Moritella viscosa Virulence

Extracellular Products and Host-Pathogen Interaction

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Thesis for the degree of Philosophiae Doctor
University of Iceland
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Sýkingarmáttur *Moritella viscosa*
seyti og samspil hýsils og sýkils

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Háskóli Íslands
Heilbrigðisvísindasvið
Læknadeild
Maí 2011

ISBN 978-9935-9024-1-2

Printing by Leturprent ehf.

Reykjavík, Iceland 2011

ÁGRIP

Vetrarsár í laxfiskum orsakast af bakteríunni *Moritella viscosa*. Sjúkdómurinn hefur valdið miklum fjárhagslegum afföllum í fiskeldi við norðanvert Atlantshaf og veldur enn umtalsverðum vandamálum, þrátt fyrir tilkomu sérvirkra bóluefna. Bakterían sýkir einnig eldisþorsk (*Gadus morhua* L.). Aukinn skilningur á líffræði og sýkingarmætti *M. viscosa*, ásamt samspili bakteríunnar við hýsil, er því mikilvægur í baráttunni gegn sjúkdómnum og við þróun bættra sjúkdómsvarna. Helstu markmið rannsóknarinnar voru að kanna sýkingarmátt *M. viscosa*, með áherslu á þætti í seyti, og samspil hýsils og sýkils.

Í fyrsta hluta rannsóknarinnar var sýkingarmáttur seytis 22ja *M. viscosa* stofna metinn, bæði *in vivo* og *in vitro*, ásamt því að ýmsir þættir í seyti voru mældir. Sýkingarhæfir stofnar framleiddu óskilgreint banvænt eitur og endurspegluðu niðurstöðurnar það mikilvægi sem seyti virðist hafa á þróun og sjúkdómsmynd vetrarsára. Seyti ósýkingarhæfra *M. viscosa* stofna drap ekki lax. Frumudrápsvirkni seytis var tengd þeirri fisktegund sem viðkomandi stofn var einangraður úr og bendir til hýsilsérhæfingar *M. viscosa*.

Því næst var helsti peptíðasi í seyti *M. viscosa* einangraður og virkni hans skilgreind. Peptíðasinn, sem nefndur var MvP1, reyndist vera áður óskilgreint vibriolysin og hafði eiginleika sem geta haft áhrif á sýkingarmátt bakteríunnar. MvP1 peptíðasinn var virkur á breiðu hitasviði og olli blæðingum og vefjaskemmdum í laxi. Eiginleikar hans eiga mögulega þátt í íferð og útbreiðslu bakteríunnar í fiski. Tilraunir til að útbúa MvP1 neikvæð stökkbrigði báru ekki árangur og var því mikilvægi peptíðasans sem sýkiþáttur ekki ákvarðaður til fullnustu.

Hæfni *M. viscosa* til að ræsa ónæmissvar hýsils var metin með örvun laxafrumulínu. Frumur voru örvaðar með mismunandi *M. viscosa* mótefnavökum og tjáning frumuboðanna IL-1β og IL-8 metin. Aukin tjáning genanna var túlkuð sem vísbending um ræsingu bólgusvars. Lifandi *M. viscosa* frumur örvuðu tjáningu beggja frumuboða, en hitadrepnar frumur og seyti örvuðu einungis tjáningu IL-8. Einangraður MvP1 peptíðasi reyndist ekki hafa mikil áhrif á tjáningu frumuboðanna. Niðurstöðurnar benda til að mótefnavakar *M. viscosa* hafi mismunandi áhrif á örvun frumuboða og gætu nýst við þróun bóluefna gegn vetrarsárum.

Með skimun genamengis *M. viscosa* var hægt að bera kennsl á tvö genasæti sem kóða fyrir seytiferli af týpu VI. Genasætin voru nefnd *mts1* og *mts2*. Virkni Mts1 seytiferlisins var staðfest í sýkingarhæfum og ósýkingarhæfum *M. viscosa* stofnum. Seytiferli af týpu VI flytja

prótein úr bakteríufrumum út í umhverfi þeirra, eða jafnvel inn í umfrymi hýsilfruma, í gegnum einskonar nál. Seytiferli af týpu VI virðast gegna ýmsum hlutverkum og geta þau m.a. átt þátt í að stuðla að sýkingarmætti baktería. Hlutverk seytiferla af týpu VI í *M. viscosa* eru þó ekki þekkt, en eru áhugavert rannsóknarverkefni.

Í heild gefa niðurstöður rannsóknanna nýja sýn á líffræði og sýkingarmátt *M. viscosa*, einkum á mikilvægi seytis í myndun sjúkdóms.

Lykilorð: *Moritella viscosa*, sýkingarmáttur, seyti, seyting, MvP1 peptíðasi

ABSTRACT

Moritella viscosa is the aetiological agent of winter ulcer disease in farmed salmonid fish. The disease has had significant economic effects on the salmon aquaculture industry in the North Atlantic, and continues to cause problems despite the availability of commercial vaccines. Infections also occur in cultured Atlantic cod (*Gadus morhua* L.). Improved understanding of *M. viscosa*, its biology, virulence mechanisms and host-pathogen interactions is therefore important in order to fight disease outbreaks and for development of effective treatments against the disease. The main objectives of this study were to examine the virulence mechanisms of *M. viscosa*, with emphasis on secreted factors and bacterial interactions with its host.

In the first part of the study, the virulence properties of extracellular products (ECP) of 22 *M. viscosa* isolates were evaluated. The *in vivo* and *in vitro* virulence of ECPs were screened and several parameters measured. Virulent isolates produced an unidentified lethal factor and the results reflected the proposed significance ECP may have on the development and pathology of winter ulcer disease. Avirulent *M. viscosa* isolates produced non-lethal ECPs. Cytotoxin production followed host species origin of isolates and may reflect host adaptation in *M. viscosa*.

Successively, the major extracellular caseinase of *M. viscosa* was isolated and characterized. The caseinase, termed MvP1, was a previously unknown vibriolysin with virulence related activities. The MvP1 peptidase, which was active over a broad temperature range, caused hemorrhages and tissue destruction in salmon and may aid in bacterial invasion and dissemination within the host. Attempts to construct MvP1 negative mutants were unsuccessful and the importance of this peptidase in *M. viscosa* virulence was therefore not fully determined.

For evaluating the ability of *M. viscosa* to trigger a host immune response, selected *M. viscosa* antigens were used to stimulate a continuous salmon cell line. Following stimulation, expression of the IL-1β and IL-8 cytokine genes was measured, as indicators of a pro-inflammatory response. Live *M. viscosa* cells induced both IL-1β and IL-8 expression, while heat-killed cells and ECP only induced the expression of IL-8. Furthermore, isolated MvP1 was not a major stimulant of pro-inflammatory gene expression. The results indicate that *M. viscosa* antigens have different stimulatory potentials, which may be of value for improved vaccine production.

Finally, two type VI secretion system (T6SS) loci were identified in the genome of *M. viscosa*, termed *mts1* and *mts2*. A functional Mts1 system was confirmed in *M. viscosa*, both in virulent and avirulent isolates. T6SSs can export proteins through a needle like structure into the environment or directly into host cells, and they have been implicated with various functions, including virulence. However, the role of T6S in *M. viscosa* is yet unknown and is an interesting research topic.

Overall, the results of the study give new insight into the biology and virulence of *M. viscosa*, especially into the importance of secreted factors on disease development.

Keywords: *Moritella viscosa*, virulence, extracellular products, secretion, MvP1 peptidase

ACKNOWLEDGMENTS

This study was carried out at the Institute for Experimental Pathology, University of Iceland, Keldur (Keldur), between 2004 and 2010. A part of the work was performed at the NRC Institute for Marine Biosciences (IMB) in Halifax, 2006, where I worked as a visiting scientist. I would like to express my sincere gratitude to all the people, institutions and research facilities who contributed to the work described in the thesis.

First of all I would like to thank my supervisor, Bjarnheiður K. Guðmundsdóttir, for all her support. Her guidance, encouragement, motivation and friendship have been invaluable. She has shown me endless patience and tried to teach me how to become an independent scientist. I would also like to thank my co-workers at Keldur for their advice, companionship and many laughs. I especially thank Þórunn Guðmundsdóttir and Johanna Schwenteit for their assistance.

My sincere gratitude goes to my collaborators outside of Keldur, at Matís ohf, IMB, University of Tromsø, the Norwegian National Veterinary Institute, Umeå University and Faculty of Physical Sciences, University of Iceland and all my co-authors, for their expertise and cooperation. Special thanks go to Ólafur H. Friðjónsson for all his advice and Laura L. Brown for welcoming me to her lab at IMB. I also thank the fish farms Stofnfiskur and Íslandslax for providing fish for experiments.

I thank the members of my doctoral committee, Valgerður Andrésdóttir, Guðmundur Ó. Hreggviðsson, Ágústa Guðmundsdóttir and Trygve Poppe for their helpful guidance throughout the study and in final preparation of the thesis. I also thank the Department of Fish Diseases at Keldur for giving me the opportunity to work in their facilities, and the School of Health Sciences, University of Iceland, for accepting me as a Ph.D. candidate.

My family and friends I wish to thank for their invaluable love, support and understanding. I am especially grateful to my parents, who raised me to believe in myself.

The work was funded by the AVS research fund of the Ministry of Fisheries in Iceland, the University fund of Eimskip, the Icelandic research fund, the University of Iceland Research Fund and the Memorial Fund of Helga Jónsdóttir and Sigurliði Kristjánsson. My stay as a visiting scientist at IMB was supported by an EMBO short term fellowship grant.

CONTENTS

ÁGRIP i
ABSTRACT iii
ACKNOWLEDGMENTS v
CONTENTS vi
LIST OF ABBREVIATIONS ix
LIST OF FIGURES xi
LIST OF TABLES xii
LIST OF PAPERS xiii
DECLARATION OF CONTRIBUTION xiv
1 INTRODUCTION 1
1.1 Aquaculture 1
1.1.1 Icelandic aquaculture 1
1.1.2 Infectious diseases in aquaculture 2
1.1.3 Prophylaxis, treatment and vaccination of fish 3
1.2 The immune system of bony fish 5
1.2.1 The innate system 5
1.2.2 The inflammatory response and pro-inflammatory cytokines 6
1.2.3 The adaptive system 7
1.2.4 Factors affecting the immune system of fish 8
1.3 Bacterial pathogenicity and virulence factors 8
1.3.1 Mechanisms of bacterial pathogenicity 9
1.4 Peptidases 14
1.4.1 Definitions and activity 14
1.4.2 Peptidase groups 15
1.4.3 Metallopeptidases 15
1.4.4 Bacterial metallopeptidases and pathogenesis 16
1.4.5 The thermolysin family 17
1.4.6 Vibriolysins 18
1.5 Winter ulcer disease 21
1.5.1 Disease occurrence 21
1.5.2 Disease symptoms and pathology 22
1.5.3 Theories on the cause of winter ulcers 23
1.6 *Moritella viscosa* 24
1.6.1 Classification 24
1.6.2 Identification and culturing 25
1.6.3 Genotypes and serotypes 26
1.6.4 Potential reservoirs and infection 27
1.6.5 Virulence and virulence factors 27
1.6.6 Treatment and vaccination 30
2 AIMS OF THE STUDY 31
3 MATERIALS AND METHODS 33
3.1 Experimental fish and injections 33
3.2 Cultivation of bacteria and identification of bacterial products 34
3.2.1 Bacterial isolates and cultivation 34

3.2.2 Antibiotic resistance 34
3.2.3 Production of extracellular products 36
3.2.4 Protein concentration 36
3.2.5 Caseinolytic activity 36
3.2.6 Esterase activity 37
3.2.7 Agar diffusion assays 37
3.2.8 Proteolytic digestion of IgM 37
3.2.9 Hemolytic assays 38
3.2.10 Cytotoxicity assays 38
3.2.11 Siderophore production assay 39
3.2.12 Acrylamide gel electrophoresis 39
3.2.13 Anti-MvP1 production 40
3.2.14 Western blotting 40
3.2.15 N-terminal sequence analysis 41
3.2.16 Mass spectrometry 41
3.2.17 Autodegradation of *M. viscosa* ECP 41
3.2.18 MvP1 peptidase purification 41
3.2.19 Fractionation of esterase activity 42
3.2.20 Preparation of antigens for SHK-1 stimulation 42
3.3 SHK-1 cell culturing and stimulation 43
3.4 DNA and RNA methods 43
3.4.1 Standard isolation and manipulation of DNA and RNA 43
3.4.2 *M. viscosa* genome 46
3.4.3 Sequencing of the *mvp1* gene and flanking regions 46
3.4.4 Screening for additional vibriolysin genes 48
3.4.5 Quantification of pro-inflammatory gene expression 48
3.4.6 MvP1 negative *M. viscosa* mutant construction 49
3.5 Statistical analysis 53
3.6 Licenses 53
4 RESULTS 55
4.1 *M. viscosa* virulence and growth 55
4.1.1 Virulence of *M. viscosa* isolates 55
4.1.2 *M. viscosa* growth 55
4.2 Virulence properties of *M. viscosa* ECP 55
4.2.1 B-ECP versus C-ECP 55
4.2.2 Virulence activities 56
4.2.3 Protein concentration, enzymatic activities and siderophore production 57
4.3 Autodegradation of *M. viscosa* ECP 58
4.4 *M. viscosa* MvP1 extracellular peptidase 60
4.4.1 Isolation 60
4.4.2 Characterization 60
4.4.3 Toxicity 61
4.4.4 Sequencing 61
4.4.5 Production of MvP1 by *M. viscosa* isolates 65
4.4.6 Construction of MvP1 negative *M. viscosa* mutants was unsuccessful 65
4.4.7 Vibriolysin genes detected in the *M. viscosa* genome 65
4.5 Protein identification in *M. viscosa* culture supernatants 67
4.6 Effects of *M. viscosa* on pro-inflammatory gene expression 68
4.6.1 *M. viscosa* cytotoxicity 68

4.6.2 EF-1A reference gene expression 69
4.6.3 Effects of *M. viscosa* cells and ECP 69
4.6.4 Effects of isolated MvP1 70
4.7 *M. viscosa* type VI secretion systems 71
4.7.1 Identification of two T6SSs in the *M. viscosa* genome 71
4.7.2 Active Mts1 T6SS in *M.viscosa* 72
5 DISCUSSION 77
5.1 Virulence properties of *M. viscosa* ECP 77
5.2 The *M. viscosa* MvP1 vibriolysin 80
5.3 *M. viscosa* stimulation of pro-inflammatory genes 83
5.4 Two T6SSs in *M. viscosa* 85
5.5 Methodological considerations 86
6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES 89
REFERENCES 91
ORIGINAL PAPERS I - IV 107

LIST OF ABBREVIATIONS

aa	amino acid
AP	alkaline phosphatase
BA	5% horse blood agar medium
B-ECP	ECP collected from broth cultures
BF-2	bluegill fry fibroblast cells
BHI	Brain-Heart infusion broth
BLAST	Basic Local Alignment Search Tool
CAS	cromoazurol S, siderophore-indicative medium
CDS	coding sequence
C-ECP	ECP produced from cellophane overlay cultures
CFU	colony forming units
CHSE	chinook salmon embryo cells
CV	column volume
dH_2O	deionised distilled water
DNA	deoxynucleicacid
DPBS	Dulbecco's phosphate buffered saline
ECP	extracellular products
EDTA	ethylenediaminetetraacetic acid
EF-1A	elongation factor-1alpha
EPC	epithelioma papulosa carpio cells
FHA	forkhead-associated domain
FPLC	fast protein liquid chromatography
HCl	hydrogen chloride
Hcp	haemolysin co-regulated protein
Ig	immunoglobulin
IL-1β	interleukin-1β
IL-8	interleukin-8
i.m.	intramuscular
i.p.	intraperitoneal
kDa	kilodaltons
LB	Luria-Bertani
LD_{50}	50 percent lethal dose
LDH	lactate dehydrogenase
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MeOH	methanol
MHC	major histocompatibility complex
MLD	minimum lethal dose
MOI	multiplicity of infection
mts1	Moritella Type Six secretion 1 locus

Mts1	Moritella Type Six secretion system 1
mts2	Moritella Type Six secretion 2 locus
MvP1	*M. viscosa* extracellular peptidase 1
mvp1	the gene encoding MvP1
MvP2	*M. viscosa* predicted extracellular peptidase 2
mvp2	the gene encoding putative MvP2
MvP3	*M. viscosa* predicted extracellular peptidase 3
mvp3	the gene encoding putative MvP3
MW	molecular weight
MWCO	molecular weight cut off
NaCl	sodium chloride
NaOH	sodium hydroxide
nt	nucleotide
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QS	quorum sensing
RNA	ribonucleicacid
SD-200 buffer	50 mM Na-phosphate (pH 7.0), 15 mM NaCl
sdm	site directed mutagenesis
SDS	sodium dodecyl sulphate
T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
T4SS	type IV secretion system
T5SS	type V secretion system
T6SS	type VI secretion system
TBS-TC	20 mM, 0.8% NaCl, 0.1% Tween20, 0.1% skimmed milk powder
TEM	transmission electron microscope
U	units

LIST OF FIGURES

Fig. 1. Use of antibiotics in Icelandic aquaculture and introduction of vaccines. 4
Fig. 2. Secretion systems in Gram negative bacteria. 13
Fig. 3. Thermolysin active site. 18
Fig. 4. Fish with winter ulcer disease. 23
Fig. 5. *M. viscosa* cells and viscosity. 25
Fig. 6. Construction of an *mvp1* deletion template. 50
Fig. 7. Growth curves of *M. viscosa* isolates. 56
Fig. 8. Isolated MvP1 peptidase. 60
Fig. 9. Salmon injected with isolated MvP1 peptidase. 61
Fig. 10. Effects of isolated MvP1 peptidase on EPC cell culture. 62
Fig. 11. Nucleotide sequence of the *mvp1* gene. 63
Fig. 12. Schematic figure of the MvP1 prepropeptide. 64
Fig. 13. Tree of vibriolysin peptidases. 64
Fig. 14. Sequence alignment of mature peptidase regions of MvP1 and MvP2. 66
Fig. 15. Sequence alignment of mature peptidase regions of MvP1 and MvP3. 67
Fig. 16. Cytotoxicity of SHK-1 cells following *M. viscosa* antigen stimulation. 69
Fig. 17. IL-1β and IL-8 expression in SHK-1 cells following stimulation with *M. viscosa* cells or ECP. 70
Fig. 18. IL-1β and IL-8 expression in SHK-1 cells following stimulation with *M. viscosa* MvP1 peptidase. 71
Fig. 19. *M. viscosa* T6SS loci. 73
Fig. 20. T6SS comparison. 74
Fig. 21. Hcp secretion in *M. viscosa*. 75

LIST OF TABLES

Table 1. Summary of main innate immune activities investigated in fish 7
Table 2. Summary of main adaptive immune activities investigated in fish 8
Table 3. LD_{50} of *M. viscosa* (isolate 3) in different fish species 28
Table 4. *M. viscosa* isolates and related bacterial strains used in the study 35
Table 5. Primers used for sequencing of *mvp1* and flanking regions 47
Table 6. Real-time Q-PCR primers 48
Table 7. Primers used for the construction of mvp1 mutagenesis templates 50
Table 8. B-ECP versus C-ECP 56
Table 9. Virulence activities of ECP 59

LIST OF PAPERS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (Papers I to IV) and previously unpublished results.

Paper I Bjornsdottir B., Gudmundsdottir T. and Gudmundsdottir B.K. (2011) **Virulence properties of *Moritella viscosa* extracellular products.** Journal of Fish Diseases, **34**, 333-343.

Paper II Bjornsdottir B., Fridjonsson O.H., Magnusdottir S., Andresdottir V., Hreggvidsson G.O. and Gudmundsdottir B.K. (2009) **Characterisation of an extracellular vibriolysin of the fish pathogen *Moritella viscosa*.** Veterinary Microbiology **136**, 326-334.

Paper III Bjornsdottir B., Fast M.D., Sperker S.A., Brown L.L. and Gudmundsdottir B.K. (2009) **Effects of *Moritella viscosa* antigens on pro-inflammatory gene expression in an Atlantic salmon (*Salmo salar* Linnaeus) cell line (SHK-1).** Fish & Shellfish Immunology **26,** 858-863.

Paper IV Bjornsdottir B., Hjerde E., Willassen N.P. and Gudmundsdottir, B.K. **Identification of type VI secretion systems in *Moritella viscosa*.** Veterinary Microbiology, submitted manuscript.

All papers are reprinted by permission of the publisher.

Additional related scientific papers published during the Ph.D. period but not included in the thesis:

Gudmundsdottir B.K. and Bjornsdottir B. (2007) **Vaccination against atypical furunculosis and winter ulcer disease of fish.** Vaccine **25,** 5512-5523.

Gudmundsdottir B.K., Bjornsdottir B., Gudmundsdottir S. and Bambir S.H.(2006) **A comparative study of susceptibility and induced pathology of cod, *Gadus morhua* (L.), and halibut, *Hippoglossus hippoglossus* (L.), following experimental infection with *Moritella viscosa*.** Journal of Fish Diseases **29,** 481-487.

DECLARATION OF CONTRIBUTION

I took part in the conception and design of the study and in writing grant applications and periodical reports. I had a major role in planning, set-up and execution of experiments and the analysis and interpretation of data, as well as in writing of all the papers.

1 INTRODUCTION

1.1 Aquaculture

Aquaculture supplies around half of the fish and shellfish consumed by humans. The importance of aquaculture has increased following decline or stagnation in the world´s fisheries and overexploitation of many fish species. Aquaculture grows more rapidly than other sectors producing animal food, and production has increased dramatically in the last 50 years both in value and volume. Asia, particularly China, is by far the biggest production region of aquacultured products, with about 89% of global aquaculture production in 2006 (Food and Agriculture Organization of the United Nations, 2009). A diverse array of taxa is being farmed and the number of aquatic species that have been domesticated is rapidly increasing (Duarte et al., 2007). The major production species in aquaculture are freshwater fish and molluscs (Food and Agriculture Organization of the United Nations, 2009). Aquaculture is an important industry in the Nordic countries, where Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) are the main cultured species (Paisley, 2010).

Negative aspects of increased aquaculture include effects on wild populations, effects on ecosystems due to effluents and waste from farms, and effects on wild species used as feed (Duarte et al., 2007). The potential of pathogen transmission between farmed and wild fish populations is also a major concern. The conservation and value of wild fish populations has been increasingly recognized, as well as the economical importance of wild sport fisheries. Topics of fish welfare and ethics in aquaculture have also gained increased attention in recent years. Husbandry techniques, disease, transport and slaughtering have been the main areas of interest, but recently the use of so-called welfare indicators has gained increased interest (Evans, 2009; Huntingford & Kadri, 2009).

1.1.1 Icelandic aquaculture

Fishing is an important industry in Iceland and the aquaculture industry is in development. Fish exports account for about 53% of the country's total exports (Paisley, 2010). Aquaculture in Iceland has mainly evolved around the production of salmonid species. The first attempt to rear fish to full size for food consumption was in 1951, when rainbow trout was imported from Denmark for farming in Reykjavik. However, larger scale farming of fish was not established in Iceland until after 1980 (Kristinsson, 1992). Currently, farming of fish is

increasing in Iceland following a decline in production in recent years. In 2009, 42 fish farms were operated in Iceland and produced over 5.000 tonnes of fish, mainly Arctic charr (*Salvelinus alpinus* L.), Atlantic cod (*Gadus morhua* L.) and Atlantic salmon. Production peaked in 2006 when about 10.000 tonnes were produced, thereof over 6.000 tonnes of salmon. Production decreased the following years, after the shut-down of two of the biggest sea-cage salmon farms in 2007 (Jonsson, 2010; Paisley, 2010).

The main production method in Iceland has been rearing of fish in land based tanks, with less focus on sea cage farming. Production of smolts for domestic production, stocking of rivers and international export have been important aspects of Icelandic aquaculture. Ocean ranching of salmon has also been attempted. Capture of wild cod for ongrowing is currently a developing industry (Kristinsson, 1992; Jonsson, 2010; Paisley, 2010). Since 1985, all Icelandic fish farms have been under obligatory and regular fish health surveillance and European Union regulations are followed (Paisley, 2010). Iceland is not a major producer of aquacultured products, but has good potential for succeeding in larger-scale aquaculture, with high quality fresh and salt water, geothermal heat, and good professional and scientific knowledge in the field.

1.1.2 Infectious diseases in aquaculture

Ideal rearing conditions are often hard to maintain during the intensive industrialised farming of fish. The fish are often subjected to suboptimal water quality, excessive handling and high biomass densities, factors that are known to stress the fish and lead to the onset of disease (Austin & Austin, 2007). Infectious diseases have hindered growth of the aquaculture industry and cause significant economic losses. Many infectious diseases have been reported in farmed fish and shellfish and the infectious agents include bacteria, viruses, parasites and fungi (Poppe, 1999). Bacteria of many taxa have been associated with fish diseases. Most are considered opportunistic pathogens that cause disease in hosts with impaired resistance, while others are considered primary pathogens with high virulence. The number of bacterial species of significant economic importance causing disease in marine aquaculture is not very high, but several diseases that have been considered as typical fresh water diseases have now become important also in marine aquaculture (Toranzo et al., 2005; Austin et al., 2007).

Bacterial infections have caused the major infectious diseases in Icelandic aquaculture. The bacterium *Aeromonas salmonicida* subsp. *achromogenes* (atypical furunculosis) has affected all species of fish cultured in Iceland and together with *Renibacterium salmoninarum*

(bacterial kidney disease), *Moritella viscosa* (winter ulcer disease), *Yersinia ruckeri* (enteric redmouth disease) and *Vibrio anguillarum* (vibriosis) have been the cause of most bacterial disease problems in the last decade (Jonsson, 2010).

Parasitic infections in Icelandic aquaculture due to Costia (*Ichtyobodo necator*) are a common problem, mainly in salmon farming. Ciliates of *Trichodina* spp. and fish lice, mainly *Caligus elongatus*, are frequently diagnosed in fish farms, among other parasites. The only viral infections reported to have caused mortalities in Icelandic aquaculture are *Reoviridae* infections in halibut (*Hippoglossus hippoglossus* L.), which have only caused minor losses (Jonsson, 2010).

1.1.3 Prophylaxis, treatment and vaccination of fish

The onset and development of an infectious disease is the result of interactions between the pathogen, the host and the environment. Therefore, multidisciplinary studies on all three aspects are necessary to increase the success of disease prevention and control. Following the increase in fish farming in recent years and the introduction of new species being farmed, new or previously unimportant infectious disease problems have emerged (Murray & Peeler, 2005).

Infectious diseases and high mortalities in aquaculture have led to the widespread use of antibiotics, which often provide a useful means of controlling many bacterial diseases. However, the use of antibiotics has many disadvantages, such as the appearance of drug resistant strains and recurrent outbreaks (Schmidt et al., 2000; Nikaido, 2009). Furthermore, antibiotics are administered to fish by incorporation into the feed, but infected fish often have reduced appetite and receive inadequate treatment. The marketing of effective vaccines has dramatically reduced the use of antibiotics in aquaculture in the last several decades (Hastein et al., 2005) (Fig 1).

Disease prevention, or prophylaxis, rather than disease treatment is a desirable method. Prophylaxis treatments of fish include improved husbandry, use of disease resistant fish strains, improved diet, use of immunostimulants, probiotics or prebiotics and vaccination (Austin et al., 2007; Merrifield et al., 2010). Vaccines have played an important role in commercial fish farming and have been a major reason for its success, especially in cultivation of salmon (Fig. 1).

Most bacterial fish vaccines are inactivated bacterial products, and many are sold as polyvalent oil-adjuvanted vaccines. Recombinant and DNA vaccines have so far not

been widely used in aquaculture (Clark & Cassidy-Hanley, 2005; Kurath, 2005; Sommerset et al., 2005). Fish vaccines are mainly administered by injection, but also by immersion/bathing or oral uptake. Vaccination by injections of adjuvanted vaccines has in most cases proven to be the most effective route. However, vaccination by injection has some disadvantages such as that small fish cannot be injected and the appearance of vaccination induced side-effects. Side-effects such as granulomas and adhesions are mainly caused by oil-adjuvants (Evensen et al., 2005). Orally administered vaccines have usually only been found to provide limited protection (Hastein et al., 2005; Austin et al., 2007). Several vaccines have been developed for use against viral diseases in aquaculture, but are usually not as effective as bacterial vaccines (Biering et al., 2005; Hammel et al., 2009). No vaccines against parasitic or fungal infections have been developed for commercial use in fish so far, although experimental parasitic vaccines have been tested (Sommerset et al., 2005; Alvarez-Pellitero, 2008).

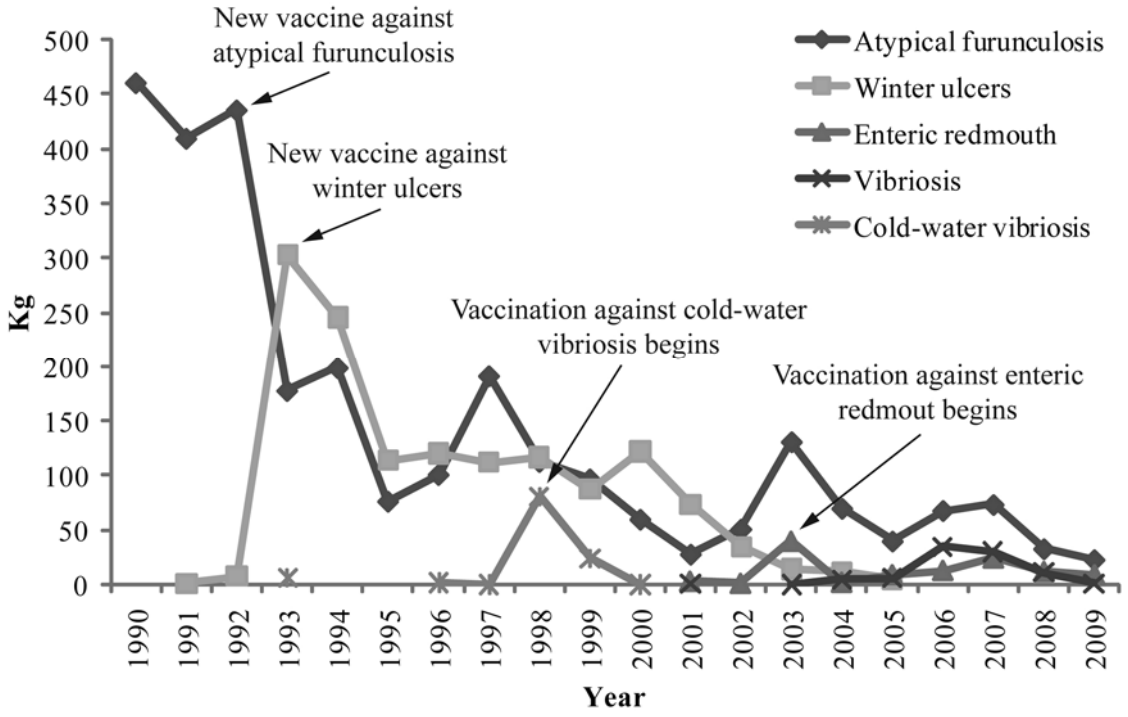

Fig. 1. Use of antibiotics in Icelandic aquaculture and introduction of vaccines.
Antibiotic treatments are divided by which disease they were prescribed against. Arrows indicate the onset of administration of new or improved vaccines. Atypical furunculosis (*Aeromonas salmonicida* subsp. *achromogenes*); winter ulcer disease (*Moritella viscosa*); enteric redmouth disease (*Yersinia ruckeri*); vibriosis (*Vibrio anguillarum*); cold-water vibriosis (*Aliivibrio salmonicida*). Figure: G. Jonsson, adapted with permission.

According to a recent report, all salmon producing countries vaccinate cultured salmon before sea transfer against bacterial and viral diseases that are relevant to the region. Multivalent injection vaccines, conferring immunity against up to six different pathogens,

including *V. anguillarum* (two serotypes), *Aliivibrio salmonicida*, *Aeromonas salmonicida* ssp. *salmonicida* and *M. viscosa* are commonly used (Hammel et al., 2009). Vaccines have been developed for use in several other species of fish, but vaccines are still needed for many cultivated species.

1.2 The immune system of bony fish

The innate immune system is present in all metazoans and works as a first line of defence. The adaptive immune system did, however, not evolve until in the gnathostomata (jawed vertebrates) and fish are the first animal phyla where the adaptive system is present (Flajnik & Kasahara, 2010).

Teleosts (bony fish) consist of evolutionary distinct families which live in very diverse habitats (Ravi & Venkatesh, 2008). The immune systems of teleost fish are therefore not homogeneous systems and they respond differently to infections and vaccinations (Magnadottir, 2006; Zapta et al., 2006). The variable success between vaccination of Atlantic salmon and Atlantic cod reflects well the differences in immune systems of fish species. Atlantic salmon often develops good immunological protection following vaccination with simple oil adjuvated inactivated bacterins, while Atlantic cod does not seem to develop a strong immunological memory or specific protective antibodies following vaccination with similar vaccines (Gudmundsdottir et al., 2009; Magnadottir, 2010). Increased knowledge on fish immune systems and development of new vaccine delivery systems is therefore needed to develop new and improved vaccines for the diverse fish species currently in production.

Considerable advances have been made in recent years in cloning and expression of genes encoding immunoregulatory molecules in fish. Much of the information on the immunology of fish comes from comparative studies to other vertebrates. However, the knowledge on the function of most of those molecules cloned from fish is still very scarce and largely restrained by the lack of specific markers for immunoregulatory peptides (Randelli et al., 2008).

1.2.1 The innate system

The innate response seems to be particularly important to fish and it appears to show more diversity and activity than in mammals (Magnadottir, 2006). Besides acting as a first line of

defence, the innate immune system also has an important role in the activation and regulation of the adaptive response (Iwasaki & Medzhitov, 2010).

The innate system is commonly divided into physical parameters, cellular factors and humoral factors. The physical parameters, epithelia and mucous surfaces, are a very important part of the fish defence system and contain several active innate defence molecules such as antibacterial peptides, lysozymes and complement proteins (Magnadottir, 2006; Magnadottir, 2010). Innate cellular defences include phagocytic neutrophils and B lymphocytes, and cytotoxic monocytes/macrophages. Fish also have non-specific cytotoxic cells (NCC) which are believed to be equivalent to mammalian natural killer (NK) cells (Yoder, 2004; Fischer et al., 2006; Li et al., 2006). The humoral part of the innate response is composed of cell receptors or secreted soluble receptors. The complement system of fish is well developed and in many ways comparable to the mammalian system. It includes the alternative, lectin and classical pathways and can lead to cell lysis or opsonization of pathogens (Boshra et al., 2006). Other innate pattern recognition proteins or receptors of the humoral innate response include several lytic enzymes, agglutinins, antimicrobial peptides, peptidase inhibitors and Toll-like receptors. Several of these parameters have been shown to have more diversity in fish than in mammals, which may give the innate immune response of fish increased recognition abilities (Alvarez-Pellitero, 2008; Aoki et al., 2008; Rebl et al., 2010) (Table 1).

1.2.2 The inflammatory response and pro-inflammatory cytokines

There is genetic and biological evidence showing that the inflammatory response and the properties of pro-inflammatory cytokines/chemokines act in similar manners in both fish and mammals (Reite & Evensen, 2006).

The pro-inflammatory cytokine interleukin-1β (IL-1β) is a central mediator of both inflammatory and other immune responses and initiates a wide variety of functions. It induces the expression of other cytokines, activates macrophages and vascular endothelium, and induces the acute phase response. IL-1β is primarily produced by monocytes and macrophages (Bird et al., 2002; Subramaniam et al., 2004). Both IL-1β and the IL-1R receptor, which binds IL-1β, have been cloned in several species of fish, including salmon (Kaiser et al., 2004; Stansberg et al., 2005; Hong & Secombes, 2009).

Table 1. Summary of main innate immune activities investigated in fish

Activity/factor	Cells involved	cDNA sequence coding for	Cellular marker
Phagocytosis	Mononuclear phagocytes, B-cells	-	mAb to Mφ, and IgM, pAb to granulocytes, neutrophils, granulin NBT, no antibodies pAb to granulin
ROS species	Mononuclear phagocytes	iNOS	NBT, no antibodies
Complement and acute-phase responses	Hepatocytes	C3, C4, C5, C7, C8, CRP, SAP	pAb to C3
Antibacterials	Various types	Families of peptides	None
Antiviral	Leucocytes, Fibroblasts	IFN-1, IFN, Mx protein	None
Enzymes	Various types	Lysozyme, caspases, peptidases	None
Inflammation, cytokines, monokines	Leucocytes	TNFα, COX-2, PLA2, TLRs, Ils (1, 6, 12, 14, 16, 17, 18, 20, 21, 22), > 16 chemokines	pAb for IL-1, pAb and mAb for TNFα
Non-specific killing	Leucocytes	NCCRP-1	mAb 5C6

COX-2, cyclooxygenase 2; CRP, C reactive protein; iNOS, inducible nitric oxide synthase; IFN, interferon; IL, interleukin; NCCRP-1, non-specific cytotoxic cells receptor protein-1; NBT, nitroblue tetrazolium; PLA2, phospholypase A2; ROS, reactive oxygen species; SAP, serum amyloid P; TLRs, Toll-like receptors; TNF, tumor necrosis factor; mAb, monoclonal antibodies; pAb, polyclonal antibodies.
Table modified and reprinted from Fish Shellfish Immunol., Vol 25, Randelli, Buoncore & Scapigliati, Cell markers and determinants in fish immunology, 326-340 (2008), with permission from Elsevier.

The pro-inflammatory cytokine interleukin-8 (IL-8 or CXCL8) is a chemokine and attracts neutrophils to the site of infection or injury and activates them to degranulate and cause tissue damage. IL-8 is produced by several cell types and is induced by different factors, including IL-1β (Kaiser et al., 2004; Laing & Secombes, 2004). IL-8 orthologues have been cloned from several fish species, including salmon (Sangrador-Vegas et al., 2002; Laing et al., 2004; Covello et al., 2009).

1.2.3 The adaptive system

Fish do not have a very strong adaptive immune response, showing reduced immunological memory and limited antibody repertoire and affinity compared to mammals. The secondary immune response of fish is also not as strong as in higher vertebrates. Bony fish do not possess proper lymph nodes and bone marrow as higher vertebrates do. However, fish develop a specific response to infections, have immunological memories and can be protected through vaccination, showing that the main features of the adaptive response are present (Magnadottir, 2010) (Table 2).

The main lymphoid tissues of fish are the head-kidney, thymus and spleen, where leucocytes are produced, differentiate and mature. Both class I and class II major

histocompatibility complex (MHC) receptors are present in teleost fish. T- and B-lymphocytes are present and expression of several cell specific markers and receptors has been confirmed (Alvarez-Pellitero, 2008; Magnadottir, 2010). So far, immunoglobulin (Ig) M and T are the only functional secreted antibodies identified in fish, although class IgD has also been identified (Randelli et al., 2008; Zhang et al., 2010).

Table 2. Summary of main adaptive immune activities investigated in fish

Activity/factor	Cells involved	cDNA sequence coding for	Cellular marker
Specific antibody, memory	B-cells	IgM, IgD, IgT, RAGs	mAb to IgM and B-cells
Cellular recognition, memory	T-cells	TcRα, β, γ, δ, CD3, RAGs	DLT15, WCL38
Specific killing	T-cells	CD8α, CD8β, MHC I	None
Helper activity	T-cells	CD4, MHC II	None
Helper activity	Th1	IFN- γ, IL-2	None
	Th2	IL-4, IL-10	None
	Leucocytes	ILs: 7, 15, 21, 22, 26, LtB	None

Ig, immunoglobulin; RAGs, recombinase-activating genes; TcR, T cell receptor; CD, cell-differentiation cluster; MHC, major histocompatibility antigen; LtB, lymphotoxin B; IFN, interferon; IL, interleukin.
Table modified and reprinted from Fish Shellfish Immunol., Vol 25, Randelli, Buoncore & Scapigliati, Cell markers and determinants in fish immunology, 326-340 (2008), with permission from Elsevier.

1.2.4 Factors affecting the immune system of fish

Various aspects affect the immune response in fish and thereby affect their resistance against infectious agents. Host factors affecting the immune response include age, maturation, sex, breed, stress and general health status. Environmental factors also greatly affect the immune system of aquacultured fish and include temperature, light, nutrition, water quality and various husbandry methods (Bowden et al., 2007; Bowden, 2008; Magnadottir, 2010).

1.3 Bacterial pathogenicity and virulence factors

Several different criteria have been used to establish a connection between an infectious disease and a certain pathogenic organism. Koch´s postulates are the most widely known criteria and have provided an invaluable tool for standardized scientific methodology. Similarly, Koch´s molecular postulates have been designed to prove that a particular gene contributes to virulence. However, the postulates cannot always be satisfied and do not apply to all infectious diseases or virulence factors and have therefore received substantial criticism and debate. Currently, a much broader definition of virulence factors is accepted, where a

virulence factor is commonly determined as “a component of a pathogen that damages the host”, although the different definitions are quite variable and still controversial (Casadevall & Pirofski, 1999; Falkow, 2004; Pallen & Wren, 2007).

Bacterial virulence factors are expressed and secreted to the outer membrane or out of the bacterial cell. Virulence factors are usually believed to aid in the colonization of the host, growth of the pathogen, evasion or inhibition of host defences, acquisition of iron and nutrition from the host and/or entry or exit of cells (for intracellular bacteria) (Casadevall et al., 1999). Extensive research has been conducted to understand the basis of bacterial virulence, which is most often complex and multifactorial. Moreover, the outcome of an encounter between a pathogen and a host is an intricate process, depending on the host´s defences, the pathogenic strain and environmental factors.

An obligate pathogen is a term used for pathogens that must cause disease in order to be transmitted from one host to another, and must also infect a host in order to survive. Opportunistic pathogens, on the other hand, cause diseases in compromised hosts, which usually do not occur in uncompromised hosts (Todar, 2007). Many of the fish pathogenic bacteria can therefore be defined as opportunistic pathogens, which cause disease problems in aquaculture where conditions are often suboptimal and host defences compromised (Austin et al., 2007).

1.3.1 Mechanisms of bacterial pathogenicity

Colonization and invasion

Colonization is the first stage of infection. The main entry sites of pathogenic bacteria in fish are believed to be through the skin, the lateral line, gills, and the digestive tract (Austin et al., 2007). Mucosal surfaces are often the first host tissue pathogens need to adhere to. On fish, the mucosal surface of the skin is continually replenished and sloughed off (Ellis, 2001). Pathogenic bacteria produce various adhesins which are surface structures or molecules that bind to a specific host surface, usually glycoproteins, called receptors. It has been demonstrated that adhesion of fish pathogenic bacteria to fish tissues acts through both specific and unspecific receptors (Vendrell et al., 2009).

Bacterial invasion is usually carried out or aided by invasins, which can promote the spread and growth of the pathogen. Most invasins are extracellular proteins that often have an effect on the pathology of an infectious disease, either directly through damage to host cells/tissues, or indirectly through stimulation of host defences. Spreading factors are a group

of invasins which affect tissue matrices and intercellular spaces, such as hyaluronic acid and collagen. Cytolysins are another group of invasins which usually create a pore in or destabilise cell membranes and are often referred to as lecithinases, phospholipases or hemolysins. Other invasins include coagulases, which cause clotting, and diverse groups of extracellular digestive enzymes, which are active against host components and are primarily thought to have nutritionally related functions but are also thought to aid in the invasion of pathogens by damaging host tissues (Pizarro-Cerda & Cossart, 2006; Todar, 2007). Numerous studies have indicated the importance of cytotoxins and spreading factors in virulence of fish pathogenic bacteria (Toranzo et al., 1983; Wang et al., 1998; Austin et al., 2007; Croxatto et al., 2007).

Evasion and inhibition of host defences

Antibacterial immune defences on epithelial surfaces of fish include mucosal antibodies and antibacterial peptides (defensins) (Cain et al., 2000; Richards et al., 2001; Boman, 2003). Following invasion the main host defences are phagocytosis, the complement system, the inflammatory response, antibody-mediated immunity and cell-mediated immunity.

Pathogenic bacteria possess inherent abilities to resist host immune components, such as polysaccharide capsules, but they also produce various specific virulence factors that help them resist phagocytosis and immune responses (Finlay & McFadden, 2006; Todar, 2007). To fight host defences on epithelial surfaces bacteria produce peptidases that specifically degrade immunoglobulins and anti-defensins which inhibit the activity of antibacterial peptides (Peterson, 2009). Avoiding phagocytosis is one of the first task successful pathogens have to accomplish within the host. They do it through diverse mechanisms that interfere with the phagocytic process, such as avoiding contact and recruitment of phagocytes, inhibiting engulfment, damaging or killing the phagocyte or even surviving within the phagocyte (Flannagan et al., 2009; Diacovich & Gorvel, 2010).

Bacterial pathogens use various ways to evade the host complement system. Complement activation can be avoided by producing capsules which hide bacterial components, by binding complement components to the lipopolysaccharide (LPS) O-antigen and thereby limiting access to the bacterial surface, or by inhibition/destruction of complement system components (DiRita, 2007; Casadevall & Pirofski, 2009).

Pathogenic bacteria also use the strategy of hiding from the host immune system by coating themselves with host proteins, using molecular mimicry or antigen variation, causing

immunosuppression or persisting at sites inaccessible to the immune response (DiRita, 2007; Casadevall et al., 2009; Elde & Malik, 2009; Peterson, 2009).

Toxins

Bacteria produce two types of toxins, exotoxins (protein toxins) and endotoxins (LPS). Exotoxins are among the most potent toxins known, per unit weight, but the functions of exotoxins for bacteria are usually unknown. A wide variety of toxins exist, demonstrating diverse modes of action. Most exotoxins are secreted proteins, with high specificity and activity, and are often major determinants of virulence. The nomenclature and grouping of exotoxins is not straight forward. For example, they can be grouped by their biological effects, such as the type of cell or tissue they affect, by their activities, or by the name of the bacterial species they are produced by. Most toxins are strongly antigenic and neutralized by specific antibodies, which make them important targets of vaccine production. Toxoids, which are detoxified versions of toxins that maintain their antigenic capacity, are often used for immunisation (Barbieri, 2007; Todar, 2007).

LPS endotoxins on the outer cell wall of Gram negative bacteria often exert profound and diverse effects on the host. The effects may be described as a series of alarm reactions such as fever, complement and macrophage activation and stimulation of B lymphocytes. At high concentrations, LPS can produce shock or bacterial sepsis and may even be lethal to the host (Wang & Quinn, 2010). LPS recognition in fish and lower vertebrates is different than in higher animals, where they can resist much higher doses of LPS without developing endotoxin shock. Toll-like receptor 4, which specifically binds the lipid A part of LPS, is the main activator of the strong immune reaction to LPS in higher vertebrates. In fish the Toll-like receptor 4 and other molecules involved in LPS recognition have not been fully established. Nonetheless, LPS exerts a wide variety of effects, including immunostimulation, in fish (Swain et al., 2008).

Secretion systems in Gram negative bacteria

Bacterial secretion systems export proteins and other molecules out of the cell cytoplasm. Gram negative bacteria have developed several different secretion systems and they are involved in transporting virulence factors and toxins into the extracellular matrix, the cell membrane, or directly into host cells (Fig. 2).

The type I secretion system (T1SS) is a relatively simple and widespread system, forming a channel which spans both the inner and outer membrane. Secreted proteins usually contain C-terminal secretion signals and translocation occurs in a one step process (Delepelaire, 2004).

The type II and type V (autotransporter) secretion systems (T2SS and T5SS) export a wide variety of proteins, including virulence factors, into the extracellular environment or the outer membrane from the periplasmic space. These secretion systems depend on the Sec dependent pathway or the twin-arginine translocation (Tat) pathway to first secrete proteins into the periplasm. The Sec pathway secretes unfolded proteins containing an N-terminal signal peptide while the Tat pathway secretes folded proteins containing two consecutive R residues in the signal sequence (Cianciotto, 2005; Dautin & Bernstein, 2007; De Buck et al., 2008; Tseng et al., 2009).

Three secretion systems, type III (T3SS), type IV (T4SS) and type VI (T6SS) can deliver effector proteins directly into host cells. T3SS delivers effector proteins through a syringe-like needle and is highly associated with bacterial virulence. The secretion complex has been shown to share evolutionary origin with the flagellum (Cornelis, 2006). T4SS delivers a variety of effector molecules, including nucleic acids, to eukaryotic host cells, yeast or other bacteria, using a system evolutionary related to bacterial conjugation systems (Cascales & Christie, 2003).

The T6S was not indentified until 2006 (Mougous et al., 2006; Pukatzki et al., 2006). It is found in many proteobacteria and is involved in several processes, including pathogenesis. The system is composed of several components which are believed to deliver proteins into the environment or directly into host cell cytoplasm, through a phage-tail-like injectisome. T6SSs consist of several core proteins, including the secreted haemolysin co-regulated protein (Hcp) and valine-glycine repeat G (VgrG) proteins. The specific function of each of the T6SS core proteins is still not well known and the mode of assembly of T6SSs is unclear (Boyer et al., 2009; Pukatzki et al., 2009). Hcp and VgrG proteins are found in culture supernatants of bacteria with T6SSs and may have dual function as part of the secretion machinery and as effector proteins (Filloux et al., 2008). Hcp forms hexameric rings that form tubes through which effector proteins are supposedly secreted (Mougous et al., 2006; Ballister et al., 2008). Several *hcp* encoding genes can be found in a bacterial strain, either as a part of or separated from a T6SS gene cluster. T6S has been shown to affect the virulence of *Edwardsiella tarda* in blue gourami fish and to modulate stress response in *V. anguillarum*

(Zheng & Leung, 2007; Weber et al., 2009). Many bacterial genomes contain more than one T6SS clusters and several T6SS subclasses have been identified. The subclasses contain different regulatory proteins, which indicates adaptation to different functions (Bingle et al., 2008; Boyer et al., 2009).

Gram negative bacteria can also release proteins and lipids through outer membrane vesicles, where a part of the outer membrane forms a sphere that detaches from the cell and contains periplasmic material, including virulence factors (Kuehn & Kesty, 2005).

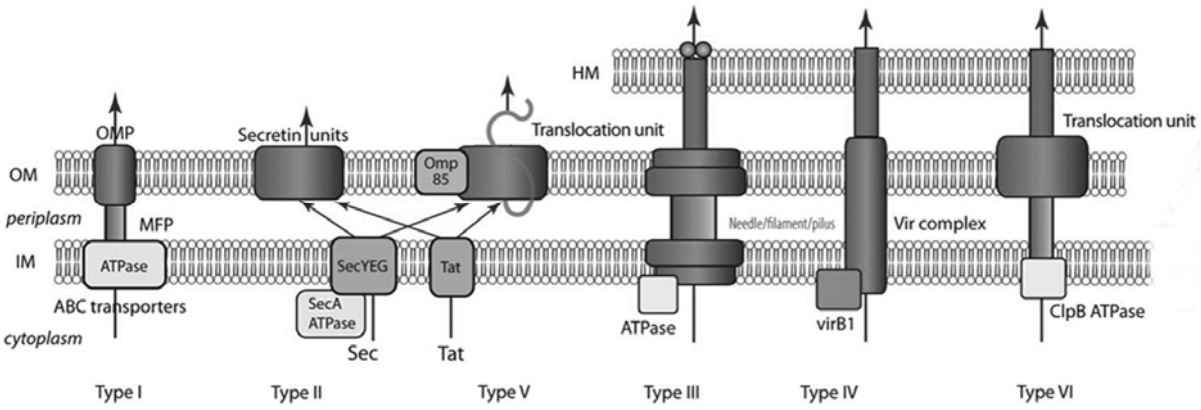

Fig. 2. Secretion systems in Gram negative bacteria.
A simplified view of each secretion system is shown. OM, outer membrane; IM, inner membrane; OMP, outer membrane protein; HM, host membrane; MFP, membrane fusion protein.
Figure modified and reprinted from BMC Microbiol., Vol 9 (Suppl 1):S2, Tseng, Tyler & Setubal, Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology (2009), with permission from BioMed Central Ltd.

Iron acquisition

Animals and bacteria require iron for metabolism and growth. Animals have evolved mechanisms of retaining iron in order to limit the availability of free iron to invading pathogens. Some pathogenic bacteria produce receptors for eukaryotic iron-binding proteins, such as transferrin, which facilitate iron acquisition. Others produce and secrete siderophores, small chelator compounds, which have such high affinity for iron that they are able to bind iron previously bound to iron-binding proteins. Specialised siderophore receptors are expressed on the bacterial cell membrane and recognize siderophores carrying bound iron. For their importance to pathogenic survival within hosts, siderophores are generally considered important virulence factors (DiRita, 2007; Casadevall et al., 2009).

Motility

Bacterial motility is driven by the action of hair like structures called flagella. Motility is often associated with bacterial virulence and enables pathogens to migrate and encounter host cells and to penetrate host cell membranes. Flagella are known to function as virulence factors, mainly at the initial phases of infection, through attachment, colonization and biofilm formation. Impairment of motility often results in attenuated virulence (Josenhans & Suerbaum, 2002; Soutourina & Bertin, 2003).

Quorum sensing

Quorum sensing (QS) is a system which bacteria use for cell-cell communication, for sensing their population density, and species present in a community. Bacteria produce small hormone-like signalling molecules, termed autoinducers, which diffuse out of the cell, and at threshold concentrations when the bacterial density has reached a certain level, bind intracellular receptors and affect a variety of regulatory processes. QS affects the expression of a wide array of molecules, including virulence factors (Waters & Bassler, 2005; Casadevall et al., 2009). QS systems have been identified in various fish pathogenic bacteria and been implicated with virulence (Milton et al., 1997; Swift et al., 1997; Bruhn et al., 2005; Ye et al., 2008; Schwenteit et al., 2011).

1.4 Peptidases

1.4.1 Definitions and activity

Proteolysis reduces proteins or peptides into smaller peptide fractions or single amino acids (aa). Peptidases play an extremely important role in biology and are essential to all kinds of living organisms. They also play important roles in many infectious diseases (Barrett, 2000; Potempa & Pike, 2005).

Peptidases are also known as proteases or proteolytic enzymes. The term protease is the most commonly used, but the term peptidase is more logical since it includes the proteolysis of both proteins and peptides. The term peptidase can be thought of as a short form of "peptide bond hydrolase", and has been used as a root for the nomenclature of all peptidase subtypes (Barrett, 2000). The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) has therefore proposed that the term

peptidase is used as the general term for all proteolytic enzymes (http://www.chem.qmul.ac.uk/iubmb/enzyme/).

1.4.2 Peptidase groups

Classification of peptidases has mainly been based on three aspects; type of proteolytic action, type of catalytic mechanism, and structural relationships between peptidases. By classification based on the type of proteolytic action, peptidases can be grouped by where in the polypeptide chain they cut. Peptidases which cut near the end of the polypeptide chain and free one or few aa are called exopeptidases. Peptidases which cut the polypeptide internally and produce a large fragment are termed endopeptidases. Classification based on the chemical nature of the catalytic site has proven valuable and currently divides peptidases into five groups. Several peptidases are still of unknown catalytic type. The five groups are; aspartic-, cysteine-, metallo-, serine-, and threonine- peptidases. Structural groupings of peptidases, which reflect the evolutionary relationships between them, are also used for classification (Barrett, 2000).

In 1993, Rawlings and Barrett suggested that peptidase classification would be based both on the catalytic mechanism of the peptidase and on structural relationships between enzymes (Rawlings & Barrett, 1993). The MEROPS database (http://merops.sanger.ac.uk) (Rawlings et al., 2010a) is based on this hierarchical classification scheme. There, peptidases are grouped into clans based on evolutionary relationships which indicate similar three-dimensional structures or conserved motifs, although they do not share significant aa similarities. Families are based on aa sequence comparison of peptidase units and each family is assigned a type example, or holotype, which all other members of the family must be statistically significantly related to (Rawlings et al., 2010b).

1.4.3 Metallopeptidases

Metallopeptidases are a diverse group of peptidases, named after the chemical nature of the catalytic site. The catalytic site contains a divalent metal ion at the active site and is usually coordinated by three aa side chain ligands. As a fourth ligand, a water molecule also coordinates with the metal ion and is essential for hydrolysis of the peptide bond. The hydrolysis is mediated by the nucleophilic attack of the water molecule on the carbonyl of the scissile bond. The metal ion is in most cases zinc, but can also be cobalt, manganese or nickel (Nagase, 2001). According to the MEROPS database, metallopeptidases are currently divided into 14 clans and 56 families.

The catalytic HEXXH metal ion binding motif is found in most metallopeptidases, where the two H´s are zinc ligands, the E has a catalytic function, and X is any amino acid. Metallopeptidases containing the HEXXH motif are grouped into clan MA, the largest metallopeptidase clan, and includes members found in all kingdoms of organisms (Nagase, 2001; Potempa et al., 2005).

1.4.4 Bacterial metallopeptidases and pathogenesis

Metallopeptidases are widespread amongst bacteria. Bacterial peptidases are predominantly extracellular and are commonly synthesised as inactive precursors whose maturation is achieved by several processing stages. Most extracellular bacterial metallopeptidases contain signal peptides and propeptides which are removed from the mature enzyme during and following secretion. Typical signal peptides are cleaved off during translocation of the prepropeptide to the periplasm in a signal peptide-dependent manner (Hase & Finkelstein, 1993; Miyoshi & Shinoda, 2000; Potempa et al., 2005). Propeptides, which are located on the N- or/and C-terminal ends of the protein, have been shown to form complexes with the mature enzyme and inhibit intracellular proteolysis and facilitate folding (Tang et al., 2003; Yeats et al., 2004; Demidyuk et al., 2008). Furthermore, C-terminal propeptides have been shown to have functions important for efficient peptidase attachment to substrates and cell membranes (Miyoshi & Shinoda, 1997).

The role of bacterial extracellular metallopeptidases in pathogenesis has been widely researched and studies providing information of their pathogenic roles have gradually accumulated. However, except in a few cases, their specific roles in pathogenesis have not yet been fully identified. Several known bacterial exotoxins are metallopeptidases, such as the neurotoxins of tetanus and botulism and the anthrax toxin lethal factor (Schmitt et al., 1999). Many peptidases are considered to have primary functions in nutrient acquisition for growth, but are believed to play direct or indirect roles in pathogenesis through aiding in the invasion and spread of the bacterium within the host, or in inhibiting the functions of the host's immune system. Bacterial extracellular peptidases often cause hemorrhagic tissue damage or necrosis through digestion of structural components of the host. They also enhance vascular permeability, which can promote spread of the bacterium, and form lesions through the activation of inflammatory mediators. Bacterial metallopeptidases can also activate exo- and enterotoxins and thereby affect pathogenesis and aid in iron acquisition. Bacterial peptidases may act specifically against the host immune system through degradation of immunoglobulins

and various components of the complement system (Harrington, 1996; Miyoshi et al., 2000; Todar, 2007; Ingmer & Brondsted, 2009).

Several extracellular metallopeptidases produced by pathogenic fish bacteria have been implicated with toxicity and virulence, although generally their specific roles are very poorly understood. These include peptidases produced by *V. anguillarum*, *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Y. ruckeri*, *Vibrio pelagius* and *Flavobacterium psychrophilum* (Cascon et al., 2000; Ostland et al., 2000; Farto et al., 2002; Fernandez et al., 2002; Denkin & Nelson, 2004; Arnadottir et al., 2009).

1.4.5 The thermolysin family

Metallopeptidases of the thermolysin family bind a single catalytic zinc ion and contain the HEXXH motif, where the third zinc ligand is an E, located 18-72 residues C-terminal to the HEXXH motif. These peptidases are also known as Glu-zincins (Adekoya & Sylte, 2009; Rawlings et al., 2010a). The family holotype, *Bacillus thermoproteolyticus* thermolysin, was one of the first metallopeptidases to be sequenced and its tertiary structure determined (Matthews et al., 1972; Titani et al., 1972). The 3D structures of vibriolysins consist of two-domains, with the active site located between the domains. The N-terminal domain carries the HEXXH motif and includes both α-helices and β-sheets. The C-terminal domain is mostly helical and carries the third zinc ligand. The HEXXH motif and the third zinc ligand are located in α-helices connected by a turn which brings them together and forms the zinc binding site (Fig. 3). Almost all identified thermolysin peptidases are bacterial enzymes, most of which are secreted. Thermolysins have also been described in fungi and plants (Adekoya et al., 2009; Rawlings et al., 2010a). Several bacterial thermolysins have been implicated with virulence and considered potential drug targets. Others have industrial applications, such as those active at extreme temperatures and pH, or have high activity in organic solvents. Thermolysins have also been used in the production process of the artificial sweetener aspartame (Adekoya et al., 2009). The vibriolysin of *Vibrio proteolyticus* has been used for removing necrotic tissue from burn wounds and cutaneous ulcers (Durham et al., 1993).

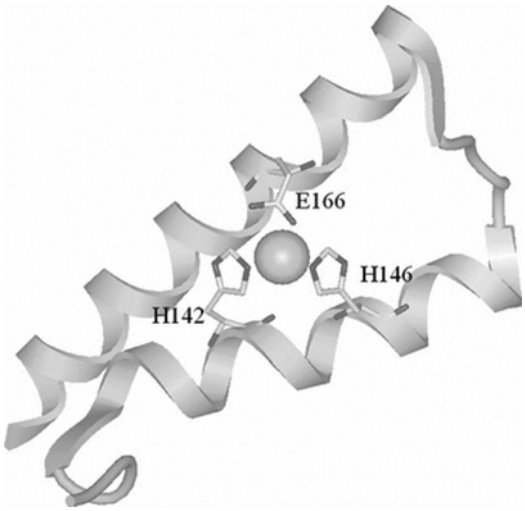

Fig. 3. Thermolysin active site.
The zinc ion and three zinc coordinating amino acids are shown.
Figure reprinted from Chemical Biology and Drug Design, Vol 73, Adekoya & Sylte, The thermolysin family (M4) of enzymes: therapeutic and biotechnological potential, 7-16 (2009), with permission from John Wiley and Sons.

1.4.6 Vibriolysins

Vibriolysins are extracellular bacterial peptidases which belong to the thermolysin peptidase family and the holotype is the *V. proteolyticus* vibriolysin. The 3D structure of a vibriolysin has not yet been determined, but homology modelling of the *Vibrio cholerae* and the Antarctic bacterium 643 vibriolysins has been performed (Adekoya et al., 2006; Lutfullah et al., 2008). Most currently known vibriolysin sequences are found in members of the genera *Vibrio*, *Shewanella* and *Pseudoalteromonas* (David et al., 1992; Rawlings et al., 2010a). The abilities of several *Shewanella* species to biodegrade organic pollutants, their energy-generating biocatalysis abilities and abilities to produce high proportions of polyunsaturated fatty acids have aroused interest in their whole genome sequencing and led to the discovery of several vibriolysin sequences (Hau & Gralnick, 2007). Other vibriolysins have not only been identified by sequencing, but have also been isolated and characterized. This is true mainly for vibriolysins produced by *Vibrio* species, several of which are pathogenic. Furthermore, vibriolysins belonging to both psychrophilic and halophilic bacteria have been described (Adekoya et al., 2006; Karbalaei-Heidari et al., 2007).

Vibriolysins produced by two human pathogens, *V. cholerae* and *Vibrio vulnificus*, have been implicated with virulence. *V. cholerae* serogroups O1 and O139 cause the water borne diarrheal disease cholera, which primarily affects the small intestine and transmits from contaminated waters and with poor sanitation (Reidl & Klose, 2002). The *V. cholerae*

hemagglutinin/protease (HA/P) vibriolysin is encoded by the *hap* gene (Hase & Finkelstein, 1991) and displays a range of potential pathogenic activities. HA/P degrades several host components such as mucin, fibronectin, and lactoferrin, has hemagglutinating activities and causes skin reaction and necrosis in a rabbit model. It can nick and thus activate the A subunit of the cholera enterotoxin and activate the El Tor cytolysin and hemolysin (Hase & Finkelstein, 1990; Nagamune et al., 1996). HA/P has also been shown to affect intercellular tight junctions and the F-actin cytoskeleton in a canine kidney epithelial cell line (Wu et al., 1998; Wu et al., 2000). However, there is no direct evidence of the involvement of HA/P in the pathogenesis of cholera, although it has been proposed that it may play a role in attachment, penetration and detachment of the intestine. A HA/P deletion mutant was fully virulent in a rabbit model (Finkelstein et al., 1992; Benitez et al., 1997; Silva et al., 2003).

V. vulnificus causes infections in humans which often occur after consumption of raw seafood or through open wounds from contact with infected water. Immune compromised individuals are especially vulnerable and can develop invasive septicaemia which can be fatal (Chakraborty et al., 1997). *V. vulnificus* has also been reported as an eel (*Anguilla japonica*) pathogen (Tison et al., 1982). Several potential virulence factors have been associated with *V. vulnificus* virulence, but the Vvp vibriolysin (also known as VvpE or vEP) has been the most extensively studied. Vvp has been proposed to play a role in pathogenesis through degradation of tissue components and causes tissue necrosis during wound infection (Kothary & Kreger, 1985; Chuang et al., 1997). The peptidase is toxic for mice and causes extensive dermonecrosis (Kothary & Kreger, 1987). Vvp has also been shown to enhance vascular permeability through generation of chemical mediators and induces severe hemorrhagic damage through digestion of the vascular basement membrane (Miyoshi et al., 1987; Miyoshi et al., 1998). It is therefore believed that the Vvp vibriolysin plays a role in edema formation, in mediating skin damage, and bacterial invasion during infection. However, Vvp mutants retained their virulence and abilities to cause tissue damage in a mouse model, indicating that the importance of Vvp in *V. vulnificus* pathogenicity may be overestimated, or that inactivation of Vvp alone may be masked by other compensatory virulence factors (Jeong et al., 2000; Shao & Hor, 2000). Recent studies indicate that Vvp may have an important role in surface adherence and colonization by facilitating swarming, and in mucosal invasion by degradation of IgA and lactoferrin (Kim et al., 2007). In eels, Vvp is not a major toxic factor or essential in producing internal pathology and lesions (Valiente et al., 2008b). It has, however, been shown to positively affect bacterial chemoattraction and attachment to eel

mucus and gills and virulence of a Vvp negative mutant was reduced by an immersion challenge (Valiente et al., 2008a).

V. anguillarum is the causative agent of vibriosis, a systemic disease of both wild and cultured marine fish, characterized by hemorrhagic septicaemia (Austin et al., 2007). *V. anguillarum* produces a vibriolysin termed EmpA (Norqvist et al., 1990) which has been implicated with virulence. EmpA negative mutants have been demonstrated to have reduced virulence following challenge experiments in salmonids (Milton et al., 1992). The peptidase is strongly induced by gastrointestinal mucus, suggesting a role for EmpA during colonization of fish intestine (Denkin & Nelson, 1999). A recombinantly expressed EmpA vibriolysin was cytotoxic to a flounder gill cell line and lethal to turbot following intraperitoneal (i.p.) injections at concentrations down to 7.6 µg protein/g fish, causing hemorrhages and necrosis at the site of injection and in the peritoneal cavity (Yang et al., 2007b). These results are thought to indicate that EmpA may promote bacterial invasion trough tissue damage, by directly degrading host tissue components. An EmpA toxoid based DNA vaccine has been demonstrated to protect Japanese flounder (*Paralichthys olivaceus* Temminck et Schlegel) against *V. anguillarum* infection (Yang et al., 2009).

Other vibriolysins which have been proposed to play a role in bacterial pathogenesis include the VFP vibriolysin of the opportunistic human pathogen *Vibrio fluvialis*, showing hemagglutinating, permeability-enhancing and hemorrhagic activities and degrades casein and elastin (Miyoshi et al., 2002). Also, the VtpA vibriolysin of the bivalve pathogen *Vibrio tubiashii* has been shown to have toxic effects on Pacific oyster larvae (*Crassostrea gigas* Thunberg) (Hasegawa et al., 2008).

Maximum expression and/or production of vibriolysins are usually reported in dense bacterial cultures and seem to be growth phase dependent. It has been reported that the HA/P, Vvp and EmpA vibriolysins are QS regulated (Croxatto et al., 2002; Jeong et al., 2003; Kawase et al., 2004; Silva & Benitez, 2004). Vibriolysins such as HA/P, Vvp and EmpA appear to be translocated to the periplasm through the Sec pathway and out of the cell through the T2SS (Sandkvist et al., 1997; Zhang et al., 2006; Park et al., 2008) . Vibriolysin peptidases mature in several processing steps, usually involving both N-terminal and C-terminal processing. Autocatalytic digestion of C-terminal propeptides has been suggested (Miyoshi et al., 2002). Recently, an extracellular peptidase, Epp, of *V. anguillarum* has been shown to be responsible for extracellular processing of EmpA through digestion of a 10 kDa C-terminal polypeptide (Varina et al., 2008).

1.5 Winter ulcer disease

1.5.1 Disease occurrence

Winter ulcer disease, caused by the pathogenic bacterium *M. viscosa*, affects farmed fish in the North Atlantic. Disease outbreaks are reported mainly in juvenile and adult fish during the winter months when temperatures go below 8 °C, in sea-cages or land facilities that take in seawater. The disease was originally identified in Norway in the 1980s in farmed Atlantic salmon and in 1990 the disease had become widespread (Lunder, 1992; Lunder et al., 1995; Benediktsdottir et al., 1998a).

Winter ulcer disease has mainly affected salmonids in Norway and Iceland (Lunder et al., 1995; Benediktsdottir et al., 1998a), but also in Scotland (Bruno et al., 1998), the Faroe Islands, Canada (Whitman et al., 2000) and Ireland (ICES mariculture committee, 2005). Outbreaks occur most commonly in Atlantic salmon and rainbow trout, (Benediktsdottir et al., 1998a; Lillehaug et al., 2003), but infections have also been reported in Atlantic cod (Colquhoun et al., 2004), seawater reared brown trout (*Salmo trutta* L.) (Heidarsdottir et al., 2008), and in captive wild-caught European plaice (*Pleuronectes platessa* L.) (Lunder et al., 2000). Isolation of *M. viscosa* from wild fish has been reported, where a low virulent isolate was cultured from the gills of a healthy lumpsucker (*Cyclopterus lumpus* L.) (Benediktsdottir et al., 2000). Experimental infections have shown that turbot (*Psetta maxima* L.) is susceptible to *M. viscosa*, but that halibut is more resistant (Table 3) (Bjornsdottir et al., 2004; Gudmundsdottir et al., 2006). Experimental infections of cod eggs and larvae with *M. viscosa* did not have negative effects on survival (Sandlund & Bergh, 2008).

Mortalities due to winter ulcer outbreaks are usually not high, ranging from 0-10%, although more severe mortalities have been reported. Infected fish may survive for long periods of time, even though open ulcers cover large areas of their body surface. When water temperatures rise above 8 °C in spring, surviving fish usually recover from the infection. Mortalities, as well as downgrading of affected ulcerated fish at slaughtering, cause significant economical losses to the farming industry. The disease is also an important animal welfare problem (Lunder, 1992; Salte et al., 1994; Lunder et al., 1995; Bruno et al., 1998).

In Iceland, skin lesions of cultured salmon caused by vibrio infections became a problem in the late 1980s, and later also in rainbow trout (Benediktsdottir et al., 1998a). Following the introduction of an effective furunculosis vaccine in 1992, a sharp increase in *M. viscosa* infections was detected in farmed salmonids (Fig. 1). All farmed salmon in Iceland

are vaccinated against winter ulcer disease before transport to seawater. However, it has proven difficult to eradicate the disease once it has become established in a farm, and infections are reported following stress or infections. Other fish farms have remained totally free of winter ulcer disease outbreaks. Following a reduction in salmon farming in recent years, winter ulcer disease has not been a problem in Icelandic aquaculture (G. Jonsson, written communication, 2010).

Winter ulcers have been recognized as the most important bacterial disease in Norwegian fish farming for several years, causing huge annual losses to the salmon farming industry (Lillehaug et al., 2003; National Veterinary Institute Norway, 2007). However, in 2009 fewer winter ulcer disease outbreaks were reported than in previous years, while infections due to *F. psychrophilum* increased (National Veterinary Institute Norway, 2010).

1.5.2 Disease symptoms and pathology

Fish affected with winter ulcer disease may develop external lesions or ulcers, often appearing along the side of the fish (Fig. 4). Ulcers initially appear as small epidermal lesions which gradually increase and can cover considerable areas of the body surface (Salte et al., 1994; Lunder et al., 1995; Bruno et al., 1998). A large proportion of the infected population may become affected by ulcers and up to 50% of fish have been reported to show extensive ulceration at slaughter (Lunder, 1992).

Lunder et al. (1995) categorised skin lesions of salmon affected with winter ulcer disease as acute or subacute; superficial lesions extending to the stratum compactum, as chronic; lesions extending beyond the stratum compactum down to the muscle, and regenerative; lesions showing granulation tissue covered with a scale-free epidermis.

External disease signs of fish with winter ulcer disease include gill pallor, subcorneal hemorrhages of the eye, exopthalmia, fin rot, and petechial hemorrhaging of the skin. Internal gross pathological changes include diffuse or petechial hemorrhages, tissue necrosis, ascites and splenomegaly. Microscopically observed signs include hemorrhages, infiltration of inflammatory cells, fibroblast proliferation and necrosis of muscle cells in acute or chronic ulcers. Bacteria are frequently detected at the edge of ulcers or invading the muscle tissue. Congestion and necrosis of the liver and spleen, necrosis of the glomeruli and tubule degeneration in kidney and epithelial sloughing and hyperplastic lamella in the gills are also frequently detected (Lunder et al., 1995; Benediktsdottir et al., 1998a; Bruno et al., 1998; Bjornsdottir et al., 2004; Gudmundsdottir et al., 2006).

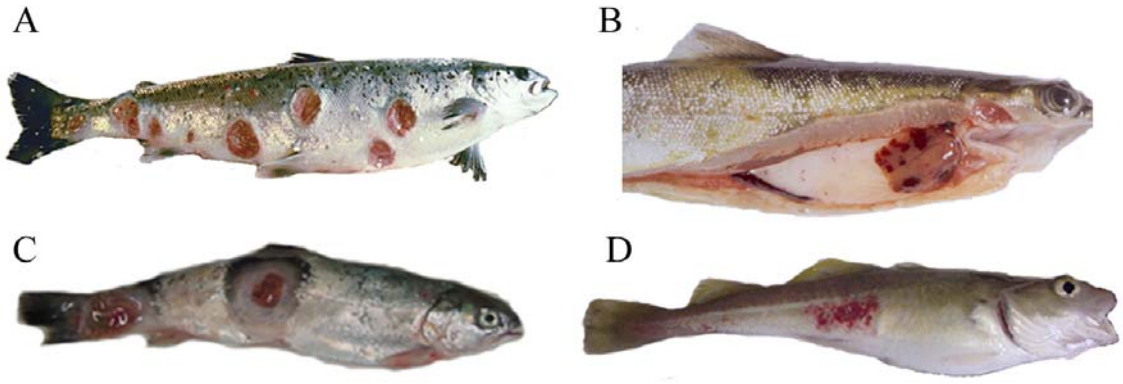

Fig. 4. Fish with winter ulcer disease.
A) Farmed Atlantic salmon showing extensive ulceration. Photo courtesy K.J. Heidarsdottir. B) Internal symptoms, including petechial hemorrhages, in experimentally infected salmon. Photo courtesy B.K. Gudmundsdottir. C) Farmed rainbow trout with ulcers. Photo courtesy G. Jonsson. D) Experimentally infected Atlantic cod showing skin ulcers. Photo courtesy B.K. Gudmundsdottir.

Pathological signs of salmonids and turbot with winter ulcer disease are comparable. Cod shows host response to *M. viscosa* infection resulting in granuloma formation in infected tissues, a known response of cod to an infection with Gram-negative bacteria (Lunder et al., 1995; Benediktsdottir et al., 1998a; Bruno et al., 1998; Greger & Goodrich, 1999; Bjornsdottir et al., 2004; Gudmundsdottir et al., 2006).

Observations of different presentations of winter ulcer disease have been reported, where high mortalities and systemic infections without ulcerations are documented, along with septicaemia or toxemia, and internal pathological changes as described above. Reports of fish with external ulcers, but showing minor internal pathological changes and low mortalities have also been made (Tunsjo, 2009).

1.5.3 Theories on the cause of winter ulcers

Different theories regarding the cause of winter ulcers have been suggested. A non-infectious aetiology was proposed by Salte et al. (1994) which speculated that ulcers were the result of a mechanical disruption of vesicles, formed from vascular thrombosis of dermal vessels, and discussed a potential role for dietary iron in the thrombotic process. They also suggested that bacteria commonly isolated from ulcers might play a role in tissue damage, but that they were not likely primary pathogens.

Infectious aetiology was strongly indicated when two dominating bacterial phena were reported being commonly isolated from salmonids during winter ulcer disease outbreaks in Norway and Iceland (Lunder et al., 1995; Benediktsdottir et al., 1998a). These phena were

later identified as *Vibrio viscosus* and *Vibrio wodanis* (now *M. viscosa* and *Aliivibrio wodanis*) (Benediktsdottir et al., 2000; Lunder et al., 2000; Urbanczyk et al., 2007). Furthermore, infection challenges showed that the phenon composing *M. viscosa* caused mortalities in salmon and induced a condition similar to winter ulcer disease (Lunder et al., 1995; Benediktsdottir et al., 1998a).

A. wodanis is commonly isolated during winter ulcer outbreaks and may even be isolated in pure cultures. Its role in winter ulcer disease has been hypothesised and discussed. Several infection studies have, however, confirmed that *M. viscosa* is the infectious agent, and that *A. wodanis* is non-pathogenic (Lunder et al., 1995; Benediktsdottir et al., 1998a; Bruno et al., 1998; Greger et al., 1999; Whitman et al., 2000; Bjornsdottir et al., 2004; Gudmundsdottir et al., 2006). An intramuscular (i.m.) infection using *A. wodanis* (up to 5 x 10^{11} colony forming units (CFU)/fish) further showed that the bacterium did not cause ulceration or produce pathological signs (Lunder et al., 1995). Therefore, the significance of *A. wodanis* for the development of winter ulcer disease is uncertain, if any.

1.6 *Moritella viscosa*

1.6.1 Classification

M. viscosa was originally classified as *Vibrio viscosus*, but was later transferred to the *Moritella* genus on the basis of 16S rRNA sequencing data (Benediktsdottir et al., 2000), along with the type strain *M. marina* (previously *V. marinus*) (Urakawa et al., 1998). The *Moritella* genus is a member of the *Moritellaceae* family (Ivanova et al., 2004), which currently includes two genera, *Moritella* and *Paramoritella*. The *Paramoritella* genus includes just one species, *Paramoritella alkaliphila*, of marine origin (Hosoya et al., 2009). The *Moritella* genus currently includes seven defined species; the type species *M. marina*, *M. yayanosii*, *M. japonica*, *M. profunda*, *M. abyssi*, *M. dasanensis*, most of which are strictly psychrophilic and barophilic species isolated from deep-sea or marine sediments (Colwell & Morita, 1964; Nogi et al., 1998; Nogi & Kato, 1999; Xu et al., 2003; Kim et al., 2008), and *M. viscosa*, the only currently known pathogenic member of the family. Additionally, several *Moritella* strains have been isolated which are not yet assigned to a species, including the fully sequenced isolate *Moritella* sp. PE36 (Xu et al., 2000; Saito & Nakayama, 2004).

1.6.2 Identification and culturing

M. viscosa is a Gram negative, psychrophilic, facultative anaerobe. The cells are none spore-forming pleomorphic rods which are motile, with a single polar flagellum (Fig. 5A). Colonies grown on solid media are grey, translucent and round, adhere to the agar surface, forming long threads when removed by an inoculation loop (Fig. 5B). The species name, *viscosa*, meaning viscous, is derived from these adherent thread-forming properties. On blood agar, β hemolysis can easily be detected underneath and/or surrounding colonies. Growth occurs at temperatures between 4 and 21 °C, although growth at 25 °C has also been reported. Sodium chloride (NaCl, 1 – 4 %) is required for growth (Benediktsdottir et al., 2000; Lunder et al., 2000).

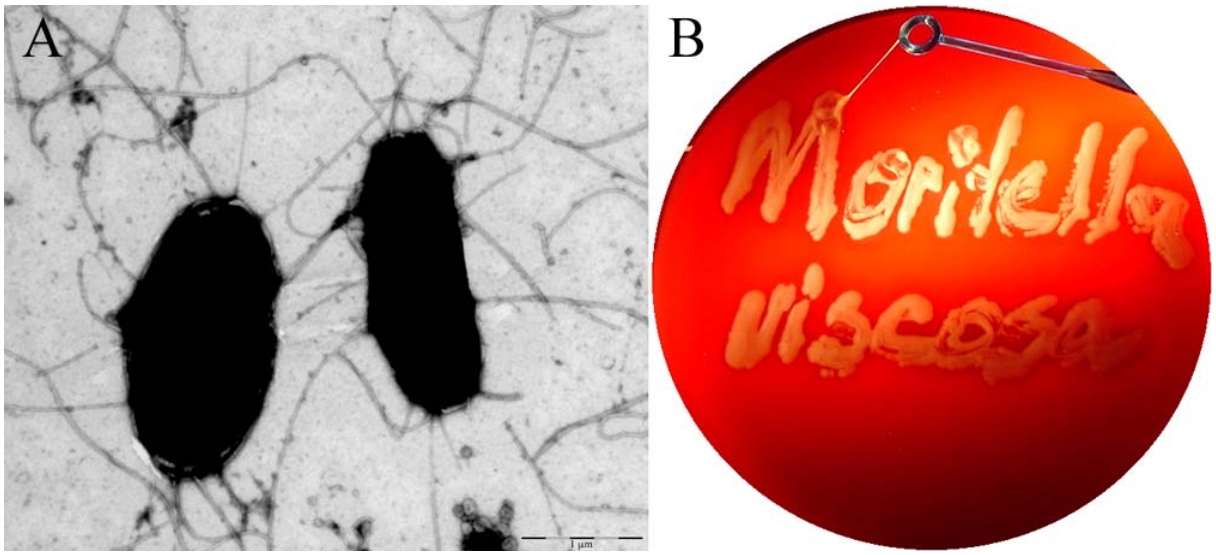

Fig. 5. *M. viscosa* cells and viscosity.
A) Transmission electron microscope (TEM) photo of *M. viscosa* cells showing flagella. Photo courtesy H.C. Winther-Larsen. B) *M. viscosa* viscosity on blood agar. Photo courtesy B.K. Gudmundsdottir.

M. viscosa produces acid from glucose and is oxidase- and catalase-positive. Most strains produce lysine decarboxylase but all are negative in arginine dihydrolase and ornithine decarboxylase tests. It degrades gelatin, casein, DNA, Tween, urea, lecithin, and starch. The bacterium is partially susceptible to O/129 vibriostatic agent and is relatively inert in standard biochemical diagnosis test (Bruno et al., 1998; Benediktsdottir et al., 2000; Lunder et al., 2000).

Standard culturing for isolation of *M. viscosa* from tissue samples is on blood agar plates supplemented with NaCl and incubation at 15 °C for 2-3 days. *M. viscosa* can be grown in conventional media supplemented with NaCl, but is very sensitive to lysis. Cultivation

temperature and NaCl concentration strongly influence cell proliferation and stability. Culturing at 4 °C compared to 15 °C increases cell stability, although highest growth rates are measured at 15 °C. NaCl concentration also affects growth and highest cell yields are obtained at 3-4 % NaCl. Addition of minerals further delays and reduces lysis. Cultivation at high temperatures and low salt concentrations upregulates the expression of several stress or heat-shock related proteins (Benediktsdottir & Heidarsdottir, 2007; Tunsjo et al., 2007).

As previously mentioned, *A. wodanis* is commonly isolated from winter ulcer disease outbreaks, either together with *M. viscosa*, in mixed cultures, or even as a pure culture (Lunder et al., 1995; Benediktsdottir et al., 1998a; Whitman et al., 2000). The common isolation of *A. wodanis* from winter ulcer disease outbreaks may be the result of inhibition of growth or overgrowth over the more slowly growing *M. viscosa* (Lunder et al., 1995; Heidarsdottir & Benediktsdottir, 2001). Therefore, isolation and diagnosis of *M. viscosa* on blood agar plates is often considered unreliable. Addition of 0.5 µg/ml of vibriostat O/129 into the blood agar medium inhibits *A. wodanis* but not *M. viscosa* growth (Heidarsdottir et al., 2001). Recently, a real-time polymerase chain reaction (PCR) was developed for identification of *M. viscosa*, depending on specific amplification of the *tonB* gene, and was found to be more sensitive than isolation by culturing (Grove et al., 2008).

1.6.3 Genotypes and serotypes

M. viscosa has been shown to be a rather homogenous species, a trait which may indicate close adaptation to the host and clinical importance (Benediktsdottir et al., 2000; Lunder et al., 2000). However, both phenotypic and genotypic analysis of *M. viscosa* isolates has revealed some strain heterogeneity. Four *M. viscosa* genotypes have been identified by AFLP DNA fingerprinting of strains from Norway and Iceland. Norwegian isolates, South West Iceland isolates and North Iceland isolates each formed one genotype and the fourth contained a low-virulent lumpsucker isolate. This indicates a common clonal origin of strains within certain geographical areas. Isolates within each AFLP group were biochemically homogenous (Benediktsdottir et al., 2000).

Different *M. viscosa* serotypes have also been identified, using polyclonal sera against whole cell samples of isolates originating from different geographical locations and host species. Serotypes partially reflected the fish species from which they were isolated, but also the country of origin. It was also speculated that the significant cross reaction of the polyclonal sera masked the presence of more serotypes. The major specific antigens of *M.*

viscosa whole cell samples were lipooligosaccharides (LOS) and a 17/19 kDa protein antigen (Heidarsdottir et al., 2008).

1.6.4 Potential reservoirs and infection

Indications of a deep sea reservoir of *M. viscosa* exist, as winter ulcer disease outbreaks have been reported following a change in depth of seawater intake from 20 meters to 90 meters (Lunder, 1992). A potential risk of *M. viscosa* infections from seawater taken from increased depths may perhaps be expected, considering the origins of other members of the *Moritella* genus. Horizontal transmission of winter ulcer disease has been demonstrated (Lunder et al., 1995), but there are no reports of vertical transmission. Salmon lice are a possible transmission vector for *M. viscosa*, as the bacterium has been isolated from salmon lice removed from salmon infected with winter ulcer disease (Lunder et al., 1995).

The gills have been proposed as a possible entry site, through real-time PCR detection of *M. viscosa* DNA in bath infected salmon. The presence of *M. viscosa* DNA was first detected in gills, and at a later time also in muscle and other tissues, showing bacterial spread and systemic infection coinciding with appearance of skin ulcers (Lovoll et al., 2009). Immunohistochemical staining of infected tissues and cell-culture model infections have indicated that *M. viscosa* does not invade host cells (Lovoll et al., 2009; Tunsjo et al., 2009). Phagocytosis of *M. viscosa* by mononuclear cells has been indicated by immunohistochemical staining (Lovoll et al., 2009).

1.6.5 Virulence and virulence factors

Experimental infections have shown that *M. viscosa* can infect and cause mortalities in salmon in concentrations as low as 14 CFU/fish (Lunder et al., 1995). *M. viscosa* strains do not seem to be host-specific, as an isolate originating from salmon in Norway has been shown to infect different fish species (Table 3). The discovery of an *M. viscosa* isolate of reduced virulence from gills of a healthy lumpsucker (Benediktsdottir et al., 2000) indicates the existence of low- or non-virulent strains in the environment.

Table 3. LD_{50} of *M. viscosa* (isolate 3) in different fish species

Fish species	LD_{50} (CFU/ml)		Reference
	i.m.	i.p.	
Salmon	2.0×10^4	8.0×10^5	B.K. Gudmundsdottir, oral communication, 2010
Cod	1.5×10^5	4.0×10^5	Gudmundsdottir et al. 2006
Halibut	1.7×10^6	6.5×10^7	Gudmundsdottir et al. 2006
Turbot	$< 1.0 \times 10^5$	3.2×10^5	Bjornsdottir et al. 2004

The isolate was grown on agar plates at 15 °C for 19 h prior to injection, and infection experiments were performed at 8-9 °C. LD_{50}, 50 percent lethal dose; CFU, colony forming units; i.m. intramuscular; i.p., intraperitoneal; salmon, *Salmo salar* L.; cod, *Gadus morhua* L.; halibut, *Hippoglossus hippoglossus* L.; turbot, *Psetta maxima* L.

The mechanisms behind *M. viscosa* virulence have not been extensively studied. Motility has been shown to be affected by temperature, and is increased at 4 °C compared to higher temperatures (Tunsjo et al., 2007), a property which may be related to bacterial virulence. However, temperature did not affect the ability of *M. viscosa* to infect a chinook salmon embryo (CHSE) cell line model, suggesting that the bacteria were fully virulent at the temperature range tested (4-15 °C) (Tunsjo et al., 2009). *M. viscosa* adherence to various salmon mucosal surfaces and to fish cell lines has been demonstrated (Knudsen et al., 1999; Tunsjo et al., 2009). Adherence to the CHSE fish-cell line was increased at 4 °C compared to higher temperatures, and a higher expression of a lateral flagellin was also detected, which may affect adherence capacity (Tunsjo et al., 2009). The same study also described the morphological changes of CHSE cells following infection with *M. viscosa*. Initially, cell-cell contact was lost and then cells retracted, rounded up and lost their attachment abilities. Disruption of F-actin microfilaments was reported and actin aggregation seen at cell edges. CHSE cell membrane disruption was also demonstrated (Tunsjo et al., 2009).

The temperature dependent outbreaks of winter ulcer disease may reflect impaired immune responses and reparative abilities of fish at low temperatures. Also, the low optimum temperatures for *M. viscosa* growth, cell stability, motility and adherence to host cells may contribute to the temperature dependence of the disease.

M. viscosa isolates have been reported to produce several extracellular products (ECP) that constitute possible virulence factors (Lunder et al., 2000). The ECP of *M. viscosa* are lethal to salmon and turbot, and cause internal disease signs similar to those detected in infected fish (Benediktsdottir & Helgason, 1997; Bjornsdottir, 2004). The ECP have cytotoxic and hemolytic activities and the toxic and lethal activities are heat sensitive, indicating that the toxic factors are proteinaceous (Benediktsdottir et al., 1997; Benediktsdottir et al., 1998b; Tunsjo et al., 2009). The ECP have also been shown to cause rapid pore formation and lysis

of CHSE cells and to severely affect cell morphology, independent of *M. viscosa* growth temperature. The same study further showed that temperature did not have a big effect on the *M. viscosa* extracellular proteome. Sixteen extracellular protein spots were identified using tandem mass (MS/MS) analysis and may have virulence related activities (Tunsjo et al., 2009).

Knowledge about the virulence factors produced by *M. viscosa* is scarce. Two *M. viscosa* isolates have been sequenced. The draft genome of the type strain, NCIMB 13584 has been automatically assembled and annotated (Grove et al., 2008), and the genome of strain 26 (06/09/139) has been sequenced and is currently being annotated (http://www.sanger.ac.uk/resources/downloads/bacteria/moritella-viscosa.html). Genome sequence information will provide new information and insight into *M. viscosa* virulence.

The viscous properties of *M. viscosa* may be due to the production of a surface polysaccharide layer, such as exopolysaccharide or capsule polysaccharide layers, which may protect the bacterial cells or have a function in attachment. Furthermore, *M. viscosa* does not seem to produce typical smooth LPS with O-polysaccharide chains, but a LOS containing a core-lipid and a short polysaccharide chain on the membrane surface (Heidarsdottir et al., 2008). Genetic systems encoding both polar and lateral flagella have been identified, as well as type IV pili systems (Tunsjo, 2009). No molecular studies have been published so far on *M. viscosa* secretion systems, but putative T1, T2 and T6 secretion systems have been identified in the genome and structures resembling membrane vesicles have been detected by electron microscopy (Tunsjo, 2009). Production of acylated homoserine lactone QS signals has so far not been detected in *M. viscosa* (Bruhn et al., 2005; Johansen et al., 2007; B.K. Gudmundsdottir, oral communication, 2010).

In a recent study, the expression profiles of 12 *M. viscosa* putative virulence genes during cell culture infection were examined. Possible repeats in toxin and cytotoxic necrotizing factor encoding genes were upregulated at the same time as cell rounding was detected, and a putative hemolysin gene was upregulated at later stages, during cell lysis. *In vivo* upregulation of the predicted cytotoxic necrotizing factor encoding gene and a lateral flagellin gene was detected in ulcers compared to kidney tissues of bath infected salmon, indicating a possible role in tissue damage (Tunsjo, 2009).

1.6.6 Treatment and vaccination

Antibiotic treatment using medicated feed is a standard procedure to treat *M. viscosa* infections in farmed fish. It has been reported that fish affected with winter ulcer disease do not feed properly and therefore do not receive adequate antibiotic treatment (Coyne et al., 2004; Coyne et al., 2006). Effective vaccines are therefore important in the fight against winter ulcer disease.

Vaccination against winter ulcer disease started in the beginning of the 1990s. Commercially available vaccines against *M. viscosa* are multivalent injection vaccines developed for vaccination of salmonids. These are oil-adjuvanted vaccines, based on formalin-inactivated bacterial cultures and are extensively used in salmonid farming (Hastein et al., 2005; Gudmundsdottir & Bjornsdottir, 2007). Vaccination experiments conducted in Atlantic salmon and rainbow trout demonstrated significant short-term and long-term protection, and cross protection between Icelandic and Norwegian strains (Greger et al., 1999). Experimental vaccinations of cod and turbot, using a commercial multivalent salmon vaccine containing *M. viscosa*, did not protect the two fish species against challenge infections (Bjornsdottir et al., 2004; Gudmundsdottir et al., 2007), demonstrating that winter ulcer vaccines developed for use in salmonids may not confer protection in other fish species.

According to a survey answered by aquaculture authorities in Norway and Iceland the effect of vaccination of salmonids against winter ulcer disease was reported to be good or acceptable. *M. viscosa* infections do, however, occur in vaccinated fish, which develop skin ulcers, and although vaccine efficiency has improved, the vaccines are not considered to provide adequate protection (Hastein et al., 2005; Gudmundsdottir et al., 2007; National Veterinary Institute Norway 2007; Lovoll et al., 2009).

Currently, winter ulcer vaccines contain only one *M. viscosa* isolate. Considering reports of different serotypes, addition of more serotypes might be important to improve vaccine efficiency. The LOS and 17/19 kDa antigen appear to be the main protective antigens, as sera of vaccinated salmon groups showing good or fair protection reacted against these antigens (Heidarsdottir et al., 2008). The 17/19 kDa protein has been characterized as an outer membrane protein (MvOmp1), showing aa similarities with *Escherichia coli* OmpA, and has been proposed to be a candidate for subunit vaccine against winter ulcer disease (Bjornsson, 2010).

2 AIMS OF THE STUDY

Winter ulcer disease, caused by *Moritella viscosa*, has had significant economic effects on the aquaculture industry, and is also an important animal welfare issue. Improved understanding of the causative agent, its virulence mechanisms, and host-pathogen interactions is therefore important in order to minimise the risk of disease outbreaks, to develop effective treatments, and to improve vaccination strategies.

The main objectives of this work were to study the virulence mechanisms of *M. viscosa*, with emphasis on secreted factors, and to look at interactions between the bacterium and its host.

The specific aims were to:

- Evaluate the virulence properties of *M. viscosa* extracellular products (Paper I).
- Isolate and partially characterize an extracellular peptidase of *M. viscosa* and to study its role in virulence (Paper II and mutant construction).
- Study the effects of selected *M. viscosa* antigens on cytotoxicity and expression of pro-inflammatory genes in a salmon cell line (Paper III).
- Identify type VI secretion systems in *M. viscosa* (Paper IV).

3 MATERIALS AND METHODS

3.1 Experimental fish and injections

Healthy unvaccinated salmon with no history of disease were used throughout the study (Papers I and II). The salmon originated from commercial fish farms and was kept in fresh water in 150 L tanks or 10 L buckets at 7-8 °C. Oxygen concentration and temperature were checked daily and the fish fed commercial pellets. Prior to handling, all fish were anaesthetized using Tricaine Methanesulfonate (MS222, 50 mg/L, Pharmaq) and for identification the fish were spot marked using Alcian blue (Sigma).

Virulence of nine *M. viscosa* isolates was evaluated by calculating the LD_{50} (Paper I). Bacterial cells were dissolved in phosphate buffered saline (PBS, Sigma) containing 0.1% peptone (Difco) and 1.5% NaCl (PBS-PNaCl). Suspensions were spread onto BHI-NaCl agar plates and cultured at 15 °C for 19 h. Bacteria were washed off the agar with PBS-PNaCl, and washed using the same buffer. Fish were i.p. injected with 0.1 ml of bacterial dilutions and monitored for 21 days. Bacterial counts were determined by plating and calculation of CFU.

Virulence of ECPs of 22 *M. viscosa* isolates and 3 other bacterial species was tested in salmon (Paper I). Two fish were i.p. injected with 0.1 ml of each ECP and monitored for up to five days. The ECPs of five isolates was further diluted 1:2, 1:4 and 1:8 in PBS-PNaCl and 0.1 ml of each dilution injected into three fish, which were monitored for three days. The virulence of differently produced ECPs of a single isolate (isolate 6) was compared in the same way. Control fish were injected with PBS-PNaCl.

Toxicity testing of *M. viscosa* ECP and isolated *M. viscosa* extracellular peptidase 1 (MvP1) was performed in salmon by i.m. and i.p. injections (Paper II). Experimental fish were injected with 0.1 ml ECP containing 2 µg protein/g fish (70 units caseinase activity) or 0.2 ml isolated MvP1 at concentrations up to 0.22 µg protein/g fish (65 units caseinase activity). Control fish were injected with 50 mM Na-phosphate (pH 7.0) containing 15 mM NaCl (SD-200 buffer, used in isolation of MvP1). Two to six fish were included in each group and monitored for up to nine days post injection.

Moribund or dead fish were collected regularly and tissue samples from head kidney streaked onto blood agar plates (blood agar base, Life Technologies, supplemented with 5%

horse blood) with 2% NaCl (BA-NaCl) to confirm the presence or absence of bacteria. Necropsy was performed on infected and injected fish and gross pathology recorded.

3.2 Cultivation of bacteria and identification of bacterial products

3.2.1 Bacterial isolates and cultivation

All *M. viscosa* isolates used in this study have been given a number and are hereafter referred to by those numbers. The *M. viscosa* isolates are listed in Table 4, as well as three other bacterial species included in the study. All isolates have been identified by 16S rRNA sequencing and/or by biochemical and phenotypical methods.

Bacterial isolates were stored at -80 °C in Brain-Heart infusion broth (BHI, BD) containing 1.5% NaCl (BHI-NaCl) and 15% glycerol. All virulent *M. viscosa* isolates were passaged in Atlantic salmon by i.p. injection and re-isolation prior to use. Bacteria were streaked onto BA-NaCl plates and incubated at 4-15 °C for up to four days. Broth cultures were performed in BHI broth containing 1.5-3.0% NaCl with shaking (200 rpm), at 4-15 °C for two to five days.

Growth curves of three *M. viscosa* isolates (1, 6 and 17) were determined (Papers I and II). The growth curve of each isolate was determined by comparing three identical broth cultures and growth estimated by plating 10-fold serial dilutions and counting CFU/ml. Absorbance of cultures was measured at 600 nm.

3.2.2 Antibiotic resistance

The resistance of *M. viscosa* (isolate 6) to several antibiotics was tested on BHI-NaCl agar at 15 °C, in order to identify antibiotics that could be used for selection of the bacterium during the construction of an MvP1 negative mutant. The tests were performed using the Kirby Bauer disc diffusion assay or by mixing antibiotics into the cooled agar (50 °C) just before pouring it into plates. The antibodies tested were: chephalotin (30 µg), ampicillin (33 µg), vibriostat O129 (150 µg), oxolinic acid (2 µg), tetracycline (30 µg), oxytetracycline (30 µg), sulphamethoxazole/trimethoprim (25 µg), trimethoprim (5 µg), sulphonamides (300 µg), furazolidone (100 µg), amoxycillin/clavulanic acid (30 µg), novobiocin (5 µg),

chloramphenicol (17 µg/ml) and kanamycin (5 – 100 µg/ml), and were purchased from Sigma or Oxoid.

Table 4. *M. viscosa* isolates and related bacterial strains used in the study

Number	Designation	Species	Origin	Place of isolation
1	NCIMB 13584^T	*Moritella viscosa*	*S. salar*	Norway
2	LFI 5006	"	"	Norway
3	F288/95	"	"	Norway
4	NVI 4731	"	"	Norway
5	NVI 5433	"	"	Norway
26	06/09/139	"	"	Norway
6	K58	"	"	SW-Iceland
7	K56	"	"	N-Iceland
8	F-3-04	"	"	E-Iceland
9	F-6-05	"	"	E-Iceland
10	990129-1/3B	"	"	Faroe Islands
11	990217-1/1A	"	"	Faroe Islands
12	MT 2528	"	"	Scotland
13	MT 2858	"	"	Scotland
14	Vvi-7	"	"	Canada
15	Vvi-11	"	"	Canada
16	NVI 5450	"	*O. mykiss*	Norway
17	NVI 4917	"	"	Norway
18	NVI 5168	"	"	Norway
19	F162/01	"	"	Iceland
20	NVI 5482	"	*G. morhua*	Norway
21	NVI 5204	"	"	Norway
22	F57	"	*C. lumpus*	Iceland
23	NCIMB 1144	*Moritella marina*	seawater/sediment	Pacific Ocean
24	JCM 10249	*Moritella japonica*	seawater/sediment	Japan
25	88/441^T	*Aliivibrio wodanis*	*S. salar*	Norway

NCIMB, The National Collection of Industrial, Marine and Food Bacteria, Aberdeen, UK; JCM, Japan Collection of Microorganisms, Saitama, Japan. Isolates 2, 4, 5, 10 - 18, 20, 21, 25 were provided by Dr. Duncan Colquhoun (National Veterinary Institute, Norway), isolate 26 was provided by Christian Karlsen (University of Tromso, Norway) and other isolates came from the Institute for Experimental Pathology, University of Iceland, Keldur. *S. salar*, *Salmo salar*; *O. mykiss*, *Oncorhynchus mykiss*; *G. morhua, Gadus morhua*; *C. lumpus*, *Cyclopterus lumpus*.

3.2.3 Production of extracellular products (Papers I-IV)

Bacterial ECP were produced both by collecting spent medium from broth cultures (B-ECP) and by the cellophane overlay method (C-ECP) (Liu, 1957). Broth cultures of BHI or Tryptone soya broth (TSB, Difco) or cellophane (Topoplast) covered agar plates, supplemented with NaCl, were inoculated with starter cultures and incubated at 4-15 °C for two to five days. For collection of ECPs, cultures were centrifuged at 4 °C and the supernatants collected and filtered through 0.2 µm filters (Whatman) and stored at 4 °C or -80 °C if not used the following two days. Purity of cultures was checked by streaking out pelletted cells onto BA-NaCl plates.

For concentration of ECP in Paper IV, supernatants were filtered on a centrifugal filter (Amicon Ultra-15, MWCO 10.000, Millipore) (3800 x g, 30 min, 10 °C) and desalted on the same filter with 15 ml PBS under the same conditions. This resulted in about 50-fold reduction of the total volume of supernatants.

3.2.4 Protein concentration (Papers I-IV)

Total protein concentration was determined using Coomassie Plus protein assay kit (Pierce) according to the manufacturer's protocol. Bovine serum albumin (Pierce) was used for plotting a standard curve. Absorbance was measured at 590 nm.

3.2.5 Caseinolytic activity

Azocasein assay (Papers I-III)

Caseinolytic activity was estimated by the azocasein assay, using azocasein (Sigma) as substrate, as previously described (Gudmundsdottir, 1996). The assay was incubated for 1 h at 30 °C, except when assaying the optimal temperature of MvP1 (Paper II) where the incubation temperature ranged between 5 and 80 °C. Sample buffers and cultivation media were used as negative controls. Absorbance was measured at 430 nm. One unit (U) caseinase activity was defined as an increase in absorbance of 0.01 under the assay conditions.

Peptidase inhibition (Papers I & II)

Samples were incubated with inhibitors for 10 min at 22 °C. Stock solutions of the dissolved metallopeptidase inhibitors EDTA (Merck) and 1,10-Phenanthroline monohydrate (OPA, Sigma Aldrich) and the serine peptidase inhibitor Phenylmethanesulphonyl fluoride (PMSF,

Sigma) were prepared and added to the samples in final concentrations of 1 or 10 mM. Remaining caseinolytic activity was then determined using the azocasein assay.

Optimal temperature and thermostability (Paper II)

For estimating the optimal temperature for MvP1, the catalytic activity of the pure peptidase was measured at different temperatures between 5 and 80 °C for 1 h, by the azocasein assay. For thermostability testing the purified protein was incubated at 30, 40, 50 and 60 °C for 30 min in SD-200 buffer and centrifuged (16.000 x g for 5 min at 4 °C) before performing azocasein assay.

3.2.6 Esterase activity (Paper I)

Esterase activity assay was based on the basic protocol of Sparks et al. (1979), using alpha-naphtyl acetate (Sigma) as substrate. Samples (30 µl) were mixed with 70 µl of the substrate (10 mM alpha-naphtyl acetate (Sigma) in 50 mM sodium phosphate buffer, pH 6) and incubated at 37 °C for 20 min. The reaction was stopped using 10 µl stop solution (0.5% Fast Blue B salt (Sigma) in 120 mM sodium dodecyl sulphate (SDS) and absorbance measured at 600 nm. Each sample was measured in triplicate. One U esterase activity was defined as an increase in absorbance of 0.01 under the assay conditions.

3.2.7 Agar diffusion assays (Paper I)

ECP degradation of egg-yolk and potato starch was evaluated by agar diffusion on plates (1% agar in PBS) containing either 5% egg-yolk emulsion (Oxoid) or 0.4% potato starch. Each sample (20µl) was pipetted in duplicate into 4 mm diameter wells in the agar and incubated at 15 °C for 30 h. Plates containing potato starch were flooded with lugol to visualize zones around the wells. The diameter of the reaction zone was used as quantitative indication of enzymatic activities. PBS-PNaCl or fresh medium were used as negative controls.

3.2.8 Proteolytic digestion of IgM (Paper II)

Isolated salmon and cod IgM (4 µg) were incubated in duplicate for 24 h at 9 °C with *M. viscosa* ECP (1.2 µg) or isolated MvP1 (0.12 µg), each containing caseinolytic activity of 65 U. As control, IgM was was incubated with SD-200 buffer. Samples were electrophorised using standard SDS-PAGE and IgM visualized using Western blot analysis (see below).

3.2.9 Hemolytic assays (Papers I & II)

Hemolytic activity was determined using salmon and sheep erythrocytes. Serial dilutions of *M. viscosa* ECPs or isolated MvP1 were incubated for 5-24 h at 22 °C in 0.5% washed red blood cells suspended in PBS. Each dilution was carried out in duplicate. PBS was used to calculate 0% lysis and deionised distilled water (dH_2O) to calculate 100% lysis. The supernatant was collected and its absorbance at 405 nm measured. Hemolytic units were defined as the dilution factor giving ≤ 50% hemolysis.

3.2.10 Cytotoxicity assays (Papers I-III)

Cytotoxic activity of selected *M. viscosa* antigens (cells, ECP and MvP1) was determined using epithelioma papulosa carpio (EPC), bluegill fry fibroblast (BF-2) and Atlantic salmon head kidney macrophage-like (SHK-1) cells.

For EPC and BF-s cells, confluent cell monolayers were prepared in 96-well flat-bottom cell culture plates containing 150 µl Eagle's minimal essential medium (Eagle's MEM, Gibco), with 10% foetal bovine serum (Gibco), penicillin (Sigma, 100 IU/ml), and streptomycin (Sigma,100 µg/ml).

As described in Paper I, medium was removed from EPC cells following incubation at 22 °C for 20 h. Then, cell monolayers were washed once with 200 µl of the previously used medium, without serum, and kept at 10 °C for 40 min. ECP samples diluted 1:20 in serum free medium were added in triplicate in total volume of 200 µl to the monolayer and incubated at 10 °C for 3 h. Cytotoxicity was assayed using a cytotoxicity detection kit (LDH, Roche), and absorbance read at 490 nm. Assay setup, controls and calculations of percent cytotoxicity were performed following the manufacturer's instructions.

As described in Paper II, medium was removed from wells of EPC and BF-2 monolayers following incubation at 23 °C for 20 h and 150 µl fresh Eagle's MEM with or without 10% foetal bovine serum added to each well, as well as 40 µl of different dilutions of ECP (22-175 µg/ml) or MvP1 (3-30 µg/ml), containing equal caseinolytic activities. SD-200 buffer was used as control and each treatment carried out in triplicate. Cells were incubated for further 24 h at 15 °C and microscopically examined.

In Paper III, the cytotoxicity of selected *M. viscosa* antigens on SHK-1 cells was determined. The cells were cultured for two days as described below in 96 well plates, washed twice with Dulbecco's phosphate buffered saline (DPBS, with Ca^{++} and Mg^{++}, Sigma) and incubated with stimulants diluted in DPBS in triplicate at 20 °C for 4, 24 or 72 h. Then,

cytotoxicity was determined using the LDH cytotoxicity detection kit, according to the manufacturer's instructions. Controls, measurements and calculations were performed as previously described (Fast et al., 2008).

3.2.11 Siderophore production assay (Paper I)

Siderophore production was estimated using cromoazurol S (CAS) a siderophore-indicative medium as previously described (Lauzon et al., 2008), with the exception that 2% NaCl and 4.5% agar were used in the medium. ECPs (30 µl) were pipetted into 4 mm diameter wells in the agar medium or bacterial colonies streaked onto the medium. CAS plates were incubated at 15 °C for 3 days. The diameter of the reaction zone around wells or bacterial growth was used as indication of siderophore production.

3.2.12 Acrylamide gel electrophoresis

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried out using the Mini-Protean II system (BioRad). Samples were reduced in sample buffer and denatured at 100 °C for 7 min before loading onto the gel. Electrophoresis was carried out in 10-16% SDS-poly-acrylamide separation gels with 4.5% stacking gels. Protein bands were visualized by silver staining (either Silver Stain Plus Kit, BioRad, or using the method of Chevallet et al. (2006) for mass spectrometry analysis). Protein bands from SDS-PAGE gels were also visualized by Western blotting (see below). Molecular weight (MW) standard proteins (BioRad and Fermentas) were run in electrophoresis gels for estimating the MW of protein bands.

Zymography (Papers I & II)

Substrate SDS-PAGE (zymography) was performed as previously described (Gudmundsdottir, 1996), using 0.03% casein, 0.1% collagen (isolated from lumpfish skin) or 0.1% gelatin as substrates in gels. Samples were diluted 1:1 or 1:4 in sample buffer without 2-mercaptoethanol and loaded onto gels without heating (native electrophoresis). Gels were incubated at 30 or 37 °C for 2 h before staining with Coomassie Brilliant Blue R (Sigma-Aldrich).

Detection of esterolytic factors in SDS-PAGE (Paper I)

Analysis of esterase activity in SDS-PAGE gels was performed using the α-naphthyl acetate-azo dye method (Lachmann et al., 1997). Following native electrophoresis, α-naphthyl acetate (Sigma) was used as substrate and the reaction visualized by pararosaniline (ICN Biomedicals). Gels were incubated with the substrate for 10 min at 37 °C.

Isoelectric focusing (Paper II)

Isoelectric focusing was carried out in Phast system (Pharmacia), using PhastGel IEF 4-6.5 (GE healthcare), and standards pH 3-10 (Pharmacia). Gels were fixed in 20% trichloroacetic acid and visualized with silver staining.

3.2.13 Anti-MvP1 production (Paper II)

Polyclonal mouse antibodies against MvP1 were produced in ascitic fluid (Overkamp et al., 1988) with some modifications (Lange et al., 2004). *M. viscosa* ECP (1 mg protein) were separated using native SDS-PAGE and Coomassie stained. The MvP1 protein band (38 kDa, isotype I1) was excised from the gel and emulsified. The protein was homogenised with Freund´s Complete Adjuvant (FCA) 1:5 and 5 female Balb/c mice each i.p. injected with 200 µl. The injection was repeated on day 15 with the protein homogenized with Freund´s Incomplete Adjuvant (FIA) 1:5 followed by 500 µl Pristane (Sigma). On day 31 the mice were again injected with MvP1 in FIA and in the following days the mice were sacrificed, ascitic fluid collected and stored at -80 °C. The specificity of the antibodies was tested by Western blotting. The injections and the collection of ascitic fluid were performed by a qualified veterinary officer.

3.2.14 Western blotting (Papers I, II and IV)

Western blotting was performed as previously described (Gudmundsdottir et al., 2003). For detection of MvP1 or IgM (Magnadottir, 1998), blots were incubated with polyclonal mouse antibodies diluted 1:2000 in 20 mM Tris (pH 8.0), 0.8% NaCl, 0.1% Tween 20, 0.1% skimmed milk powder (TBS-TC), followed by incubation with goat anti-mouse immunoglobulin antibodies conjugated with alkaline phosphatase (AP, Dako) diluted 1:1000 in TBS-TC.

Immunoblot detection of Hcp was performed using polyclonal rabbit antibodies raised against *V. anguillarum* Hcp (Vts subclass T6SS) (Weber et al., 2009). *V. anguillarum* Hcp

antibodies were diluted 1:10000 in TBS-TC, followed by incubation with AP conjugated goat anti-rabbit immunoglobulin antibodies (Dako) diluted 1:1000 in TBS-TC.

3.2.15 N-terminal sequence analysis (Papers II & IV)

Proteins were separated by standard SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Proteins were stained with Coomassie blue R350 in dH_2O/methanol/acetic acid (45:45:10) and the membrane destained in the same solution without the dye. Protein bands of interest were excised from gels and N-terminal aa sequence analysis carried out at the Institut Pasteur, Paris, by automated Edman degradation using an Applied Biosystems 494 automatic sequencer.

3.2.16 Mass spectrometry

Protein bands visualized by silver staining were trypsinised and identified using MALDI-TOF mass spectrometry as described (Shevchenko et al., 1996) with modifications (Gylfason et al., 2010). Iodoacetamide was used as alkylating agent, giving carboxyamidomethyl cysteine. Proteins were identified by the Mascot online program (www.matrixscience.com), using the following search parameters; Proteobaceria; database: NCBInr; missed cleavages: maximum of one by trypsin; fixed modifications: cysteine carbamidomethylation; variable modification: methionine oxidation; peptide mass tolerance: ± 35 ppm.

3.2.17 Autodegradation of *M. viscosa* ECP

Autodegradation of B-ECP of isolate 6 due to metallopeptidase activities was evaluated. Samples diluted 10:1 in dH2O, 10 mM OPA or methanol (MeOH, used as OPA solvent) were incubated at 15 °C for 5 days in duplicate. A frozen sample diluted 10:1 in dH2O was also kept at -80 °C. Following incubation, protein content, caseinolytic activities and protein profiles of samples were evaluated.

3.2.18 MvP1 peptidase purification (Paper II)

B-ECP proteins of *M. viscosa* isolate 6 were precipitated by adding ammonium sulphate to 85% saturation in an ice bath with constant stirring for 30 min. The precipitate was recovered by centrifugation (10000 x g for 10 min at 4 °C), dissolved in 20mM Tris buffer (pH 8.0) and filtered through a 0.2 µm filter. Then, precipitated proteins were desalted using PD-10 columns (Amersham Biosciences) according to the manufacturer's instructions at 4 °C, using 20 mM Tris buffer (pH 8.0) for equilibration and elution. Ion-exchange and gel filtration

chromatography were performed using ÄKTA fast protein liquid chromatography (FPLC) (Amersham Pharmacia Biotech, UPC-900 Monitor, P-920 Pump, Frac-950 Fraction Collector) at 15 °C. Buffers used for FPLC were made with dH_2O, filtered through a 0.2 µm membrane filter (Millipore), degassed for 10 min and cooled to 15 °C prior to use. Elution profiles were recorded with a mercury lamp set at 280 nm. Desalted precipitated ECP proteins were filtered through a 0.2 µm filter and 1 ml loaded onto a MonoQ HR 5/5 column (Pharmacia) at a flow rate of 1 ml/min. The column was equilibrated with 20 mM Tris (pH 8.0). Eluent buffer (20 mM Tris (pH 8.0) containing 1 M NaCl) was applied at a linear gradient of 0-80% in 18 column volumes. Eluate fractions of 0.5 ml were collected and analyzed for caseinolytic activity. Pooled fractions showing caseinolytic activity from several MonoQ runs were concentrated using Amicon Ultra-15 filter (Millipore, MW cut off (MWCO) 10,000) and centrifuged (2000 x g for 13 min at 4 °C). Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) was equilibrated and eluted with SD-200 buffer and 0.5 ml of concentrated fractions (sterilefiltered through a 0.2 µm filter) loaded onto the column at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and analyzed for caseinolytic activity. Fractions with major caseinolytic activities were pooled and used as the purified enzyme, following analysis with SDS-PAGE and Western blotting.

3.2.19 Fractionation of esterase activity (Paper I)

C-ECP of isolate 6 was concentrated fourfold in polyethylenglycol 6000 (PEG, Merck) in a dialysis tube (MWCO 12000, Sigma) at 4 °C. The ECP was desalted and fractionated on a MonoQ anion exchange column as previously described for MvP1 isolation. Eluate fractions were analyzed for esterase activity, using the microwell esterase assay or the α-naphthyl acetate-azo dye method.

3.2.20 Preparation of antigens for SHK-1 stimulation (Paper III)

M. viscosa (isolate 6) cells and ECP were harvested from a logarithmic phase culture, grown for 48 h in BHI broth containing 2% NaCl at 4 °C. Cells were washed in DPBS, resuspended in the same buffer and kept on ice. For heat-killing of cells, washed cell suspension was heated to 60 °C in a water bath for 1 h. CFUs were estimated by plate counting. ECPs were isolated from cells through centrifugation (3200 x g, 10 min, 4 °C) and filtration (0.2 µm). The MvP1 peptidase was isolated from a stationary phase culture as previously described.

The stability of MvP1 activity was tested in the various assay media and buffers used in cell stimulation assays. MvP1 stability in DPBS and SHK-1 cell culture medium (see

below), both fresh and conditioned, was compared to MvP1 stability in SD-200 buffer at 20 °C for up to 72 h. Medium collected from SHK-1 culture flasks after 3 days of culturing was used as conditioned medium. MvP1 was diluted 1:1 in the respective buffer or medium and caseinolytic activity measured in duplicate using the azocasein assay.

3.3 SHK-1 cell culturing and stimulation (Paper III)

SHK-1 cells were cultured in 75 cm^2 flasks (Costar, Fisher Scientific) at 18 °C as described by Fast et al. (2005), without antibiotics. Cells (passage 61-63) were seeded in 25 cm^2 flasks (Costar, Fisher Scientific) at approximately 1 x 10^6 cells/flask 72 h before stimulation, and cultured at 20 °C. Then, medium was removed and 5 ml fresh medium, containing the stimulants added. Stimulations were performed in two separate experiments. In experiment 1, SHK-1 cells were stimulated with *M. viscosa* cells, either live or heat-killed, with estimated multiplicity of infection (MOI) of 2, or ECP in final concentrations of 0.01 or 0.05 µg protein/ml. In experiment 2, isolated MvP1 was added in final concentrations of 0.1, 0.4, 0.7 and 1.0 µg protein/ml. Stimulated cell cultures were incubated at 20 °C for 4, 24 or 72 h, and cells incubated with medium and dilution buffer only as controls. Each treatment was carried out in triplicate.

3.4 DNA and RNA methods

3.4.1 Standard isolation and manipulation of DNA and RNA

Isolation of M. viscosa *genomic DNA*

M. viscosa cells were cultured in 20 ml BHI-NaCl at 15 °C for 24 h (200 rpm). Purgene DNA purification Kit (D-5000A, Gentra Systems) was used for genomic DNA isolation according to the manufacturer's instructions. Finally, 1 mM EDTA was added to the rehydrated DNA and the DNA stored at -20 °C.

Isolation of plasmids

The GeneJet Plasmid Miniprep Kit (Fermentas) was used for routine isolation of plasmids from *E. coli* cultures. For medium scale purifications of plasmids, a PureLink Plasmid

Midiprep Kit (Invitrogen) was used. In each case the manufacturer´s instructions were followed.

Isolation of RNA

Total RNA was isolated using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions, and as described by Fast et al. (2008) and stored at -80 °C.

DNA and RNA quantitation

DNA and RNA concentrations were determined using NanoDrop-1000 (v3.2.1, Thermo Scientific) spectrophotometer according to the manufacturer´s instructions.

Polymerase chain reaction

PCR was performed using Taq (Amersham), Teg (Prokaria) or Phusion Hot Start proofreading (Finnzymes) polymerases and cycling conditions followed standard laboratory practices. Briefly, the basic reaction mix contained PCR buffer, deoxyribonucleotide triphosphate (dNTP) mix, specific or arbitrary forward and reverse primers, polymerase and template DNA. Various thermal cycling programs were used and parameters, such as annealing temperature, cycle number etc., were adjusted to the primers used and the type of DNA template being amplified.

For PCR on colonies of *E. coli* or *M. viscosa*, colonies were picked from agar plates and dissolved in 10 µl dH_2O. One µl of the solution was used as template for PCR and the initial denaturing step of the thermal cycling program extended to 10 min.

Cycle sequencing

Standard sequencing by the dideoxy method was performed using BigDye® Terminator sequencing kit (Applied Biosystems), and the sequencing reaction mix prepared according to the manufacturer's instructions. Products from sequencing reactions were analyzed on either an ABI PRISM 310 or ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Standard single letter codes are used to refer to nucleotides (nt) and aa.

Oligonucleotide primers

Universal M13 primers (forward and/or reverse) were used for amplification and sequencing of cloned DNA fragments in commercial plasmids. Other oligonucleotide primers used in this

study are listed below where appropriate. Primers were designed using the SECentral computer program (Scientific & Educational Software) or the Primer3 (http://frodo.wi.mit.edu/primer3) program.

Agarose gel electrophoresis

DNA electrophoresis was performed using standard procedures and carried out in 0.6-1.2% agarose (Le-agarose, Seakem) gels made up with 0.5x Tris-Borate-EDTA (TBE) buffer and ethidium bromide.

DNA purification from agarose gels

Two methods were used to purify DNA from electrophorised agarose gels, the QIAquick Gel Extraction Kit (Qiagen) and the GFX^{TM} PCR, DNA and Gel Band Purification Kit (Amersham Bioscienses), following the manufacturer´s instructions.

Restriction digests and DNA ligation

Restriction digests were performed using enzymes and buffers from Fermentas and New England Biolabs, according to the manufacturer's specifications. DNA ligations were performed using T4 DNA ligase (Fermentas) as described by the manufacturer.

Computer analysis of sequences

Short DNA sequences were analyzed using Sequencher 4.6 (Scientific & Educational Software). Annotation of T6SSs (Paper IV) was performed as previously described (Hjerde et al., 2008) using the Artemis DNA Sequence Viewer and Annotation Tool (Sanger). The NCBI Basic Local Alignment (BLAST) and the EBI FASTA search tools were used for identification and comparison of protein sequences. Conserved domains were detected using CD (NCBI). ClustalW (EBI) was used for multiple sequence alignments. Putative open reading frames (ORFs) were identified by the SoftBerry FGENESB program (www.softberry.com). Protein motifs were identified using Pfam (Sanger) and transmembrane domains were identified with TMHMM (CBS). The ProtParam program (ExPASy) was used for MW computation and SignalP 3.0 (CBS) for predicting the location of a signal peptide cleavage site.

The BioEdit sequence alignment editor software was used to compose a protein database from the draft genome of *M. viscosa* isolate 1 (see below) and to screen for various

aa sequences in the *M. viscosa* genome. Comparison of *M. viscosa* isolate 26 T6SS loci against previously described T6SSs was facilitated by using The Artemis Comparison Tool (ACT, Sanger), which enabled the visualization of BLASTN and TBLASTX comparisons. Prediction of antigenic peptides was performed using a computer program found at http://imed.med.ucm.es/Tools/antigenic.pl. GenBank accession numbers of genes and proteins are given in brackets where appropriate.

3.4.2 *M. viscosa* genome

The draft genome of *M. viscosa* isolate 1, annotated using BAsys automatic annotation (Grove et al., 2008), was kindly provided by Duncan Colquhoun at the Norwegian Veterinary Institute, and used to screen for several *M. viscosa* genes. The genome of *M. viscosa* isolate 26 was used for genomic screening of T6SS (Paper IV). The sequence assembly, which is in its finishing phase, can be accessed at: http://arctic.imb.fm.uit.no/blast/.

3.4.3 Sequencing of the *mvp1* gene and flanking regions (Paper II)

Degenerate primers were designed for PCR amplification of a sequence from *mvp1*, the gene encoding MvP1. Primer construction was based on N-terminal sequencing results and the third zinc ligand motif of thermolysin peptidases. The amplified PCR product from the genome of *M. viscosa* isolate 6 (508 nt), was cloned into pCR®2.1-TOPO® (Invitrogen) and sequenced.

A series of nested PCR amplifications and subsequent sequencing was performed to determine the flanking sequences of the 508 nt product. Gene specific 5´ biotin labelled primers and arbitrary primers (Arb1 or Arb2) were used for first round PCR amplification. Amplified PCR products were purified and biotin labelled PCR products further purified using Dynabeads® M-280 Streptavidin (Dynal Biotech). Second round PCR amplification was performed using nested gene specific primers, which bound upstream of the previously used gene specific primers, and Arb 3, a nested primer to Arb1 and Arb2. PCR products were purified, cloned into pCR®4-TOPO® (Invitrogen) and transformed into One Shot® TOP10 competent *E. coli* (Invitrogen). Cloned inserts were sequenced, using M13 primers. The sequence information was then used to generate new gene specific primers for the next nested PCR round until the complete gene and flanking regions were obtained.

For confirmation, the *mvp1* gene and its flanking regions were PCR amplified from genomic DNA, using primers containing *Eco*RI and *Bam*HI recognition sequences, and cloned into pUC19 (Invitrogen), using *Eco*RI and *Bam*HI. Chemically competent DH5α *E.*

coli cells (Invitrogen) were transformed with the resulting ligation product, the plasmid isolated and both strands of the cloned insert sequenced.

A list of primers used for sequencing the *mvp1* gene and its flanking regions is given in Table 5.

Table 5. Primers used for sequencing of *mvp1* and flanking regions

No.	Name	Sequence 5´-3´	Application
1	mvp1-N5-f	GGMTTYGGTGGTAACGARAAAACRGG	Amplification of a 508 nt fragment of *mvp1*
2	mvp1-I492-r	GCTTCACCWGCCATATCAGARAAYGCTTC	
3	Arb1-f/r	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	Arbitrary primers for outward sequencing
4	Arb2-f/r	GGCCACGCGTCGACTAGTANNNNNNNNNNACGCC	of *mvp1* and flanking regions.
5	Arb3-f/r	GGCCACGCGTCGACTAGTAC	Nested primer for Arb1 and Arb2.
6	mvp1-1bio-r	ATTACCAGACTGCCCTACATTC	Specific primers for outward sequencing of
7	mvp1-2bio-f	TTCAGCTCATGAAGTTAGTC	*mvp1* and flanking regions.
8	mvp1-3-r	CAAGTATCCGAAATCAGTACC	Even numbered primers are designed for
9	mvp1-4-f	GGTTTATGAGGCTAAATCAG	upstream amplification, odd numbered
10	mvp1-5bio-r	CAACAGCACCATGAGCATTC	primers are designed for downstream
11	mvp1-6bio-f	GTACTGACAGTGGTGATGAG	amplification.
12	mvp1-7-r	CCGTCAGAATAACGAGATATAATT	Primers marked bio are 5´ biotyn labelled
13	mvp1-8-f	CAGGTGCCAGTGACATTC	and other primers are nested.
14	mvp1-9bio-r	ACACCACCATCACTATCG	
15	mvp1-10bio-f	CGGTTACAGTACTGCATCTG	
16	mvp1-11-r	GACCACACTAAGACCTTC	
17	mvp1-12-f	TGATGTGGAGCTAAATATTCA	
18	mvp1-13bio-r	GTTGTTGCTGAAGATATTGC	
19	mvp1-15a-r	GCTGATAATTGTTGAATATTGTC	
20	mvp1-EcoRI-f	CGGAATTCGACGTACCGCACCAAATCC	Ligation of *mvp1*and flanking regions into
21	mvp1-BamHI-r	CGGGATCCTATTAGCTAAATTATCTGCTG	pUC19. Restriction sites are underlined.
22	mvp1-425-f	CAGTCGCTTACAATTTTCAGTT	Sequencing of *mvp1*and flanking regions in
23	mvp1-990-f	TCTCGATAGTGATGGTGGTG	pUC19.
24	mvp1-1478-f	TGAATGTAGGGCAGTCTGG	Primers no. 8, 9, 17 and 19 were also used.
25	mvp1-3100-r	GCCAACGTCGATAGAGAAAC	
26	mvp1-2654-r	GTATAGCTGCCGACCAAACT	
27	mvp1-2190-r	GCTCCAATAGAGTTGGTTGG	
28	mvp1-1794-r	GTACGATTCACCATCACCAA	
29	mvp1-446-r	AACTGAAAATTGTAAGCGACTG	

f, forward primers; r, reverse primers.

3.4.4 Screening for additional vibriolysin genes

The draft genome of *M. viscosa* isolate 1 was screened for the presence of additional vibriolysin genes. Two vibriolysin genes were identified (see results), one of which was amplified from isolate 6 using two primer pairs: vl2a-f, 5´-ACAACTCTTGGGGCTGTCAC-3´; vl2a-r, 5´-AGACGCGTATAGCCTTCACC-3´and vl2b-f, 5´-GAAACGGAAGGGACTACTGC-3´; vl2b-r, 5´-TCACATCAGCAACGATACCG-3´, amplifying 1500 and 760 nt sequences from the gene, respectively.

3.4.5 Quantification of pro-inflammatory gene expression (Paper III)

Following stimulation of SHK-1 cells, expression of genes encoding the pro-inflammatory cytokines interleukin-1β (IL-1β) and interleukin-8 (IL-8) was evaluated. For reverse transcription, total RNA (2.0 µg) was dissolved in molecular biological grade water and two-step reverse transcription real-time Q-PCR carried out using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR green (Invitrogen) on an iCycler iQ Real-Time detection system (BioRad). The manufacturer's instructions were followed, with some exceptions (Fast et al., 2008). Primers for real-time Q-PCR amplification of the genes encoding elongation factor-1alpha (EF-1A), which was used as a reference gene, IL-1β, and IL-8 were as described in Table 6. Plasmid vectors with cloned PCR products of each primer pair were used as standards for real-time Q-PCR (Fast et al., 2005). To ensure that genomic DNA was not quantified in real-time Q-PCR the EF-1A primers were designed to span an intron/exon splice site and single-product amplification confirmed through melt curve analysis. Cycle conditions were as previously described (Fast et al., 2008). Tenfold dilutions of standards and a blank without cDNA were run in each PCR, along with the duplicate samples.

Table 6. Real-time Q-PCR primers

Gene	Accession number	Primer	Sequence (5`- 3`)
EF-1A	**AF321836**	EF-1A forward	GTGGAGACTGGAACCCTGAA
		EF-1A reverse	CTTGACGGACACGTTCTTGA
IL-1β	**AY617117**	IL-1β forward	CGTCACATTGCCAACCTCAT
		IL-1β reverse	ACTGTGATGTACTGCTGAAC
IL-8	**BT046706**	IL-8 forward	GAATGTCAGCCAGCCTTGTC
		IL-8 forward	TCCAGACAAATCTCCTGACCG

The relationship between the threshold cycle (C_t) and log(RNA) was linear (-3.18 < slope < -3.02) for all reactions. The gene expression results of IL-1β and IL-8 in stimulated cells is presented as fold expression compared to expression in control cells and expressed as

means ± SEM. Expression of IL-1β and IL-8 was calculated as relative to the expression of the EF-1A reference gene. Due to significant differences in the expression of EF-1A between sampling times, the relative expression of IL-1β and IL-8 was calculated from the mean expression of EF-1A at each time point, for each experiment, with values from control and stimulated cells combined.

3.4.6 MvP1 negative *M. viscosa* mutant construction

Mutant construction was not successfully completed. Below is a description of template constructions and attempted bacterial conjugations.

Construction of an in-frame mvp1 deletion template

A 2421 bp region, including the *mvp1* gene, was amplified from *M. viscosa* genomic DNA of isolate 6 using the "nested" forward and reverse primers (Fig. 6 and Table 7). The product was purified from agarose gel, A-overhangs added and cloned into pCR®2.1-TOPO® and transformed into One Shot® TOP10 cells. Next, the upstream and downstream sequences of the mature peptidase region of *mvp1* were amplified from the isolated Topo vector using the *Xba*I-5-f and soe-5-r and the soe-3-f and *Sac*I-3-r primer pairs. The two resulting amplified products, 593 bp upstream and 544 bp downstream of the mature peptidase region were then spliced together in a splice overlap extension PCR (Horton & Pease, 1991), which was possible since the soe primers were designed to have overlapping extensions (Table 7). This resulted in an in-frame deletion of the mature peptidase region of the *mvp1* gene (nt 449-1518). A Phusion proofreading polymerase was used in all PCR amplifications.

Table 7. Primers used for the construction of mvp1 mutagenesis templates

Primer	Sequence 5´-3'	Application
mvp1-nested-f	AACGGATTCAGGTGTTGAGTGTTCG	Amplification of a 2421 bp region of
mvp1-nested-r	TTCAGCCTCTGTAGCCAACGTCG	*M. viscosa* DNA including the mvp1 gene.
mvp1-*Xba*I-5-f	TCTAGACCACCACTACAGCTACCGCCGCTC	Amplification of a 593 bp region upstream of
mvp1-soe-5-r	**CAGGACTGGCATTAGAATT**AAAGCCAGTGGCATCGGC	the mature peptidase region. Underlined bases represent a *Xba*I restriction site and bold bases overlap with a region amplified by the mvp1-soe-3-f primer.
mvp1-soe-3-f	**GCCGATGCCACTGGCTTT**AATTCTAATGCCAGTCCTG	Amplification of a 544 bp region downstream
mvp1-*Sac*I-3-r	GAGCTCCAGGTGAAGACTCAGCACCATGCTG	of the mature peptidase region. Underlined bases represent a *Sac*I restriction site and bold bases overlap with at region amplified by the mvp1-soe-5-r primer.
mvp1-ala-f	GGATGTTTCAGCTCATGcAGTTAGTCACGGTTTTAC	Site directed mutagenesis of the active site
mvp1-ala-r	GACTAACTgCATGAGCTGAAACATCCAAgCTTACTAATG	glutamic acid residue in the HEVSH motif
mvp1-gln-f	GGATGTTTCAGCTCATcAAGTTAGTCACGGTTTTAC	of *mvp1*. Mutagenized positions are denoted
mvp1-gln-r	GACTAACTTgATGAGCTGAAACATCCAAgCTTACTAATG	in lower case font and silent *Hind*III
mvp1-asp-f	GGATGTTTCAGCTCATGAcGTTAGTCACGGTTTTAC	restriction sites are underlined.
mvp1-asp-r	GACTAACgTCATGAGCTGAAACATCCAAgCTTACTAATG	
mvp1-sdm559-f	TTGGTGGTGTGGTATTTGATATG	Amplification of a 559 bp region surrounding
mvp1-sdm559-r	CAGTTAGTATTAGCGCTCCAATAGAG	the HEVSH motif where site directed mutagenesis was constructed.
pDM4-f	TAACGGCAAAAGCACCGCCGGACATCA	Sequencing of templates ligated into pDM4.
pDM4-r	ACATGTGGAATTGTGAGCGGATAACAA	

f, forward primers; r, reverse primers.

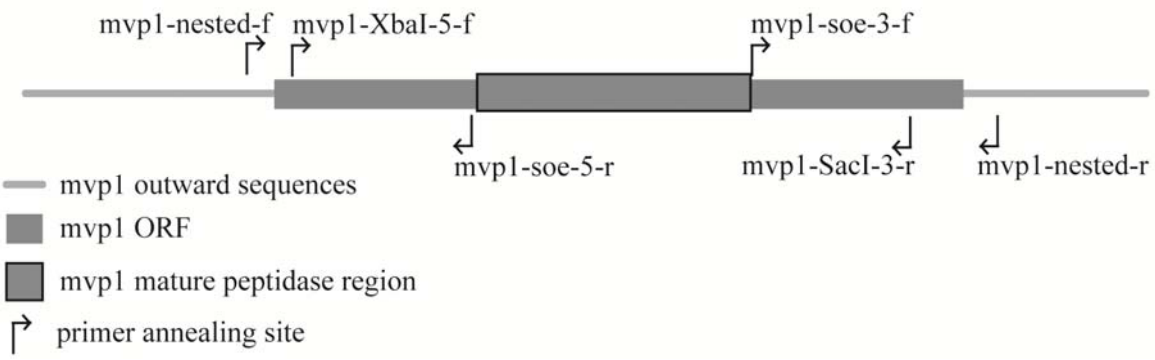

Fig. 6. Construction of an *mvp1* deletion template.
The figure shows the relevant genomic region and the primers used. ORF, open reading frame.

Construction of mvp1 site directed mutagenesis templates

Site directed mutagenesis (sdm) was applied to exchange the glutamic acid (E), which is an active site residue in the HEVSH motif (nt 1054-1056) of MvP1. Three different templates were constructed where E was replaced with: alanine (A), a small residue; aspartic acid (D), an acidic residue; or Glutamine (Q), a residue similar in size to glutamic acid. The construction was based on a method described by (Zheng et al., 2004). The 2421 bp product, cloned into Topo vector (created during deletion mutant construction) was amplified with mvp1-ala, -gln or -asp forward and reverse primers (Table 7), which amplified the whole plasmid, creating a single base mutation of the glutamic acid residue. The primers also inserted a single base mutation creating a silent HindIII restriction site, used for initial verification of the mutation. Parental DNA was restricted using DpnI and the plasmids transformed into One Shot® TOP10 cells. Transformed plasmids were isolated and amplified with sdm559 forward and reverse primers and the isolated amplificates restricted with HindIII for verification of sdm. For final verification the plasmids were sequenced using the sdm 559 forward and reverse primers. A Phusion proofreading polymerase was used in all amplifications.

Cloning of templates into pDM4 suicide vector

The templates created above (in-frame *mvp1* deletion and sdm), along with the wild-type template, were amplified with the XbaI-5-f and SacI-3-r primers and a Phusion polymerase. A-overhangs were added and the templates cloned into a pCR®2.1-TOPO® vector. The cloned templates were restricted out with *Xba*I and *Sac*I restriction enzymes. The pDM4 suicide plasmid (Milton et al., 1996), which requires the *pir* gene for replication, was also restricted with the same enzymes. Restricted plasmid and gel purified templates were then ligated together using T4 ligase. The ligation mix was subsequently cut with *Sph*I, which cut the pDM4 linker region, resulting in a mix containing only circular pDM4 plasmids with template inserts.

Transformation into donor strain

The pDM4 plasmids with inserted templates were transformed into *E.coli* DH5α λpir (Biomedical) cells, containing the *pir* gene necessary for replication of pDM4. The pDM4 plasmid contains a chloramphenicol resistance gene and transformed cells were selected on Luria Bertani (LB) agar plates containing 25 µg/ml chloramphenicol. The pDM4 plasmids

with inserts were subsequently isolated and transformed into the donor strain *E. coli* S17-1 λpir (Simon et al., 1983; Milton et al., 1992), containing chromosomally integrated RP4 transfer functions. Finally, isolated plasmids were sequenced using pDM4 forward and reverse primers.

Bacterial conjugation

Bacterial conjugation attempts were performed in order to transfer the pDM4 plasmid containing the in-frame *mvp1* deletion template from the S17-1 donor strain to the *M. viscosa* recipient strain (isolate 6). The aim was to create an *mvp1* deletion mutant and subsequently construct sdm mutants and a complementation mutant.

Briefly, both the donor strain and the recipient strain were grown to mid-logarithmic phase, mixed together and pelletted and spotted onto agar plates, and conjugation allowed to progress for up to 22 h. Then, the bacterial pellet was scraped off the agar, diluted and spread out onto agar plates containing kanamycin (selection of *M. viscosa*) and chloramphenicol (selection of single crossover transconjugants). Plates were incubated for up to 12 days but no transconjugant *M. viscosa* colonies were detected. Several different methods were tested in order to obtain transconjugated colonies. The *M. viscosa* recipient strain was grown in different media (BHI, LB or TSB) containing 2.0, 2.5 or 3.0% NaCl at 4, 12 or 15 °C. The donor strain and the recipient strain were mixed together in various different ratios (10:1 down to 0.25:1, respectively) and in varying total culture volumes (0.5 – 10 ml). Conjugation was performed on different agar media (BA-NaCl, BHI, LB and TSB) containing 2.0, 2.5 or 3.0% NaCl and the plates incubated at 12 to 20 °C for 10 to 22 h. Selection plates were either BHI, LB or TSB agar medium containing 2.0, 2.5 or 3.0% NaCl and the concentration of chloramphenicol and kanamycin ranged from 5 µg/ml up to 50 µg/ml.

If any transconjugated colonies had been detected the allelic exchange for the in-frame *mvp1* deletion would have been completed as described by (Milton et al., 1996), by utilizing the *sacB* gene located on the pDM4 plasmid. The *sacB* gene, which is lethal for gram-negative bacteria (Gay et al., 1985), can be induced by the addition of 5% sucrose to the medium. Thus, only strains lacking the *sacB* gene (and the integrated pDM4 plasmid) would have grown on the medium.

3.5 Statistical analysis

In Paper I, statistical comparison of isolate growth was performed using one-way ANOVA (post-hoc test Fisher's PLSD) and Spearman r test to analyze correlation. In Paper III, statistical analyses on cytotoxicity assay data and SHK-1 cells gene expression data were performed using two-way ANOVA (variables: antigen, sampling time, and interaction). Tukey HSD test was used to discern differences among means for those effects that had significant differences and the data segregated by variable and compared to the relative control. Results were considered significant if $p < 0.05$.

3.6 Licenses

The study was approved and performed according to the Icelandic Animal Research Authority (approval no. YDL 03080041/023BE and 1205-1001).

4 RESULTS

This chapter describes the main results of the study, published in Papers I-IV, along with some previously unpublished results.

4.1 *M. viscosa* virulence and growth (Paper I)

4.1.1 Virulence of *M. viscosa* isolates

Out of 22 *M. viscosa* isolates tested (Table 4), 20 were found to be virulent and were passed in salmon. Only two Canadian salmon isolates (14 and 15) were non-virulent and could not be passed in salmon, although injected in doses up to 8 x 10^8 CFU/fish. Re-isolated bacteria from kidney were used for LD_{50} determinations, ECP production and growth curve determination. The LD_{50} of 9 *M. viscosa* isolates was determined by i.p. injections and was as follows: isolate 1, 6 x 10^5; isolates 6, 10 and 20, 2 x 10^5; isolate 12, 2 x 10^4; isolate 17, 7 x 10^5; isolates 5, 21 and 22, > 2 x 10^6 CFU/fish.

4.1.2 *M. viscosa* growth

Growth curves of three isolates were compared; the Norwegian salmon isolate 1, the Icelandic salmon isolate 6 and the Norwegian rainbow trout isolate 17 (Fig. 7). After 48 h of cultivation all the isolates were in stationary phase with CFU counts that did not differ significantly; isolate 1, 2.9 x 10^9 CFU/ml, absorbance 0.89; isolate 6, 2.1 x 10^9 CFU/ml, absorbance 0.87; isolate 17, 3.1 x 10^9 CFU/ml, absorbance 0.96.

4.2 Virulence properties of *M. viscosa* ECP (Paper I)

4.2.1 B-ECP versus C-ECP

The ECPs of isolate 6 produced by the two different production methods, broth culture (B-ECP) or cellophane overlay (C-ECP), were compared (Table 8). C-ECP had higher minimal lethal dose than B-ECP, was non-hemolytic and showed very little cytotoxic activity. The protein concentration of C-ECP was higher than that of B-ECP and it showed higher

caseinase and esterase activity. In the following sections of the thesis the term ECP is used instead of B-ECP, unless otherwise noted.

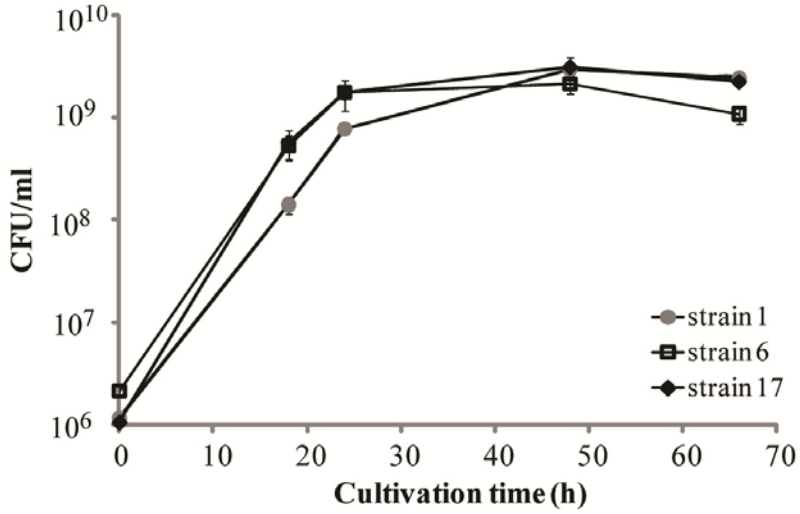

Fig. 7. Growth curves of *M. viscosa* isolates.
Growth of three *M. viscosa* isolates (CFU/ml ± SD) originating from salmon in Norway (isolate 1) and Iceland (isolate 6), and from rainbow trout in Norway (isolate 17) in BHI-NaCl broth cultures at 15 °C. CFU, colony forming units.

Table 8. B-ECP versus C-ECP
Comparison of broth culture produced ECP (B-ECP) and cellophane overlay produced ECP (C-ECP) of *M. viscosa* isolate 6.

	Protein conc µg/ml ± SD	MLD µg prot/fish	Cytotoxicity* % ± SD	Hemolysis** units	Caseinase activity units ± SD	Esterase activity units ± SD
B-ECP	135 ± 9.6	3	31 ± 3.6	512	8.6 ±0.2	24.1 ± 1.4
C-ECP	617 ± 13.1	62	2 ± 0.7	< 2	117.3 ± 1.8	479.6 ± 11.3

ECP, extracellular products; SD, standard deviation of measurements; MLD, minimum lethal dose.
* Cytotoxicity against EPC (epithelioma papulosa carpio) cell monolayer measured by the LDH assay.
** Hemolytic activity against salmon erythrocytes. Sample dilution which caused ≤ 50% hemolysis was defined as its hemolytic units.

4.2.2 Virulence activities

Lethality in salmon

Lethalities of ECPs of isolates 1 to 25 (Table 4) were checked in salmon juveniles (Table 9). ECPs of *M. viscosa* isolates 5, 14 and 15 were non-lethal, ECPs of isolates 2 and 7 killed one out of two fish and ECPs of the remaining isolates killed both injected fish. Fish injected with

non-*M. viscosa* ECPs survived the injection and showed no apparent disease signs at the end of the study. ECPs of isolates 1, 6, 9, 10 and 17 were injected into salmon in serial dilutions and the three day minimum lethal dose (MLD) determined (Table 9). Out of the five ECPs tested, ECP of isolate 1 was the most lethal.

Cytotoxic and hemolytic activity

Cytotoxicity values and hemolytic units for ECPs of isolates 1 to 25 are shown in Table 9. ECPs of *M. viscosa* isolates from salmon were significantly less cytotoxic and had lower hemolytic activities than ECPs of isolates from other fish species. However, ECPs of isolate 5 (from salmon) and isolate 20 (from cod) did not follow this pattern. A significant correlation ($p < 0.0001$) was obtained between the cytotoxic and hemolytic activities of the *M. viscosa* isolates.

4.2.3 Protein concentration, enzymatic activities and siderophore production

The protein concentration of *M. viscosa* ECPs varied from 85 to188 µg/ml, except for isolate 7, which had a significantly lower protein concentration of 50 µg/ml (Table 9).

All *M. viscosa* ECPs showed a weak positive caseinase signal in the azocasein assay, ranging from 3.3 to 14.9 caseinase units, except for ECP of isolate 7, which had the highest caseinase activity of 37.1 caseinase units (Table 9). All *M. viscosa* isolates had similar band patterns in a casein zymogram (Paper I). OPA inhibition of ECPs of isolates 2 and 6 resulted in complete caseinase activity inhibition in casein zymograms, suggesting a metallopeptidase nature of all bands.

Esterase activity of varying levels was detected in ECPs of all *M. viscosa* isolates using the microwell esterase assay (ranging from 13.3 to 24.1 esterase units) (Table 9). However, no band containing esterase activity was detected in ECPs in α-naphthyl acetate-azo dyed gels (Paper I).

Degradation of egg-yolk and potato starch was measured in agar diffusion assays. Degradation of egg-yolk was detected in ECPs of all isolates (ranging from 0.7 to 1.4 cm), except for ECP of isolate 7, which was negative (Table 9). Starch degradation was detected in ECPs of all *M. viscosa* isolates and the reaction zone ranged from 1.5 to 2.3 cm, but no degradation of starch was detected in ECPs of non-*M. viscosa* isolates (Table 9).

Siderophore production was detected in ECPs of all *M. viscosa* isolates. Halos around bacterial growth were ≤ 1 mm for all bacterial isolates, except for isolates 6 and 8, which had

larger halos, and isolates 10, 11 and 23, where yellow colour was only detected underneath the bacterial growth.

Isolate origin, lethality or hemolytic- and cytotoxic activitiy could not be connected to protein concentration, measured enzymatic activities or siderophore production of ECPs.

4.3 Autodegradation of *M. viscosa* ECP

The ECP of *M. viscosa* were autodegraded. Freshly thawed ECP of isolate 6 had protein content of 170 µg protein/ml and caseinase activity of 17 U. These values were set as 100%. Following incubation at 15 °C for 5 days the protein content of dH_2O and MeOH diluted samples had decreased to 55 and 44% of the original concentration, respectively, while samples inhibited by the metallopeptidase inhibitor OPA had 81% residual protein concentration. The H_2O and MeOH diluted samples showed unchanged caseinase activity following the 5 day incubation, but 6% residual activity remained in samples containing OPA. No further protein degradation was measured at day 10, while caseinase activity remained unchanged (results not shown).

Table 9. Virulence activities of ECP

Protein concentration, lethality, cytotoxicity, hemolytic activities and enzymatic assays of extracellular products.

	Isolate no	Protein conc µg/ml ± SD	Lethality in salmon no dead (dod)	MLD µg prot/fish	Cytotoxicity* % ± SD	Hemolysis** units	Caseinase activity units ± SD	Esterase activity units ± SD	Egg-yolk degrad diam (cm)	Starch degrad diam (cm)
M. viscosa *S. salar* origin	1	166 ± 7.9	2/2 (2,2)	< 2	10 ± 6.4	512	9.8 ± 0.5	19.5 ± 1.5	1.0	2.3
	2	85 ± 6.4	2/2 (2,3)	na	32 ± 6.8	512	7.1 ± 0.3	19.4 ± 0.6	1.1	2.0
	3	93 ± 8.9	1/2 (3)	na	32 ± 0.4	256	9.4 ± 0.6	20.9 ± 0.8	1.0	2.0
	4	159 ± 13.5	2/2 (2,2)	na	1 ± 0.8	64	12.4 ± 0.1	15.5 ± 0.7	0.7	2.1
	5	138 ± 8.8	0/2 (na)	na	92 ± 13.3	4096	7.2 ± 0.4	18.0 ± 0.4	1.2	1.6
	6	135 ± 9.6	2/2 (2,3)	3	31 ± 3.6	512	8.6 ± 0.2	24.1 ± 1.4	1.0	1.8
	7	50 ± 7.4	1/2 (4)	na	2 ± 0.5	< 2	37.1 ± 1.9	14.4 ± 0.4	0.0	1.9
	8	97 ± 15.8	2/2 (2,2)	na	31 ± 5.6	512	8.4 ± 1.4	22.8 ± 1.0	1.1	1.7
	9	97 ± 13.9	2/2 (2,2)	5	34 ± 5.1	512	5.9 ± 0.7	22.3 ± 1.0	1.3	1.5
	10	139 ± 10.6	2/2 (2,2)	4	15 ± 4.6	256	6.8 ± 0.7	13.3 ± 0.8	1.2	1.6
	11	188 ± 3.6	2/2 (2,2)	na	12 ± 4.6	256	6.4 ± 0.3	16.8 ± 0.8	1.2	1.6
	12	158 ± 19.0	2/2 (2,3)	na	31 ± 4.8	512	11.9 ± 0.3	20.6 ± 1.6	0.9	2.2
	13	104 ± 6.3	2/2 (2,3)	na	24 ± 2.0	512	11.3 ± 0.4	15.6 ± 0.3	1.0	1.9
	14	162 ± 20.6	0/2 (na)	na	1 ± 0.1	256	14.9 ± 0.4	20.9 ± 1.4	1.0	1.7
	15	128 ± 2.6	0/2 (na)	na	2 ± 0.5	64	3.3 ± 0.6	19.0 ± 0.1	1.4	1.6
M. viscosa non-*S. salar* origin	16	117 ± 21.0	2/2 (2,2)	na	64 ± 10.8	4096	12.3 ± 0.3	18.8 ± 0.4	1.1	1.8
	17	128 ± 4.0	2/2 (1,1)	6	58 ± 4.1	4096	13.3 ± 0.4	23.7 ± 1.3	1.2	1.7
	18	93 ± 4.2	2/2 (1,1)	na	70 ± 1.8	8192	3.7 ± 0.1	14.0 ± 0.7	1.1	1.7
	19	99 ± 18.6	2/2 (2,2)	na	59 ± 2.3	4096	5.2 ± 0.6	13.6 ± 0.3	0.9	1.5
	20	144 ± 0.5	2/2 (2,3)	na	22 ± 2.7	512	6.7 ± 0.1	18.1 ± 0.7	1.0	2.0
	21	131 ± 17.9	2/2 (1,2)	na	53 ± 5.6	4096	4.0 ± 0.5	16.4 ± 0.2	0.9	1.9
	22	86 ± 13.5	2/2 (4,4)	na	134 ± 7.1	4096	9.8 ± 0.1	15.1 ± 0.6	1.1	1.6
non-*M. viscosa*	23	268 ± 13.4	0/2 (na)	na	0 ± 0.2	< 2	0.0 ± 0.2	10.6 ± 0.6	0.7	0.0
	24	88 ± 8.0	0/2 (na)	na	2 ± 0.1	256	0.0 ± 0.2	12.3 ± 0.8	0.9	0.0
	25	193 ± 10.9	0/2 (na)	na	0 ± 0.4	512	0.0 ± 0.2	7.2 ± 0.2	0.6	1.7

SD, standard deviation of measurements; dod, day of death; MLD, minimum lethal dose; na, not applicable.

* Cytotoxicity against EPC (epithelioma papulosa carpio) cell monolayer measured by the LDH assay

**Hemolytic activity against salmon erythrocytes. Sample dilution which caused ≤ 50% hemolysis was defined as its hemolytic units

4.4 *M. viscosa* MvP1 extracellular peptidase

4.4.1 Isolation (Paper II)

The *M. viscosa* extracellular peptidase 1 (MvP1) was isolated from ECP of isolate 6 in three different isotypes, I1, I2 and I3. The I1 and I2 isotypes appeared as 38 and 36 kDa bands in SDS-PAGE, but as 40 and 38 kDa bands in casein zymogram, respectively. The I3 isotype was seen as a 35 kDa band (Fig. 8). All three isotypes were active (Fig. 8) and confirmed as MvP1 by Western blotting, using polyclonal antibodies raised against the 38 kDa MvP1 band (results not shown).

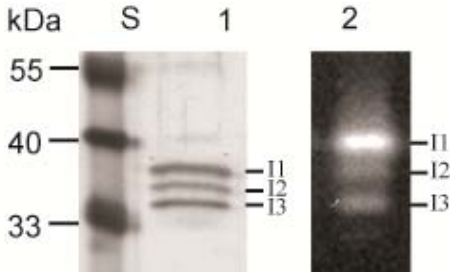

Fig. 8. Isolated MvP1 peptidase.
Three isolated MvP1 isotypes, termed I1, I2 and I3. Lane S, molecular weight standards; lane 1, silver staining; lane 2, casein zymogram.

Total recovery of peptidase activity following isolation was 1.4% and the relative purification was 510-fold. Specific activity of the three isolated MvP1 isotypes was calculated 1020 caseinase U/mg isolated protein, and based on that the percentage of MvP1 in total extracellular proteins was estimated from 10 to 17%.

4.4.2 Characterization (Paper II)

Extracellular peptidase production was observed at the end of the exponential growth phase of isolate 6, and cultures from 4, 9 and 15 °C produced the same caseinase band pattern in a casein zymogram. The isolated MvP1 peptidase degraded casein, lumpfish skin collagen and gelatin, and identical band patterns were observed in zymograms containing the three different substrates.

The metallopeptidase inhibitors OPA and EDTA, at 1 mM concentrations, reduced caseinolytic activity by 73 and 16%, respectively. At 10 mM, OPA completely inhibited MvP1 activity. The serine peptidase inhibitor PMSF did not affect caseinolytic activity.

The optimum reaction temperature for purified MvP1 was 40 °C and at 5 °C 35% of the relative activity still remained. Following incubation at 50 °C for 30 minutes, peptidase activity dropped significantly, and at 60 °C no residual activity was measured. Two isoelectric points were determined in a sample of the purified MvP1 peptidase, containing the I1 and I2 isotypes, with calculated pI of 4.4 and 4.2. Isolated MvP1 partially degraded both salmon and cod IgM heavy chain (Paper II).

4.4.3 Toxicity (Paper II)

At concentrations up to 0.22 µg/g fish the isolated MvP1 peptidase was non-lethal to salmon. However, extensive tissue necrosis and hemorrhages were seen at the injection site following injection with MvP1 in concentrations as low as 0.06 µg/g fish, and ascites formation was detected after i.p. injection (Fig. 9).

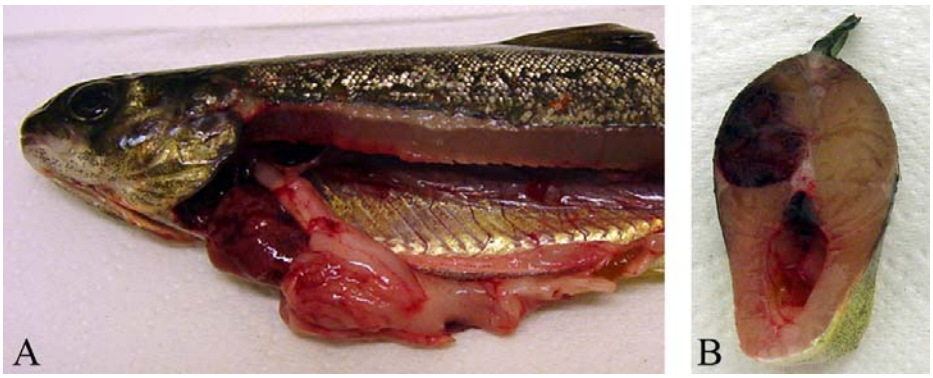

Fig. 9. Salmon injected with isolated MvP1 peptidase.
Internal hemorrhages and tissue necrosis following A) i.p. injection; B) i.m. injection of isolated MvP1 peptidase.

Cytotoxic activity of purified MvP1 against EPC and BF-2 cells was evaluated. The effects observed in both cell types were similar. The MvP1 peptidase did not cause prominent cell death, which was only detectable at higher concentrations, but caused detachments from well edges and ruptures in the cell monolayer (Fig. 10). MvP1 was non-hemolytic against salmon and sheep erythrocytes.

4.4.4 Sequencing (Paper II)

A potential *mvp1* ORF of 2.205 nt encoding a 734 aa polypeptide with calculated MW of 79.095 was identified (Fig. 11). The nt sequence of the *mvp1* gene and flanking sequences have been deposited in the GenBank database (accession number EU876833). The gene contained a putative signal sequence, with a predicted

cleavage site after Ala-25. The determined aa sequence for the N-terminal end of MvP1 (ADATGFGGNEKTGKYHYGTDF) was found within the polypeptide, starting at Ala-211. A conserved zinc-binding motif of metallopeptidases, HEXXH, was found within *mvp1* (aa 351-355), and also a third zinc ligand motif, GXXNEXXSD (aa 371-379) (Fig. 11). A potential Shine-Dalgarno (SD) sequence (nt: ggagac) was identified upstream of the predicted Met start codon (nt -10 to -5). An inverted repeat sequence, characteristic of a Rho-independent transcription determinator was found downstream of the stop codon (nt 2227-2243).

The MvP1 prepropeptide contained several conserved domains which could be divided into four regions; a signal peptide, an N-terminal propeptide that contains an FTP domain (aa 64-114) and a PepSy domain (aa 127-205), the mature peptidase, consisting of an M4 domain (aa 217-360) and an M4 C-terminal domain (aa 362-506), and finally a C-terminal propeptide, containing two PPC domains (aa 543-612 and 650-719) (Fig. 12).

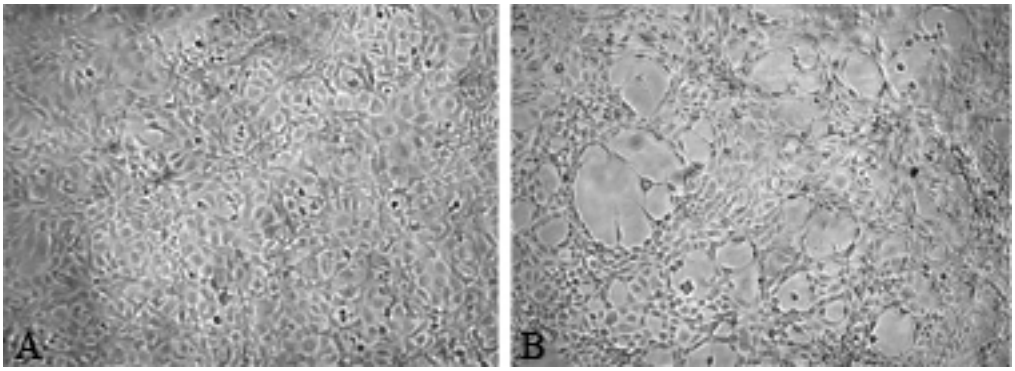

Fig. 10. Effects of isolated MvP1 peptidase on EPC cell culture.
A) control cells and B) cells incubated with isolated MvP1 at 15 °C for 24h. EPC, epithelioma papulosa carpio.

Fig. 11. Nucleotide sequence of the *mvp1* gene.
Deduced amino acids of the MvP1 prepropeptide are shown below the first nucleotide of each codon. The first nt of the Met initiation codon is set as +1 and the initiation Met is set as the first aa. A putative SD sequence is double-underlined and a predicted signal peptidase cleavage site is shown with a vertical arrow. The determined N-terminal amino acid sequence of MvP1 is underlined. A HEXXH zinc-binding motif is shaded and a third zinc ligand-motif is boxed. A putative Rho-independent transcription determinator (inverted repeat sequence) is indicated by convergent arrows.

The calculated size of the MvP1 protein from the N-terminal end was 56 kDa, whereas the purified peptidase was seen as a 38 kDa band or smaller by SDS-PAGE. This suggests that MvP1 undergoes polypeptide removal from the carboxy terminal. The translated protein sequence of the predicted mature peptidase (aa 217-506) had highest similarity with vibriolysins (EC 3.4.24.25, Fig. 13). It showed highest percent sequence similarity with the MprI peptidase of *Pseudoalteromonas piscicida* (80% sequence identity, BAB79615) (Paper II).

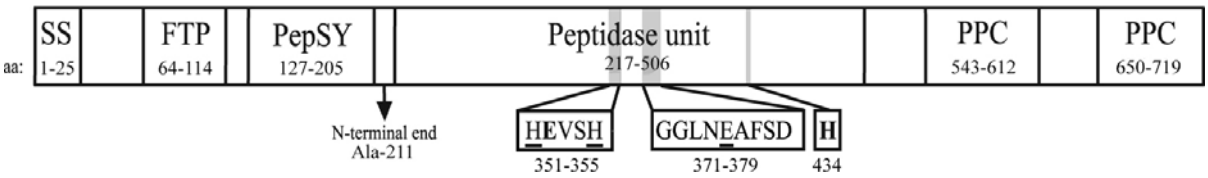

Fig. 12. Schematic figure of the MvP1 prepropeptide.
The figure shows the domains and motifs of the MvP1 prepropeptide, with amino acid numbering below. An arrow indicates the determined N-terminal end of the peptidase. Predicted metal ligands are underlined and active site residues are shown in bold. SS, signal sequence; FTP; fungalysin/thermolysin propeptide motif; PepSY; peptidase propeptide and YPEB domain; PPC, bacterial pre-peptidase C-terminal domain; aa, amino acid

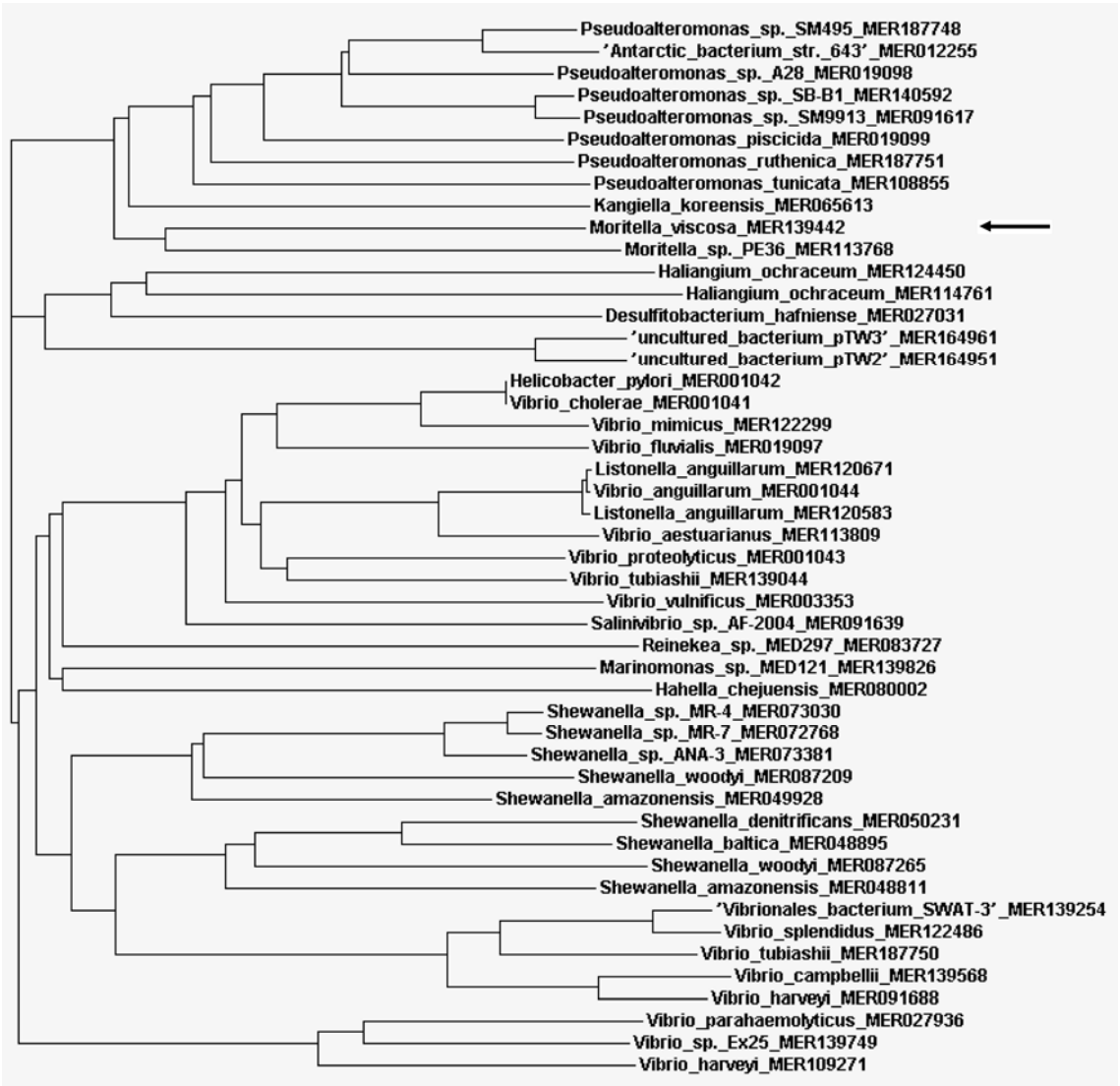

Fig. 13. Tree of vibriolysin peptidases.
A difference matrix is calculated from sequence alignment of peptidase units and an evolutionary tree generated by using the UPGMA algorithm. An arrow indicates the location of the *Moritella viscosa* MvP1 vibriolysin. Numbers behind organism names indicate the respective MEROPS identifier. Taken from the MEROPS database (http://merops.sanger.ac.uk, page created April 30th 2010).

4.4.5 Production of MvP1 by *M. viscosa* isolates (Paper I)

The ECPs of 22 *M. viscosa* isolates originating from different host species and geographical locations were screened for MvP1 production. All isolates produced the 40 and 38 kDa bands, detected by casein zymogram, and were positive in an anti-MvP1 Western blot. ECPs of the *M. marina*, *M. japonica* and *A. wodanis* isolates did not produce the 40 and 38 kDa bands and responded negatively in an anti-MvP1 Western blot.

4.4.6 Construction of MvP1 negative *M. viscosa* mutants was unsuccessful

To further investigate the virulence functions of the MvP1 peptidase, MvP1 negative *M. viscosa* mutant construction was attempted.

Resistance of *M. viscosa* against an antibiotic which the donor strain S17-1 was sensitive to needed to be identified for successful *M. viscosa* selection following conjugation. *M. viscosa* isolate 6 was sensitive to all antibiotic tested, except kanamycin, which was thereafter used for selection. Isolate 6 was resistant to kanamycin at concentrations up to 100 µg/ml, while isolate 1 was less kanamycin resistant, capable of growing at 50 µg/ml, but not at 100 µg/ml (at 2% NaCl).

An in-frame *mvp1* deletion template and three site directed mutagenesis templates were successfully created, cloned into the pDM4 suicide vector and transformed into the S17-1 donor strain. However, despite multiple attempts at varying conditions no chloramphenicol resistant transconjugant *M. viscosa* colonies were detected following bacterial conjugation of the S17-1 donor strain and the *M. viscosa* 6 recipient strain. Construction of MvP1 negative mutants was therefore unsuccessful and not completed.

4.4.7 Vibriolysin genes detected in the *M. viscosa* genome

Screening of the genome of *M. viscosa* isolate 1 revealed an *mvp1* gene showing only one aa difference to the MvP1 of isolate 6 (aa 29 from T to I). The screening also revealed two more vibriolysin genes containing the HEVSH motif.

The first gene (1866 nt), hereafter termed *mvp2*, showed 84% aa identity (90% aa similarity) with the mature peptidase region of a putative metalloproteinase of *Moritella* sp. PE36 (ZP_01898184), and 73% aa identity (86% aa similarity) with the mature peptidase region of MvP1 (Fig. 14). The predicted protein (MvP2) contained

similar domain structure as MvP1, but only one PPC domain was present on the C-terminal end. Sequences (1500 and 760 nt) from the gene were successfully amplified from isolate 6 using primers having 55% nt identity or less with *mvp1* sequences. Antigenic peptide prediction of the mature peptidase region of both MvP1 and the predicted MvP2 peptidase revealed that the sequences had several common predicted epitopes with high aa sequence similarities (Fig. 14).

```
MvP1 GGNEKTGKYHYGTDFGYLNVGQSGNNCIMNNTNVKTINLNHGTNGSSAFSFTCPENTVKS 60
MvP2 GGNEKTGEYRYGLDFKPLNVSQSGDTCTMSNKQLKTVNLNHETEGTTAFSFTCPENTAKQ 60
     *******:*:** **  ***.***:.* *.*.::**:**** *:*::**********.*.

MvP1 INGAFSPLNDAHYFGGVVFDMYNDWINTAPLSFQLKMRVHYSKDYENAFWDGTAMTFGDG 120
MvP2 INGAYSPLNDAHFFGGVIFDMYKDWLNVSPLTSQLVMRVHYDKNYENAFWNGESMTFGDG 120
     ****:*******:****:****:**:*.:**: ** *****.*:******:* :******

MvP1 ESYFYPLVSLDVSAHEVSHGFTEQNSGLVYEAKSGGLNEAFSDMAGEAAEFFMSGTNDWQ 180
MvP2 ADTFYPLVSLDVSAHEVSHGFTEQNSGLEYKGKSGGLNESFSDMAGEAAEFYMNGSNDWL 180
      . ************************ *:.*******:**********:*.*:***

MvP1 VGAQIFKGNGALRYMDEPTRDGKSIDHQSNYNSGMDVHNTSGVYNKAFYNLATTVGWDTK 240
MvP2 VGAQILKGSGGLRSMSNPTMDGNSIDNQADYSNIMNVHHSSGVYNKAFYNLATTSGWDTE 240
     *****:**.*.** *.:** **:***:*::*.. *:**::*************** ****:

MvP1 KAFIVYAKANQLYWSANTNWDEAGNGVMDAACDLGYSTDEVKASLAAVGI 290
MvP2 KAFVLYATANQLYWTPNSNWDDAGNGVMDAACDLGYSVDDVLASLTAVGI 290
     ***::**.******:.*:***:***************.*:* ***:****
```

Fig. 14. Sequence alignment of mature peptidase regions of MvP1 and MvP2.
Predicted epitopes are underlined.

The second gene (5400 nt), termed *mvp3*, showed 68% aa identity (83% aa similarity) with the mature peptidase region of a *Shewanella violacea* thermolysin (BAJ03844), and 55% aa identity (74% aa similarity) with the mature peptidase region of MvP1 (Fig. 15). The domain structure of the predicted protein (MvP3) included an N-terminal FTP domain (aa 72-114), a mature peptidase (M4 domain aa 224-367 and M4 C-terminal domain aa 369-510) and a C-terminal HYR domain (aa 1105-1182). Antigenic peptide prediction of both MvP1 and MvP3 peptidase regions revealed some convergence of predicted antigenic peptides of high sequence similarities (Fig. 15).

```
Mvp1 GGNEKTGKYHYGTDFGYLNVGQSGNNCIMNNTNVKTINLNHGTNGSSAFSFTCPENTVKS 60
Mvp3 GGNAKVGQYEYGTHYTALDVTQNGNDCLLENKNVRTVDLQHGTTNNDTFAFTCSRNEYKE 60
     *** *.*:*.***.:  *:* *.**:*:::*.**:*::*:***....:*:***..*  *.
Mvp1 INGAFSPLNDAHYFGGVVFDMYNDWINTAPLSFQLKMRVHYSKDYENAFWDGTAMTFGDG 120
Mvp3 INGAYSPLNDAHGFGNAVFDMYRDWYGLAPLTFQLVMKVHYNNNYANAFWDGSSMTFGDG 120
     ****:******* **..*****.** . ***:*** *:***.::* ******::******
Mvp1 ESYFYPLVSLDVSAHEVSHGFTEQNSGLVYEAKSGGLNEAFSDMAGEAAEFFMSGTNDWQ 180
Mvp3 DNNIYPLVSLDIVAHEVSHGFTDQNSDLIYNGQSGGINESFSDMAGETAELFVRGSNDWL 180
     :. :*******: *********:***.*:*:.:***:**:*******:**:*: *:***
Mvp1 VGAQIFKGNGALRYMDEPTRDGKSIDHQSNYNSGMDVHNTSGVYNKAFYNLATTVGWDTK 240
Mvp3 IGADITKNGGAFRFFEDPTLDGKSIGHAENYYENIDVHHSSGVFNRAFYILSNTPGWNIK 240
     :**:* *..**:*:::** *****.* .** ..:***::***:*:*** *:.* **: *
Mvp1 KAFIVYAKANQLYWSANTNWDEAGNGVMDAACDLGYSTDEVKASLAAVGI 290
Mvp3 KAFSIMLDANRFYWTQSTDFVDGACGAINASVDKGYDAFPVINAFSQ--- 287
     *** :  .**::**: .*:: :.. *.::*: * **.:  *  :::
```

Fig. 15. Sequence alignment of mature peptidase regions of MvP1 and MvP3.
Predicted epitopes are underlined.

4.5 Protein identification in *M. viscosa* culture supernatants

Six proteins were identified from the culture supernatant of *M. viscosa*, using N-terminal sequencing or mass spectrometry. The translated aa sequences of all six proteins are given in the appendix.

A protein band of approximately 32 kDa had the N-terminal sequence YDNYSLFSD and matched to the predicted N-terminal end of a single hypothetical secreted protein found in the genome of *M. viscosa* isolate 1. The gene was amplified from the genome of isolate 6, cloned into a Topo vector and sequenced. The nt sequence has been submitted to GenBank (accession number HM453331). The deduced aa sequence had highest similarity with a hypothetical protein of *Vibrio* sp. AND4 (accession number EDP60703), showing 53% aa identity (73% aa similarity).

A second band of approximately 33 kDa had the N-terminal sequence AEVYNN, matching to the predicted N-terminal end of a single hypothetical secreted protein found in the *M. viscosa* genome. The gene encoded for an outer membrane protein (OmpH) and the deduced aa sequence had 79% identity (94% similarity) with a predicted outer membrane protein of *Moritella* sp. PE36 (accession number ZP_01900001).

A third band of approximately 23 kDa had the N-terminal sequence ANKRSVM, and matched to a single translated sequence in the *M. viscosa* genome. The gene encoded for a stringent starvation protein A (SspA), a cytoplasmic RNA polymerase-associated regulatory protein important for stress responses during

stationary phase or under nutrient-limited conditions. The deduced aa sequence had 100% identity with a *Moritella* sp. PE36 *sspA* gene (accession number ZP_01899250).

A fourth band of approximately 25 kDa had the N-terminal sequence ATPHINA, matching to a single translated sequence in the *M. viscosa* genome. The gene (*deoD*) encoded for a putative purine nucleoside phosphorylase, a cytoplasmic enzyme acting in nucleotide transport and metabolism. The deduced aa sequence had 98% identity (100% similarity) with a predicted purine nucleoside phosphorylase of *Moritella* sp. PE36 (accession number ZP_01896245).

A fifth band of approximately 34 kDa had the N-terminal sequence SNQLEQL, matching to a single translated sequence in the *M. viscosa* genome. The gene encoded for a putative transaldolase, a cytoplasmic enzyme involved in carbohydrate transport and metabolism. The deduced aa sequence had 97% identity (99% similarity) with a predicted transaldolase of *Moritella* sp. PE36 (accession number ZP_01899105).

A single protein was identified by Mascot search using peptide mass spectra acquired from MALDI-TOF analysis. The exited band of approximately 52 kDa gave a significant Mascot score of 102 ($p < 0.05$) with seven peptide mass values matched and 19% sequence coverage with the *Moritella* sp. PE36 dihydrolipoamide dehydrogenase (accession number ZP_01898040). The cytoplasmic enzyme is predicted to have functions in energy production and conversion. The gene encoding the dehydrogenase was identified in the *M. viscosa* genome and the deduced aa sequence had 98% identity (99% similarity) with the dehydrogenase of *Moritella* sp. PE36.

4.6 Effects of *M. viscosa* on pro-inflammatory gene expression (Paper III)

4.6.1 *M. viscosa* cytotoxicity

The effects of *M. viscosa* antigens on SHK-1 cell viability were determined (Fig. 16). Live *M. viscosa* cells caused significant cytotoxicity in SHK-1 cells at 72 h in all densities tested, MOI 6, 3 and 0.3, and at 24 h with the highest density. Heat-killed *M. viscosa* cells did not affect SHK-1 cell death at any time point. *M. viscosa* ECP caused significant cell death at 24 and 72 h in a concentration of 0.5 µg protein/ml, but a concentration of 0.05 µg protein/ml ECP did not affect cell viability. The MvP1

peptidase did not cause significant cell death at a 1 µg protein/ml concentration. Cytotoxicity assay results were used to determine the upper limits of *M. viscosa* antigen doses used in cell stimulation experiments.

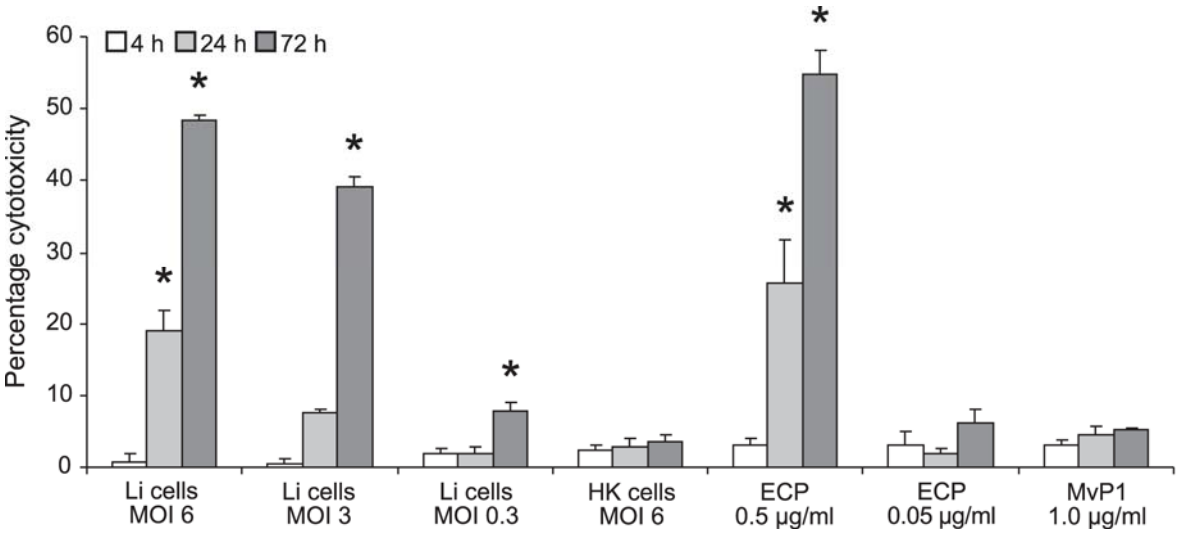

Fig. 16. Cytotoxicity of SHK-1 cells following *M. viscosa* antigen stimulation.
Mean percent cell death (±SEM) in SHK-1 cells treated with *M. viscosa* antigens for 4, 24 and 72 h. Li, live; HK, heat-killed; MOI, multiplicity of infection; ECP, extracellular products; MvP1, peptidase. Concentration of ECP and MvP1 is given in µg protein/ml. An asterisk signifies a significant difference from negative controls ($p < 0.05$).

4.6.2 EF-1A reference gene expression

The mean expression of the EF-1A reference gene was calculated from values of both stimulated and unstimulated cells at each time point. Following stimulation using *M. viscosa* cells or ECP, EF-1A expression levels were not significantly different. However, following stimulation with the MvP1 peptidase, EF-1A expression values were significantly different between sampling times (Paper III). To compensate for differences in the expression of the reference gene, the average copy number at each time point was used to calculate the relative expression of both IL-1β and IL-8.

4.6.3 Effects of *M. viscosa* cells and ECP

Mean relative expression of IL-1β in control cells, following stimulation with *M. viscosa* cells or ECP, was $5.2 \times 10^{-4} \pm 0.84 \times 10^{-4}$, $4.9 \times 10^{-4} \pm 0.95 \times 10^{-4}$ and $5.4 \times 10^{-4} \pm 0.5 \times 10^{-4}$ at 4, 24 and 72 h, respectively. The values did not differ significantly. Stimulation with live *M. viscosa* cells for 24 h caused a significant 7.1-fold increase in IL-1β expression, while heat-killed cells did not cause a significant increase in IL-1β

expression, with only a two-fold increase. ECP did not cause a significant increase in the expression of IL-1β (Fig. 17A).

Mean relative expression of IL-8 in control cells was $2.1 \times 10^{-2} \pm 0.21 \times 10^{-2}$, $2.0 \times 10^{-2} \pm 0.34 \times 10^{-2}$ and $1.0 \times 10^{-2} \pm 0.07 \times 10^{-2}$ at 4, 24 and 72 h, respectively, with no significant differences. Live and heat-killed *M. viscosa* cells caused a significant increase in the expression of IL-8, showing 9.3- and 6.6-fold expression, respectively. The difference between stimulation with live and heat-killed cells was not significant. *M. viscosa* ECP (0.01 µg protein/ml) caused a significant 34.1-fold increase in IL-8 expression at 72 h, but not at 4 or 24 h. At a concentration of 0.05 µg protein/ml, ECP caused a significant increase in IL-8 expression at all sampling times (5.8 to 19.1-fold) (Fig. 17B).

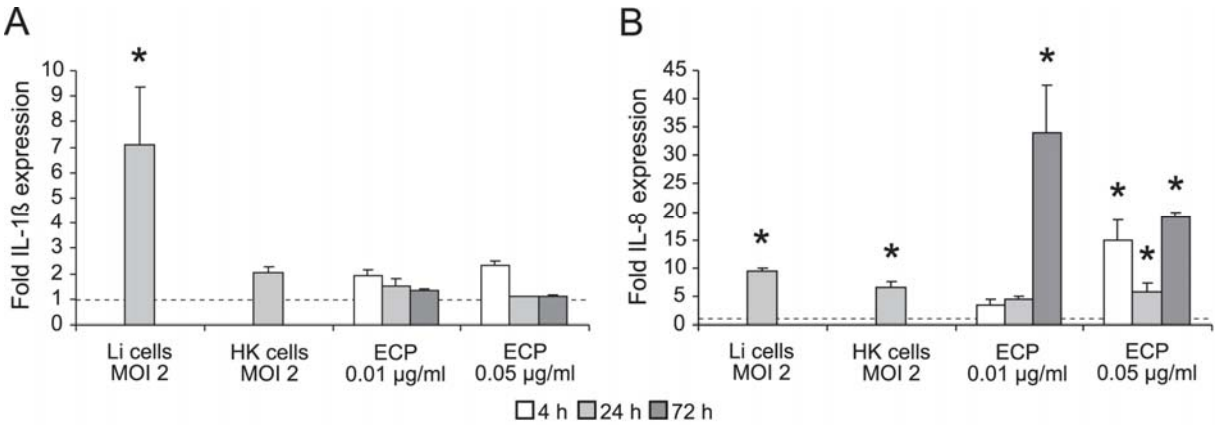

Fig. 17. IL-1β and IL-8 expression in SHK-1 cells following stimulation with *M. viscosa* cells or ECP.
A) Fold IL-1β and B) IL-8 expression in stimulated SHK-1 cells compared to control cells, following stimulation for 4, 24 and 72 h. Expression was calculated as relative to the expression of a reference gene. Li, live; HK, heat-killed; MOI, multiplicity of infection; ECP, extracellular products. Concentration of ECP is given in µg protein/ml. An asterisk signifies a significant difference from the relative control ($p < 0.05$) and a dotted line signifies the expression in control cells. Note the different Y-axis scales.

4.6.4 Effects of isolated MvP1

Following stimulation with isolated MvP1 peptidase, mean relative IL-1β expression in control cells was $6.5 \times 10^{-4} \pm 1.9 \times 10^{-4}$, $1.2 \times 10^{-3} \pm 0.29 \times 10^{-3}$ and $8.9 \times 10^{-4} \pm 0.88 \times 10^{-4}$ at 4, 24 and 72 h, respectively. No significant differences were measured between control values. Only cells stimulated with the highest concentration of MvP1 showed a significant 2.6-fold increase in IL-1β expression at 4 h (Fig. 18A).

Mean relative expression of IL-8 in control cells was $8.0 \times 10^{-3} \pm 0.47 \times 10^{-3}$, $7.4 \times 10^{-3} \pm 4.1 \times 10^{-3}$ and $1.2 \times 10^{-2} \pm 0.25 \times 10^{-2}$ at 4, 24 and 72 h, respectively, and did

not show any significant differences. IL-8 expression exhibited dose-dependence on the concentration of MvP1 at 4 and 24 h, but was only significantly higher from control cells at the highest concentration (1.0 µg protein/ml MvP1), or 4.9- and 9.4-fold, respectively. No significant differences were detected at 72 h (Fig. 18B).

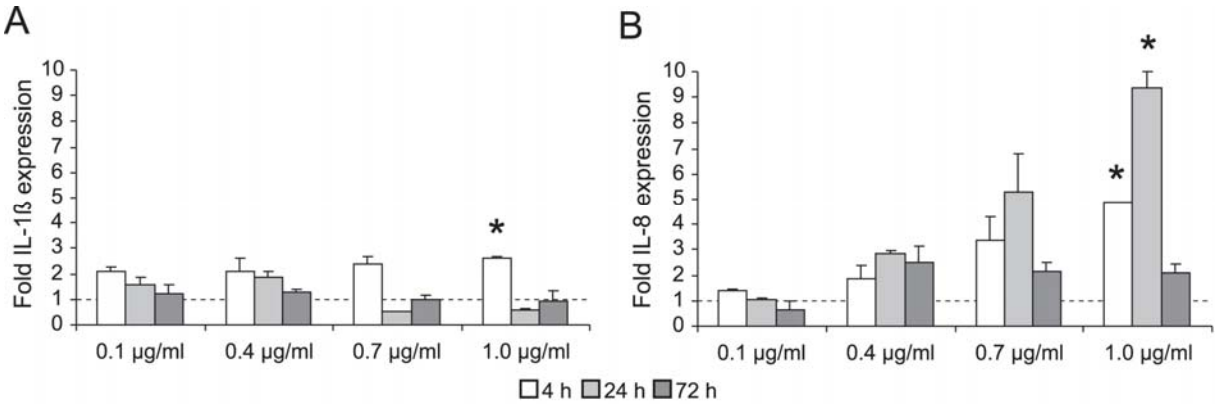

Fig. 18. IL-1β and IL-8 expression in SHK-1 cells following stimulation with *M. viscosa* MvP1 peptidase.
A) Fold IL-1β and B) IL-8 expression in stimulated SHK-1 cells compared to control cells, following stimulation for 4, 24 and 72 h. Expression was calculated as relative to the expression of a reference gene. Concentration of MvP1 is given in µg protein/ml. An asterisk signifies a significant difference from the relative control ($p < 0.05$) and a dotted line signifies the expression in control cells.

4.7 *M. viscosa* type VI secretion systems (Paper IV)

4.7.1 Identification of two T6SSs in the *M. viscosa* genome

Two putative T6SSs were identified in the genome of *M. viscosa* isolate 26, termed Moritella Type Six Secretion 1 and 2 (*mts1* and *mts2*). The *mts1* locus consisted of 23 predicted coding sequences (CDSs) organized into three operons and four single genes (Fig. 19A). The *mts2* locus was predicted to consist of 27 CDSs organized into five operons and one single gene (Fig. 19B). Most of the genes identified in the two T6SS loci are T6S specific, including those encoding Hcp (*mts1-M* and *mts2-M*) and VgrG (*mts1-V* and *mts2-V*). The predicted VgrG proteins do not carry C-terminal extensions with potential effector domains. Genes encoding putative VgrG or Hcp were not identified outside of the *mts1* or *mts2* loci.

Homologous CDSs in the two *mts* loci were less than 25% identical to each other at the aa level, except for *mts1-X* and *mts2-X* encoding ClpV ATPases, which

were 42% identical. This lack of sequence conservation, together with different structural organization between the two loci suggests that they have been acquired in separate events, rather than having arisen from a duplication event.

The *M. viscosa mts* loci were compared against other T6SS loci. The *mts1* locus showed sequence similarity and synteny to a T6SS of the fish pathogen *Aliivibrio salmonicida* (Fig. 20A). The *mts2* locus showed high similarities and synteny to a *Vibrio parahaemolyticus* T6SS locus (Fig. 20B). The *mts1* was similar to a T6SS locus found in the genome of the non-pathogenic *Moritella* sp. PE36, but no system similar to *mts2* was identified in the genome (results not shown).

4.7.2 Active Mts1 T6SS in *M.viscosa*

V. anguillarum (Vts T6SS) anti-Hcp antibodies bound strongly to two protein bands in the supernatant of *M. viscosa* isolate 1 (Fig. 21). The bands were approximately 14 and 15 kDa, and had the same N-terminal aa sequence; (AS)IYMRID, where the first two aa were not determined with full certainty. The *M. viscosa* genome (isolate 26) gave only one match to the IYMRID sequence, which was the gene encoding *mts1-M* (Hcp). Hcp proteins of Mts1 T6SS were detected in culture supernatants and cell pellets of all ten *M. viscosa* isolates tested (isolates 1, 2, 6, 7, 14, 15, 17, 20, 22 and 26; results not shown). Immunodetection of bands, representing any of the N-terminally determined Hcp proteins, was defined as positive production.

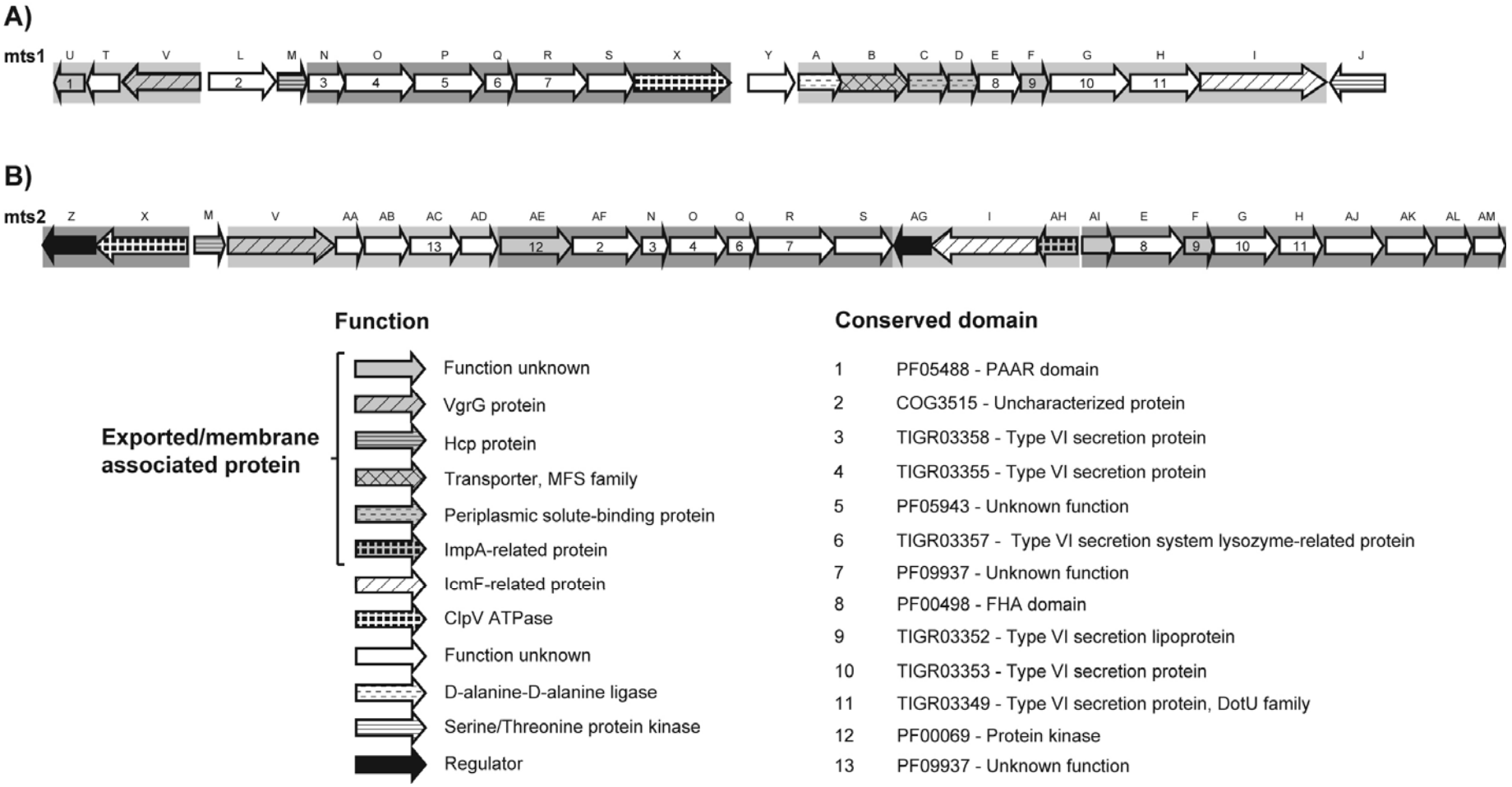

Fig. 19. *M. viscosa* T6SS loci.

Genetic organization of *M. viscosa mts1* (A) and *mts2* (B) T6SS loci. Different arrows indicate functional annotations associated with genes. Numbers indicate conserved domains found in genes with no functional annotation. Grey boxes mark the boundaries of predicted operons within each *mts* loci. T6SS, type VI secretion system; *mts*, Moritella Type Six secretion locus.

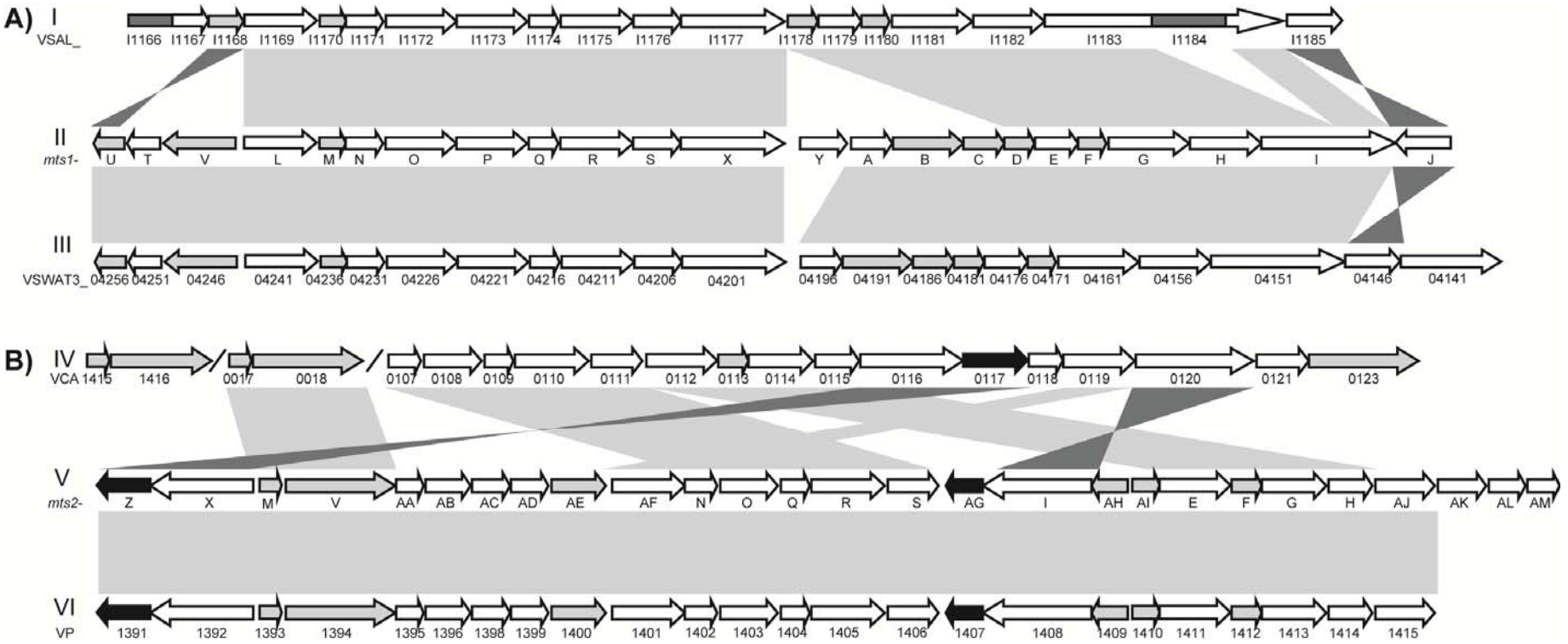

Fig. 20. T6SS comparison.

Comparison of *Moritella viscosa* A) *mts1* (II) against *Aliivibrio salmonicida* LFI1238 (I) and *Vibrionales* bacterium SWAT-3 (III), and B) *mts2* (V) against *Vibrio cholerae* N16962 (IV) and *Vibrio parahaemolyticus* RMID 2210633 (VI) type VI secretion system (T6SS) loci. Grey arrows indicate putative exported or membrane associated proteins, white arrows indicate putative cytosolic proteins, black arrows indicate regulators and dark grey boxes indicate insertion sequence elements. For the comparison, light grey boxes indicate similar regions between T6SSs and dark grey hour-shaped boxes indicate similar regions, but in opposite directions. *mts*, Moritella Type Six secretion locus.

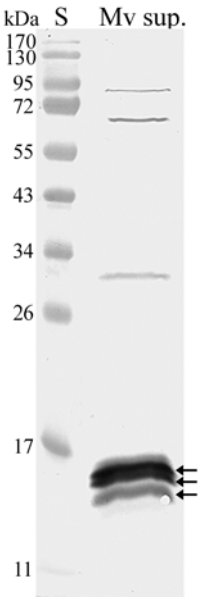

Fig. 21. Hcp secretion in *M. viscosa*.
Immunoblot of *M. viscosa* culture supernatant using *Vibrio anguillarum* (Vts T6SS) anti-Hcp antibodies. Bands identified as Hcp are indicated with arrows. Lane S, protein standard; lane Mv sup., *M. viscosa* supernatant. T6SS, type VI secretion system; Hcp, hemolysin coregulated protein.

5 DISCUSSION

The study was aimed at improving the understanding of *M. viscosa* pathogenicity, with emphasis on secreted factors. Virulence properties of *M. viscosa* ECP were evaluated and demonstrated the importance they may have on the development of winter ulcer disease. Indications of host adaptation were observed, as isolates originating from salmon were less cytotoxic than isolates originating from other host species. Isolates showing different virulence in salmon, as identified in the study, may be useful tools for future research on *M. viscosa* virulence (Paper I). A previously unknown extracellular vibriolysin, MvP1, with virulence related activities was identified and characterized (Paper II). Furthermore, two additional vibriolysins, MvP2 and MvP3, were genetically identified. Attempts to construct MvP1 negative mutants were unsuccessful and the importance of this peptidase in *M. viscosa* virulence was therefore not fully determined. The effects of selected *M. viscosa* antigens on cytotoxicity and pro-inflammatory gene expression in a salmon cell-culture model were measured (Paper III). Live *M. viscosa* cells and ECP were cytotoxic, but heat-killed cells and the MvP1 peptidase were non-cytotoxic. Live *M. viscosa* cells induced IL-1β expression but heat-killed cells did not. Furthermore, MvP1 was not the main secreted stimulant of pro-inflammatory gene expression. Finally, two T6SS loci were identified in *M. viscosa* (Paper IV). The systems were genetically described and compared to previously reported T6SSs. Active Mts1 T6S was found in both virulent and avirulent isolates, while function of the *mts2* locus was not confirmed. The specific roles of T6SSs in *M. viscosa* are not elucidated.

5.1 Virulence properties of *M. viscosa* ECP

The virulence properties of *M. viscosa* ECP were evaluated in Paper I. The natural variability of *M. viscosa* isolates from winter ulcer disease outbreaks of different geographical locations and host origins was used to shed light on adaptation of isolates and potential virulence mechanisms.

The two ECP production methods applied affected the properties of the ECPs. Broth cultured ECP (B-ECP) had much lower protein concentration but was more

virulent than cellophane produced ECP (C-ECP) of the same isolate. B-ECP was therefore used for all following virulence assays, unless otherwise noted.

Out of 22 *M. viscosa* isolates, all except two were found to be virulent when i.p. injected into salmon and the LD_{50} of nine selected isolates ranged from 2 x 10^4 to > 2 x 10^6 CFU/fish. Previously reported LD_{50} determinations for *M. viscosa* have been from i.m. injections, giving lower LD_{50} values (Benediktsdottir et al., 1998a; Benediktsdottir et al., 2000). Two Canadian *M. viscosa* isolates from salmon were found to be non-virulent. Furthermore, the respective ECPs were non-lethal to salmon. The low-virulent isolate 5 also produced non-lethal ECP. This indicates the importance *M. viscosa* ECP may have on the onset and/or development of winter ulcer disease.

Production of ECPs was performed at 15 °C, a higher temperature than at which the bacterium infects fish. Temperature selection was based on data showing that temperature has little effect on production of *M. viscosa* extracellular proteins and toxic factors (Tunsjo et al., 2007; Tunsjo et al., 2009). To compare isolate virulence to ECP virulence, isolates were also cultured at 15 °C prior to injection, which may have reduced their virulence properties (Tunsjo et al., 2007; Tunsjo et al., 2009).

Lethality of ECPs of all 22 isolates was tested in salmon. Lethality was not correlated with ECP protein concentration, indicating a difference in production of certain unknown toxins between isolates. ECPs of *M. marina*, *M. japonica* and *A. wodanis* were non-lethal and showed negative or low cytotoxic and hemolytic activities, reflecting the non-pathogenic nature of the species.

M. viscosa isolates from salmon showed lower cytotoxic and hemolytic activities than isolates from other species of fish. ECPs of isolate 5 (from salmon) and isolate 20 (from cod) did, however, deviate from this pattern. The consistency between cytotoxic and hemolytic activities of *M. viscosa* ECPs indicates that the same bacterial product/s is responsible for membrane disruption of both EPC cells and salmon erythrocytes. The differences observed in hemolytic and cytotoxic activities may be the result of *M. viscosa* host adaptation. *M. viscosa* serotypes have been found to partially follow host species origin of isolates (Heidarsdottir et al., 2008), whereas genotypes have been shown to reflect different geographical origins of isolates (Benediktsdottir et al., 2000). A recent publication has now revealed that *M. viscosa* consist of two genetically and phenotypically distinct clusters (Grove et al., 2010). One cluster, which includes the type strain, contains isolates from Atlantic salmon in Norway, Scotland and

the Faroe Islands. The other "variant" cluster contains isolates from Icelandic and Canadian salmon, isolates from Norwegian and Icelandic rainbow trout and isolates from lumpsucker and cod. Only two isolates from Norwegian salmon grouped within the variant cluster. The apparent host adaptation in hemolytic and cytotoxic activities of ECP clearly does not follow this clustering.

Cytotoxic- and hemolytic activities did not represent the lethal factor secreted by *M. viscosa* isolates. Furthermore, different virulence of ECPs of *M. viscosa* isolates could not be explained by any of the activities or factors that were measured. The study showed that *M. viscosa* secrets an unidentified toxic factor, which previously has been identified as a protein, as the lethal activity is heat sensitive (Benediktsdottir et al., 1997).

ECPs of all *M. viscosa* isolates showed caseinase activity and the band pattern in zymograms was very similar for all isolates. Esterase activity was detected by the microwell assay in B-ECPs of all isolates, but no bands were detected in α-naphthyl acetate-azo dyed gels using the same substrate. Fractionation of C-ECP, containing 36 and 33 kDa esterase bands, indicated that the microwell assay detected esterase activity which is not stained by the gel assay method (Paper I).

ECP of an N-Iceland salmon isolate (7) behaved differently from the other ECPs. High caseinase activity of the isolate ECP may have caused protein degradation which could explain its low protein concentration and reduced activities. Previously, salmon isolates from N-Iceland have been shown to form a distinct genotype and showed biochemical differences and distinct whole cell protein profiles compared to other *M. viscosa* isolates (Benediktsdottir et al., 2000).

Protein identification

Production of six *M. viscosa* proteins found in culture supernatants was confirmed by N-terminal sequencing or mass spectrometry. The genes encoding all six proteins had previously been identified by genomic sequencing. Four of the proteins are predicted to be found in the cytoplasm, one is an outer membrane protein and only one, a protein of unknown function, is likely to be secreted. It therefore seems that cell lysis had occurred in culture supernatants used for protein identification.

ECP autodegradation

Preliminary work with *M. viscosa* cultures and ECP revealed considerable autolysis of bacterial products, even during storage at low temperatures (4 °C). This autolysis was proposed to be caused by extracellular peptidases and raised interest in studying the major secreted peptidases of *M. viscosa*, and to study their possible roles in virulence.

Autodegradation of ECP was measured following incubation at 15 °C and compared to ECP incubated with a specific metallopeptidase inhibitor. Following five day incubation, protein concentration of ECP had halved, compared to less than 20% decrease in protein content of ECP with restricted metallopeptidase activity. Therefore, the major part of ECP protein autodegradation appeared to be caused by one or more metallopeptidases.

5.2 The *M. viscosa* MvP1 vibriolysin

Isolation and characterization

The major extracellular *M. viscosa* peptidase, MvP1, was isolated and characterized as a vibriolysin (Paper II). Three active isoforms of MvP1 of different MW were identified, termed I1, I2 and I3, mainly appearing after precipitation or storage of ECP and indicating degradation of a single polypeptide. The isolation of two or more isotypes of the same vibriolysin has been described previously (Wu et al., 1996; Miyoshi et al., 2002). MvP1 production seems to be universal in *M. viscosa* and was identified in all 22 isolates tested (Paper I). The MvP1 peptidase was active over a broad temperature range and was found to have relatively high activity at low temperatures, which probably reflects the low temperatures at which the bacterium is found and causes infections.

Isolated MvP1 caused extensive tissue necrosis and hemorrhages in salmon, but was non-lethal at the concentrations tested. ECP containing equal caseinolytic activities were, however, lethal. MvP1 does therefore not appear to be the lethal factor in *M. viscosa* ECP. The necrotic and hemorrhagic damage caused by MvP1 may be due to degradation of structural components and possibly has an effect on ulcer development. Bacterial peptidases which cause tissue damage and degradation of host tissues have been suggested to play roles in pathogenesis by facilitating invasion of the pathogen (Miyoshi et al., 2000). MvP1 did not cause prominent cell death in cell cultures but

affected cell-cell adhesions. Disruption of epithelial cell adhesions may aid the invasion and dispersion of *M. viscosa* in its host. MvP1 partially degraded salmon and cod IgM heavy chain, which might have an effect on the host immune system. The MvP1 peptidase may thus contribute to the pathogenesis of *M. viscosa* by obstructing the host's immune response.

The gene encoding the MvP1 peptidase was sequenced. Sequence alignments show that MvP1 is a new vibriolysin belonging to the thermolysin family of zinc metallopeptidases. The deduced aa sequence contained the conserved HEXXH zinc-binding motif of metallopeptidases (Rawlings & Barrett, 1995) and a third zinc ligand motif GXXNEXXSD, that is characteristic of the thermolysin family (Miyoshi et al., 2000). The aa sequence showed highest similarity to several vibriolysins from *Pseudoalteromonas* species (77-80% aa identity and 89-91% aa similarity).

The MvP1 prepropeptide consisted of a signal peptide, an N-terminal propeptide, a mature peptidase, and a C-terminal propeptide. The signal peptide is most likely cleaved off in the periplasmic space, and subsequently both the N-terminal and the C-terminal propeptides are cleaved off to form the mature peptidase. A recent study revealed that a *V. anguillarum* protease, termed Epp, cleaves a 10 kDa C-terminal propeptide of its secreted EmpA vibriolysin and thereby processing it to the mature enzyme (Varina et al., 2008). A gene encoding a predicted extracellular protein showing 68% aa identity (82% aa similarity) with the Epp peptidase was identified in the *M. viscosa* genome of isolate 1, indicating that the MvP1 peptidase may be processed in a similar way. The FTP and PepSY domains found within the N-terminal propeptide of MvP1 may have inhibitory or chaperone activities (Yeats et al., 2004; Marchler-Bauer et al., 2007; Demidyuk et al., 2008). The mature peptidase region consists of a M4 domain and a M4 C-terminal domain that is unique for the thermolysin family. Two PPC domains, which are not often found in mature peptidases, were detected in the C-terminal propeptide of MvP1.

No regulatory motifs or promoter sequences were determined upstream of the *mvp1* encoding gene. Previous studies have shown that several vibriolysin genes are under QS regulation (Croxatto et al., 2002; Kawase et al., 2004; Silva et al., 2004). No acylated homoserine lactone QS signals have been detected in *M. viscosa* (Bruhn et al., 2005; Johansen et al., 2007; B.K. Gudmundsdottir, oral communication, 2010), but genomic screening indicates that *M. viscosa* may contain a LuxS/PQ homolog QS

system (C. Karlsen, written communication, 2010), which would most likely utilize an autoinducer-2 type signal molecule.

Additional vibriolysin genes in the M. viscosa genome

Polyclonal antibodies raised against MvP1 isotype I1 bound all detectable caseinolytic bands secreted by *M. viscosa* at 4, 9 and 15 °C. It was therefore concluded that MvP1 was the only detectable caseinolytic enzyme secreted by the bacterium at the experimental conditions. After the publication of Paper II, two additional vibriolysin encoding genes were detected in the *M. viscosa* genome. Both genes encode for hypothetical vibriolysin peptidases, showing significant aa identity and common predicted epitopes with the MvP1 peptidase. Whether they are expressed, translated and secreted in active forms is not known. However, if they were present in the ECP of isolate 6, the antibodies raised against MvP1 may have cross reacted with them. Therefore, it cannot be excluded that additional peptidases, other than MvP1, were produced by *M. viscosa*.

Unsuccessful MvP1 mutant construction

To further identify the importance and role of the MvP1 vibriolysin in *M. viscosa* virulence, MvP1 negative mutant construction was initiated. The aim was to compare growth, peptidase profiles and virulence of wild-type isolates and MvP1 negative mutants. To the best of my knowledge, genetic manipulation of members of the *Moritellaceae* family has not been described so far.

M. viscosa mutant construction was not successfully completed and no transconjugant single crossover mutants were detected following repeated conjugation experiments. Attempts by others, using conjugation, transposon delivery or electroporation have also not been successful in constructing *M. viscosa* mutants (Tunsjo, 2009 ; H. Sorum and E. Benediktsdottir, written communications 2010). Genetic modification of *M. viscosa* therefore seems to be a complicated task, for reasons yet unknown, and the ideal method has not been identified.

The lack of identification of selective medium or antibiotic resistance for *M. viscosa* may be a hindering factor in mutant construction, for selection of transconjugant *M. viscosa* cells. Of all the antibiotics tested, *M. viscosa* was only resistant to kanamycin, which was subsequently used in the conjugation protocol. However, isolate

6 was only partially resistant, and isolate 1 was only slightly resistant. Furthermore, kanamycin resistance genes were not identified in the draft genome of isolate 1. Kanamycin may therefore not be ideal for selection of *M. viscosa* following conjugation.

For construction of site directed mutagenesis templates we chose to mutate the active site residue (E). This was based on previously published mutations on the EmpA vibriolysin of *V. anguillarum* (Yang et al., 2007a). Changing the active site E to A or K in recombinantly expressed EmpA resulted in a complete loss of proteolytic and cytotoxic activities. The importance of this residue for structural stability of the enzyme was also shown, through reduced binding of native EmpA antibodies.

Reduced virulence has been detected in *V. vulnificus* Vvp vibriolysin mutants following immersion challenges in eels (Valiente et al., 2008a) and in a *V. anguillarum* EmpA mutant following immersion challenges in rainbow trout (Milton et al., 1992). Whether MvP1 negative *M. viscosa* mutants would have shown reduced virulence in salmon is unknown.

5.3 *M. viscosa* stimulation of pro-inflammatory genes

The effects of selected *M. viscosa* antigens on pro-inflammatory gene expression in a salmon cell line model were evaluated in Paper III. Previously to this study, very little was known about the immunological response of fish to *M. viscosa* infection and the antigens responsible for triggering the host response. Information on which bacterial antigens trigger the fish immune response may be especially useful for the development of improved vaccines against winter ulcer disease. The model used in this study was a continuous cell line derived from Atlantic salmon head kidney leucocytes, SHK-1 cells, which have been used as a model system to study salmon immune responses (Fast et al., 2005; Fast et al., 2007; Martin et al., 2007a; Martin et al., 2007b). The pro-inflammatory cytokines IL-1β and IL-8 were chosen as indicators of an inflammatory response, which triggers a wide range of immune responses, and their expression following antigen stimulation measured using real-time Q-PCR.

Cytotoxic effects of *M. viscosa* antigens on SHK-1 cells were evaluated. Live cells were highly cytotoxic but heat-killed cells had no significant effect on cell

viability. Our results indicate that heat treatment of cells may have impaired the activity of important cytotoxins. *M. viscosa* ECP were also highly toxic, supporting previous findings of the study (Paper I) which indicate that virulence is related to ECP secretion. Isolated MvP1 had no significant effect on cell viability. Therefore, one or more unidentified components of the ECP are responsible for cell death.

Following 24 h of stimulation live *M. viscosa* cells caused a significant increase in IL-1β expression, but heat-killed cells or ECP did not. The different effects of live and heat-killed *M. viscosa* cells on IL-1β expression in SHK-1 cells can be the result of several different scenarios. Heat killing of bacterial cells may have destroyed stimulatory antigens. Some cytotoxicity, although not significant, may have been associated with live cells and dead/dying SHK-1 cells may therefore have acted as danger signals inducing IL-1β expression in the culture. Interaction of live *M. viscosa* cells with cellular membranes and/or ECP production may also be reasons for the differences. Gene expression may also have been stimulated through synergistic effects of *M. viscosa* cells and ECP. IL-8 expression was significantly increased by live and heat-killed cells and ECP. A higher concentration of ECP stimulated IL-8 expression faster than a lower concentration and the effect lasted throughout the experiment, for up to 72 h.

SHK-1 cells were stimulated with isolated MvP1 at concentrations which are believed to be within biologically relevant limits. However, concentrations of isolated MvP1 were up to 200-fold higher than would be expected from the ECP used to stimulate cells. Only the highest concentration of MvP1 stimulated IL-1β and IL-8 expression. Therefore, the increased expression of IL-8 seen after stimulation with ECP has not been caused by MvP1, or unknown factors in the ECP have enhanced the stimulatory effects of MvP1. A previous study has shown that the *V. cholerae* HA/P vibriolysin does not induce IL-8 expression or affect cell viability in human intestinal epithelial cells (Zhou et al., 2004).

IL-1β expression in SHK-1 cells has been examined in two previous studies by Fast et al. (2005; 2007), showing a rapid increase in IL-1β expression following LPS stimulation. LOSs produced by *M. viscosa* were presumably present on both live and heat killed cells, and since heat killed cells did not stimulate IL-1β expression the LOS does not seem to have affected IL-1β expression under the assay conditions. Recent studies by Lovoll et al. (2009) and Ingerslev et al. (2010) have shown systemic

upregulation of both IL-1β and IL-8 expression in *M. viscosa* infected salmon, but delayed upregulation of IL-1β and C3 expression in head kidney suggested that *M. viscosa* may have some escape mechanisms to evade the immune system.

Cytokine production is highly regulated and they usually have short half-lives. Prolonged expression of pro-inflammatory cytokines can lead to local tissue damage. Following stimulation with ECP, IL-8 expression was up-regulated for up to 72 h and therefore the pathology observed in fish suffering from winter ulcer disease may possibly be associated with the host's prolonged inflammatory reaction to ECP.

5.4 Two T6SSs in *M. viscosa*

Two potential *M. viscosa* T6SSs were identified by genomic screening in Paper IV, termed *mts1* and *mts2*. Both loci carried a copy of each of the 13 conserved core genes identified in a genome wide analysis of 176 T6SSs (Boyer et al., 2009). The two putative VgrG proteins, encoded by *mts1-V* and *mts2-V*, had the signature of phage-related baseplate assembly proteins involved in forming the small spike at the end of the tail of phage P2 (Haggard-Ljungquist et al., 1995), and may function in puncturing host cells. However, they did not carry C-terminal extensions with functional domains as described for several VgrG proteins (reviewed in Pukatzki et al., 2009). Also considered as core genes are *mts1-E* and *mts2-E* genes coding for proteins with forkhead-associated (FHA) domains (Durocher & Jackson, 2002), and *mts1-J* and *mts2-AE* coding for protein kinases. These genes may play an important role in T6SS regulation. In *Pseudomonas aeruginosa* PpkA/PppA/Fha1 play a crucial role in controlling activity of T6S at the post-translational level (reviewed in Filloux et al., 2008). There, Hcp secretion requires phosphorylation of Fha1 by PpkA, and is prevented by dephosphorylation through PppA. Similarly, FHA domain proteins and protein kinases encoded by the *mts* loci may be involved in post-translational regulation of the two *M. viscosa* T6SSs. However, both *mts1* and *mts2* lack the phosphatase with antagonistic activities to the *mts*-encoded kinases. It is possible that genes encoding phosphatases are located outside the *mts* loci in *M. viscosa*, or that genes with no known function within the *mts* loci are capable of dephosphorylating the FHA domain proteins.

More species specific CDSs were also identified. The *mts2-AA*, *mts2-AB*, *mts2-AC* and *mts2-AD* have previously been found exclusively in Vibrio species. They have

no predicted functions. The genetic organization of these CDSs together with high synteny with other T6SSs indicates a role in T6S. The CDSs *mts2-AK*, *mts2-AL* and *mts2-AM* have not been associated with T6SSs previously, but are organised in an operon together with *mst2-AI*, *mts2-EFGH* and *mts2-AJ*. In *V. anguillarum*, secretion of Hcp is decreased when *vtsEFGH* homologous to *mts2-EFGH* are mutated (Weber et al., 2009), and suggests that these CDSs play a part in type VI secretion.

Function of the Mts1 T6SS was confirmed by identification of *in vitro* secretion of Mts1 type Hcp. Furthermore, the Mts1 system was functional in all *M. viscosa* isolates tested, both virulent and avirulent, and originating from various host species and geographical locations. The role of the Mts1 system is unknown. Function of the other *M. viscosa* T6SS loci, *mts2*, was not confirmed. It is possible to speculate whether Mts1, which is present in all isolates tested, as well as in the non-pathogenic *Moritella* sp. PE36, has non-virulent related functions, while Mts2, which is not found in *Moritella* sp PE36, may be involved in virulence.

5.5 Methodological considerations

The methods applied in the study have their advantages and limitations. A collection of isolates was used in Paper I to study the virulence properties of *M. viscosa*. Using the natural variability of isolates from different geographical location and host species can be useful in studying pathogenicity and virulence factors, especially when genetic manipulation is not possible. It must be noted that the ECP were collected from broth cultures, and that factors only secreted *in vivo* are not produced. However, since the ECP were lethal and highly toxic, lethal factors and toxins are produced under the *in vitro* conditions applied. Proteomic comparison of lethal and non-lethal ECP would be an interesting next step in identifying important *M. viscosa* virulence factors.

The MvP1 peptidase was shown to have virulence related activities (Paper II). An MvP1 deletion mutant could not be constructed and therefore the importance of the vibriolysin in *M. viscosa* virulence could not be fully identified. This is a significant drawback to the study. The toxicity and lethality of ECP treated with OPA metallopeptidase inhibitor could have been evaluated *in vivo*. This method has been used to study the virulence of bacterial extracellular peptidases in fish and shrimp (Lee et al., 1997; Ostland et al., 2000). The disadvantage of OPA treatment is that it can affect other enzymes dependent on metal ions for their activity, which possibly are of

importance for *M. viscosa* virulence. Production of active MvP1 was not demonstrated *in vivo*. An attempt to identify MvP1 in tissue samples of experimentally injected salmon, through casein zymogram or immunodetection, based on the method described by Rockey et al. (1988), was not successful. Expression of the *mvp1*gene has, however, been demonstration in ulcer and kidney tissue following salmon bath infection (Tunsjo, 2009).

The expression of pro-inflammatory genes following stimulation with *M. viscosa* antigens was measured in a cell line model using real-time Q-PCR (Paper III). This widely used method has been used in both diagnostic and basic research, where it is commonly used to quantify gene expression. The technique has many possible sources of errors, such as in sample preparation, normalization and data analysis. In Paper III the reference gene EF-1A, used for normalization, was not found to be stably expressed in the SHK-1 cell line. Calculations of expression relative to EF-1A at each timepoint were done to compensate for the problem. It is also important to note that the method measures gene expression, which does not directly translate to protein levels. Furthermore, cell stimulation was performed at 20 °C, whereas *M. viscosa* causes infections at temperatures below 8 °C. Therefore, cells may have responded differently to antigen stimulation at lower temperatures. Antigens were produced from 4 °C cultures so that they would represent factors produced at those temperatures.

Two potential *M. viscosa* T6SSs were identified by genomic screening in Paper IV. Function of the Mts1 T6SS was confirmed, adding considerable value to the results. Function of the other T6SS loci, *mts2*, was not confirmed and may depend on construction of T6SS mutants. Also, Hcp proteins of some T6SSs may only be secreted in small amounts and need host cell contact for active secretion.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

M. viscosa and winter ulcer disease have had considerable effects on salmonid farming in the North Atlantic in the last two decades. Initial studies focused on describing the disease and on identifying the aetiological agent. Information on isolation, cultivation, geographical distribution and host range of the bacterium has also accumulated. More recently, studies have focused on the infection process and virulence mechanisms.

The results of this study add to our previous understanding of the pathogen, with emphasis on secreted factors and bacterial interactions with its hosts. The importance of extracellular factors produced by *M. viscosa* on virulence and development of winter ulcer disease, including the secretion of lethal and cytotoxic factors, has been demonstrated. It also revealed interesting differences in host adaptation which may be important for the development of improved vaccines. A secreted novel vibriolysin, termed MvP1, was isolated and identified. It is an enzyme with a broad temperature range and a virulence factor. The studies reveal that it causes necrotic and hemorrhagic tissue damage and may have an important role in *M. viscosa* invasiveness and in establishing infection. Two additional vibriolysins were genetically identified but their expression was not confirmed. Stimulation experiments, using a salmon cell line model, showed that live *M. viscosa* cells but not heat-killed cells or ECP induced an IL-1β response. It would be of interest to further examine these differences, especially with improved vaccine strategies in mind, such as the potential use of live attenuated vaccines. A prolonged inflammatory reaction was observed following stimulation of salmon cells with ECP. This indicates that clinical signs of winter ulcer disease may be associated with the host's reaction as well as direct effects of the bacterium. Studies on finding the main inflammatory-stimulating factors in ECP would also be of interest. Several *M. viscosa* ECP proteins were identified for the first time, but their biological roles were not tested. Finally, two potential T6SS were described in *M. viscosa* and the functionality of one of them confirmed. Identification of effector molecules and unfolding the role of T6SSs in *M. viscosa* are of great interest.

The forthcoming publication of the whole genome of *M. viscosa* and annotation of the encoding genes will add greatly to our knowledge on the pathogen and provide a

very useful tool for future research. However, this will also make the development of techniques for genetic manipulation of *M. viscosa* even more important.

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ORIGINAL PAPERS I - IV

I

PAPER I

Virulence properties of *Moritella viscosa* extracellular products

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Published in: Journal of Fish Diseases, 2011, **34**, 333-343

Journal of Fish Diseases 2011, **34**, 333–343 doi:10.1111/j.1365-2761.2011.01246.x

Virulence properties of *Moritella viscosa* extracellular products

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Abstract

Moritella viscosa is the causative agent of winter ulcer disease of marine fish. Knowledge of its pathogenicity is limited and there are no reports comparing the virulence properties of a collection of bacterial isolates. The *in vivo* and *in vitro* virulence of the extracellular products (ECP) of 22 *M. viscosa* isolates was screened. Two non-virulent Canadian isolates and a Norwegian isolate with reduced virulence produced non-lethal ECP. Correlation was obtained between cytotoxin and haemolysin production of *M. viscosa*. Isolates from salmon produced ECP with lower cytotoxic and haemolytic activities than ECP of isolates originating from other hosts. Correlation was not found between lethality of ECPs in salmon and cytotoxic or haemolytic activities. All isolates secreted esterases and a metallopeptidase (MvP1), degraded starch and produced siderophores. Variable levels of ECP protein concentration, different enzymatic activities and siderophore production could not explain differences in virulence. The results show that virulent *M. viscosa* isolates secrete a lethal toxic factor of unknown nature and that cytotoxin production may reflect host adaptation. Cell-culture models may not be optimal for determining the virulence of *M. viscosa*, as no association between cytotoxicity and bacterial virulence was obtained. Non-virulent strains may be useful in future research on *M. viscosa* virulence, as construction of mutants has not been successful.

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Keywords: cytotoxicity, ECP, extracellular products, lethality, *Moritella viscosa*, virulence.

Introduction

Winter ulcer disease is caused by the bacterium *Moritella viscosa* and affects fish reared in marine waters at temperatures below 8 °C. Winter ulcer disease has mainly affected salmonids in Norway and Iceland (Lunder, Evensen, Holstad & Hastein 1995; Benediktsdottir, Helgason & Sigurjonsdottir 1998), but also in Scotland (Bruno, Griffiths, Petrie & Hastings 1998), the Faroe Islands, Canada and Ireland (Whitman, Backman, Benediktsdottir, Coles & Johnson 2000; ICES 2005). Outbreaks occur most commonly in Atlantic salmon, *Salmo salar* L., but also in rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Benediktsdottir *et al.* 1998; Lillehaug, Lunestad & Grave 2003). Furthermore, *M. viscosa* infections have been reported in farmed Atlantic cod, *Gadus morhua* L. (Colquhoun, Hovland, Hellberg, Haug & Nilsen 2004), seawater-reared brown trout, *Salmo trutta* L. (Heidarsdottir, Gravningen & Benediktsdottir 2008), and in captive wild-caught European plaice, *Pleuronectes platessa* L. (Lunder, Sorum, Holstad, Steigerwalt, Mowinckel & Brenner 2000). Isolation of *M. viscosa* from wild fish has been reported once, where a low virulent isolate was cultivated from the gills of a healthy lumpsucker, *Cyclopterus lumpus* L. (Benediktsdottir, Verdonck, Sproer, Helgason & Swings 2000).

Moritella viscosa-infected fish often develop external lesions or ulcers. Internal pathological changes include haemorrhages, tissue necrosis and ascites (Lunder *et al.* 1995). Mortalities caused by *M. viscosa* infections are usually not high, but the economic effects are often severe, because of

Journal of Fish Diseases

lowered market value of affected fish. The disease is also a serious fish welfare problem (Greger & Goodrich 1999).

Four *M. viscosa* genotypes have been identified by AFLP DNA fingerprinting of strains from Norway and Iceland (Benediktsdottir *et al.* 2000). Norwegian isolates, South West Iceland isolates and North Iceland isolates each formed one genotype and the fourth contained a low-virulent lumpsucker isolate. Different *M. viscosa* serotypes have also been identified, partially reflecting the host species origin of isolates (Heidarsdottir *et al.* 2008).

Moritella viscosa virulence is poorly understood. Gills have been proposed as a potential site of entry (Lovoll, Wiik-Nielsen, Tunsjo, Colquhoun, Lunder, Sorum & Grove 2009) and the bacterium adheres to fish cells and disrupts the cytoskeleton (Tunsjo, Paulsen, Berg, Sorum & L'abee-Lund 2009). The ECP of an Icelandic *M. viscosa* isolate was lethal to salmon juveniles, had cytotoxic effects and stimulated interleukin-8 gene expression in fish cell-line models, and caused internal disease signs similar to those detected in infected fish (Bjornsdottir, Fast, Sperker, Brown & Gudmundsdottir 2009a; Bjornsdottir, Fridjonsson, Magnusdottir, Andresdottir, Hreggvidsson & Gudmundsdottir 2009b). The ECP of a Norwegian *M. viscosa* isolate caused pore formation and lysis of fish cells (Tunsjo *et al.* 2009). An extracellular vibriolysin, termed MvP1, has been isolated from *M. viscosa* ECP (Bjornsdottir *et al.* 2009b). The vibriolysin is non-lethal at concentrations up to 0.22 $\mu g\ g^{-1}$ in salmon, but causes haemorrhages and tissue necrosis, affects cell–cell adhesions and degrades components of the connective tissue.

The aim of this study was to study the virulence of *M. viscosa* by comparing enzymatic activities, siderophore production and the *in vivo* and *in vitro* effects of ECP of 22 *M. viscosa* isolates, originating from different geographical locations and host species. The possibility of using a cytotoxicity cell-culture model instead of fish infection experiments for virulence studies was also investigated.

Materials and methods

Bacterial isolates and culturing

All isolates used in the study are listed in Table 1 and have been identified by 16S rRNA sequencing and/or standard biochemical and phenotypical methods (Lunder *et al.* 2000). Bacteria were routinely cultured on 5% horse blood agar supplemented with 2% NaCl (BA-NaCl, prepared at our laboratory, ISO 17025 accredited) at 15 °C for 3 days. Isolates were stored frozen in brain-heart infusion broth (BHI, BD, Vistor hf.) containing 2% NaCl and 15% glycerol at −80 °C. Prior to ECP preparation and calculation of the 50% lethal dose (LD_{50}), isolates 1–13 and 16–22 were passed by intraperitoneal (i.p.) injection into salmon juveniles. Isolates 14 and 15 did not infect the fish.

Growth curves of isolates 1, 6 and 17 were determined. Colonies from BA-NaCl plates were inoculated into 3 mL BHI medium containing 2% NaCl (BHI-NaCl) and cultured at 15 °C (150 rpm) for 16 h. The absorbance at 600 nm of each culture was adjusted to approximately 0.6 with fresh medium and 50 μL of the preculture was used to inoculate 35 mL of fresh medium at 15 °C (200 rpm). Each isolate was grown in triplicate cultures and bacterial concentrations determined by plating of tenfold serial dilutions and counting colony forming units (CFU) mL^{-1}.

Experimental fish

Atlantic salmon juveniles, with no history of disease, from a commercial salmon farm were used for this study. Fish used for virulence testing of isolates had a mean weight of 25 g ± 11 g and fish used for testing of ECP lethality were 31 g ± 11 g. The fish were kept in 150 L tanks in aerated water at 7–8 °C and fed commercial pellets (Laxá hf.) Prior to injection, all fish were anaesthetized using MS222 (Pharmaq, Vistor hf.) and marked with Alcian blue (Sigma, Gróco ehf.).

Virulence determination

The LD_{50} of *M. viscosa* isolates 1, 5, 6, 10, 12, 17, 20, 21 and 22 was determined. Cultures were scraped off freshly grown BA-NaCl plates, dissolved in phosphate buffered saline (PBS; Sigma) containing 0.1% peptone (Difco, Gróco ehf.) and 1.5% NaCl (PBS-PNaCl). The suspensions were spread onto BHI-NaCl agar plates (15 cm diameter) and cultured at 15 °C for 19 h. Then, bacteria were washed off the agar with PBS-PNaCl, washed twice by centrifugation and tenfold dilutions prepared, using the same buffer. Fish were i.p. injected with 0.1 mL of bacterial dilutions and monitored for 21 days. Bacterial counts were determined by plating and counting of CFUs.

Table 1 Bacterial isolates used in the study

Number	Designation	Species	Origin	Place of isolation	References
1	NCIMB 13584^T	*Moritella viscosa*	*Salmo salar*	Norway	a, b, c, d, e, f, g, h
2	LFI 5006	"	"	Norway	e, h
3	F288/95	"	"	Norway	c, i, j, k
4	NVI 4731	"	"	Norway	
5	NVI 5433	"	"	Norway	
6	K58	"	"	SW-Iceland	a, c, g, l, m
7	K56	"	"	N-Iceland	c, g
8	F-3-04	"	"	E-Iceland	
9	F-6-05	"	"	E-Iceland	
10	990129-1/3B	"	"	Faroe Islands	
11	990217-1/1A	"	"	Faroe Islands	
12	MT 2528	"	"	Scotland	
13	MT 2858	"	"	Scotland	
14	Vvi-7	"	"	Canada	
15	Vvi-11	"	"	Canada	
16	NVI 5450	"	*Oncorhynchus mykiss*	Norway	
17	NVI 4917	"	"	Norway	
18	NVI 5168	"	"	Norway	
19	F162/01	"	"	Iceland	g
20	NVI 5482	"	*Gadus morhua*	Norway	
21	NVI 5204	"	"	Norway	
22	F57	"	*Cyclopterus lumpus*	Iceland	c, g
23	NCIMB 1144	*Moritella marina*	Seawater/sediment	Pacific Ocean	
24	JCM 10249	*Moritella japonica*	Seawater/sediment	Japan	
25	88/441^T	*Aliivibrio wodanis*	*Salmo salar*	Norway	

NCIMB, The National Collection of Industrial, Marine and Food Bacteria, Aberdeen, UK; JCM, Japan Collection of Microorganisms, Saitama, Japan; Isolates 2, 4, 5, 10–18, 20, 21, and 25 were provided by Dr Duncan Colquhoun (National Veterinary Institute, Norway) and other isolates came from the Institute for Experimental Pathology, University of Iceland; a, Benediktsdottir *et al.* (1998); b, Greger & Goodrich (1999); c, Benediktsdottir *et al.* (2000); d, Lunder *et al.* (2000); e, Tunsjo *et al.* (2007); f, Benediktsdottir & Heidarsdottir (2007); g, Heidarsdottir *et al.* (2008); h, Tunsjo *et al.* (2009); i, Bjornsdottir *et al.* (2004); j, Bruhn, Dalsgaard, Nielsen, Buchholtz, Larsen & Gram (2005); k, Gudmundsdottir *et al.* (2006); l, Bjornsdottir *et al.* (2009a); m, Bjornsdottir *et al.* (2009b).

ECP preparation

Extracellular products (ECP) were produced both by collecting spent medium from BHI-NaCl broth cultures (B-ECP) and by cultivation on cellophane-covered BHI-NaCl agar plates (C-ECP). For preparation of B-ECP, isolates were grown as described for growth curve determination for 48 h, until absorbance at 600 nm reached approximately 0.9 (stationary growth phase). For preparation of C-ECP, colonies from BA-NaCl plates were dissolved in PBS-PNaCl at absorbance at 600 nm of approximately 0.6. Two mL of the bacterial suspension was then cultured on cellophane (Topiplast) covered BHI-NaCl agar plates (15 cm diameter) at 15 °C. After 2 days of culture, 1 mL PBS-PNaCl was added on top of the cellophane cultures. The bacterial culture in stationary growth phase was finally scraped off the plates after 3 days of culture, using 5 mL PBS-PNaCl. B-ECP and C-ECP cultures were centrifuged (1700 ***g***, 20 min, 4 °C) and the supernatants filtered (Whatman, Gróco ehf., 0.45 μm). ECP were frozen in aliquots at −80 °C until used. ECP of each isolate were produced from single cultures from either culture method. Repetitions were performed to verify results of some strains.

ECP lethality assays

Virulence of undiluted B-ECP obtained from cultures with cell densities ranging from 10^9 to 5×10^9 CFU mL^{-1} of all isolates was tested by i.p. injection of 0.1 mL of each ECP into two fish, and the fish were monitored for 5 days. B-ECP of isolates 1, 6, 9, 10 and 17 were further diluted 1:2, 1:4 and 1:8 in PBS-PNaCl and 0.2 mL of each dilution injected into three fish, which were monitored for 3 days. Simultaneously, the virulence of B-ECP and C-ECP of isolate 6 was compared in the same way. The fish were injected with PBS-PNaCl as a control. The fish were monitored regularly and moribund and dead fish collected and checked for internal disease signs. At the end of the experiments, all remaining fish were killed and checked for pathological changes. Kidney samples

of 20 untreated or ECP-injected fish were streaked onto BA-NaCl plates to check that the fish were not carrying an underlying bacterial infection. The study was approved by and performed according to the Icelandic Animal Research Authority (approval no. 1205-1001).

Cytotoxicity assay

Cytotoxicity of ECP was assayed on *epithelioma papillosum cyprini* (EPC) cells (passage 251). Confluent cell monolayers (6.3×10^4 cells per well) were prepared in 96-well flat-bottom cell culture plates in Eagle's minimal essential medium, as previously described (Bjornsdottir *et al.* 2009b). Following incubation at 22 °C for 20 h, cell medium was removed and the monolayer washed with 200 μL of the previously used medium, without serum. Then, 200 μL of each ECP sample, diluted 1:20 in serum free medium was added in triplicate to the EPC monolayer and incubated at 10 °C for 3 h. Cytotoxicity was assayed using a cytotoxicity detection kit (LDH; Roche, Lyra ehf.), and the absorbance of samples read at 490 nm. Assay setup, controls and calculations of percent cytotoxicity were performed following the manufacturer's instructions.

Haemolytic assay

Haemolytic activity of ECP against salmon and sheep erythrocytes was evaluated as previously described (Bjornsdottir *et al.* 2009b) in serial dilutions. Plates containing washed erythrocytes and ECP dilutions were incubated at 22 °C for 7 h before percent haemolysis was calculated. Haemolytic units (HU) were defined as the dilution factor giving ≤50% haemolysis.

Protein concentration and enzymatic assays

Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Gróco ehf.) and caseinolytic activity measured using azocasein as substrate, both as previously described (Bjornsdottir *et al.* 2009b).

Esterase activity assay was based on a previously described protocol (Sparks, Willis, Shorey & Hammock 1979), using alpha-naphthyl acetate as a substrate, or by enzymatic staining in SDS-PAGE (see below). In a microwell plate, 30 μL of sample was mixed with 70 μL of the substrate [10 mM alpha-naphthyl acetate (Sigma) in 50 mM sodium phosphate buffer, pH 6] and incubated at 37 °C for 20 min. The reaction was stopped using 10 μL stop solution [0.5% Fast Blue B salt (Sigma) in 120 mM SDS] and absorbance measured at 600 nm. Each sample was measured in triplicate. One unit of caseinase or esterase activity was defined as an increase in absorbance of 0.01 under the assay conditions.

Degradation of egg-yolk and potato starch was evaluated by agar diffusion on plates (1% agar in PBS) containing either 5% egg-yolk emulsion (Oxoid, Vistor hf.) or 0.4% potato starch, which putatively indicate the respective lecithinase and amylase activity of samples. Twenty microlitres of each sample was pipetted in duplicate into 4-mm diameter wells that were made in the agar and incubated at 15 °C for 30 h. Plates containing potato starch were flooded with lugol to visualize zones around the wells. The diameter of the reaction zone was used as a quantitative indication of the relative enzymatic activities.

PBS-PNaCl or fresh medium were used as negative controls in all assays.

Electrophoresis, zymography, α-naphthyl acetate-azo dye and Western blotting

Standard SDS-PAGE was carried out (Laemmli 1970) in 12% acrylamide gels. Western blotting was performed as previously described using polyclonal anti-MvP1 antibodies (Bjornsdottir *et al.* 2009b).

Analysis of esterase activity in SDS-PAGE gels was performed using the α-naphthyl acetate-azo dye method as described (Lachmann, Wagner, Hadge & Drossler 1997) where, following native electrophoresis, α-naphthyl acetate (Sigma) was used as substrate and the reaction visualized by pararosaniline (ICN Biomedicals, Gróco ehf.). Gels were incubated with the substrate for 10 min at 37 °C.

Analysis of caseinolytic proteases was performed using zymography in acrylamide gels containing 0.1% casein as previously described (Gudmundsdottir 1996), with the exception that gels were incubated for 2 h at 37 °C before staining with Coomassie Brilliant Blue R (Sigma-Aldrich, Gróco ehf.). Metallopeptidase inhibition in zymograms was performed using 1,10-phenanthroline (Sigma-Aldrich), which was added to samples and to washing- and incubation buffers to a final concentration of 10 mM.

Siderophore production assay

Siderophore production was estimated using siderophore-indicative medium, cromoazurol S (CAS), as previously described (Lauzon, Gudmundsdottir, Pedersen, Budde & Gudmundsdottir 2008), with the exception that 2% NaCl and 4.5% agar were used in the medium. Medium was used as a negative control and isolate 6 as a positive control (based on pre-experiments). ECP samples were pipetted into 4-mm diameter wells in the agar medium, 30 μL per well and bacterial colonies streaked onto the medium from BA-NaCl plates, and incubated at 15 °C for 3 days. The diameter of the yellow reaction zone around the wells or bacterial growth was used as an indication of siderophore production.

Fractionation of esterase activity

C-ECP of isolate 6 was concentrated fourfold in polyethyleneglycol 6000 (Merck, Cetus) in a dialysis tube (MWCO 12000; Sigma) at 4 °C. The ECP was desalted and fractionated on a MonoQ anion exchange column as previously described (Bjornsdottir *et al.* 2009b). Eluate fractions were analysed for esterase activity, using the microwell esterase assay or the α-naphthyl acetate-azo dye method.

Data analysis

Data are presented as means ± SD for measurements. Statistical analysis of data was performed using: the Kolmogorov and Smirnov method to test for Gaussian distributions; the Mann–Whitney test to compare medians; the Spearman *r*-test to analyse correlation and one-way ANOVA (*post hoc* test Fisher's PLSD) for isolate growth comparison.

Results

Virulence of *Moritella viscosa* isolates

Twenty of 22 *M. viscosa* isolates were found to be virulent and were passed in salmon. Infected fish showed internal disease signs, i.e. haemorrhages (including petechial haemorrhages), tissue necrosis and ascites. Bacteria were re-isolated from kidney and used for LD_{50} determinations, ECP production and growth curve determination. Only the two Canadian salmon isolates (14 and 15) were non-virulent and could not be passed in salmon, although injected in doses up to 8×10^8 CFU per fish. The injected fish were anaesthetised 5–8 days post-injection and showed no signs of infection and the bacterium could not be re-isolated from kidney. The LD_{50} of 9 *M. viscosa* isolates was determined by i.p. injections and was as follows: isolate 1, 6×10^5 CFU per fish; isolates 6, 10 and 20, 2×10^5 CFU per fish; isolate 12, 2×10^4 CFU per fish; isolate 17, 7×10^5 CFU per fish; isolates 5, 21 and 22, $> 2 \times 10^6$ CFU per fish. Mortalities started on day 4 after injection and lasted until day 17. The bacterium was re-isolated from kidney of moribund and dead fish, but not from kidney of surviving fish.

Moritella viscosa growth

Growth curves of the Norwegian salmon isolate 1, the Icelandic salmon isolate 6 and the Norwegian rainbow trout isolate 17 were compared. At the 48 h timepoint, all three isolates were in stationary phase with CFU counts that did not differ significantly ($P < 0.05$); isolate 1, 2.9×10^9 CFU mL^{-1}, absorbance 0.89; isolate 6, 2.1×10^9 CFU mL^{-1}, absorbance 0.87; isolate 17, 3.1×10^9 CFU mL^{-1}, absorbance 0.96. Therefore, B-ECP of all isolates were subsequently collected after 48 h of cultivation with absorbance values ranging from 0.87 to 1.08.

Comparison of B-ECP and C-ECP

The ECP of isolate 6, produced by the two different production methods, were compared (Table 2). C-ECP of isolate 6 had over 4.5× higher protein concentration than B-ECP, but had a much higher minimal lethal dose, was non-haemolytic and showed very little cytotoxic activity. However, caseinase and esterase activity of C-ECP was 13 and 19× higher than in B-ECP, respectively. Using the α-naphthyl acetate-azo dye method, esterase bands of 36 and 33 kDa were detected in C-ECP but not in B-ECP.

Lethality in salmon

Lethalities of B-ECP of all isolates in salmon juveniles are shown in Table 3. Fish dying from B-ECP injection showed internal pathological changes indistinguishable from those seen in i.p. infected fish. Fish surviving the injection were anaesthetized after 5 days and those injected with *M. viscosa* ECP

Table 2 Comparison of broth culture-produced ECP (B-ECP) and cellophane overlay-produced ECP (C-ECP) of *Moritella viscosa* isolate 6

	Protein conc. μg mL^{-1} ± SD	Min. lethal dose μg protein per fish	Cytotoxicity[a] % ± SD	Haemolysis[b] units	Caseinase activity units ± SD	Casein zymogram bands (kDa)	Esterase activity units ± SD	Azo dye esterase bands (kDa)
B-ECP	135 ± 9.6	3	31 ± 3.6	512	8.6 ± 0.2	40, 38, 30, 29	24.1 ± 1.4	no band
C-ECP	617 ± 13.1	62	2 ± 0.7	<2	117.3 ± 1.8	40, 38, 35, 29, 20	479.6 ± 11.3	36, 33

SD, standard deviation for measurements; ECP, extracellular products; EPC, *epithelioma papillosum cyprini.*
[a]Cytotoxicity against EPC cell monolayer measured using the LDH assay.
[b]Haemolytic activity against salmon erythrocytes. Sample dilution which caused ≤50% haemolysis was defined as its haemolytic units.

had internal haemorrhages. All fish injected with non-*M. viscosa* B-ECP survived the injection and showed no internal disease signs at the end of the study. Bacteria were not cultivated from the kidney of any fish tested.

B-ECP of isolates 1, 6, 9, 10 and 17 were injected into salmon in serial dilutions and the 3-day minimum lethal dose (MLD) determined (Table 3). The MLD of all 5 isolates killed three of three fish, except for isolate 9, where two of three fish receiving the minimal dilution died. B-ECP of isolate 1 was the most lethal of the five B-ECP tested.

Cytotoxicity

Cytotoxicity values for B-ECP of all isolates are shown in Table 3. B-ECP of *M. viscosa* isolates from salmon were significantly less cytotoxic than B-ECP of isolates from other fish species ($P = 0.0059$). However, B-ECP of isolate 5 (from salmon) showed high cytotoxicity and B-ECP of isolate 20 (from cod) showed low cytotoxicity. B-ECP of four salmon isolates were non-cytotoxic (≤2% cytotoxicity). These were isolates 4 and 7 and the avirulent Canadian isolates 14 and 15.

Haemolytic activity

The haemolytic activities of B-ECP against salmon erythrocytes are shown in Table 3. B-ECP of *M. viscosa* isolates from salmon were significantly less haemolytic (HU ≤ 512) than B-ECP of isolates originating from other species of fish (HU ≥ 4096) ($P = 0.0003$). However, high haemolytic activity was measured in B-ECP of isolate 5 (from salmon) and low haemolytic activity in ECP of isolate 20 (from cod). The only non-haemolytic B-ECP was that of the Icelandic salmon isolate 7. Significant correlation ($P < 0.0001$) was obtained between cytotoxic and haemolytic activity produced by the *M. viscosa* isolates. B-ECP showed lower haemolytic activities against sheep erythrocytes than against salmon erythrocytes. The haemolytic activities of B-ECP against sheep erythrocytes ranged from 4 to 16 HU for all *M. viscosa* isolates, but B-ECP of non-*M. viscosa* isolates were non-haemolytic against sheep erythrocytes.

Protein concentration and enzymatic assays

Protein concentration of *M. viscosa* B-ECP varied from 85 to 188 μg mL^{-1}, except for isolate 7, which had a significantly lower protein concentration of 50 μg mL^{-1} (Table 3).

All *M. viscosa* B-ECP showed a weak positive caseinase signal in the azocasein assay, ranging from 3.3 to 14.9 caseinase units, except for B-ECP of isolate 7, which had the highest caseinase activity of 37.1 caseinase units (Table 3). Non-*M. viscosa* B-ECP were negative in the azocasein assay. Casein zymography showed that all *M. viscosa* B-ECP had the band pattern 40, 38, 30 and 29 kDa, with B-ECP of isolates 2, 4, 10, 13, 17, 18, 19, 21 and 22 having an additional band of 32 kDa. The B-ECP of *Moritella marina* and *Aliivibrio wodanis* produced no caseinolytic bands but three weak bands of 52, 33 and 32 kDa were seen in the B-ECP of *Moritella japonica*. All *M. viscosa* isolates were positive in an anti-MvP1 Western blot, but non-*M. viscosa* ECP did not bind anti-MvP1 antibodies. OPA inhibition of B-ECP of isolates 2 and 6 resulted in complete caseinase activity inhibition in casein zymograms, suggesting a metallopeptidase nature of all bands.

Esterase activity of varying levels was detected in B-ECP of all *M. viscosa* isolates using the microwell esterase assay (ranging from 13.3 to 24.1 esterase units) (Table 3). However, no band containing esterase activity was detected in B-ECP in α-naph-

Table 3 Protein concentration, lethality, cytotoxicity, haemolytic activities and enzymatic assays of extracellular products of bacterial isolates, cultivated in broth cultures (B-ECP)

Strain no.	Protein conc. μg mL^{-1} ± SD	Lethality in salmon no. dead (dod)	Min. lethal dose μg protein per fish	Cytotoxicity[a] % ± SD	Haemolysis[b] units	Caseinase activity units ± SD	Esterase activity units ± SD	Egg-yolk degrad. zone diam (cm)	Starch degrad. zone diam (cm)
Moritella viscosa									
Salmo salar origin									
1	166 ± 7.9	2/2 (2,2)	<2	10 ± 6.4	512	9.8 ± 0.5	19.5 ± 1.5	1.0	2.3
2	85 ± 6.4	2/2 (2,3)	na	32 ± 6.8	512	7.1 ± 0.3	19.4 ± 0.6	1.1	2.0
3	93 ± 8.9	1/2 (3)	na	32 ± 0.4	256	9.4 ± 0.6	20.9 ± 0.8	1.0	2.0
4	159 ± 13.5	2/2 (2,2)	na	1 ± 0.8	64	12.4 ± 0.1	15.5 ± 0.7	0.7	2.1
5	138 ± 8.8	0/2 (na)	na	92 ± 13.3	4096	7.2 ± 0.4	18.0 ± 0.4	1.2	1.6
6	135 ± 9.6	2/2 (2,3)	3	31 ± 3.6	512	8.6 ± 0.2	24.1 ± 1.4	1.0	1.8
7	50 ± 7.4	1/2 (4)	na	2 ± 0.5	<2	37.1 ± 1.9	14.4 ± 0.4	0.0	1.9
8	97 ± 15.8	2/2 (2,2)	na	31 ± 5.6	512	8.4 ± 1.4	22.8 ± 1.0	1.1	1.7
9	97 ± 13.9	2/2 (2,2)	5	34 ± 5.1	512	5.9 ± 0.7	22.3 ± 1.0	1.3	1.5
10	139 ± 10.6	2/2 (2,2)	4	15 ± 4.6	256	6.8 ± 0.7	13.3 ± 0.8	1.2	1.6
11	188 ± 3.6	2/2 (2,2)	na	12 ± 4.6	256	6.4 ± 0.3	16.8 ± 0.8	1.2	1.6
12	158 ± 19.0	2/2 (2,3)	na	31 ± 4.8	512	11.9 ± 0.3	20.6 ± 1.6	0.9	2.2
13	104 ± 6.3	2/2 (2,3)	na	24 ± 2.0	512	11.3 ± 0.4	15.6 ± 0.3	1.0	1.9
14	162 ± 20.6	0/2 (na)	na	1 ± 0.1	256	14.9 ± 0.4	20.9 ± 1.4	1.0	1.7
15	128 ± 2.6	0/2 (na)	na	2 ± 0.5	64	3.3 ± 0.6	19.0 ± 0.1	1.4	1.6
M. viscosa									
non-*S. salar* origin									
16	117 ± 21.0	2/2 (2,2)	na	64 ± 10.8	4096	12.3 ± 0.3	18.8 ± 0.4	1.1	1.8
17	128 ± 4.0	2/2 (1,1)	6	58 ± 4.1	4096	13.3 ± 0.4	23.7 ± 1.3	1.2	1.7
18	93 ± 4.2	2/2 (1,1)	na	70 ± 1.8	8192	3.7 ± 0.1	14.0 ± 0.7	1.1	1.7
19	99 ± 18.6	2/2 (2,2)	na	59 ± 2.3	4096	5.2 ± 0.6	13.6 ± 0.3	0.9	1.5
20	144 ± 0.5	2/2 (2,3)	na	22 ± 2.7	512	6.7 ± 0.1	18.1 ± 0.7	1.0	2.0
21	131 ± 17.9	2/2 (1,2)	na	53 ± 5.6	4096	4.0 ± 0.5	16.4 ± 0.2	0.9	1.9
22	86 ± 13.5	2/2 (4,4)	na	134 ± 7.1	4096	9.8 ± 0.1	15.1 ± 0.6	1.1	1.6
non-*M. viscosa*									
23	268 ± 13.4	0/2 (na)	na	0 ± 0.2	<2	0.0 ± 0.2	10.6 ± 0.6	0.7	0.0
24	88 ± 8.0	0/2 (na)	na	2 ± 0.1	256	0.0 ± 0.2	12.3 ± 0.8	0.9	0.0
25	193 ± 10.9	0/2 (na)	na	0 ± 0.4	512	0.0 ± 0.2	7.2 ± 0.2	0.6	1.7

SD, standard deviation for measurements; dod, day of death; na, not applicable; ECP, extracellular products; EPC, *epithelioma papillosum cyprini*.

[a]Cytotoxicity against EPC cell monolayer measured using the LDH assay.

[b]Haemolytic activity against salmon erythrocytes. Sample dilution which caused ≤50% haemolysis was defined as its haemolytic units.

thyl acetate-azo dyed SDS-PAGE gels. Concentrated C-ECP of isolate 6, containing 740.3 esterase U, produced esterase bands with apparent molecular weight of 36 and 33 kDa in a α-naphthyl acetate-azo dyed gel. Following anion exchange chromatography, esterase activity measured by the microwell esterase assay was detected in two peaks. A minor peak eluted at about 0.30 M NaCl and gave esterase activity of 171.0 U, while a major peak eluted at about 0.37 M NaCl and gave esterase activity of 439.5 U. The α-naphthyl acetate-azo dye detected the 36 and 33 kDa esterase bands in the minor peak, but no esterase band was stained from the major peak by this method, suggesting at least two different esterases. Screening of C-ECP in α-naphthyl acetate-azo dyed gels revealed the 36 and 33 kDa esterase bands in all 22 *M. viscosa* isolates.

Degradation of egg-yolk and potato starch was measured in agar diffusion assays. Degradation of egg-yolk was detected in B-ECP of all isolates (ranging from 0.7 to 1.4 cm), except for B-ECP of isolate 7, which was negative (Table 3). Starch degradation was detected in B-ECP of all *M. viscosa* isolates and the reaction zone ranged from 1.5 to 2.3 cm (Table 3).

Neither isolate origin, lethality nor haemolytic- and cytotoxic activity could be connected to the protein concentration or the measured enzymatic activities of B-ECP.

Siderophore detection

Weak yellow halos were detected around wells with B-ECP of all isolates, except for the type strain of *M. marina* (23). Halos around bacterial growth were ≤1 mm for all isolates, except 6 and 8, which had larger halos, and isolates 10, 11 and 23, where yellow colour was only detected underneath the bacterial growth.

Discussion

This study focused on examining the virulence of the extracellular products of 22 *M. viscosa* isolates. Nineteen isolates produced one or more extracellular factors that were lethal to salmon. However, the lethal activity could not be determined and did not correlate with cytotoxic- and haemolytic activities or measured enzymatic activities of the ECP. The three isolates that did not produce lethal ECP originated from two non-virulent Canadian isolates and a single Norwegian isolate with reduced virulence.

The bacterial cultures used in the study represent the currently known geographical distribution of *M. viscosa* and the fish species affected by the bacterium. Also included were two non-pathogenic species of *Moritella*, and one type culture of *A. wodanis*, which is non-pathogenic but commonly isolated from fish with winter ulcer disease (Lunder *et al.* 1995; Benediktsdottir *et al.* 1998).

All except two of the *M. viscosa* isolates included in the study were found to be virulent when i.p. injected into salmon and the LD_{50} of nine selected isolates ranged from 2×10^4 to $> 2 \times 10^6$ CFU per fish. Previously reported LD_{50} determinations for *M. viscosa* have been from intramuscular (i.m) injections, ranging from 10^3 CFU per fish for isolates 6 and 7 and up to 2×10^6 CFU per fish for isolate 22 (Benediktsdottir *et al.* 1998, 2000). The low virulence of isolate 22 has been confirmed in this study. The results of this study indicate that the LD_{50} of *M. viscosa* is higher for i.p. than i.m. infections. Previously we have found that the LD_{50} of isolate 3, following i.m. injections in salmon, cod and turbot, *Psetta maxima* L., was around 10^5 CFU per fish, whereas in halibut, *Hippoglossus hippoglossus* (L.), the LD_{50} was around 8×10^7 CFU per fish (Bjornsdottir, Gudmundsdottir, Bambir, Magnadottir & Gudmundsdottir 2004; Gudmundsdottir, Bjornsdottir, Gudmundsdottir & Bambir 2006). The isolates were cultured at 15 °C prior to LD_{50} estimation using i.p. injection so that bacterial virulence could be more easily compared with ECP virulence, which was also produced by culturing at 15 °C and i.p. injection into salmon. However, bacterial virulence may be reduced at 15 °C at which temperature *M. viscosa* mobility and adhesion to fish cells is decreased compared with lower temperatures (Tunsjo, Paulsen, Mikkelsen, L'abee-Lund, Skjerve & Sorum 2007; Tunsjo *et al.* 2009). Temperature selection was based on data showing that temperature elevation from 4 to 15 °C has little effect on the production of *M. viscosa* extracellular proteins and toxic factors, but that the bacterium grows faster at the higher temperature (Tunsjo *et al.* 2007, 2009).

Two *M. viscosa* isolates originating from salmon in Canada were found to be non-virulent. Furthermore, the respective B-ECP were non-lethal to salmon, although internal haemorrhages were observed in injected fish. Moreover, the low-virulent isolate 5 was found to produce non-lethal ECP. This indicates the importance *M. viscosa* ECP may have in the onset and/or development of

winter ulcer disease. So far, efforts to construct *M. viscosa* mutants have been unsuccessful (data not shown). Therefore, non- or low-virulent isolates may be useful in future research in *M. viscosa* virulence and in detecting potentially important virulence factors.

The two ECP production methods affected the properties of the ECP considerably. B-ECP had a much lower protein concentration, was more lethal to salmon juveniles and had higher cytotoxic and haemolytic activities than C-ECP of the same isolate. Environmental influence on cells during the two cultivation methods is quite different, which explains the difference in protein expression. Cell density is higher on cellophane covered agar plates than in broth cultures. Higher protein concentration can at least partly be explained by proteins from lysed cells, as *M. viscosa* is very sensitive to lysis at high cell density (Benediktsdottir & Heidarsdottir 2007; Tunsjo *et al.* 2007). Cell–cell communication, which is dependent on cell density, can also affect protein expression. Furthermore, different incubation times can have an influence, as proteins secreted by the cells accumulate in the cell environment and some expressed proteins may be degraded. Stained SDS-PAGE of B-ECP and C-ECP from the same isolates revealed different band patterns, although common bands were also present (data not shown).

Lethality of B-ECP of all isolates was tested in salmon juveniles. Lethality was not correlated with total protein concentration of ECP, indicating a difference in production of certain unknown toxins between isolates. Isolate 22 has previously been described as a low-virulent isolate and not found to cause petechial internal haemorrhages in salmon (Benediktsdottir *et al.* 2000). However, in this study, both fish infected with the isolate and fish injected with B-ECP of the isolate showed similar pathological changes as fish injected with fully virulent *M. viscosa* isolates. The two fish injected with B-ECP of isolate 22 did, however, not die until 4 days post-injection, which was later than most fish injected with B-ECP of other *M. viscosa* isolates. B-ECP of *M. marina*, *M. japonica* and *A. wodanis* were non-lethal and showed negative or low cytotoxic and haemolytic activities, reflecting the non-pathogenic nature of the species.

A pattern between host species origin of isolates and haemolytic and cytotoxic activities of B-ECP was detected, where isolates from salmon showed lower cytotoxic and haemolytic activities than isolates from other species of fish. B-ECP of isolate 5 (from salmon) and isolate 20 (from cod) did, however, deviate from this pattern. To better survive within the host bacteria can alter their production of secreted factors. Reducing the production of cytotoxins/haemolysins may give *M. viscosa* an advantage during the infection of salmon. The differences observed in haemolytic and cytotoxic activities may therefore be the result of *M. viscosa* host adaptation. Previously, *M. viscosa* serotypes have been found to partially follow host species origin of isolates (Heidarsdottir *et al.* 2008). However, *M. viscosa* genotypes and the size of a 17/19 kDa antigen have been shown to reflect the different geographical origins of isolates (Benediktsdottir *et al.* 2000; Heidarsdottir *et al.* 2008). The consistency between cytotoxic and haemolytic activities of *M. viscosa* B-ECP indicates that the same bacterial product/s is responsible for membrane disruption of both EPC cells and salmon erythrocytes. It also appears that the B-ECP contain a haemolytic factor without cytotoxic activity against EPC cells, as three of four B-ECP with no detectable cytotoxic activity induced haemolysis.

B-ECP of isolate 5 which showed high cytotoxic and haemolytic activities was non-lethal to salmon, whereas B-ECP of isolate 4 which showed low cytotoxic and haemolytic activities was lethal. This indicates that the cytotoxic- and haemolytic activities do not represent the lethal factor secreted by the isolates. Furthermore, the different virulence of B-ECP of *M. viscosa* isolates could not be explained by any of the various activities/factors that were measured. This study shows that *M. viscosa* secretes an unidentified toxic factor and a previous study indicates that it is a protein, as the lethal activity is heat sensitive (Benediktsdottir & Helgason 1997). Previously, increased expression of putative virulence genes has been measured following infection of a cell-culture model with *M. viscosa* cells, including genes encoding a putative haemolysin, type VI pili, repeats in toxin, cytotoxic necrotizing factor and phospholipase (Tunsjo 2009).

Caseinase activity was detected in B-ECP of all *M. viscosa* isolates and the caseinase band pattern in zymograms was very similar for all isolates. The MvP1 peptidase has previously been suggested to act as a virulence factor because of its tissue degrading abilities and thereby possibly aiding in invasion and dissemination of the bacterium within its host (Bjornsdottir *et al.* 2009b). Therefore, its importance in virulence may have been overlooked

by i.p. injection, as performed in this study. In a previous study, including 20 *M. viscosa* isolates from salmon and a single isolate from plaice in Norway, all isolates were found to have identical peptidase profiles in gelatin zymography (Lunder *et al.* 2000). The active MvP1 peptidase was not detected in B-ECP of *M. marina*, *M. japonica* and *A. wodanis*, species which are not known to cause disease.

Esterase activity was detected by the microwell assay in B-ECP of all isolates, but no bands were detected in α-naphthyl acetate-azo dyed gels using the same substrate. Fractionation of C-ECP, containing 36 and 33 kDa esterase bands, indicated that the microwell assay detected esterase activity which is not stained by the gel assay method. The presence of esterase bands in α-naphthyl acetate-azo dyed gels in C-ECP but not in B-ECP could therefore be explained by increased production of the enzymes in C-ECP, or that the bands may have been intracellular enzymes from lysed bacterial cells.

B-ECP of isolate 7, originating from salmon in North Iceland, behaved differently from other B-ECP included in the study. It had significantly higher caseinase activity and lower protein concentration than other *M. viscosa* B-ECP and did not degrade egg-yolk. It was non-cytotoxic and non-haemolytic and killed only one of two injected fish. The high caseinase activity of B-ECP of isolate 7 may have caused protein degradation which could explain its low protein concentration and reduced activities. In a previous study (Benediktsdottir *et al.* 2000) the same isolate, along with other *M. viscosa* isolates from salmon in North Iceland, formed a distinct genotype and showed some differences in biochemical tests and whole cell protein profile from other *M. viscosa* isolates.

This study demonstrates the importance of factors secreted by *M. viscosa* in infection and development of winter ulcer disease. Lethality of ECP did not correlate with the *in vitro* cytotoxic virulence of ECP, indicating that the extracellular lethal factor is not a cytotoxin/membrane damaging factor. Cytotoxicity assay using cell-culture models may therefore not be suitable for predicting *M. viscosa* virulence, although the two non-virulent isolates did not secrete cytotoxins. It must, however, be noted that the study evaluated ECP virulence based on factors produced by broth culturing, which may not represent all factors produced *in vivo*.

Acknowledgements

The authors wish to thank Dr Duncan Colquhoun for providing bacterial isolates, Stofnfiskur for providing experimental fish and Dr Sigurdur Helgason for providing EPC cell cultures. This study was funded by the AVS R&D Fund of Ministry of Fisheries in Iceland.

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Received: 4 August 2010
Revision received: 18 November 2010
Accepted: 14 December 2010

II

PAPER II

Characterisation of an extracellular vibriolysin of the fish pathogen *Moritella viscosa*

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Published in: Veterinary Microbiology, 2009, **136**, 326-334

Veterinary Microbiology 136 (2009) 326–334

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Characterisation of an extracellular vibriolysin of the fish pathogen *Moritella viscosa*

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ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 25 November 2008
Accepted 28 November 2008

Keywords:
Moritella viscosa
MvP1
Extracellular products
Vibriolysin
Virulence factor

ABSTRACT

Moritella viscosa causes winter ulcer disease in salmonids. The aim of the present work was to isolate and partially characterise an extracellular peptidase from *M. viscosa*, and to study its role in virulence. The peptidase, termed MvP1, was a 38-kDa metallopeptidase produced in late exponential growth. The optimum temperature for MvP1 was 40 °C, but the enzyme was active over a broad range of temperatures. MvP1 was non-lethal to salmon at concentrations up to 0.22 μg/g fish, but extracellular products were lethal to salmon. MvP1 degraded casein, gelatin and collagen from lumpfish skin. It caused considerable tissue necrosis and hemorrhages at the site of injection, and affected cell–cell adhesions in EPC and BF-2 cell lines, but was not highly cytotoxic. The peptidase partially degraded fish IgM heavy chain but was non-hemolytic. The *mvp1* gene was sequenced and encoded a 734-aa polypeptide containing a signal sequence, an N-terminal propeptide, a mature peptidase domain and a C-terminal propeptide. The MvP1 propeptide undergoes both N-terminal and C-terminal processing and different C-terminal processing results in the formation of several active isoforms of the mature peptidase. The catalytic domain showed highest sequence similarity with several vibriolysins (EC 3.4.24.25) originating from *Pseudoalteromonas* strains, showing up to 80% aa identity. The results indicate that MvP1 is a previously unknown vibriolysin that might affect *M. viscosa* virulence by aiding in the invasion and dissemination of the bacterium in its host, by causing tissue destruction.

1. Introduction

Moritella viscosa causes winter ulcer disease in fish reared at low temperatures in North Atlantic countries, mainly in Norway (reviewed in Gudmundsdottir and Bjornsdottir, 2007). Large ulcers often appear on infected fish, and hemorrhages and tissue necrosis are detectable internally (Lunder et al., 1995; Bruno et al., 1998). The disease causes significant mortalities and financial losses in Atlantic salmon, *Salmo salar* (L.) and rainbow trout, *Oncorhynchus mykiss* (Walbaum), and causes infections in Atlantic cod, *Gadus morhua* (L.). Furthermore, the bacterium has been isolated from several other fish species (reviewed in Gudmundsdottir and Bjornsdottir, 2007). Lipooligosaccharides and a 17–19 kDa outer membrane antigen have recently been shown to be protective antigens (Heidarsdottir et al., 2008).

Pathogenic bacteria produce a variety of virulence factors that affect their hosts, allowing the bacteria to multiply and produce a disease. Several extracellular

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doi:10.1016/j.vetmic.2008.11.020

bacterial peptidases, mainly metallopeptidases, have been shown to have either direct or indirect roles in pathogenesis and virulence, although most are known for nutrient acquisition for bacterial growth (Miyoshi and Shinoda, 2000). The *Pseudomonas aeruginosa* elastase and *Vibrio vulnificus* and *Vibrio cholerae* vibriolysins have been implicated with bacterial virulence. They cause various actions, such as tissue destruction and hemorrhages, and are believed to play roles in colonization, invasiveness, and dissemination within the host (Booth et al., 1984; Finkelstein et al., 1992; Komori et al., 2001; Silva et al., 2003; Miyoshi, 2006).

This paper describes the isolation and characterisation of a previously unknown extracellular peptidase from the fish pathogenic bacterium *M. viscosa*.

2. Materials and methods

2.1. Bacterial strain, growth conditions, and production of ECP

Moritella viscosa, strain K58, was isolated and identified in 1992 at the Institute for Experimental Pathology, University of Iceland, from the head kidney of diseased Atlantic salmon from a farm in SW-Iceland (Benediktsdottir et al., 1998, 2000). The strain was passaged in salmon prior to the study, by reisolation from kidney of i.p. infected fish. The bacterium was grown on 5% horse blood agar supplemented with 2% NaCl at 4 °C for 4 days. *M. viscosa* growth curve was determined by averaging three identical batch cultures cultivated in Brain-Heart infusion broth (BHI, BD) containing 1.5% NaCl (BHI-NaCl) at 9 °C, with agitation (200 rpm). Growth was estimated in log CFU/ml by serial dilution and plate counting.

Extracellular products (ECP) were isolated from batch cultures grown at 4 or 9 °C for 5 days by centrifugation, and then filtered (Whatman, 22 μm). For isolation of ECP, used for toxicity testing, *M. viscosa* was cultivated using the cellophane overlay method (Liu, 1957) on Tryptone soya broth (Difco) agar with 1.5% NaCl at 15 °C for 3 days.

2.2. Proteolytic activity, protein content, and inhibition studies

Caseinolytic activity was assayed using azocasein (Sigma) as previously described (Gudmundsdottir, 1996), and incubated at 30 °C for 60 min. Samples were assayed in duplicate and each duplicate measured twice. One unit (U) of caseinase activity was defined as an increase in absorbance of 0.01 under the assay conditions.

Protein content was measured with a protein assay kit (Coomassie Plus, Pierce) and bovine serum albumin (Sigma) used for plotting a standard curve. Absorbance was measured at 590 nm and all samples measured in duplicate.

The pure peptidase was incubated with inhibitors for 10 min at 22 °C. Stock solutions of the dissolved metallopeptidase inhibitors EDTA (Merck) and OPA (1,10-phenanthroline) (Sigma–Aldrich) and the serine peptidase inhibitor PMSF (Sigma) were prepared and added to the MvP1 peptidase in final concentrations of 1 or 10 mM. Remaining caseinolytic activity was determined using azocasein assay.

2.3. Peptidase purification

ECP proteins were precipitated by adding ammonium sulphate to 85% saturation. The precipitate was recovered by centrifugation and dissolved in 20 mM Tris (pH 8). Precipitated proteins were desalted using PD-10 columns (Amersham Biosciences), equilibrated with the same buffer at 4 °C. Desalted samples were loaded onto a MonoQ HR 5/5 column (Pharmacia), preequilibrated with 20 mM Tris (pH 8). The column was eluted at 15 °C with a linear gradient of 0–0.8 M NaCl in the same buffer. Eluate fractions were collected and analyzed for caseinolytic activity. Pooled fractions containing the highest caseinolytic activities were concentrated by ultrafiltration using Amicon Ultra-15 filter (Millipore, MWCO 10,000) at 4 °C. Concentrated fractions were further fractionated on a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech), equilibrated and eluted with 50 mM Na-phosphate buffer (pH 7), containing 15 mM NaCl (SD-200 buffer) at 15 °C. Fractions with major caseinolytic activities were pooled and used as the purified enzyme, following analysis with SDS-PAGE and Western blotting.

2.4. Electrophoresis and isoelectric focusing

Standard SDS-PAGE was carried out according to the Laemmli method (Laemmli, 1970) in 12% polyacrylamide gels and protein bands visualised by silver staining (Silver Stain Plus Kit, BioRad). Zymography was performed as previously described (Gudmundsdottir, 1996), using 0.03% casein, 0.1% collagen (isolated from lumpfish skin), or 0.1% gelatin as substrates. Gels were incubated for 2 h at 30 °C.

Isoelectric focusing was carried out in Phast system (Pharmacia), using PhastGel IEF 4–6.5 (GE healthcare), and standards pH 3–10 (Pharmacia). Gels were fixed in 20% trichloroacetic acid and visualised with silver staining.

2.5. Immunoreactivity of MvP1 isoforms

Isolated MvP1 was electrophorised and visualised using Western blotting as described previously (Gudmundsdottir et al., 2003). Blotted membranes were incubated with polyclonal mouse antibodies against the 38 kDa MvP1 isotype (I1, see definition in Section 3.2) that were prepared in mouse ascitic fluid using a previously described method (Gudmundsdottir et al., 2003), and with an estimated dose of 10 μg MvP1 injected each time. Antibodies were diluted 1:2000. Washed membranes were then incubated with goat anti-mouse antibodies conjugated with alkaline phosphatase (Dako), diluted 1:1000.

2.6. Optimal temperature and thermostability

For estimating the optimal temperature for MvP1, the catalytic activity of the pure peptidase was measured at different temperatures between 5 and 80 °C for 1 h, by the azocasein assay. For thermostability testing the purified protein was incubated at 30, 40, 50 and 60 °C for 30 min in

SD-200 buffer and then centrifuged before performing the azocasein assay. Samples were measured in duplicates.

2.7. Toxicity testing

Healthy unvaccinated Atlantic salmons from a commercial fish farm were used for toxicity testing of *M. viscosa* ECP, and isolated MvP1. The fish were 46 g (±6 g) and kept in fresh water in a 0.15 m^3 tank at 8 °C. Intramuscular (i.m.) and intraperitoneal (i.p.) injections with 0.1 ml ECP (2 μg/g fish (70 U)) or 0.2 ml MvP1 (up to 0.22 μg/g fish (65 U)) were performed. Two to six fish were included in each group and groups were identified with Alcian blue markings. The fish were monitored for up to 9 days and complete necropsy performed on all fish. Fish were injected with SD-200 buffer as control.

2.8. Cytotoxicity assay

Cytotoxic activity of *M. viscosa* ECP and purified MvP1 was determined using epithelioma papulosa carpio (EPC) and bluegill fry fibroblast (BF-2) cells. Confluent cell monolayers were prepared in 96-well flat-bottom cell culture plates containing 150 μl Eagle's minimal essential medium (Eagle's MEM, Gibco), with 10% foetal bovine serum (Gibco), penicillin (100 IU/ml), and streptomycin (100 μg/ml), and cultured at 23 °C for 20 h. Then, medium was removed from wells and 150 μl of fresh Eagle's MEM with or without 10% foetal bovine serum added, as well as 40 μl of different dilutions of ECP (22–175 μg/ml) or MvP1 (3–30 μg/ml), containing equal caseinolytic activities (50 U in highest concentrations). Each treatment was carried out in triplicate and SD-200 buffer used as control. The cells were incubated for further 24 h at 15 °C and microscopically examined.

2.9. Proteolytic digestion of IgM

Isolated salmon and cod IgM (4 μg) (Magnadottir, 1998) were incubated in duplicate for 24 h at 9 °C with *M. viscosa* ECP (1.2 μg) or isolated MvP1 (0.12 μg), each containing caseinolytic activity of 65 U. Incubation with SD-200 buffer was used as control. Samples were then electrophorised using standard SDS-PAGE and IgM visualised using Western blot analysis as described in Section 2.5, with the exception that membranes were incubated with polyclonal mouse antibodies against salmon or cod IgM (Magnadottir, 1998), diluted 1:2000.

2.10. Hemolytic assay

Hemolytic activity was determined using salmon and sheep erythrocytes. Serial dilutions of *M. viscosa* ECP and isolated MvP1 were incubated for 5 and 24 h at 22 °C, in 0.5% washed red blood cells suspended in phosphate buffered saline (PBS, Sigma). Each dilution was carried out in duplicate. PBS was used to calculate 0% lysis and dH_2O to calculate 100% lysis. Absorbance of the supernatant was measured at 405 nm, and hemolysis of each sample dilution calculated.

2.11. N-terminal sequence analysis

MvP1 was blotted on to a polyvinylidene fluoride membrane (Millipore) and the N-terminal aa sequence of the 38 kDa isotype (I1, see definition in Section 3.2) determined at the Institut Pasteur, Paris by automated Edman degradation.

2.12. DNA sequencing and computer analysis

Degenerate primers were designed for PCR amplification of a sequence from *mvp1*, the gene that encodes MvP1. Construction of primers was based on N-terminal sequence analysis and the third zinc ligand motif of thermolysin peptidases. A list of primers used in this study is given in Table 1. The 508-nt PCR product was cloned into pCR®2.1-TOPO® (Invitrogen) and sequenced by the dideoxy method using BigDye® Terminator sequencing kit (Applied Biosystems) and universal M13 primers. Then, a series of nested PCR amplifications and subsequent sequencing was performed to determine flanking sequences. Gene-specific 5′ biotin-labelled primers and arbitrary primers (Arb1 or Arb2) were used for first round PCR amplification. PCR products were purified using the Qiaquick PCR purification Kit (Qiagen) and biotin labelled PCR products further purified using Dynabeads® M-280 Streptavidin (Dynal Biotech). Second round PCR amplification was performed using nested gene-specific primers, which were designed to bind upstream of the previous gene-specific primers, and also a nested primer to Arb1 and Arb2 (Arb3). PCR products were purified using GFX™ PCR, DNA and Gel Band Purification Kit (Amersham Bioscienses), cloned into pCR®4-TOPO® (Invitrogen) and transformed into One Shot® TOP10 competent *Escherichia coli* (Invitrogen). Cloned inserts were PCR amplified using M13 primers and cycle sequenced. The sequence information was used to generate new gene-specific primers for the next nested PCR round until the complete gene and flanking regions were obtained. All PCR cycling conditions followed standard laboratory practices. For confirmation of *mvp1* sequencing, the gene and its flanking regions were PCR amplified from genomic DNA, using primers containing EcoRI and BamHI recognition sequences, and cloned into pUC19 (Invitrogen), using EcoRI and BamHI. Chemically competent DH5α *E. coli* cells (Invitrogen) were transformed with the resulting ligation product, and both strands of the cloned insert sequenced. DNA sequences were analyzed using Sequencher 4.6 (Scientific & Educational Software). Nucleotide acid and translated amino acid sequences of the *mvp1* gene and flanking sequences have been deposited in the GenBank database under accession number EU876833. The NCBI Basic Local Alignment Search Tool (BLAST) was used for comparing protein sequences and CD search for detecting conserved domains. Putative open reading frames (ORF) were identified by the SoftBerry FGENESB program. The ProtParam program (ExPASy) was used for MW computation and SignalP 3.0 (CBS) for predicting the location of a signal peptide cleavage site.

Table 1
Oligonucleotide primers used in the study.

No.	Name	forward (f) reverse (r)	5′- 3′	Application
1	mvp1-N5-f	f	GGMTTYGGTGGTAACGARAAAACRGG	Amplification of a 508-nt fragment of *mvp1*
2	mvp1-I492-r	r	GCTTCACCWGCCATATCAGARAAYGCTTC	
3	Arb1	f/r	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	Arbitrary primers for outward sequencing of mvp1 *and flanking regions*
4	Arb2	f/r	GGCCACGCGTCGACTAGTANNNNNNNNNNACGCC	
5	Arb3	f/r	GGCCACGCGTCGACTAGTAC	Nested primer for Arb1 and Arb2
6	mvp1-1bio	r	ATTACCAGACTGCCCTACATTC	Specific primers for outward sequencing of mvp1 and flanking regions.
7	mvp1-2bio	f	TTCAGCTCATGAAGTTAGTC	
8	mvp1-3	r	CAAGTATCCGAAATCAGTACC	Even numbered primers are designed for upstream amplification, odd numbered primers are designed for downstream amplification. Primers marked bio are 5′ biotin labelled and other primers are nested
9	mvp1-4	f	GGTTTATGAGGCTAAATCAG	
10	mvp1-5bio	r	CAACAGCACCATGAGCATTC	
11	mvp1-6bio	f	GTACTGACAGTGGTGATGAG	
12	mvp1-7	r	CCGTCAGAATAACGAGATATAATT	
13	mvp1-8	f	CAGGTGCCAGTGACATTC	
14	mvp1-9bio	r	ACACCACCATCACTATCG	
15	mvp1-10bio	f	CGGTTACAGTACTGCATCTG	
16	mvp1-11	r	GACCACACTAAGACCTTC	
17	mvp1-12	f	TGATGTGGAGCTAAATATTCA	
18	mvp1-13bio	r	GTTGTTGCTGAAGATATTGC	
19	mvp1-15a	r	GCTGATAATTGTTGAATATTGTC	
20	mvp1-EcoRI	f	CGGAATTCGACGTACCGCACCAAATCC	Ligation of *mvp1* and flanking regions into pUC19
21	mvp1-BamHI	r	CGGGATCCTATTAGCTAAATTATCTGCTG	Restriction sites for cloning are underlined
22	mvp1-425	f	CAGTCGCTTACAATTTTCAGTT	Sequencing of *mvp1* and flanking regions in pUC19. Primers no. 8, 9, 17 and 19 were also used
23	mvp1-990	f	TCTCGATAGTGATGGTGGTG	
24	mvp1-1478	f	TGAATGTAGGGCAGTCTGG	
25	mvp1-3100	r	GCCAACGTCGATAGAGAAAC	
26	mvp1-2654	r	GTATAGCTGCCGACCAAACT	
27	mvp1-2190	r	GCTCCAATAGAGTTGGTTGG	
28	mvp1-1794	r	GTACGATTCACCATCACCAA	
29	mvp1-446	r	AACTGAAAATTGTAAGCGACTG	

2.13. Licenses

The study was approved by and performed according to the Icelandic Animal Research Authority (approval no. YDL 03080041/023BE).

3. Results

3.1. M. viscosa cultivation and peptidase production

M. viscosa generation time in BHI-NaCl at 9 °C was 2 h and 48 m, as calculated from plate counts in the log phase (11 and 17 h timepoints) of all three cultures (Fig. 1). Under these conditions the cultures reached a maximum of 1.2×10^{10} CFU/ml after 96 h of cultivation.

Peptidase production in the ECP was observed at the end of the exponential growth phase. The activity was detected after 35 h of cultivation by casein zymogram and after 43 h when using azocasein assay. Maximum caseinase activity (71 U) was measured at the final timepoint at 111 h and two caseinolytic bands were detected in a zymogram of ECP collected at 35 h or later. Occasionally, a third faint band of a smaller size was also identified in stationary phase ECP (data not shown). Peptidase production was also identified in ECP from cultures grown at 4 and 15 °C, where the same band pattern was observed. Polyclonal antibodies raised against the 38 kDa MvP1 band bound to all caseinolytic bands detected in ECP (data not shown).

3.2. MvP1 isolation

Following ECP precipitation, several caseinolytic bands of varying numbers ranging from 24 to 40 kDa were detectable in a zymogram (data not shown). After ion-exchange FPLC,

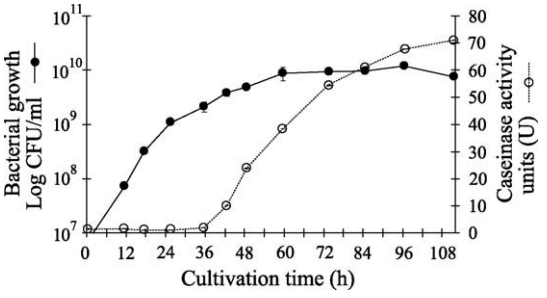

Fig. 1. Growth curve of *M. viscosa* K58 (●) (± S.D.), and production of caseinolytic peptidase (○) in BHI-NaCl at 9 °C. The figure shows the average from three parallel batch cultures.

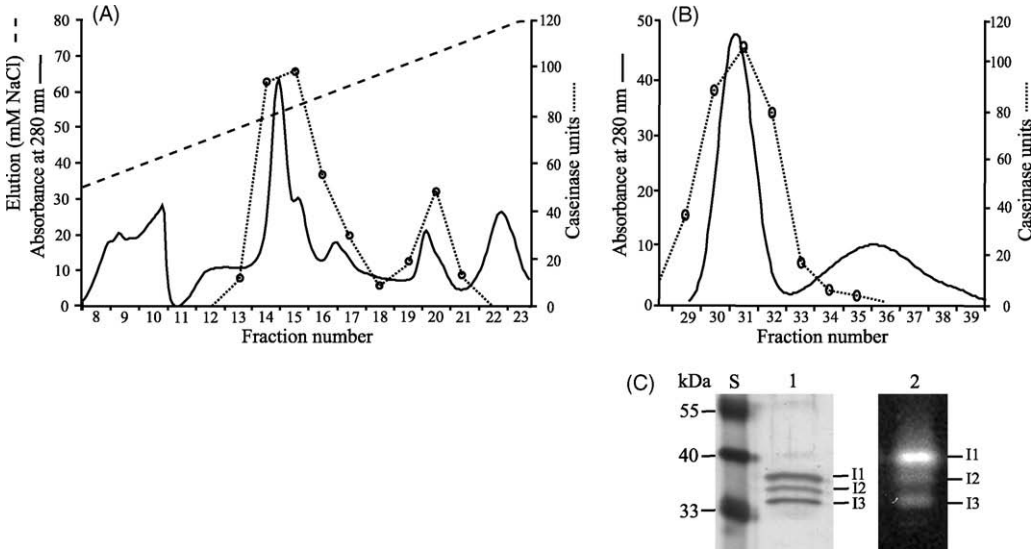

Fig. 2. Purification of the extracellular *M. viscosa* K58 MvP1 peptidase. (A) MonoQ HR 5/5 anion exchange chromatography of precipitated ECP proteins. (B) Superdex 200 gel filtration chromatography of the major caseinolytic peak (fractions 14–15) from A. (C) Three active MvP1 isotypes (fractions 30–32) from B, termed I1, I2 and I3. The I1 and I2 isotypes appeared as 38 and 36 kDa bands in SDS-PAGE, but as 40 and 38 kDa bands in casein zymogram, respectively. Both methods produced a 35 kDa band of I3. Lane S, molecular weight standards; lane 1, silver staining; lane 2, casein zymogram.

Table 2
Summary of the purification of *M. viscosa* MvP1 peptidase.

Purification step	Total protein (mg)	Total proteolytic units	Specific activity (units/mg)	Recovery (%)	Relative purification
Extracellular products	17.68	3224	4	100	1
1 Amm. sulf. precipitation	2.84	473	54	13.0	14
2 PD-10 gel filtration	2.14	464	50	12.8	13
3 Mono Q ion exchange	0.46	219	221	6.0	56
4 Ultrafiltration	0.36	66	364	1.8	92
5 Superdex 200 gel filtration	0.05	51	2020	1.4	510

ECP proteins eluted as several peaks with caseinolytic activity eluting in two peaks (Fig. 2A). The major caseinolytic peak (fractions 14–15) typically produced three protein bands, hereafter termed MvP1 isotypes I1, I2 and I3. The I1 and I2 isotypes appeared as 38 and 36 kDa bands in SDS-PAGE, but as 40 and 38 kDa bands in casein zymogram, respectively. Both methods produced a 35-kDa band of I3 (Fig. 2C). The minor peak (fraction 20) produced mainly the smaller caseinolytic bands. After further separation of the major peak by gel filtration on Superdex 200, the MvP1 peptidase eluted in one peak (fractions 30–31) (Fig. 2B). Silver staining of the peak revealed one to three protein bands (I1, I2 and/or I3) varying between purifications. The protein bands were all confirmed as MvP1 by Western blotting and were all active (Fig. 2C). Specific activity of the three isolated MvP1 isotypes was calculated 1020 U/mg isolated protein, using caseinase units. Total recovery of peptidase activity from ECP was 1.4% and the relative purification was 510-fold (Table 2). The percentage of MvP1 in total extracellular proteins was estimated to be from 10 to 17%, based on the specific activity of MvP1.

3.3. MvP1 characterisation

The MvP1 peptidase degraded casein, lumpfish skin collagen and gelatin, and the same band pattern was observed in zymograms containing the three different substrates. The metallopeptidase inhibitors OPA and EDTA, at 1 mM concentrations, reduced activity by 73 and 16%, respectively. At 10 mM, OPA inhibited MvP1 activity completely but 21% of the activity still remained after treatment with 10 mM EDTA. The serine peptidase inhibitor PMSF did not affect MvP1 activity at 1 mM concentration.

Optimum reaction temperature for the purified MvP1 peptidase was around 40 °C (Fig. 3). The peptidase showed

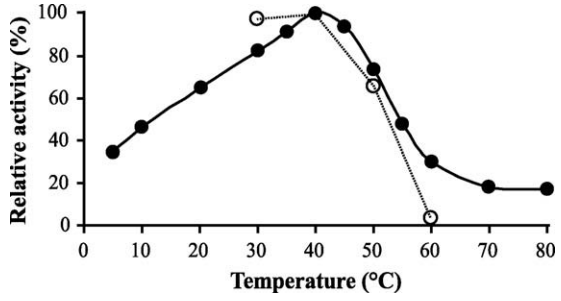

Fig. 3. Temperature effect on MvP1 activity. (●) Activity range and optimum temperature measured by incubating isolated MvP1 in azocasein sample mixture at a given temperature for 60 min. (○) Thermostability measured by incubating isolated MvP1 at a given temperature for 30 min prior to measuring azocasein activity. Activity at 40 °C was set as 100%.

activity between 5 and 60 °C and at 5 °C 35% of the relative activity still remained. At temperatures of 45 °C or higher, the activity declined rapidly. MvP1 thermostability is also shown in Fig. 3. After incubation at 50 °C for 30 min, peptidase activity dropped significantly, and at 60 °C no residual activity was measured. In a sample of the purified MvP1 peptidase, containing the I1 and I2 isotypes, two isoelectric points could be detected with calculated pI of 4.4 and 4.2.

Isolated MvP1 was non-lethal to salmon at concentrations up to 0.22 μg/g fish. However, the peptidase caused considerable hemorrhages and extensive tissue necrosis at the site of injection in concentrations as low as 0.06 μg/g fish, and ascites formation was detected after i.p. injection. The ECP of *M. viscosa* were lethal to salmon, at 2 μg/g fish, causing death in all injected fish within 72 h. Extensive tissue necrosis and hemorrhages were seen around the site of injection, and hemorrhages were also seen in various internal organs and tissues, in both i.m. and i.p. injected fish. Ascites was seen in the peritoneal cavity of all fish, and petechial hemorrhages in liver and perivisceral fat were seen in two out of four fish.

Cytotoxic activity of *M. viscosa* ECP and purified MvP1 against EPC and BF-2 cells was tested. Each dilution of ECP and isolated MvP1 contained equal caseinolytic activities. Serum decreased the toxic effects of ECP and MvP1, but did not hinder them completely. Effects seen in both cell types were similar. ECP caused cell death, ruptures in the cell monolayer, and detachment from the edges of the wells. The MvP1 peptidase did not cause prominent cell death, which was only detectable at the higher concentrations. However, MvP1 caused detachments from well edges and ruptures in the cell monolayer.

Isolated MvP1 partially digested both salmon and cod IgM heavy chain, but IgM was not affected when incubated with ECP, containing equal caseinolytic activity (56 U) (Fig. 4).

MvP1 was non-hemolytic against salmon and sheep erythrocytes, but ECP were strongly hemolytic against salmon erythrocytes (1:126 dilution caused 20 and 89% lysis after 5 and 24 h, respectively), and weakly hemolytic against sheep erythrocytes (1:4 dilution caused 2 and 18% lysis after 5 and 24 h, respectively).

3.4. *mvp1* sequencing

In total, 3.562-nt were sequenced. A potential *mvp1* ORF of 2.205-nt encoding a 734-aa polypeptide with calculated MW of 79.095 was found (Fig. 5). A putative signal sequence was detected, with a predicted cleavage site after Ala-25. The previously determined aa sequence for the N-terminal end of MvP1 (ADATGFGGNEKTGKYHYGTDF) was found within the polypeptide, starting at Ala-211. The highly conserved HEXXH zinc-binding motif of metallopeptidases was found in *mvp1* (aa 351–355), and also the third zinc ligand motif GXXNEXXSD (aa 371–379). A possible Shine–Dalgarno (SD) sequence (ggagac) was detected upstream of the predicted Met start codon (nt −10 to −5). Another, less possible, Met start codon was also found (nt −12 to −10), that partially overlapped with the predicted ribosomal binding site. An inverted repeat sequence, characteristic of a Rho-independent transcription determinator was found downstream of the stop codon (nt 2227–2243).

Several conserved domains were identified within the MvP1 prepropeptide, which could be divided into four regions; a signal peptide, an N-terminal propeptide that contains both an FTP domain (aa 64–114) and a PepSy domain (aa 127–205), the mature peptidase, consisting of an M4 domain (aa 217–360) and an M4 C-terminal domain (aa 362–506), and finally a C-terminal propeptide, containing two PPC domains (aa 543–612 and 650–719).

The calculated size of the protein sequence from the N-terminal end was 56 kDa, whereas the purified peptidase was seen as a 38 kDa band or smaller by SDS-PAGE. This suggests that MvP1 undergoes polypeptide removal from the carboxy terminal by at least 18 kDa. BLAST search revealed that the translated protein sequence of the predicted mature peptidase unit (aa 217–506) had highest similarity with vibriolysin (EC 3.4.24.25). It showed

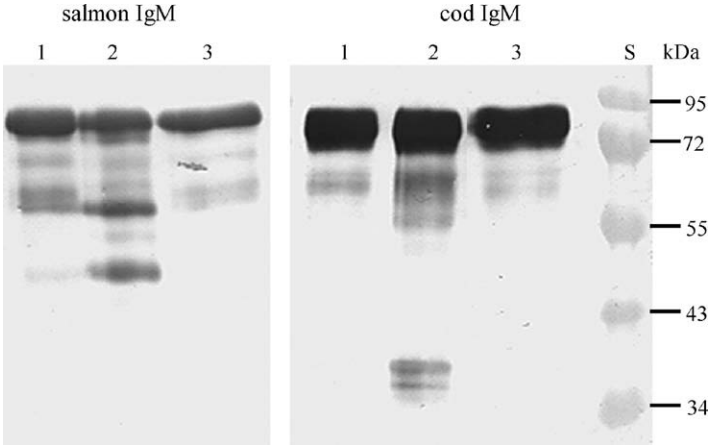

Fig. 4. Western blot analysis of salmon and cod IgM. Lane 1, untreated IgM; lane 2, IgM incubated with MvP1 peptidase; lane 3, IgM incubated with ECP; lane S, molecular weight standards as indicated in kDa. Incubation was at 9 °C for 24 h.

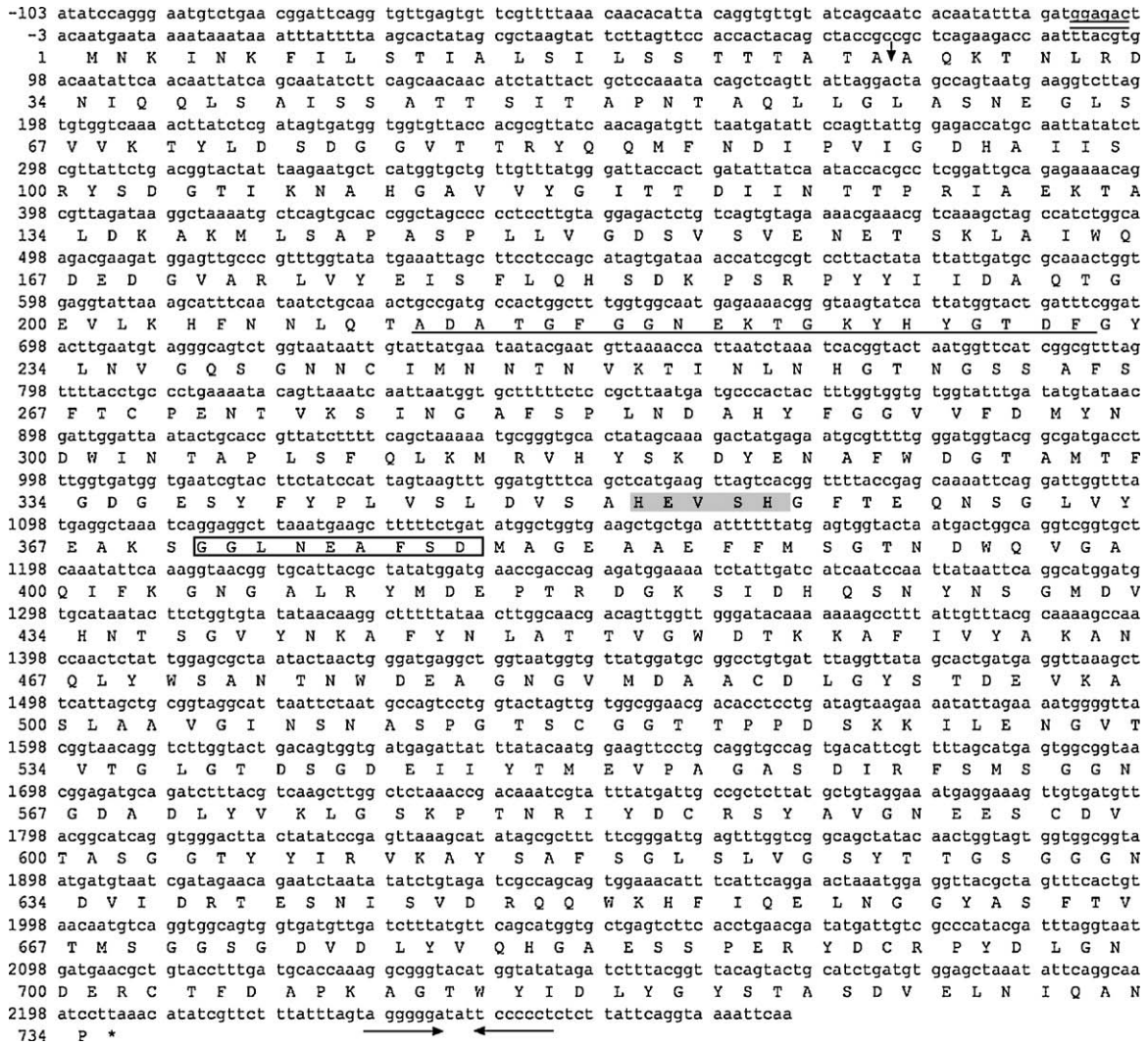

Fig. 5. Nucleotide sequence of the *mvp1* gene. Deduced amino acids of the MvP1 prepropeptide are shown below the first nucleotide of each codon. The first nt of the Met initiation codon is set as +1 and the initiation Met is set as the first aa. A putative SD sequence is double-underlined and the predicted signal peptide cleavage site is shown with a vertical arrow. The N-terminal amino acid sequence of MvP1 is underlined. The HEXXH zinc-binding motif is shaded and the third zinc ligand-motif is boxed. An inverted repeat sequence, characteristic of a Rho-independent transcription determinator, is indicated by convergent arrows.

highest percent sequence similarity with the MprI peptidase of *Pseudoalteromonas piscicida* (80% sequence identity, accession number BAB79615) and five other *Pseudoalteromonas* vibriolysins (77–79% sequence identity, accession numbers ACI28452, BAG70540, BAB85124, ABL06977, and EAR27939). Significant sequence similarity of the complete *mvp1* ORF extended throughout the entire ORFs of the *Pseudoalteromonas* peptidases. Two partial putative ORFs were also detected in the 3.562-nt sequence. A 483-nt sequence was found 59-nt downstream of *mvp1*, and showed highest aa similarity (40% sequence identity) with an aminopeptidase of *P. piscicida* (MprII, accession number BAB79616). A signal peptide with a predicted cleavage site after Ala-20 was detected in the sequence. A 212-nt sequence found 603-nt upstream of *mvp1*, encoded on the opposite strand and transcribed in the other direction, was identical with a predicted proline aminopeptidase sequence from *Moritella* sp. PE36 (accession number EDM65361).

4. Discussion

This paper describes the isolation and partial characterisation of MvP1, an extracellular metallopeptidase from the fish pathogen *Moritella viscosa*. The peptidase has virulence related activities and is able to degrade host tissue and IgM. It shows highest aa sequence similarity with *Pseudoalteromonas* vibriolysins. We also showed that the *M. viscosa* ECP are lethal to salmon.

MvP1 was the only detectable caseinolytic enzyme secreted by *M. viscosa* at 4, 9 and 15 °C. Several active isoforms of MvP1 were identified, mainly appearing after precipitation and other handling, indicating peptidase degradation of a single polypeptide. The isotypes also

appeared through natural processing during storage of ECP. The isolation of two or more isotypes of the same vibriolysin has been described previously (Wu et al., 1996; Miyoshi et al., 2002).

Total recovery of peptidase activity after isolation was 1.4%, and most of the activity was lost during the precipitation step, where only 13% of the activity measured in freshly collected ECP was recovered. The MvP1 is an abundant protein in the ECP of *M. viscosa*, as the estimated percentage of the peptidase ranged from 10 to 17%. To our knowledge, this is the first protein isolated and characterised from *M. viscosa*.

Optimum reaction temperature for isolated MvP1 was around 40 °C, but activity was detected over a broad temperature range. The relatively high activity of MvP1 at low temperatures probably reflects the low temperatures at which the bacterium is found and causes infections. The peptidase was not highly thermostable, showing decreased stability at 50 °C and no residual activity at 60 °C.

MvP1 was non-lethal to salmon, but caused extensive tissue necrosis and hemorrhages. However, ECP containing equal caseinolytic activities, were lethal to salmon and produced internal disease signs similar to those seen in fish infected with live bacteria. The ECP were isolated from a cellophane overlay culture, representing partially restricted nutrition, which resembles the host environment. The LD_{50} of the ECP of a Norwegian *M. viscosa* strain, F288/95, has been estimated in salmon, 0.3 μg/g fish, (Bjornsdottir, B., et al., unpublished results). Thus, MvP1 does not appear to be the lethal factor in *M. viscosa* ECP, or its effects may be enhanced by other unknown factors in the ECP. The necrotic and hemorrhagic damage caused by MvP1 may be due to the digestion of structural components, and possibly affect the development of ulcers that are often associated with *M. viscosa* infections. It has been proposed that bacterial peptidases, causing tissue damage and degradation of host tissues, play roles in pathogenesis by facilitating invasion of the pathogen (Miyoshi and Shinoda, 2000).

The isolated MvP1 peptidase did not cause prominent cell death in the two fish cell lines tested, EPC and BF-2, but seems to affect cell–cell adhesions. Disruption of epithelial cell adhesions can potentially aid the invasion and dispersion of *M. viscosa* in its host. *M. viscosa* ECP were more cytotoxic than isolated MvP1, and caused cell death and prominent morphological changes, indicating that MvP1 is not the major cytotoxic factor.

Salmon and cod IgM heavy chain was partially degraded by isolated MvP1, which might have an effect on the host immune system. The MvP1 peptidase may thus contribute to the pathogenesis of *M. viscosa* by obstructing the host's immune response. However, ECP containing equal caseinolytic activity did not degrade IgM. Thus, it seems that one or more factors in the ECP hindered the degradation. Whether this effect would be observed *in vivo* is not known. MvP1 was non-hemolytic against salmon and sheep erythrocytes, while hemolytic activity was detected in the ECP, especially against salmon erythrocytes.

The structural gene of the MvP1 peptidase was sequenced. Sequence alignments indicate that MvP1 is a member of the thermolysin family of zinc metallopeptidases. Inhibition studies confirmed the metallopeptidase nature of MvP1. The sequence contained the highly conserved HEXXH zinc-binding motif of metallopeptidases (Rawlings and Barrett, 1995), and the third zinc ligand motif GXXNEXXSD, that is characteristic of the thermolysin family (Miyoshi and Shinoda, 2000). The translated aa sequence showed highest similarity to vibriolysin peptidases (EC 3.4.24.25) that belong to the thermolysin family. Highest similarity was found to several vibriolysins from different *Pseudoalteromonas* species. According to the MEROPS database, vibriolysins originating from several non-pathogenic environmental bacteria, and pathogenic vibrios, causing diseases in both humans and marine organisms, have been described (Rawlings et al., 2008). Several vibriolysins have been implicated with virulence, although their functions are not fully understood, and they do not seem to be essential for bacterial virulence (Finkelstein et al., 1992; Miyoshi et al., 2002; Silva et al., 2003; Denkin and Nelson, 2004; Miyoshi, 2006).

The MvP1 prepropeptide can be divided into four regions, a signal peptide, an N-terminal propeptide, a mature peptidase, and a C-terminal propeptide. The determined N-terminal sequence of MvP1 started at aa 211, showing that an N-terminal propeptide of about 20 kDa is cleaved off to form the mature peptidase. Further N-terminal processing is unlikely, as the N-terminal end is found to be similar to previously described N-terminal ends of several metallopeptidases, and because it is located only 6 aa upstream of the predicted mature peptidase motif. Also, it seems that a C-terminal propeptide of at least 18 kDa is cleaved off the mature peptidase, since the apparent size of isolated MvP1 is 38 kDa or smaller, whereas the calculated size of the propeptide starting from the N-terminal end is 56 kDa. The presence of various active MvP1 isoforms of different sizes suggests that the C-terminal propeptide is cleaved off at different sites and results in the formation of the different isoforms of the mature peptidase. Propeptide processing at both the N- and C-terminal end has been described for several vibriolysins (David et al., 1992; Miyoshi et al., 1997; Lee et al., 2002; Miyoshi et al., 2002).

The N-terminal propeptides of thermolysins are known to inhibit peptidase activity in the cytoplasm and assist in protein folding (Tang et al., 2003; Chang et al., 2007). Two conserved domains were found within the N-terminal propeptide of MvP1. A FTP domain (Fungalysin/Thermolysin propeptide motif), which is believed to inhibit the peptidase and/or have chaperone activity, and a PepSY domain (Peptidase propeptide and YPEB domain), which is proposed to have an inhibitory function (Yeats et al., 2004; Marchler-Bauer et al., 2007; Demidyuk et al., 2008). The mature peptidase region consists of a M4 domain and a M4 C-terminal domain that is unique for the thermolysin family (Rawlings et al., 2008). Two PPC domains (bacterial pre-peptidase C-terminal domains) were detected in the C-terminal propeptide of MvP1, which are often not found in mature peptidases. The function of the C-terminal propeptide of thermolysin like peptidases is not known,

but it is believed to aid in enzyme binding to insoluble substrates (Demidyuk et al., 2008).

A partial ORF, found downstream of *mvp1*, had highest sequence similarity with the MprII aminopeptidase from *P. piscicida.* In *P. piscicida* the *mprII* gene is found 137-nt downstream of the previously mentioned vibriolysin encoding gene *mprI* (Miyamoto et al., 2002). Another partial ORF, found upstream of *mvp1*, was identical with a sequence of a predicted proline aminopeptidase from *Moritella* sp. PE36, which is not found in association with a predicted vibriolysin.

The results of the present study indicate that MvP1 is a vibriolysin, which causes necrotic and hemorrhagic tissue damage and degrades host components. It is therefore concluded that MvP1 may have an important role in *M. viscosa* invasiveness and in establishing infection.

Acknowledgments

We wish to thank Islandsbleikja (Samherji hf) for supplying salmon, Bergljot Magnadottir for isolated IgM and IgM antibodies, and Unnur Steingrimsdottir for lumpfish collagen. The EPC and BF-2 cells were a generous gift from the Danish Institute for Food and Veterinary Research. We also thank Jon Oskar Jonsson for his help with temperature effect assays. This work was supported by the AVS research fund, the Eimskip fund of the University of Iceland and the Rannis research fund.

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III

PAPER III

Effects of *Moritella viscosa* antigens on pro-inflammatory gene expression in an Atlantic salmon (*Salmo salar* Linnaeus) cell line (SHK-1)

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Published in: Fish & Shellfish Immunology, 2009, **26**, 858-863

Fish & Shellfish Immunology 26 (2009) 858–863

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Effects of *Moritella viscosa* antigens on pro-inflammatory gene expression in an Atlantic salmon (*Salmo salar* Linnaeus) cell line (SHK-1)

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ARTICLE INFO

Article history:
Received 15 January 2009
Received in revised form
19 March 2009
Accepted 20 March 2009
Available online 2 April 2009

Keywords:
Moritella viscosa
Extracellular products
MvP1 vibriolysin
Atlantic salmon
SHK-1
Gene expression
Immune response
Pro-inflammatory cytokine
Interleukin-1β (IL-1β)
Interleukin-8 (IL-8)

ABSTRACT

Moritella viscosa is the causative agent of winter ulcer disease in salmonids reared in North-Atlantic countries. In this study the effects of selected *M. viscosa* antigens on cytotoxicity and pro-inflammatory gene expression in an Atlantic salmon (*Salmo salar* Linnaeus) macrophage-like cell line (SHK-1) were examined. SHK-1 cells were stimulated with live and heat-killed bacterial cells, extracellular products (ECP) and an extracellular vibriolysin, termed MvP1. Following incubation, cytotoxicity and expression levels of interleukin-1β (IL-1β) and interleukin-8 (IL-8) were examined at different time points. Both live *M. viscosa* cells and ECP were cytotoxic, but neither heat-killed cells, nor the MvP1 peptidase caused cell death. Expression levels of both IL-1β and IL-8 increased significantly after stimulation with live cells, but heat-killed cells only caused increased IL-8 expression. ECP did not affect IL-1β expression, but did stimulate IL-8 expression. The isolated MvP1 peptidase stimulated both IL-1β and IL-8 expression at the highest concentration tested. This study reveals a difference in the induction of pro-inflammatory gene expression in salmon SHK-1 cells between live and heat-killed *M. viscosa* cells, and also that an unknown secreted factor is the main stimulant of IL-β and IL-8 expression.

1. Introduction

Moritella viscosa causes winter ulcer disease in farmed salmonids in North-Atlantic countries, at temperatures below 10 °C. Infections in Atlantic cod (*Gadus morhua* Linnaeus) have also been reported [1]. Infected fish often develop ulcers, which can extend deep into the underlying musculature, and the disease poses a serious fish welfare problem. Internally, hemorrhages and tissue necrosis are observed in various tissues and organs [2,3].

The virulence mechanisms of *M. viscosa* are still poorly understood. Lipooligosaccharides and a 17–19 kDa outer membrane antigen seem to be potential protective antigens in salmon [4]. *M. viscosa* extracellular products (ECP) cause mortalities in Atlantic salmon (*Salmo salar* Linnaeus), and internal disease signs similar to those seen in infected fish. Additionally, ECP have been shown to have both cytotoxic and hemolytic activities [5].

A metallopeptidase, termed MvP1, was isolated from the ECP of *M. viscosa*, and partially characterised. The peptidase, which is a vibriolysin (EC 3.4.24.25), is non-lethal to salmon at concentrations below 0.22 μg protein/g fish, but has virulence-related activities. It causes extensive hemorrhaging and tissue necrosis in salmon, degrades host tissue components and IgM, and affects cell–cell adhesion. It has been proposed that MvP1 aids in the invasion and dispersion of *M. viscosa* in its host [5].

A continuous cell line derived from Atlantic salmon head kidney leucocytes, SHK-1, has been used as a model system to study salmon immune responses at both the translational and transcriptional levels, under various conditions [6–9]. The cell line displays macrophage-like properties and is able to phagocytose the fish pathogen *Aeromonas salmonicida*, but does not exhibit bactericidal activity or have a macrophage-like appearance [10]. The cell line expresses both major histocompatibility complex class I and II [11], and C-type lectin receptors [12].

Most infectious agents induce an inflammatory response in their host; this response initiates activation of the innate immunological response. Cytokines such as interleukin-1β (IL-1β) and

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doi:10.1016/j.fsi.2009.03.015

interleukin-8 (IL-8), which promote the inflammation process, are called pro-inflammatory cytokines; they are good markers of the inflammatory response. Both genetic and biological evidence imply that the fundamental properties of the pro-inflammatory cytokines in mammals and teleost fish are similar [13–17]. IL-1β is an important and central mediator of immune and inflammatory responses and initiates a wide variety of functions, such as the expression of other cytokines and macrophage activation. It is primarily produced by monocytes and macrophages [14,15]. IL-8 (also known as CXCL8) is a chemokine, which attracts neutrophils to the site of infection or injury. It is produced by many different cell types and can be induced by different factors, including IL-1β [17].

The aim of this study was to examine the effects of selected *M. viscosa* antigens on gene expression of the pro-inflammatory cytokines IL-1β and IL-8 in an Atlantic salmon cell line, SHK-1, and to determine their potential role in triggering the host immune response following infection with *M. viscosa*.

2. Materials and methods

2.1. Moritella viscosa culturing and isolation of antigens

M. viscosa strain K58 was used in the current study. The strain originates from the head kidney of Atlantic salmon suffering from winter ulcer disease in Iceland [18,19]. This strain was passaged in salmon prior to the study, by re-isolation from kidney of intraperitoneally (i.p.) infected fish. The bacterium was grown in batch cultures, following cultivation on 5% horse blood agar supplemented with 2% NaCl (BA-NaCl) at 4 °C. For each batch culture, Brain–Heart infusion broth (BHI, BD, Franklin Lakes, NJ, USA) containing 1.5% NaCl (BHI-NaCl) was inoculated with strain K58 (1% inoculation from a starter culture), and cultivated at 4 °C with agitation (200 rpm). *M. viscosa* cells and ECP were harvested from a logarithmic phase culture, grown for 48 h. Cells were washed twice in Dulbecco's phosphate buffered saline (DPBS, with Ca^{++} and Mg^{++}, Sigma, Mississauga, ON, Canada), re-suspended in the same buffer and kept on ice. A part of the washed cell suspension was heat-killed in a water bath at 60 °C for 1 h. Colony forming units (cfu) were estimated by plate counting on BHI-NaCl agar at 4 °C. ECP were isolated from cells through centrifugation (3200 × g, 10 min, 4 °C) and filtration (22 μm, Whatman, Dassel, Germany). The MvP1 peptidase was isolated from a stationary phase culture as previously described [5]. Protein content of *M. viscosa* ECP and isolated MvP1 was measured in duplicates using a Bradford protein assay kit (Coomassie Plus, Pierce, Rockford, IL, USA) and bovine serum albumin (Sigma) used for plotting a standard curve. Absorbance was measured at 590 nm and each sample measured in duplicate.

2.2. M. viscosa growth in cell cultures, MvP1 stability and cytotoxicity assay

The ability of *M. viscosa* to grow at 20 °C in BHI-NaCl, SHK-1 cell cultures and cell culture medium alone was tested at 4 and 24 h. Bacterial numbers were estimated by plating out ten-fold dilutions of the medium onto BA-NaCl plates.

The stability of MvP1 activity in DPBS and culture medium, both fresh and conditioned, was verified for up to 72 h at 20 °C. Medium collected from SHK-1 culture flasks after 3 days of culturing was used as conditioned medium. MvP1 was diluted 1:1 in the respective buffer or medium. The buffer used for MvP1 isolation (50 mM Na-phosphate buffer, pH 7, containing 15 mM NaCl) was used as a control. Caseinolytic activity in each sample was measured in duplicate using the azocasein assay, as previously described [5], and one unit of azocasein activity defined as an increase in absorbance of 0.01 under the assay conditions at 430 nm.

Concentration of antigens used for SHK-1 stimulation was determined via a cytotoxicity detection kit (LDH, Roche, Basel, Switzerland), as stimulants should not affect cell viability. The assay was performed in triplicate in 96 well plates, according to the manufacturer's instructions, with 5×10^3 SHK-1 cells/well. After culturing for 2 days, under the conditions described below, cells were washed twice with DPBS and incubated with stimulants diluted in DPBS at 20 °C for 4, 24 or 72 h. Controls, lactate dehydrogenase measurements and calculation of percentage cytotoxicity were performed as described by Fast et al. [20].

2.3. SHK-1 cell culture and stimulation

SHK-1 cells were cultured at 18 °C in 75 cm^2 flasks (Costar, Fisher Scientific, Ottawa, ON, Canada), as described by Fast et al. [6], without antibiotics. Cells used in this study were passaged between 61 and 63 times. SHK-1 cells were seeded in 25 cm^2 flasks (Costar, Fisher Scientific) at approximately 1×10^6 cells/flask, 72 h before stimulation, and cultured at 20 °C. Then, medium was removed from each flask and 5 ml fresh medium, containing the stimulants, was added. The stimulations were performed in two separate experiments. In experiment 1, SHK-1 cells were stimulated with *M. viscosa* cells, either live or heat-killed, with estimated multiplicity of infection (MOI) of 2, or ECP in final concentrations of 0.01 or 0.05 μg protein/ml. In experiment 2, isolated MvP1 was added in final concentrations of 0.1, 0.4, 0.7 and 1.0 μg protein/ml. Cultures were incubated with stimulants at 20 °C for 4, 24 or 72 h, and cells incubated with medium and dilution buffer only were used as control. Each treatment was carried out in triplicate.

2.4. RNA isolation, cDNA synthesis, and real-time Q-PCR

Following stimulation, total RNA was isolated from SHK-1 cells using 3 ml TRIzol Reagent (Invitrogen, Burlington, ON, Canada), according to the manufacturers instructions, and as described by Fast et al. [20]. RNA concentrations were determined using a NanoDrop-1000 (v3.2.1, Thermo Scientific, Delaware, CO, USA) spectrophotometer, and samples were stored at −80 °C until reverse transcription. For reverse transcription, 2.0 μg total RNA from each sample were used, dissolved in molecular biological grade water. Two-step reverse transcription real-time Q-PCR was carried out using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR green (Invitrogen) on an iCycler iQ Real-Time detection system (BioRad, Mississauga, ON, Canada). The manufacturer's instructions were followed, with the exceptions described by Fast et al. [20]. Following first strand synthesis, samples were stored at −20 °C until use in real-time Q-PCR. Primers for real-time Q-PCR of elongation factor-1A (EF-1A), which was used as a reference gene, IL-1β, and IL-8 were as described in Table 1, and based on previously published Atlantic salmon sequences. The PCR products of each primer pair were cloned and sequenced, and isolated plasmid

Table 1
Real-Time Q-PCR primer sequences.

Gene	Accession number GenBank	Primer	Sequence (5′–3′)
EF-1A	AF321836	EF-1A-forward	GTG GAG ACT GGA ACC CTG AA
		EF-1A-reverse	CTT GAC GGA CAC GTT CTT GA
IL-1β	AY617117	IL-1β-forward	CGT CAC ATT GCC AAC CTC AT
		IL-1β-reverse	ACT GTG ATG TAC TGC TGA AC
IL-8	BT046706	IL-8-forward	GAA TGT CAG CCA GCC TTG TC
		IL-8-reverse	TCC AGA CAA ATC TCC TGA CCG

vectors used as standards for real-time Q-PCR as previously described [6]. The EF-1A primers were designed to span an intron/exon splice site and single-product amplification was confirmed through melt curve analysis, to ensure that genomic DNA was not quantified in real-time Q-PCR. Cycle conditions were as described by Fast et al. [20]. Ten-fold dilutions of the standards (between 1 and 10^{-5} ng) and a blank without cDNA were run in each real-time Q-PCR, along with the duplicate samples. The relationship between the threshold cycle (C_t) and log(RNA) was linear ($-3.18 <$ slope < -3.02) for all reactions.

2.5. Data analysis

All data are presented as means ± SEM. Statistical analyses on cytotoxicity assay data and gene expression data were performed using two-way ANOVA (variables: antigen, sampling time, and interaction). The Tukey HSD test was then used to discern differences among means for those effects that had significant differences ($p < 0.05$) (SPSS 15.0 for Windows) and the data were segregated by variable and compared to the relative control.

Expression of IL-1β and IL-8 was calculated as relative to the expression of the EF-1A reference gene. Due to significant differences in the expression of EF-1A between sampling times, the relative expression of IL-1β and IL-8 was calculated from the mean expression of EF-1A at each time point, for each experiment, with values from control and stimulated cells combined. Gene expression of IL-1β and IL-8 in stimulated cells is presented as fold expression compared to expression in control cells.

3. Results

3.1. M. viscosa antigens

M. viscosa cfu were counted on agar plates and the MOI calculated. No colonies grew on plates streaked out with heat-killed cells. The protein content of freshly collected ECP was estimated at 120 μg/ml, and caseinase activity of 15 U. The protein content of isolated MvP1 was estimated at 25 μg/ml and caseinase activity of 50 U.

3.2. M. viscosa growth in cell cultures, MvP1 stability and cytotoxicity assay

M. viscosa cells were unable to grow in SHK-1 cell culture medium at 20 °C. After 4 and 24 h of incubation only 2 and 0.5% of the initial bacterial numbers were culturable, respectively. Attempts to culture *M. viscosa* from SHK-1 cell cultures were unsuccessful. However, the bacterium grew normally in BHI-NaCl at 20 °C (results not shown).

The MvP1 peptidase was stable and retained its caseinolytic activity at the experimental temperature in the buffers and medium used, as shown in Fig. 1.

The effects of *M. viscosa* antigens on SHK-1 cell viability are shown in Fig. 2. *P*-values from two-way ANOVA were significant ($p = 0.000$) for all variables. Live *M. viscosa* cells caused significant cytotoxicity in SHK-1 cells after 72 h incubation in all densities tested, MOI 6, 3 and 0.3, and after 24 h with the highest density. However, heat-killed *M. viscosa* cells did not affect SHK-1 cell death at any time point when tested in the same densities. Only the results from the highest density of heat-killed cells (MOI 6) are shown in Fig. 2. *M. viscosa* ECP caused significant cell death after 24 and 72 h of incubation at a concentration of 0.5 μg protein/ml, but a concentration of 0.05 μg protein/ml ECP did not affect cell viability. The MvP1 vibriolysin did not cause significant cell death at a 1 μg protein/ml concentration. The results of the cytotoxicity assay were subsequently used to determine the upper limits of *M. viscosa* antigen doses used in cell stimulation experiments, so that they would not affect the viability of the SHK-1 cells.

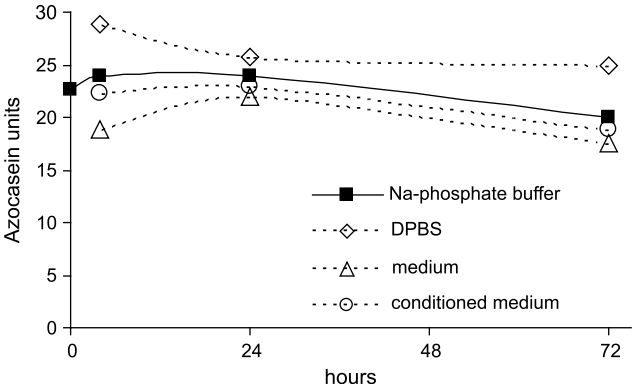

Fig. 1. MvP1 stability in buffers and tissue culture medium used during SHK-1 stimulation. MvP1 activity against azocasein was measured at each time point at 20 °C. DPBS, Dulbecco's phosphate buffered saline.

3.3. EF-1A as a reference gene

EF-1A was chosen as a reference gene for this study, and the mean expression of EF-1A at each time point was calculated from values of both stimulated and unstimulated cells. In experiment 1 the average expression was $5.4 \times 10^8 \pm 0.91 \times 10^8$, $4.7 \times 10^8 \pm 0.29 \times 10^8$ and $4.8 \times 10^8 \pm 0.36 \times 10^8$ copy numbers per μg RNA at 4, 24 and 72 h, respectively. *P*-values from two-way ANOVA were non-significant for all variables: 0.052, 0.181, and 0.490 for antigen, sampling time, and interaction, respectively. However, in experiment 2, mean EF-1A copy numbers per μg RNA were $1.2 \times 10^9 \pm 0.07 \times 10^9$, $6.7 \times 10^8 \pm 0.72 \times 10^8$ and $6.0 \times 10^8 \pm 0.89 \times 10^8$ at 4, 24 and 72 h, respectively. Sampling time was found to have a significant effect on expression ($p = 0.000$), where the expression at 4 h was higher than at 24 and 72 h. Antigen type or interaction between antigen and sampling time were not found to have significant effect on EF-1A expression ($p = 0.107$ and 0.062, respectively). Due to the differences in the expression of EF-1A, the average copy number at each time point was used to calculate the relative expression of both IL-1β and IL-8.

3.4. Stimulation experiment 1; M. viscosa cells and ECP

The mean relative expression of IL-1β in control cells in experiment 1 was $5.2 \times 10^{-4} \pm 0.84 \times 10^{-4}$, $4.9 \times 10^{-4} \pm 0.95 \times 10^{-4}$, $5.4 \times 10^{-4} \pm 0.5 \times 10^{-4}$ at 4, 24 and 72 h, respectively. The values did not differ significantly. The type and dose of antigen was found to have a significant effect on IL-1β expression ($p = 0.000$), but sampling time ($p = 0.486$) or interaction ($p = 0.892$) did not have a significant effect. Following stimulation with live *M. viscosa* cells for 24 h, expression of IL-1β increased significantly, and was 7.1 times higher than in control cells, despite a high degree of variability in the treatment group. Heat-killed cells did not cause a significant increase in IL-1β expression compared to control cells, with only a two-fold increase. ECP did not cause a significant increase in the expression of IL-1β, causing only a 2.3-fold or lower increase (Fig. 3A).

The mean relative expression of IL-8 in control cells was $2.1 \times 10^{-2} \pm 0.21 \times 10^{-2}$, $2.0 \times 10^{-2} \pm 0.34 \times 10^{-2}$, $1.0 \times 10^{-2} \pm 0.07 \times 10^{-2}$ at 4, 24 and 72 h, respectively, with no significant differences. Significant differences on IL-8 expression were found for all variables: antigen and interaction ($p = 0.000$),

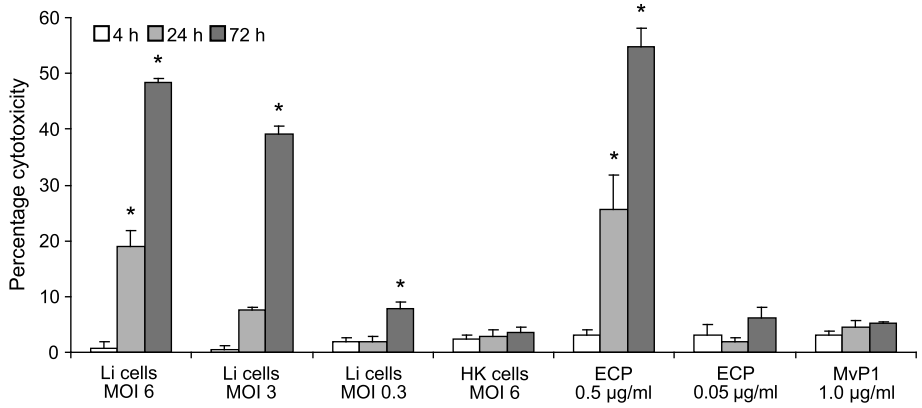

Fig. 2. Mean percent cell death (±SEM) in SHK-1 cells treated with *M. viscosa* antigens for 4, 24 and 72 h. Li, live; HK, heat-killed; MOI, multiplicity of infection; ECP, extracellular products; MvP1, vibriolysin. Concentration of ECP and MvP1 is given in µg protein/ml. An asterisk signifies a significant difference from negative controls ($p < 0.05$).

and sampling time ($p = 0.008$). Both live and heat-killed cells caused increased expression of IL-8, showing 9.3- and 6.6-fold increased expression, respectively. The difference between stimulation with live and heat-killed cells was not significant. *M. viscosa* ECP (0.01 µg protein/ml) caused a significant 34.1-fold increase in IL-8 expression at 72 h, but not at 4 or 24 h. At a higher concentration (0.05 µg protein/ml), ECP caused a significant increase in IL-8 expression from control cells at all sampling times (5.8–19.1-fold) (Fig. 3B), but there was not a significant difference between the three sampling times at that concentration.

3.5. Stimulation experiment 2; M. viscosa MvP1 vibriolysin

In experiment 2, the mean relative IL-1β expression in control cells was $6.5 \times 10^{-4} \pm 1.9 \times 10^{-4}$, $1.2 \times 10^{-3} \pm 0.29 \times 10^{-3}$, $8.9 \times 10^{-4} \pm 0.88 \times 10^{-4}$ at 4, 24 and 72 h, respectively. There was no significant difference between the control values. Significant differences on IL-1β expression were found for all variables: antigen dose ($p = 0.007$), sampling time ($p = 0.036$) and interaction ($p = 0.004$). The change in IL-1β expression of MvP1 stimulated cells ranged from 0.5 to 2.6-fold, and only cells stimulated with the highest concentration showed a significant increase in expression at 4 h (Fig. 4A).

The mean relative expression of IL-8 in control cells was $8.0 \times 10^{-3} \pm 0.47 \times 10^{-3}$, $7.4 \times 10^{-3} \pm 4.1 \times 10^{-3}$, $1.2 \times 10^{-2} \pm 0.25 \times 10^{-2}$ at 4, 24 and 72 h, respectively, and did not show any significant differences. All variables were found to have a significant effect on IL-8 expression: antigen dose ($p = 0.000$), sampling time ($p = 0.016$) and interaction ($p = 0.001$). The expression of IL-8 exhibited dose-dependence on the concentration of MvP1 at both 4 and 24 h, but was only significantly higher from control cells at the highest concentration (1.0 µg protein/ml MvP1), or 4.9- and 9.4-fold, respectively. After stimulation for 24 h with 0.7 µg protein/ml MvP1, two of the samples showed a 6.3- and 7.3-fold increase in IL-8 expression, while the third sample showed only a 2.3-fold increase in expression. Therefore, the data were not significantly different from control cells. No significant differences were detected at 72 h (Fig. 4B).

4. Discussion

In this study, the effects of *M. viscosa* antigens on pro-inflammatory gene expression in an Atlantic salmon macrophage-like cell line (SHK-1) were evaluated. The results show that live *M. viscosa* cells and MvP1 peptidase caused increased expression of IL-1β, but heat-killed cells or ECP did not. However, all the antigens stimulated expression of IL-8. Furthermore, both live *M. viscosa* cells and ECP affected the SHK-1 cell viability.

EF-1A, which is an important part of the translational machinery in eukaryotes, was chosen as a reference gene in this study. EF-1A has previously been used successfully as reference gene in expression experiments in Atlantic salmon [20–22]. In our study, however, some significant differences in expression levels were observed. EF-1A expression was significantly higher at 4 h than at

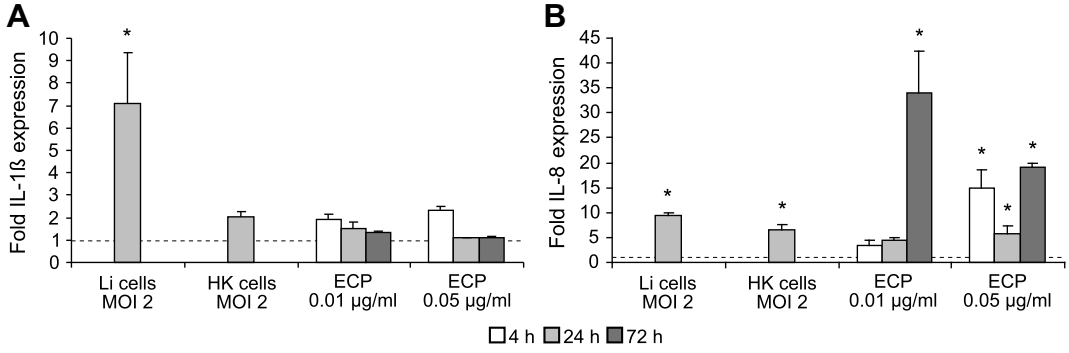

Fig. 3. Fold IL-1β (A) and IL-8 (B) expression in stimulated SHK-1 cells compared to control cells, following stimulation with *M. viscosa* cells or ECP for 4, 24 and 72 h. The expression is calculated as relative to the expression of the EF-1A reference gene. Li, live; HK, heat-killed; MOI, multiplicity of infection; ECP, extracellular products. Concentration of ECP is given in µg protein/ml. An asterisk signifies a significant difference from the relative control ($p < 0.05$) and a dotted line signifies the expression in control cells. Note the different *Y*-axis scales.

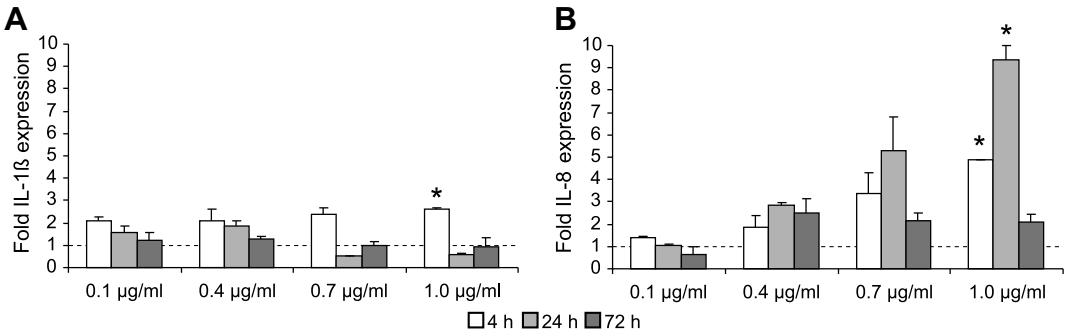

Fig. 4. Fold IL-1β (A) and IL-8 (B) expression in stimulated SHK-1 cells compared to control cells, following stimulation with the MvP1 vibriolysin for 4, 24 and 72 h. The expression is calculated as relative to the expression of the EF-1A reference gene. Concentration of MvP1 is given in µg protein/ml. An asterisk signifies a significant difference from the relative control ($p < 0.05$) and a dotted line signifies the expression in control cells.

24 or 72 h in cell stimulation experiment 2, but no significant differences were detected in experiment 1. In order to correct for the differences, the average EF-1A expression of both unstimulated and stimulated cells at each time point in each experiment was used to calculate the relative expression of IL-1β and IL-8. The increased expression of EF-1A in experiment 2 at 4 h could possibly be due to some short-term effects of handling of the cells or addition of medium. Also, EF-1A may have variable expression throughout the life cycle of SHK-1 cells, and that in experiment 2 sampling was performed at a time of increased expression. Further research into the temporal variation of EF-1A in SHK-1 cells would be valuable.

The cytotoxic effects of *M. viscosa* antigens on SHK-1 cells were evaluated, to determine their effect on cell viability during stimulation experiments. Live cells were highly cytotoxic, but heat-killed cells had no effect on cell viability. Since ECP isolated from *M. viscosa* were also highly toxic, it may suggests that at least some of the bacterial virulence is related to ECP secretion. However, our results show that only a small percentage of the live cells survived in the cell culture medium. Another possibility is that the heat treatment, during killing of *M. viscosa*, affected the ability of some bacterial antigens to cause cytotoxicity. Isolated MvP1 peptidase had no significant effect on cell viability. The peptidase was shown to be active at the experimental conditions, therefore, one or more unidentified components of the ECP are responsible for cell death. Microscopic examination of cells revealed that cell morphology was slightly affected by live cells, ECP, and MvP1 at concentrations that did not affect viability. Only heat-killed cells had no detectable effect on cell morphology. It has been shown previously that *M. viscosa* ECP are cytotoxic to EPC and BF-2 fish cell lines, and that MvP1 affects cell–cell adhesion in the same cells [5].

Based on cytotoxicity assay results, SHK-1 cells were stimulated with *M. viscosa* cells at MOI 2. Cells were stimulated for 24 h, but not for longer, as incubation with live cells at MOI of only 0.3 resulted in significant cell death at 72 h. At 24 h, live cells caused a significant increase in IL-1β expression, but heat-killed cells did not. Due to how highly cytotoxic the ECP were to SHK-1 cells at a concentration of 0.5 µg protein/ml, cells were subsequently only stimulated with a concentration of 0.05 or 0.01 µg protein/ml. At those concentrations the ECP did not affect IL-1β expression. Some cytotoxicity, although not significant, may have been associated with live cells at MOI 2. Subsequently these dead or dying SHK-1 cells may have acted as danger signals inducing IL-1β expression in the remaining culture. Interaction of *M. viscosa* with cellular membranes and/or continuous ECP production of live cells may also be reasons for the differences in IL-1β expression between SHK-1 cells stimulated with heat-killed and live cells. Yet another possibility is that gene expression was only stimulated through the synergistic effects of *M. viscosa* cells and ECP. The continuous production and release of ECP by live cells in culture with SHK-1 cells may have allowed a higher, or more stimulatory, concentration to be achieved with minimal cytotoxicity, whereas ECP at the concentrations tested on their own did not stimulate IL-1β expression. It should also be noted that the ECP used in the stimulation were from an exponential phase broth culture cultivated at 4 °C, whereas during the stimulation, *M. viscosa* cells were in contact with salmon cells at 20 °C. The possible factors that the cells may have produced during stimulation may thus have been different from the factors present in broth culture ECP. However, as previously discussed, the heat killing of bacterial cells may have destroyed a stimulatory antigen(s).

In this study, SHK-1 cells were stimulated at 20 °C, whereas *M. viscosa* usually causes infections at temperatures below 10 °C. Therefore, it is important to note that the cells can respond differently to the antigen stimulation at lower temperatures. However, the antigens were produced from 4 °C cultures, so that they would represent factors produced at those temperatures. Temperature has been shown to have a pronounced effect on expression levels of IL-1β in rainbow trout head kidney leucocytes, where expression at 22 °C was significantly higher than at both 4 and 14 °C, following LPS stimulation for 4 h [23].

Both live and heat-killed cells caused significant increases in IL-8 expression at 24 h *M. viscosa* ECP caused a significant increase in IL-8 expression at 72 h in both concentrations, but also at 4 and 24 h in the higher concentration. Therefore, a lower concentration of ECP did not affect IL-8 expression as quickly as the higher concentration, which stimulated expression throughout the experiment.

In experiment 2, SHK-1 cells were stimulated with MvP1, an extracellular *M. viscosa* vibriolysin. MvP1 has virulence-related activities and may have a role in bacterial invasion and dispersion [5]. MvP1 did not affect SHK-1 cell viability, and could thus be used in much higher concentrations for stimulation than total ECP, which were highly cytotoxic. Therefore, cells were stimulated with up to 200-fold higher concentrations of isolated MvP1 than would be expected from the ECP in experiment 1. However, the concentrations are believed to be within biologically relevant limits, as MvP1 is a major component of in vitro produced ECP [5]. The relative production of MvP1 in vivo is, however, not known.

At the highest concentration, MvP1 stimulated IL-1β expression at 4h, but not at any other concentrations or time points. Similarly, IL-8 expression increased significantly only after stimulation with the highest MvP1 concentration, both at 4 and 24 h. Thus, the increased expression of IL-8 seen after stimulation with ECP has not been caused by MvP1, or unknown factor or factors in the ECP have enhanced the stimulatory effects of MvP1. Also, the long-term

increase in IL-8 expression seen after ECP stimulation is not detected after MvP1 stimulation, where the expression increase is short-term. A previous study has shown that another vibriolysin, *Vibrio cholerae* hap, does not induce IL-8 expression or affect cell viability in human intestinal epithelial cells [24]. High variability was seen in IL-8 expression after 24 h stimulation with 0.7 μg protein/ml MvP1, where two samples showed a clear increase in expression, but one sample showed only a minor increase. This may indicate that during that time, cells were going through a shift in expression. This may also apply to other time points where gene expression was highly variable.

Several reports have been published on the effects of bacterial products on IL-1β expression in Atlantic salmon. IL-1β expression in SHK-1 cells has been examined in two previous studies by Fast et al. [6,7]. Both studies showed a rapid increase in IL-1β expression following LPS stimulation for 4 h. In another study, Fast et al. [20] found that LPS stimulation of head kidney macrophages isolated from Atlantic salmon increased IL-1β expression after 24 h, but not after 1 or 3 h *M. viscosa* produces LOS [4], which presumably were present on both live and heat-killed cells. Therefore, since heat-killed cells did not stimulate IL-1β expression, the LOS do not seem to have affected IL-1β expression under the assay conditions. A multivalent salmon vaccine, containing an oil adjuvant and selected bacterial antigens, including *M. viscosa*, has also been shown to stimulate increased IL-1β response in head kidney of i.p. and intramuscularly (i.m.) vaccinated Atlantic salmon at 24 h post injection, but not at 4 or 6 h [25]. Our results of increased IL-1β expression at 24 h following stimulation with live cells, and at 4 h following stimulation with MvP1 are in agreement with the previous studies, where the changes in expression seem to be rapid and short-term.

To our knowledge, the expression of IL-8 in Atlantic salmon in response to bacterial antigens has not been reported previously. IL-8 expression has been shown to be up-regulated in rainbow trout following bacterial infections [26], and also in pink salmon (*Oncorhynchus gorbuscha* Walbaum) and chum salmon (*Oncorhynchus keta* Walbaum) following vaccination against *A. salmonicida* [27]. Increased expression of pro-inflammatory genes thus seems to be a common response in salmonids to bacteria, as in higher vertebrates.

Cytokines usually have short half-lives in vivo and are highly regulated, both at the transcriptional and the post-transcriptional levels. Therefore, their increase following infection or tissue damage is usually only detectable over a limited time. High and prolonged expression of pro-inflammatory cytokines can lead to local tissue damage. Following stimulation with 0.05 μg protein/ml ECP, IL-8 expression was up-regulated for up to 72 h. Therefore, the pathology observed in fish suffering from winter ulcer disease may possibly be associated with the host's prolonged inflammatory reaction to ECP, and not only with direct effects of the bacterium.

The results of the study show that *M. viscosa* antigens have different stimulatory effects on pro-inflammatory gene expression in SHK-1 cells. It would be of interest to examine further the differences between the ability of live and heat-killed cells in inducing IL-1β expression, and the possible importance for vaccine production. Further studies on finding the main inflammatory-stimulating factors in ECP would also be of interest.

Acknowledgments

This study was supported by the AVS research fund, the Eimskip fund of the University of Iceland, the Rannis research fund, and the EMBO short-term fellowship fund. We also thank Thorarinn Sveinsson for his assistance with statistical analysis.

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IV

PAPER IV

Identification of type VI secretion systems in *Moritella viscosa*

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Submitted for publication in Veterinary Microbiology

Identification of type VI secretion systems in *Moritella viscosa*

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Abstract

The study describes the identification of type VI secretion systems (T6SSs) in *Moritella viscosa*, the aetiological agent of winter ulcer disease. Despite the availability of commercial vaccines, *M. viscosa* causes significant financial losses in salmonid farming. The T6SS transports bacterial proteins from the cell into the environment or directly into host cells, and has been implicated with bacterial virulence. Therefore, information on the presence of a T6SS in *M. viscosa* is of interest and could give further insight into the biology of the bacterium.

The genome of *M. viscosa* 06/09/139 was screened for homology with known T6SS encoding genes. Two genetically distinct loci, termed Moritella Type Six Secretion 1 and 2 (*mts1* and *mts2*), were identified as encoding putative T6SSs. Each locus contained known T6S core genes. The *mts2* locus contained species specific genes, some of which have not previously been connected with T6S. The predicted *mts1* and *mts2* T6SS loci were compared to previously described T6SSs. The *mts1* locus showed sequence homology and synteny to T6SSs of the fish pathogen *Aliivibrio salmonicida* and a non-pathogenic *Moritella* sp. PE36. The *mts2* locus was more similar to a *Vibrio parahaemolyticus* T6SS. A functional Mts1

T6SS was confirmed through identification of secreted Hcp protein in both virulent and avirulent *M. viscosa* isolates. The results show that *M. viscosa* has at least one functional T6SS, but the role of the secretion system and possible connections with virulence need further examination.

Keywords: *Moritella viscosa*, winter ulcer disease, Type VI secretion system

1. Introduction

The Gram negative bacterium *Moritella viscosa* is the causative agent of winter ulcer disease of farmed fish. The disease affects Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss* L.) and Atlantic cod (*Gadus morhua* L.) reared in the North Atlantic Ocean during winter months with temperatures below 8°C. The disease causes mortalities and financial losses, as large ulcers often appear on infected fish, besides being a fish welfare problem (Lunder et al., 1995; Benediktsdottir et al., 2000; Colquhoun et al., 2004). Commercial vaccines against *M. viscosa* are available, but do not provide full protection against the disease. Increased understanding of *M. viscosa* biology and pathogenesis is therefore important.

Bacteria have evolved different mechanisms to translocate proteins across cell membranes. Currently, six classes of secretion systems are known in Gram-negative bacteria, termed type I to type VI. The type VI secretion system (T6SS) has been identified within the genomes of many pathogenic and non-pathogenic proteobacteria (Filloux et al., 2008). The functions and components of the T6SS are still poorly understood, but it is believed to deliver proteins into the environment or directly into host cells through a phage-tail-like injectisome (Pukatzki et al., 2009). T6S is important for virulence of several pathogenic bacteria (reviewed in Bingle et al., 2008). Alternate roles related to bacterial fitness in the environment, such as quorum sensing (Weber et al., 2009), biofilm formation (Vaysse et al.,

2009) and antimicrobial activity (Hood et al., 2010) have also been demonstrated. Several conserved core proteins are encoded within T6SS clusters, including the secreted proteins Hcp (hemolysin coregulated protein) and VgrG (valine glycine repeat protein G) (Boyer et al., 2009). Hcp forms hexameric rings and is presumed to build the channel used for secretion. Hcp secretion is T6SS dependent and is a reliable indicator of active T6S (reviewed in Pukatzki et al., 2009). VgrG proteins may function to puncture host cells and deliver C-terminal effector domains into host cells (Pukatzki et al., 2007). The expression and assembly of T6SSs are under tight control, both at the transcriptional and the post-translational level (Bernard et al., 2010). Many bacteria contain more than one T6SS loci, which often have different gene organization and are described as different sub-groups. The systems are often independently regulated, indicating different roles of the systems (Boyer et al., 2009). T6SSs have been genetically identified in several marine bacteria (Persson et al., 2009; Vaysse et al., 2009). Two T6SSs have been identified in the genome of the non-pathogenic *Moritella* sp. PE36 (Persson et al., 2009).

The aim of the study was to identify T6SSs in the fish pathogenic bacterium *M. viscosa*, both genetically and on a protein level, and to compare the identified systems to previously described T6SSs.

2. Materials and methods

2.1. Analysis of T6SSs in the M. viscosa *genome*

The sequence assembly of the *M. viscosa* 06/09/139 genome is in its finishing phase and can be accessed at: http://arctic.imb.fm.uit.no/blast/. Genomic sequences were annotated as described previously (Hjerde et al., 2008) using the Artemis DNA Sequence Viewer and Annotation Tool (Rutherford et al., 2000). Coding sequences (CDSs) of T6SS loci were identified through extensive homology searches with known T6SS related proteins performed

against the genome sequence of *M. viscosa* 06/09/139, using BLASTP (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988) search programs. An operon was predicted as a chain of functionally related genes transcribed in the same direction with intergenic distances less than 200 base pairs. Protein motifs were identified using Pfam (Finn et al., 2010), transmembrane domains were identified with TMHMM (Krogh et al., 2001) and signal sequences were identified with SignalP (3.0) (Emanuelsson et al., 2007). Naming of *M. viscosa* T6SS CDSs was based on the naming system assigned to *Vibrio anguillarum vts* T6SS (Weber et al., 2009; Debra L. Milton, personal communication).

2.2. Comparison of M. viscosa *T6SSs to previously identified T6SSs*

Comparison of *M. viscosa* 06/09/139 T6SS loci against previously described T6SSs of the fish pathogen *Aliivibrio salmonicida* LFI1238 (FM178379), the *Vibrionales* bacterium SWAT-3 (NZ_AAZW01000012), *Vibrio cholerae* N16961 (AE003853) and *Vibrio parahaemolyticus* RIMD 2210633 (BA000031) was facilitated by using The Artemis Comparison Tool (Carver et al., 2005), which enabled the visualization of BLASTN and TBLASTX comparisons.

2.3. M. viscosa *isolates, growth conditions and preparation of supernatants and cells*

The study included 10 *M. viscosa* isolates (Table 1). Isolates were streaked onto 5% horse blood agar supplemented with 2% NaCl (BA-NaCl) and incubated at 15 °C for three days. Duplicate broth cultures of each isolate were produced. Single colonies were inoculated into 20 ml brain-heart infusion broth (BD) containing 2% NaCl (BHI-NaCl) and cultured at 15 °C (200 rpm) for 38 h. Bacterial density was estimated by plating and counting of colony forming units (CFU)/ml.

Following the addition of a protease inhibitor cocktail (P8340, 1:1000 dilution, Sigma), cultures were centrifuged (1800 x g, 25 min, 4 °C). Cells were re-suspended in 2 ml phosphate buffered saline (PBS, Sigma). Supernatants (15 ml) were filtered (Whatman, 0.45 µm), concentrated on a centrifugal filter (Amicon Ultra-15, MWCO 10.000, Millipore) (3800 x g, 30 min, 10 °C) and desalted on the same filter with 15 ml PBS under the same conditions. This resulted in about 50-fold reduction of the total volume of supernatants. Protein concentration of samples was determined using the Coomassie Plus protein assay kit (Pierce).

2.4. Electrophoresis and electroblotting

Samples of supernatants and cells were boiled in reducing buffer (100 °C, 7 min) and electrophorised (200V, 65mA, 50 min) in 14% SDS-PAGE gels (Laemmli, 1970). Between 10 and 32 µg protein of each supernatant sample or between 1.2 x 10^6 and 1.4 x 10^8 bacterial cells were loaded onto gels. Electrophorised proteins were transferred to a PVDF membrane (porablot, Macherey-Nagel) using a wet-electroblotting system (100V, 250mA, 1h, BioRad).

2.5. Detection of Hcp proteins

Immunoblot detection of Hcp proteins was performed using polyclonal rabbit antibodies raised against *V. anguillarum* Hcp (Vts T6SS) (Weber et al., 2009). Membranes with blotted proteins were incubated with Hcp antibodies diluted 1:10000 over night at 4 °C. Alkaline phosphatase conjugated polyclonal goat anti-rabbit immunoglobulins (1: 1000, Dako) were used as secondary antibodies and visualisation of bound antibodies performed using an NBT/BCIP stock solution (Roche). For N-terminal analysis, blotted proteins were visualised using Coomassie blue staining. Protein bands representing bands bound by Hcp antibodies were excised from the membranes and their N-terminal amino acid (aa) sequences determined by automated Edman degradation at the Institut Pasteur, Paris.

3. Results and discussion

3.1. Identification of two T6SSs in the M. viscosa *genome*

Two putative T6SSs were identified in the *M. viscosa* 06/09/139 genome, termed Moritella Type Six Secretion 1 and 2 (*mts1* and *mts2*). In total, 23 coding sequences (CDSs) were predicted in the *mts1* locus. The structural organization of CDSs suggests that the locus consist of three operons and four single genes (Fig. 1A). The *mts2* locus contains 27 CDSs organized into five putative operons and one single gene (Fig. 1B). Both the *mts1* and *mts2* loci carry a copy of each of the 13 conserved core genes found in a genome wide analysis where 176 T6SSs were studied (Boyer et al., 2009). Most of these genes are specific to T6SSs, including those encoding VgrG (*mts1-V* and *mts2-V*) and Hcp (*mts1-M* and *mts2-M*). The two VgrG proteins encoded by *mts1-V* (668 aa) and *mts2-V* (689 aa) are relatively small and do not carry C-terminal extensions with functional effector-domains as described in Pukatzki et al. (2009). Genes encoding putative VgrG or Hcp were not identified outside of the *mts1* or *mts2* loci.

Also considered as core genes are *mts1-E* and *mts2-E* encoding proteins with a forkhead-associated (FHA) domain, and *mts1-J* and *mts2-AE* encoding protein kinases. The genes may play a crucial role in controlling T6SS activity at the post-translational level as in *Pseudomonas aeruginosa* (reviewed in Filloux et al., 2008). However, both *mts* lack a phosphatase with antagonistic activities to the *mts*-encoded kinases. It is possible that genes encoding phosphatases are located outside the *mts* loci, or that genes with no known function within the *mts* loci are capable of dephosphorylating FHA domain proteins.

Species specific CDSs were also identified. *mts2-AA*, *mts2-AB*, *mts2-AC* and *mts2-AD* are found exclusively in some *Vibrio* species, including *V. parahaemolyticus* (Fig. 2). They have no functional annotation, but are in an operon downstream of *mts2-V*. The genetic organization of these CDSs together with high synteny with other T6SSs indicates a role in

T6S. Three CDSs *mts2-AK*, *mts2-AL* and *mts2-AM* have no functional homologs and have not been associated with T6SSs previously. The CDSs are organized in an operon together with *mts2-AI, mts2-EFGH and mts2-AJ*. In *V. anguillarum* secretion of Hcp was decreased when *vtsEFGH* homologous to *mts2-EFGH* was mutated (Weber et al., 2009). This suggests that these three CDSs play a part in T6S and that they may be specific to *M. viscosa*.

3.2. Comparison of M. viscosa *T6SSs to other T6SSs*

Homologous CDSs in the two *mts* secretion systems were less than 25% identical to each other at the amino acid level, except for *mts1-X* and *mts2-X* encoding ClpV ATPases which were 42% identical. The lack of sequence conservation and structural organization between the *mts1* and *mts2* suggest that they have been acquired in separate events, rather than having arisen from a duplication event. This has also been observed for other species with more than one T6SS (Bingle et al., 2008). Boyer et al. (2009) classified different T6SSs into five sub-groups. For example, *V. parahaemolyticus* carries two T6SSs classified as sub-groups I and V. *mts1* is structurally and sequence similar to the *V. parahaemolyticus* T6SS of sub-group I (Fig. 2), while *mts2* is more similar to the *V. parahaemolyticus* T6SS of sub-group V.

The *M. viscosa mts* secretion systems were compared against a T6SS of the fish pathogen *A. salmonicida* (belonging to sub-group I). The *mts1* locus showed higher sequence similarities and synteny to the *A. salmonicida* T6SS (Fig. 2) than *mts2*. The *mts1* is also very similar to one of the two T6SSs identified in *Moritella* sp. PE36 (Persson et al., 2009), but no system similar to *mts2* is present in the genome of this non-pathogenic *Moritella*.

3.3. Active Mts1 T6SS in M. viscosa

M. viscosa NCIMB 13584^{T} supernatant bound *V. anguillarum* (Vts T6SS) anti-Hcp antibodies (Fig. 3). The N-terminal aa sequence of two supernatant bands (14 and 15 kDa) was

determined. Both bands had the sequence; (AS)IYMRID, where the first two aa were not determined with full certainty. The *M. viscosa* 06/09/136 genome gave only one match to the IYMRID sequence, which was the *mts1-M* gene, encoding an Hcp protein. *In vitro* expression of Mts1 T6SS Hcp was therefore confirmed and a functional *M. viscosa* T6SS identified. The different size of the bands may be due to different processing or degradation of the C-terminal end. Subsequently, Hcp proteins of Mts1 T6SS were detected in culture supernatants and cell pellets of all ten *M. viscosa* isolates (results not shown). Active Mts1 T6SS was thus confirmed in all *M. viscosa* isolates tested, both virulent and avirulent.

The role of the Mts1 T6SS in *M. viscosa* is unknown, and may not necessarily be involved in virulence. Opinions of much broader roles of T6SSs in bacteria have recently risen (Schwarz et al., 2010). The EVP T6SS of the fish pathogen *Edwardsiella tarda* has been shown to affect virulence in blue gourami fish. However, unlike this study, EVP T6SS was only present in virulent isolates (Zheng and Leung, 2007). The Vts T6SS of *V. anguillarum* was not found to affect virulence in trout (Weber et al., 2009). The function of the *mts-2* T6SS in *M. viscosa* remains to be confirmed. It is possible to speculate whether the Mts1 T6SS, which is present in both virulent and avirulent *M. viscosa* isolates as well as in the non-pathogenic *Moritella* sp. PE36, has non-virulent related functions, while the Mts2 T6SS, which is not found in *Moritella* sp. PE36, may be involved in virulence.

Acknowledgments

The Hcp antiserum was a generous gift from Prof. Debra L. Milton, University of Umeå, which also provided valuable assistance and advice. The authors also thank Thorunn Gudmundsdottir for her assistance with Hcp screening. The study was supported by the University of Iceland Research Fund.

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Figure captions

Fig. 1. Genetic organization of *Moritella viscosa mts1* (A) and *mts2* (B) type VI secretion system loci. Different arrows indicate functional annotations associated with genes. Numbers indicate conserved domains found in genes with no functional annotation. Grey boxes mark the boundaries of predicted operons within each *mts* loci. For simplicity the *mts* prefix is omitted.

Fig. 2. Comparison of *Moritella viscosa* A) *mts1* (II) against *Aliivibrio salmonicida* LFI1238 (I) and *Vibrionales* bacterium SWAT-3 (III), and B) *mts2* (V) against *Vibrio cholerae* N16961 (IV) and *Vibrio parahaemolyticus* RIMD 2210633 (VI) type VI secretion system (T6SS) loci. Grey arrows indicate putative exported or membrane associated proteins, white arrows indicate putative cytosolic proteins, black arrows indicate regulators and dark grey boxes indicate insertion sequence elements. For the comparison, light grey boxes indicate similar regions between T6SSs and dark grey hour-shaped boxes indicate similar regions, but in opposite directions.

Fig. 3. Immunoblot of *Moritella viscosa* culture supernatant using *Vibrio anguillarum* (Vts T6SS) anti-Hcp antibodies. Lane S, protein standard; lane Mv sup., *M. viscosa* supernatant. Bands identified as Hcp proteins are indicated with arrows. T6SS, type VI secretion system; Hcp, hemolysin coregulated protein.

Table 1. *Moritella viscosa* isolates used in the study.

Designation	Origin	Place of isolation	Virulence
NCIMB 13584^{T}	*S. salar*	Norway	virulent[a]
LFI 5006	"	Norway	virulent[b]
06/09/139	"	Norway	virulent[c]
K58	"	SW-Iceland	virulent[a]
K56	"	N-Iceland	virulent[d]
Vvi-7	"	Canada	avirulent[a]
Vvi-11	"	Canada	avirulent[a]
NVI 4917	*O. mykiss*	Norway	virulent[a]
NVI 5482	*G. morhua*	Norway	virulent[a]
F57	*C. lumpus*	Iceland	reduced[a,d]

NCIMB, The N*ational Collection* of *Industrial*, Marine and Food *Bacteria*. Isolates LFI 5006, Vvi-7, Vvi-11, NVI 4917 and NVI 5482 were provided by Dr. Duncan Colquhoun (National Veterinary Institute, Norway) and isolate 06/09/139 was provided by Christian Karlsen (University of Tromsø, Norway). Other isolates came from the Institute for Experimental Pathology, University of Iceland.
a, Bjornsdottir et al., 2011; b, Tunsjo et al., 2009; c, Christian Karlsen, personal communication; d, Benediktsdottir et al., 2000. *S. salar*, *Salmo salar*; *O. mykiss*, *Oncorhynchus mykiss*; *G. morhua*, *Gadus morhua*; *C. lumpus*, *Cyclopterus lumpus*.

A)
mts1
B)
mts2
Function
Exported/membrane associated protein
Function unknown
VgrG protein
Hcp protein
Transporter, MFS family
Periplasmic solute-binding protein
ImpA-related protein
IcmF-related protein
ClpV ATPase
Function unknown
D-alanine-D-alanine ligase
Serine/Threonine protein kinase
Regulator
Conserved domain
1 PF05488 - PAAR domain
2 COG3515 - Uncharacterized protein
3 TIGR03358 - Type VI secretion protein
4 TIGR03355 - Type VI secretion protein
5 PF05943 - Unknown function
6 TIGR03357 - Type VI secretion system lysozyme-related protein
7 PF09937 - Unknown function
8 PF00498 - FHA domain
9 TIGR03352 - Type VI secretion lipoprotein
10 TIGR03353 - Type VI secretion protein
11 TIGR03349 - Type VI secretion protein, DotU family
12 PF00069 - Protein kinase
13 PF09937 - Unknown function

Fig. 1.

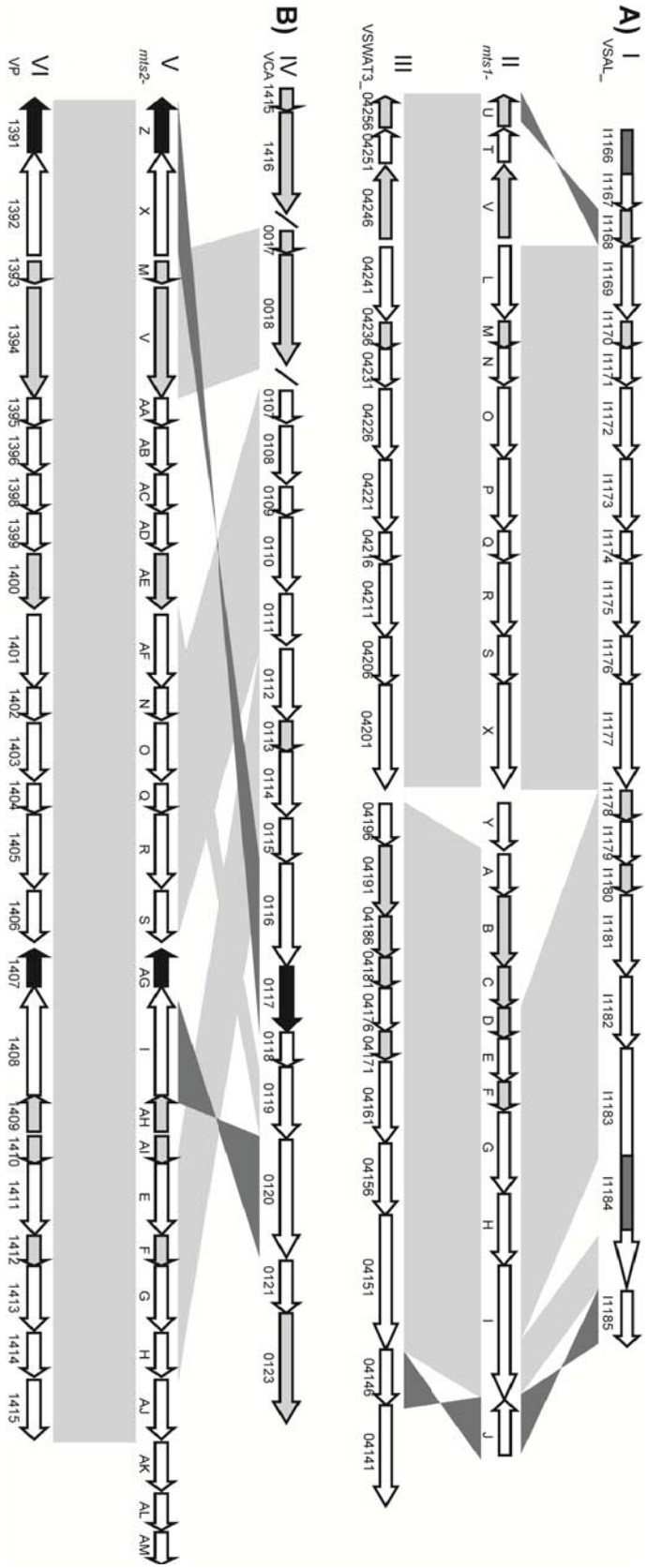

Fig. 2.

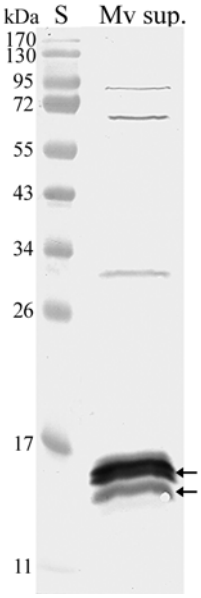

Fig. 3

APPENDIX

Translated amino acid sequences of *M. viscosa* proteins identified from culture supernatants (described in section 4.5).

M. viscosa secreted protein precursor, accession HM453331 (the determined N-terminal end is underlined):

MNLNKTLIAIAIAFTFNAHAYDNYSLFSDGIDQNLVTANLSRLVKLTKGDEVKSAYVYDDNGRITQRDYT
DSTFDYTYNSDGTIKSARALMKRNPNSFIKHTKENFISEYIYDSEGKVIREDKKVFNDLDTNFEGVPKAT
YQIMYLYNNTGQLTTRDQVLQNGELGVNLKVNYTYYDNGRLKDIQEIKASLESHKNTLLLKYYENGEIKQ
ATQTIFGSGTKTFDIEYVTDLSMIYGVYYVDPLKEWKVDMDFFGLAFHPIKKLTETAGDVITSYDYTYQN
DDSDFLADTLSLKVVSKHYKFNNEYKMTNQP

M. viscosa outer membrane protein (OmpH) (the determined N-terminal end is underlined):

MKKTILATAILLAGAANAAEVYNNEGTTVSLGGSFRGNVVIADSDNVNFQDAGSRFDIKAVKELDDGVKA
FGQMEIKYNGKDGDTLFVNKAFVGFEHDVYGKLVLGKKLGLNDDLVMNDFSYENGVYSHQDAAAGSDTQD
QLEYTKTFGGASVVVGLIDQDTYSIGGTYEVAGLSLGLAYNIKNDADAAGSDNSALIVGAQYALDALTLG
AQYQTLEVADVDTSAYGIGVHYALGQAGVYAMYDILEVAKVDGSELVIGADYEIVKDVKTYVEFNSSDSD
AKANSDETVWLGARVYF

M. viscosa stringent starvation protein A (the determined N-terminal end is underlined):

MALAANKRSVMVLYSEPTDLYSHQVRIVLAEKGVSVDIHQVDRNNLPEDLIDLNPYQTVPTLIDRELTLY
NSRIIMEYLDERFPHPPLMPVYPVSRGSSRLMMHRIENDWYSLVTKIMKGSVEEAAVARKQLQEALMSIS
PIFAEYPYFMSEEFSLVDCYMAPLLWRLPELGIDLPGQAASELKNYMLRVFDRESFQASLTEQEREMRML
M

M. viscosa purine nucleoside phosphorylase (the determined N-terminal end is underlined):

MATPHINAEKGDFAETVLFPGDPLRAKYIAETFLEDIKQVNDVRNMLGFTGTYKGKRISVMGSGMGIPSC
SIYAKELITEFGVKNLIRIGSCGAISTDVKVRDVVIGMGACTDSAVNRARFDGYDFAAIASWELLSKVTR
AAKACNIDAKVGNIFSADLFYTPKPELFDSMEKLGVLGVEMEAAGLYGVAAEFGANAICICTVSDHIRTG
EVTTAEERQLTFNDMIIMALESVLIED

M. viscosa transaldolase (the determined N-terminal end is underlined):

MSNQLEQLSKLTTIVADTGDVDAIKLYKPQDATTNPSLILKAAALPAYQPLVAQAIAYAKENATPETQVA
LACDKLAVLIGKEILTTIPGRISTEVDARLSYDTEASVKQALLLIELYKAVGIEKENILIKLASTWEGIR
AAEQLEAMGINCNLTLLFSFAQAQACAEAGVFLISPFVGRIMDWHKAKTGETYEGANDPGVISVSTIYNY
YKASGYNTVVMGASFRNTGEILALAGCDRLTIAPQLLEELQNSSEPVAQALFSEAEQVAPKAKLTEAAFR
WDHSQDPMAVEKLAEGIRNFAIDQNKLEAMIADLF

M. viscosa dihydrolipoamide dehydrogenase (identified by MALDI-TOF mass spectrometry):

MSNEVKAQVVVLGAGPAGYSAAFRAADLGLETVIIERYNTLGGVCLNVGCIPSKALLHVAKVIEEAKSLA
DHGIVFGEPQTDITKIRSWKEKVVGQLTGGLGGMAKMRKVKVVEGLAQFTGANTIEATDRDGNVTTVTFD
NAIIAAGSRPVKLPFIPHEDPRVWDSTDALELKEVPGKLLVLGGGIIGLEMGTVYSALGSDIDVVEFADQ
LVPAADKDIVKIYAKAVKNKFNVMLSTKVTAVDAKEDGLYVTFEGKKAPAEPVRYDAVLVAVGRVPNGLG
LNAEKAGITVTERGFIETNKTMSTNVPHIYAIGDIVGQPMLAHKGVHEGHVAAENIAGKKHFFDPKVIPS
IAYTEPEMAWAGLTEKEAKEQGVNYEAAVFPWAASGRAIASDASNGMTKLLFNKDTNRIIGGAMVGTNAG
ELLGEVCLAIEMGCDAEDIALTIHAHPTLHESVGMAAEIYEGSITDLPNAKAIKKK