our study we have identified two large subunit proteins (L7/L12 complex and L19) and two small subunit proteins (S13 and S2).

The 50S ribosomal protein L7/L12 refers to proteins L7 and L12. The two proteins are identical except that L7 is acetylated at the N-terminal. The protein L7/L12 form dimers and serves as an important component of the ribosomal stalk. The ribosomal stalk is a finger like projection present in the large ribosomal subunit, which is essential for attachment of translational factors. This stalk contains several copies of L7/L12 dimers attached to L10. The L10 protein binds to rRNA at the base of the ribosomal stalk next to another ribosomal protein L11. The L12 protein is found in multiple copies on the ribosome (Mandava et al.

2012). This protein is important for protein synthesis. It has been reported that the L7/L10 dimer is essential for controlling translational accuracy (Kirsebom and Isaksson 1985). This is also required for stimulation of GTPase activity of translational factors (EF-Tu, IF-2, EF-G) (Mandava et al. 2012). Removal of one copy of L7/L12 protein in *Escherichia coli* led to compromised growth rate in the bacteria as compared to growth rate of the parental strain. Variation in number of L7/L10 proteins has been reported from mass spectrometric analysis of an archaea *Methanosarcina sp.* This species showed two and three dimers of L7/L10 in lag phase and exponential growth phase respectively. The same author also suggested that in thermophils L7/L12 increases with increase in temperature (Gordiyenko et al. 2010). Also there is a study on *E. coli* showing increase in the copies of L7 protein when the bacteria were cultured under stressful conditions (Gordiyenko et al. 2008). From above cited statements it is clear that the number of ribosomal protein L7/L12 varies in different environmental conditions and growth phase. However, the exact functional importance of containing multiple copies of L7/L12 has not yet been resolved. In our study the control did not show this protein whereas the galectin-3C treated bacterial proteome showed a clear spot containing the L7/L12 protein (SS1) in the gel. There was no difference in the culture conditions (temperature, shaking) for both control and galectin-3C treated bacteria except the absence of presence of galectin-3C respectively. Hence, we speculate that the appearance of the L7/L12 could be due to a inhibition of growth of *M. viscosa* cased by galectin-3C or a bactericidal effect as mentioned above with a direct effect on the ribosomes. Increased expression of L7/L12 could be a strategy for the bacteria to handle the stress caused by host galectin-3C proteins.

The 50S ribosomal protein L19 is found in the subunit interface and interacts with rRNA of both subunits, and with ribosomal proteins L3 and L14. It has RNA chaperone activity (Semrad et al. 2004)

The 30S ribosomal protein S13 is located on the head region of the small subunit of the ribosome and plays an important role in interaction of the small and large subunits of ribosome. It helps in stabilizing the translocation of the subunits during translation. It has been found that mutation of this protein shows impairment in translation (Cukras and Green 2005).

The 30S ribosomal protein S2 is required for binding of S1 ribosomal protein to the 30S subunit. This is a highly conserved zinc binding protein and the expression is autoregulated (Katayama et al. 2002; Aseev et al. 2008). This protein is very sensitive to proteolysis. It can

crosslink to other proteins S1, S3, S5, S8 and can bind to 16S RNA (Wittmann-Liebold and Bosserhoff 1981).

Transcription termination factor rho is a rho dependent transcription terminator. Rho is a hexameric prokaryotic RNA binding protein which binds to the transcription terminator and stops the process of transcription. It plays important role in regulation of gene expression (Ciampi 2006).

Multidrug transporter proteins are used by bacteria to remove the antimicrobial drugs from the cell interior. They are special drug transporters which can handle various types of structurally unrelated foreign proteins. In our study expression of this protein in bacteria grown in galectin-3C supplemented medium shows that galectin-3C might stress the bacteria, it could also indicate that galectin-3C enters the bacterial cell.

Most of the proteins identified are directly or indirectly (via transcription) related to protein synthesis in bacteria such as ribosomal proteins and termination factor. Expression of these proteins in galectin-3C treated medium suggests that it might be because of the antimicrobial activity of galectin-3C. This study insights several speculations. In a similar study crude Atlantic salmon mucus was incubated with *Vibrio salmonicida*. Proteomic analysis of the *V. salmonicida* resulted in a wide difference of proteins being identified including ribosomal proteins (L10, S2), transcription factor (arginine repressor) and transport proteins (Raeder et al. 2007). Since the fold change is not given in the paper, it is not possible to compare it to the present galectin-3C study. Interestingly it has been shown that 2D gel analysis of treated bacteria can be used to identify the molecular drug mechanisms (Bandow et al. 2003). It is thus tempting to speculate that the identification of a multidrug transporter protein suggests that galectin-3C can enter bacteria and that the target could be the ribosomes, however further studies is needed to confirm or reject this assumption.

Galectin-3 is found in the cytosol but it is also involved in RNA splicing in the nucleus. The secretory pathway of galectin-3 out of the cell and into the nucleus is still unknown as they do not have secretion signal sequence or nuclear import sequences. There is a study on the intracellular pathogen *Shigella* where galectin-3 was localized in the membranes of phagosome and also in the rest of the endocytic pathway. In this study galectin-3 was found in the vicinity of bacteria which had capacity of lysing the phagolysosome. Here galectin-3 serves as a vacuole lysis marker (Paz et al. 2010). These results show that galectin-3 also has functions other than the extracellular ones studies in this thesis.

5.5. Methodological attributes

The practical methods used in the study are mucus sample preparation for purification, affinity chromatography, quantitative real time PCR, 2D gel electrophoresis, and bacterial incubation and sample preparation for 2D analysis. In addition sample analysis and bioinformatics were used. Mucus was sampled from anaesthetized Atlantic salmon by scrapping the dorsal and ventral body part using a sterile glass slide. The anal region was avoided to prevent faecal contamination. Other method for sampling mucus is putting the fish in plastic bags containing ammonium bicarbonate. Then the bag is agitated for a minute and the fish removed. the mucus with buffer is then used for further studies (Easy et al. 2012). Both techniques have drawbacks. Scrapping could give cell contamination and eg. Presence of cytoskeletal and cellular proteins whereas in the plastic bag method faecal contamination is unavoidable. Even if the scrapping method could give contaminating proteins, we succeeded in purifying galectin-3, a known secreted protein, through affinity purification. In teleosts galectins has been purified using a variety of adsorbents. Galectin-1 has been purified from extract of whole zebrafish using lactosyl sepharose affinity column (Ahmed et al. 2004). Alfa lactose agarose affinity chromatography followe with Sephacryl S-200 gel filtration was used for purification of galectin-1 from Atlantic cod skin mucus (Rajan et al 2013). In our study affinity chromatography using α-lactose agarose coupled with Sephadex gel filtration was employed for galectin-3C purification. The purification technique involved batch binding of the prepared crude mucus with α-lactose agarose resin (De La Hoz et al. 1986). This method allows the purification of large volume of mucus samples at one time which was favourable in our study. The batch bounded mucus was eluted manually in a gravity purification column. For desalting, a small gravity purification column was employed in the study. The purified sample was run in SDS-PAGE, the band was excised and subjected for ESI-Quad-TOF and Q-Exactive LC-MS/MS analysis.

Proteomics could also be done in a gel free setup, where MS is used directly to analyse the sample. Gel-based and gel-free analyses both have weaknesses and strengths. One known weakness for gel based techniques is that not all proteins are visible. Low abundance and hydrophobic proteins are largely not seen. The gel free techniques give larger datasets and the analysis of such data to give biological sense can be challenging. A proteomics study using non-gel methods showed another weaknesses of the gel free techniques: increased galectin-3

expression after sea lice infection was seen, but since gels were not used, we cannot know if it is the full length or our novel ~15 kD galectin-3C (Provan et al. 2013)

Sample preparation for 2D was performed in two stages. First 3 ml of bacterial culture was pelleted and washed in a low salt buffer and stored at -80ºC. Second the pellet was lysed and sonicated to rupture the cell wall of bacteria and release of bacterial proteins in the solution. 3 ml of culture yielded a very small pellet and subsequently it yielded a small volume of sample. But this problem was solved by using the very sensitive fluorescent stain Sypro Ruby. Using less quantity of sample also yielded very good gels with clear spots, smearing and streaking were observed when high concentration of protein was loaded for use with the less sensitive Coomassie stain. However, gels stained with Sypro Ruby needed a safe fluorescent imager for spot excision, Coomassie stained gels can be seen with the naked eye under normal light making spot excision more convenient. The sensitive Sypro Ruby stain binds in a linear fashion to proteins whereas Coomassie's binding to proteins is linear over a less wide range of concentrations. (Berth et al. 2007).

6. Conclusion

Galectin-3 is a β-galactoside binding lectin with a variety of roles in immune defense. In this work, a novel galectin-3C isoform was purified from skin mucus of Atlantic salmon. The purified galectin-3C has only the C-terminal domain containing the carbohydrate recognition domain, and lacks the N-terminal end. The galectin-3C showed hemagglutination activity against horse red blood cells. The hemagglutination was calcium independent and inhibited by lactose. The *leg3* mRNA was expressed in all tissues tested; skin, gill, muscle, head kidney, stomach, foregut, hindgut, spleen, and head kidney. High expression levels in skin and gill indicate a role of galectin-3C in innate immune defense. The galectin-3C successfully agglutinated the Gram negative bacterium *Moritella viscosa*. There was an increased expression of multidrug transporter protein and ribosomal subunits, and a reduced number of bacteria in galectin-3C incubated samples. Based on these data we speculate that the galectin-3C acts as an antimicrobial agent and plays important role in immune defense of Atlantic salmon.

7. Suggestions for future studies

- Recombinant galectin-3 (both intact and C-terminal alone; galectin-3C) of Atlantic salmon could be prepared and a comparative study on biochemical and functional activities of the two recombinant forms could be done.
- From the present study we know that the galectin-3C activity was inhibited by lactose. A carbohydrate specificity test using various sugars could be done to know its binding specificity with other sugars.
- 2D analysis of additional time points from galectin-3 incubated and control samples could be done to see the changes in protein expression during different growth phases.
- Expression of *leg3* in infected fish tissue could be studied to see the difference in healthy and infected tissue, also the expression level could be studied for different environmental conditions such as temperature changes.
- Tissue distribution and localization of galectin-3 in embryonic stages of Atlantic salmon could be analysed.
- Bacterial enumeration on agar plates and additional qPCRs could be done to reveal the exact number of cells present at different time points.

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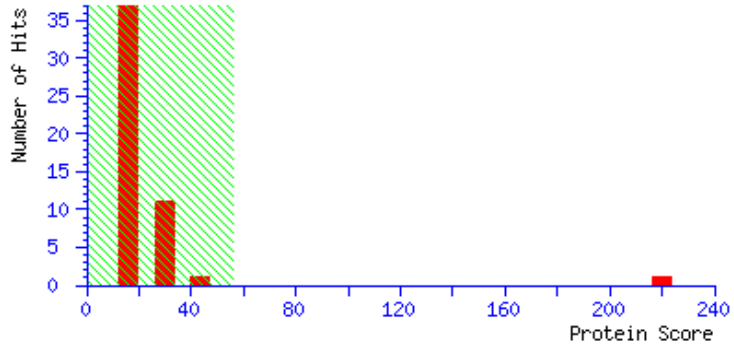
Appendix I

MASCOT search result for galectin-3C

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 56 indicate identity or extensive homology (p<0.001).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. gi|213514684 Mass: 29580 Score: 220 Matches: 7(1) Sequences: 5(1) emPAI: 0.27
Galectin-3 [Salmo salar]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
142	492.2697	982.5248	982.5157	9.26	0	35	0.26	1	U	K.MFTINLTK.G + Oxidation (M)
173	552.7857	1103.5568	1103.5360	18.9	0	(29)	1.3	1	U	R.NSQIGNTWGK.E
174	552.7857	1103.5568	1103.5360	18.9	0	36	0.28	1	U	R.NSQIGNTWGK.E
198	573.2550	1144.4954	1144.4893	5.40	0	(51)	0.0057	1	U	K.IMCTNSEFK.V + Oxidation (M)
199	573.2550	1144.4954	1144.4893	5.40	0	54	0.0025	1	U	K.IMCTNSEFK.V + Oxidation (M)
216	620.3179	1238.6212	1238.5826	31.2	0	90	9.3e-07	1	U	K.GNDIAMHVNPR.F + Oxidation (M)
263	667.9924	2000.9554	2000.9203	17.5	0	5	2e+02	9	U	R.EHNHFPFIQGQPFEMK.I + Oxidation (M)

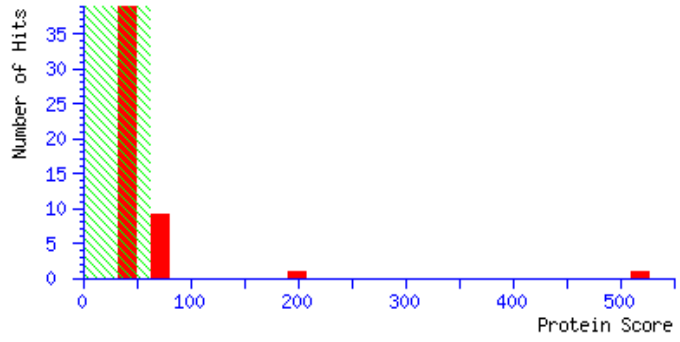
Appendix II

MASCOT results for identified spots from 2D analysis of *Moritella viscosa*

Spot SS1

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 62 indicate identity or extensive homology (p<0.01).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. gi|493844189 Mass: 12164 Score: 518 Matches: 7(5) Sequences: 6(4) emPAI: 4.39
50S ribosomal protein L7/L12 [Alteromonadales]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
109	548.3195	1094.6244	1094.6335	-8.31	0	63	0.013	1	U	K.ALVEAAPTPVK.E
137	630.8039	1259.5932	1259.6245	-24.81	0	76	0.0008	1	U	K.DLTEAGAEVEVK.-
145	454.2239	1359.6499	1359.6881	-28.13	1	80	0.00028	1	U	K.EGVSKEEAEALAK.D
152	718.3592	1434.7038	1434.7355	-22.03	0	93	1.5e-05	1	U	K.TEFDVILTGAGANK.V
173	659.9810	1976.9212	1976.9513	-15.24	0	157	4e-12	1	U	K.FGVTAAAAMVAGPAAEAAEEK.T + Oxidation (M)
174	989.4747	1976.9348	1976.9513	-8.33	0	(99)	3.1e-06	1	U	K.FGVTAAAAMVAGPAAEAAEEK.T + Oxidation (M)
221	700.0721	2796.2593	2796.3119	-18.80	0	49	0.2	1	U	K.DQILDAIAEMSVMDVVALVEAMEEK.F + 3 Oxidation (M)

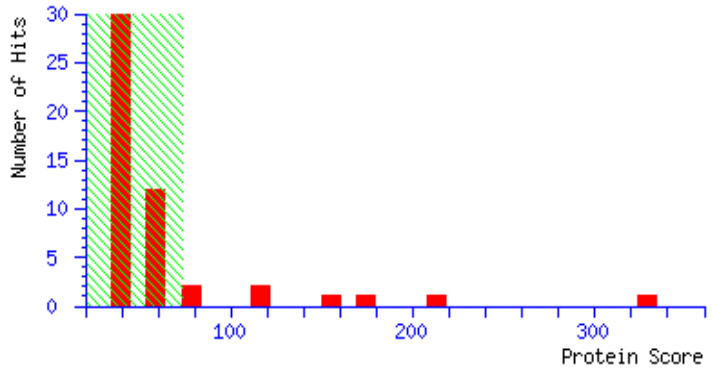
2. gi|77361355 Mass: 15718 Score: 193 Matches: 4(2) Sequences: 3(2) emPAI: 0.55
50S ribosomal protein L9 [Pseudoalteromonas haloplanktis TAC125]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
59	488.2740	974.5334	974.5470	-13.95	0	31	34	1		-.MQVILLDK.I + Oxidation (M)
126	578.3169	1154.6192	1154.6407	-18.62	0	(61)	0.025	1	U	K.IADQLVAAQAR.A
127	578.3169	1154.6192	1154.6407	-18.62	0	72	0.002	1	U	K.IADQLVAAQAR.A
155	729.3823	1456.7500	1456.7773	-18.72	0	90	3e-05	1	U	R.DIADAISAVGVEVAK.S

Spot SS2

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 73 indicate identity or extensive homology (p<0.001).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. gi|489051983 Mass: 13317 Score: 328 Matches: 6(2) Sequences: 5(1) emPAI: 0.68
50S ribosomal protein L19 [Pseudoalteromonas haloplanktis]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
60	473.2338	944.4530	944.4814	-30.07	0	26	1e+02	1	U	K.ALEDEQLK.T
62	480.7357	959.4568	959.4672	-10.80	0	59	0.049	1		K.ISNGEGVER.V
113	587.8424	1173.6702	1173.6758	-4.69	0	55	0.11	1	U	R.LQAFEGVVIAK.R
162	547.6251	1639.8535	1639.8934	-24.33	0	129	3.1e-09	1	U	R.VFQTHSPLIDSVAVK.R
163	547.6251	1639.8535	1639.8934	-24.33	0	(87)	5.2e-05	1	U	R.VFQTHSPLIDSVAVK.R
194	885.7931	2654.3575	2654.3803	-8.62	1	60	0.016	1	U	K.ALEDEQLKTDVPAFGPGDTVVVQVK.V

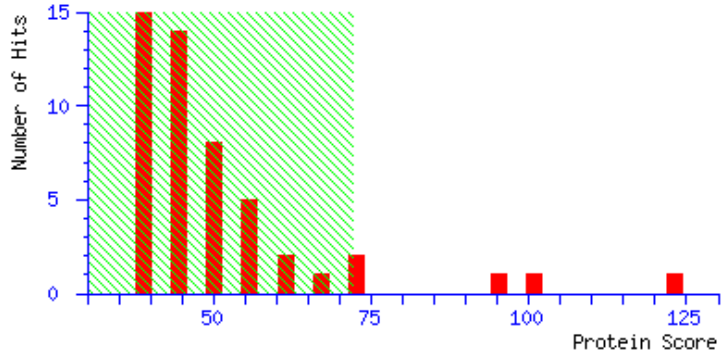
2. gi|493844189 Mass: 12164 Score: 206 Matches: 5(2) Sequences: 4(2) emPAI: 0.75
50S ribosomal protein L7/L12 [Alteromonadales]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
95	548.3134	1094.6122	1094.6335	-19.46	0	76	0.00074	1	U	K.ALVEAAPTPVK.E
125	630.8034	1259.5922	1259.6245	-25.60	0	86	8.1e-05	1	U	K.DLTEAGAEVEVK.-
141	454.2271	1359.6595	1359.6881	-21.07	1	9	4.2e+03	1	U	K.EGVSKEEAEALAK.D
210	933.0995	2796.2767	2796.3119	-12.59	0	(13)	7.5e+02	1	U	K.DQILDAIAEMSVMDVVALVEAMEEK.F + 3 Oxidation (M)
211	933.1017	2796.2833	2796.3119	-10.23	0	36	4.1	1	U	K.DQILDAIAEMSVMDVVALVEAMEEK.F + 3 Oxidation (M)

Spot SS4

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 72 indicate identity or extensive homology (p<0.001).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. gi|77361713 Mass: 13162 Score: 123 Matches: 1(1) Sequences: 1(1) emPAI: 0.30
30S ribosomal protein S13 [Pseudoalteromonas haloplanktis TAC125]
Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
☑	165	730.3895	1458.7644	1458.7566	5.40	0	123	1.4e-08	1	U	K.IGELSDETLDVLR.D

Proteins matching the same set of peptides:
gi|315127934 Mass: 13218 Score: 123 Matches: 1(1) Sequences: 1(1)
30S ribosomal protein S13 [Pseudoalteromonas sp. SM9913]
gi|490726712 Mass: 13204 Score: 123 Matches: 1(1) Sequences: 1(1)
30S ribosomal protein S13 [Pseudoalteromonas]
gi|498049569 Mass: 13217 Score: 123 Matches: 1(1) Sequences: 1(1)
30S ribosomal protein S13 [Pseudoalteromonas citrea]
gi|515080439 Mass: 13262 Score: 123 Matches: 1(1) Sequences: 1(1)
30S ribosomal protein S13 [Pseudoalteromonas haloplanktis]
gi|584418556 Mass: 13132 Score: 123 Matches: 1(1) Sequences: 1(1)
30S ribosomal protein S13 [Pseudoalteromonas lipolytica SCSIO 04301]

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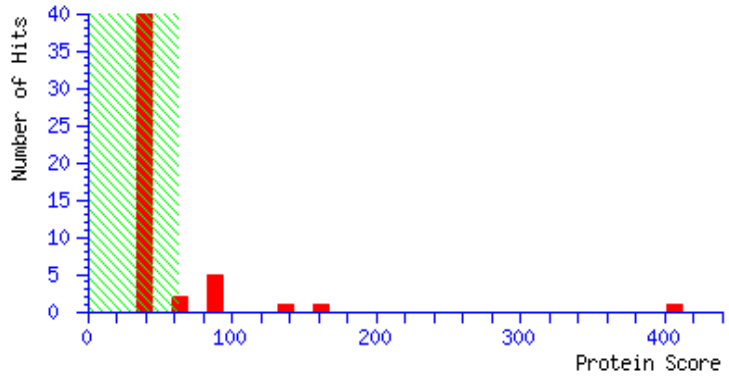
2. gi|77361720 Mass: 14053 Score: 98 Matches: 2(1) Sequences: 2(1) emPAI: 0.28
30S ribosomal protein S8 [Pseudoalteromonas haloplanktis TAC125]
Check to include this hit in error tolerant search

	Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
	85	489.7474	977.4802	977.4852	-5.05	0	19	4.1e+02	2	U	K.VSVTMPNSK.L + Oxidation (M)
☑	157	688.3643	1374.7140	1374.7143	-0.20	0	79	0.00042	1	U	M.SLQDPIADLFTR.I

Spot SS8

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 63 indicate identity or extensive homology (p<0.01).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. gi|515077714 Mass: 26438 Score: 407 Matches: 7(2) Sequences: 7(2) emPAI: 0.50
multidrug transporter [Pseudoalteromonas haloplanktis]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
109	637.8565	1273.6984	1273.7030	-3.59	0	63	0.016	1	U	K.IQNGINVEFLK.T
112	642.8162	1283.6178	1283.6220	-3.24	0	19	3.4e+02	1	U	R.VANEFPMSFVK.N + Oxidation (M)
114	646.8400	1291.6654	1291.6660	-0.39	0	44	1.3	1	U	K.DINGIYLIDEK.K
134	474.2315	1419.6727	1419.7106	-26.73	0	41	2.3	1		M.AEQQVQPLHNEK.H
150	535.2883	1602.8431	1602.8631	-12.48	0	63	0.014	1	U	K.TQHLVPVVAHEFAR.V
169	678.3856	2032.1350	2032.1456	-5.22	1	77	0.00039	1	U	K.YLADKELLVAQTLTVDIK.G
171	685.6781	2054.0125	2054.0361	-11.48	0	101	1.9e-06	1	U	K.EALVSFIEFSHVTEAFTK.Y

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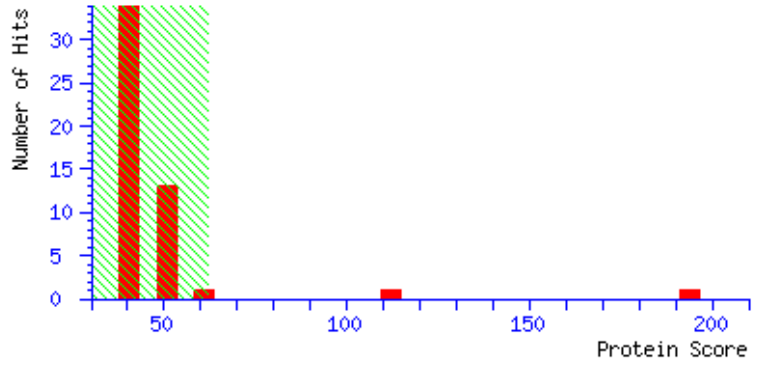
2. gi|557381106 Mass: 26633 Score: 162 Matches: 3(1) Sequences: 3(1) emPAI: 0.14
SapC [Pseudoalteromonas luteoviolacea]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
134	474.2315	1419.6727	1419.7106	-26.73	0	41	2.3	1		M.AEQQVQPLHNEK.H
149	796.8914	1591.7682	1591.7729	-2.94	0	103	1.2e-06	1	U	K.LNELSDEDFLELR.K
150	535.2883	1602.8431	1602.8631	-12.47	0	18	5e+02	4	U	K.SQHLIPVVAHEFAR.V

Spot SS9

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 62 indicate identity or extensive homology (p<0.01).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. gi|518271872 Mass: 26819 Score: 194 Matches: 3(1) Sequences: 3(1) emPAI: 0.30
30S ribosomal protein S2 [Moritella marina]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
36	425.7037	849.3928	849.4014	-10.10	0	52	0.22	1	U	K.EALMNTR.E + Oxidation (M)
152	939.9397	1877.8648	1877.8643	0.31	0	88	3.3e-05	1	U	R.NQDIAAQAESDFIEEAK.-
180	606.0614	2420.2165	2420.2410	-10.12	0	54	0.073	1	U	K.TMGGLPDVIFIIDADHEHIAIK.E + Oxidation (M)

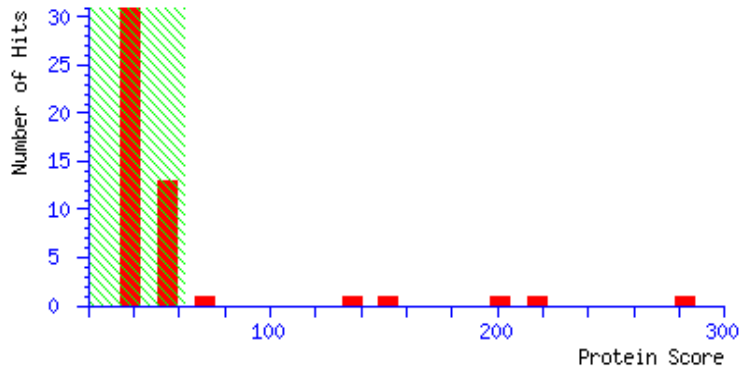
2. gi|515080529 Mass: 30667 Score: 114 Matches: 2(1) Sequences: 2(1) emPAI: 0.12
hypothetical protein [Pseudoalteromonas haloplanktis]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
109	598.3198	1194.6250	1194.6245	0.49	0	82	0.00019	1	U	K.SIATVDTFVSR.N
211	801.9075	3203.6009	3203.6211	-6.30	0	32	8	1	U	R.NGTSKPALLGQGLEISGPTHPNDLFVGEEAR.F

Spot SS10

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 62 indicate identity or extensive homology (p<0.01).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



1. gi|515080255 Mass: 47117 Score: 282 Matches: 6(2) Sequences: 6(2) emPAI: 0.17
transcription termination factor Rho [Pseudoalteromonas haloplanktis]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
52	520.8145	1039.6144	1039.6026	11.4	0	46	0.61	1		R.VLDLASPIGR.G
105	568.8088	1135.6030	1135.5873	13.8	0	23	1.7e+02	1		R.AYNTVIPSSGK.V
128	659.8082	1317.6018	1317.5798	16.7	0	58	0.043	1		K.MDEVIYEEFK.G + Oxidation (M)
142	739.8721	1477.7296	1477.7049	16.8	0	72	0.0019	1		K.GEVIASTFDEPASR.H
148	556.3043	1665.8911	1665.8838	4.35	0	71	0.0019	1	U	K.ILFENLTPLHANER.L
169	757.0483	2268.1231	2268.0910	14.2	0	12	1.1e+03	6	U	R.SSEASYLAGPDDIYISPSQIR.R

2. gi|491634854 Mass: 47155 Score: 224 Matches: 4(2) Sequences: 4(2) emPAI: 0.16
Transcription termination factor Rho [Pseudoalteromonas luteoviolacea]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
105	568.8088	1135.6030	1135.5873	13.8	0	23	1.7e+02	1		R.AYNTVIPSSGK.V
128	659.8082	1317.6018	1317.5798	16.7	0	58	0.043	1		K.MDEVIYEEFK.G + Oxidation (M)
142	739.8721	1477.7296	1477.7049	16.8	0	72	0.0019	1		K.GEVIASTFDEPASR.H
148	556.3043	1665.8911	1665.8838	4.35	0	71	0.0019	1		K.ILFENLTPIHANER.L

Appendix III

Bacterial agglutination using DAPI (4',6-diamidino-2-phenylindole) fluorescent stain.

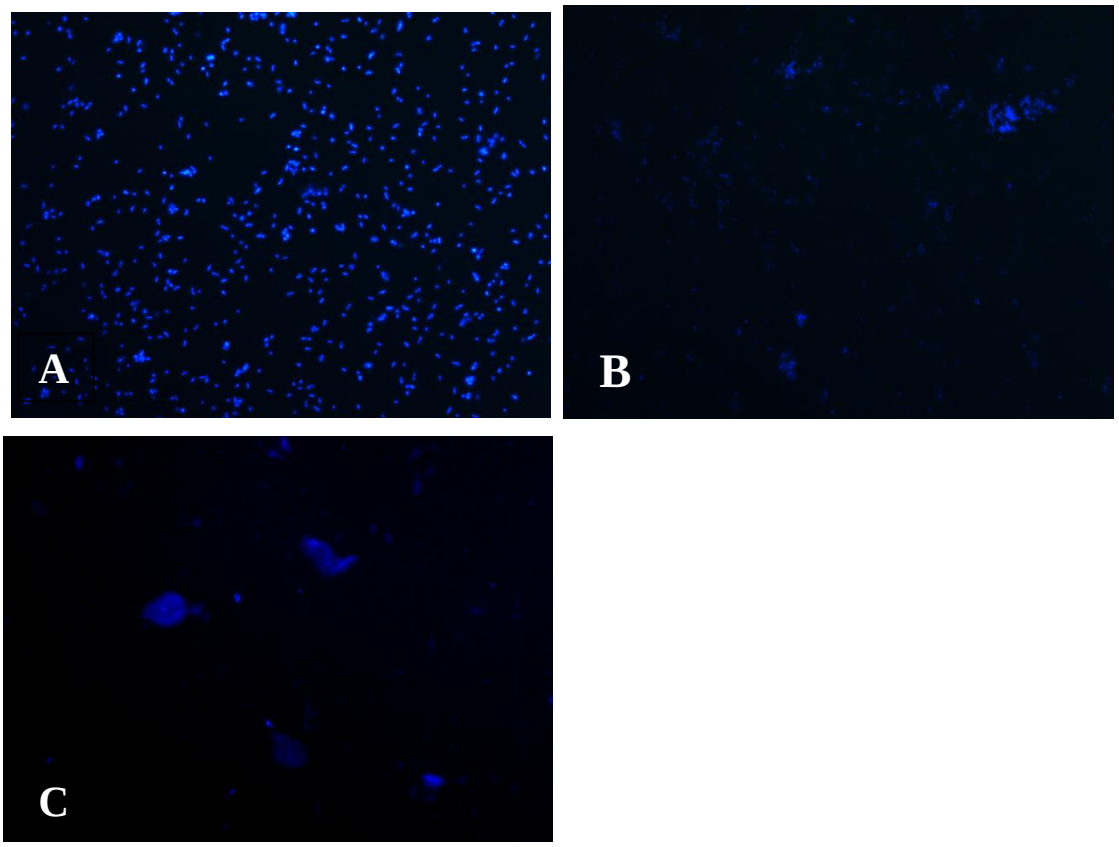


Figure.11. Bacterial agglutination of *Moritella viscosa* A. control (PBS) with no agglutination B *M.viscosa* agglutinated by crude mucus. C. Agglutination by galectin-3C. Bacterial culture (OD_{590} : 0.8) was washed and fixed with 4% paraformaldehyde for 16h at 4ºC. Fixed cells were washed in PBS and labelled with DAPI fluorescent stain for 10 min. Labeled cells were washed again and incubated with galectin-3C for 2 h. Agglutination was observed under fluorescence microscope. Protocol was followed according to (Rajan et al 2013).