Virulence Properties of *Moritella viscosa* Extracellular Products

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Moritella viscosa

- Causes **Winter ulcer disease** in fish reared in the N-Atlantic Ocean during winter months (<8 °C)
  - Infects Atlantic salmon and rainbow trout
  - Also infects Atlantic cod
- Ulcers appear on the surface of infected fish
- Financial losses due to mortality and downgrading of infected fish
- Fish welfare problem
- Farmed salmonids are extensively vaccinated
- *M. viscosa* virulence not extensively studied
- Extracellular products (ECP) are lethal and produce internal disease symptoms

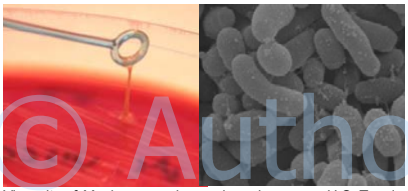
Atlantic salmon suffering from winter ulcer disease

Aims

- Evaluate the virulence properties of *M. viscosa* extracellular products
- Identify extracellular virulence factors
- Determine whether a cytotoxic cell-culture model can be used for virulence studies
- Detect type VI secretion systems in *M. viscosa*

Strains

- 22 *M. viscosa* strains
  - 4 fish species
  - 5 geographical locations
- 2 Environmental, non-pathogenic strains
  - *Moritella marina*
  - *Moritella japonica*
- 1 *Aliivibrio wodanis*
  - isolated from fish with WUD



Viscosity of *M. viscosa* and scanning microscpoy. H.S. Tunsjø.

Designation	Species	Origin	Place of isolation
NCIMB 13584	*Moritella viscosa*	*S. salar*	Norway
NVI 5433	"	*S. salar*	Norway
LFI 5006	"	*S. salar*	Norway
F288/95	"	*S. salar*	Norway
NVI 4731	"	*S. salar*	Norway
K58	"	*S. salar*	SW-Iceland
K56	"	*S. salar*	N-Iceland
F-3-04	"	*S. salar*	E-Iceland
F-6-05	"	*S. salar*	E-Iceland
5351	"	*S. salar*	Canada
5352	"	*S. salar*	Canada
990129-1/3B	"	*S. salar*	Faroe Islands
990217-1/1A	"	*S. salar*	Faroe Islands
MT 2528	"	*S. salar*	Scotland
MT 2858	"	*S. salar*	Scotland
NVI 5450	"	*O. mykiss*	Norway
NVI 4917	"	*O. mykiss*	Norway
NVI 5168	"	*O. mykiss*	Norway
F162/01	"	*O. mykiss*	Iceland
NVI 5482	"	*G. morhua*	Norway
NVI 5204	"	*G. morhua*	Norway
F57	"	*C. lumpus*	Iceland
NCIMB 1144	*Moritella marina*	seawater	Japan
JCM 10249	*Moritella japonica*	seawater sediment	Japan
88/411	*Aliivibrio wodanis*	*S. salar*	Norway

Testing strain virulence

- *M. viscosa* strains passed in salmon (i.p. injections at 8 °C in seawater)
- Two Canadian salmon strains (5351 and 5352) were non-virulent (MLD > 8 x 10^8 CFU/fish)
- The LD_{50} of nine *M. viscosa* strains determined:
  - 10^4 - 10^5 CFU/fish
  - > 10^6 CFU/fish (reduced virulence)
  - non-virulent

Designation		Species	Origin	Place of isolation
NCIMB 13584	★	*Moritella viscosa*	*S. salar*	Norway
NVI 5433	★	"	*S. salar*	Norway
LFI 5006		"	*S. salar*	Norway
F288/95		"	*S. salar*	Norway
NVI 4731		"	*S. salar*	Norway
K58	★	"	*S. salar*	SW-Iceland
K56		"	*S. salar*	N-Iceland
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NVI 5450		"	*O. mykiss*	Norway
NVI 4917	★	"	*O. mykiss*	Norway
NVI 5168		"	*O. mykiss*	Norway
F162/01		"	*O. mykiss*	Iceland
NVI 5482	★	"	*G. morhua*	Norway
NVI 5204	★	"	*G. morhua*	Norway
F57	★	"	*C. lumpus*	Iceland
NCIMB 1144		*Moritella marina*	seawater	Japan
JCM 10249		*Moritella japonica*	seawater sediment	Japan
88/411		*Alitivibrio wodanis*	*S. salar*	Norway

ECP production and assays

- ECP production:
  - Collection of spent medium from broth cultures
    BHI-NaCl, 15 °C, 48 h, 200 rpm

- *In vivo* and *in vitro* virulence:
  - Lethal activity in salmon
    i.p. injection
  - Cytotoxicity against EPC cell monolayer
    LDH assay kit
  - Hemolytic activity against salmon erythrocytes

- Enzymatic and siderophore assays:
  - Caseinase assay and casein zymogram
  - Esterase assay and Azo-dye of SDS-PAGE
  - Degradation of egg-yolk and starch
  - Siderophore detection on CAS medium

ECP lethality in salmon

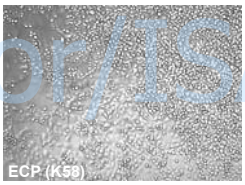
0.1 ml i.p. injection

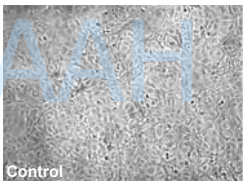
- ECPs of three *M. viscosa* strains non-lethal
  - Two non-virulent Canadian salmon strains
  - One low-virulent Norwegian salmon strain
- ECPs of non-*M. viscosa* strains non-lethal
  - No internal symptoms detected

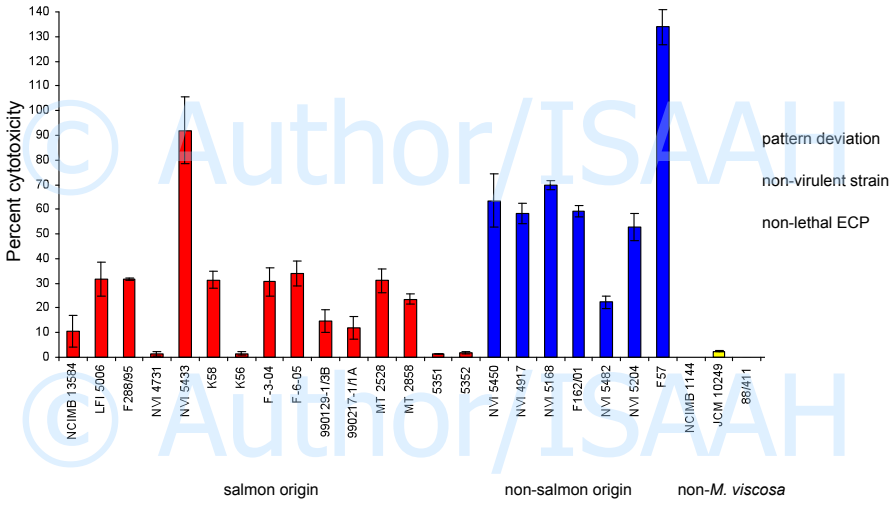
	Strain designation	Lethality in salmon # dead (dod)
M. viscosa *S. salar* origin	NCIMB 13584	2/2 (2,2)
	LFI 5006	2/2 (2,3)
	F288/95	1/2 (3)
	NVI 4731	2/2 (2,2)
	NVI 5433	0/2 (na)
	K58	2/2 (2,3)
	K56	1/2 (4)
	F-3-04	2/2 (2,2)
	F-6-05	2/2 (2,2)
	990129-1/3B	2/2 (2,2)
	990217-1/1A	2/2 (2,2)
	MT 2528	2/2 (2,3)
	MT 2858	2/2 (2,3)
	5351	0/2 (na)
	5352	0/2 (na)
M. viscosa non-*S. salar*	NVI 5450	2/2 (2,2)
	NVI 4917	2/2 (1,1)
	NVI 5168	2/2 (1,1)
	F162/01	2/2 (2,2)
	NVI 5482	2/2 (2,3)
	NVI 5204	2/2 (1,2)
	F57	2/2 (4.4)
non-*M. viscosa*	NCIMB 1144	0/2 (na)
	JCM 10249	0/2 (na)
	88/411	0/2 (na)

Cytotoxicity

against EPC cells (LHD kit)



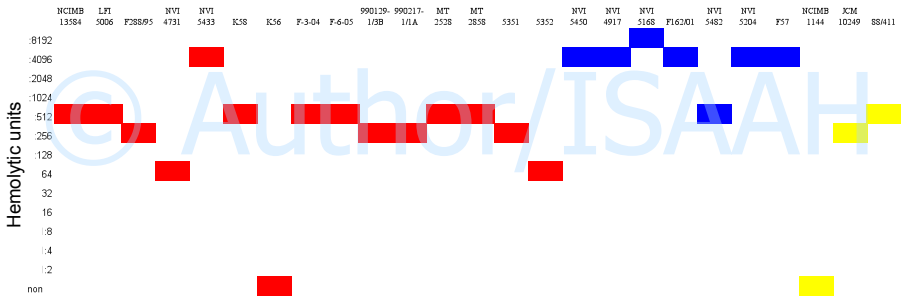




Hemolytic activity

against salmon erythrocytes

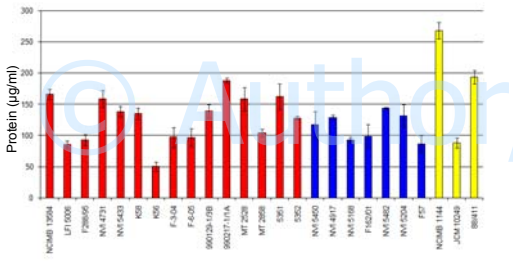
pattern deviation



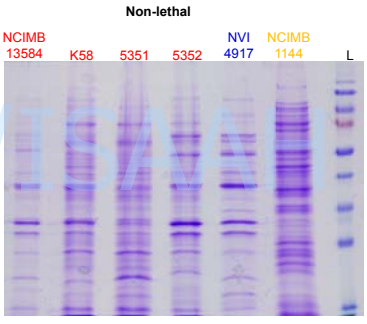
salmon origin non-salmon origin non-*M. viscosa*

Hemolytic unit: ECP dilution which gave ≤ 50% hemolysis

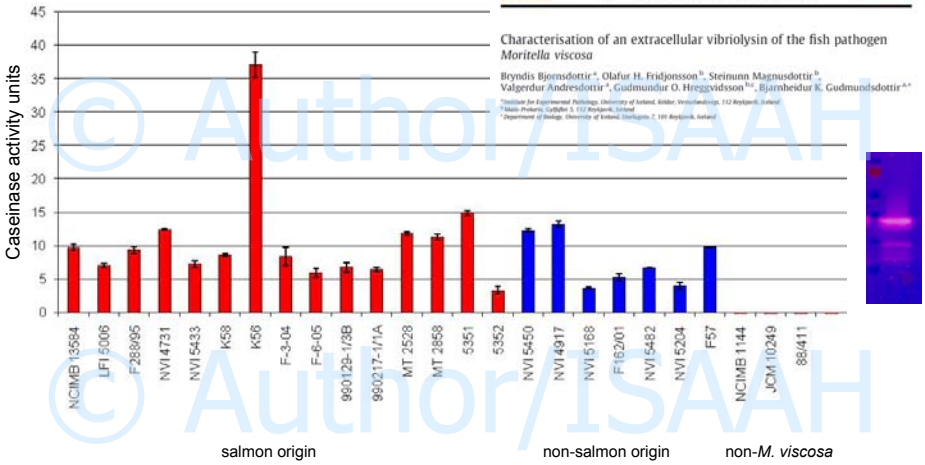
Total protein and protein profiles



salmon origin non-salmon origin non-*M. viscosa*



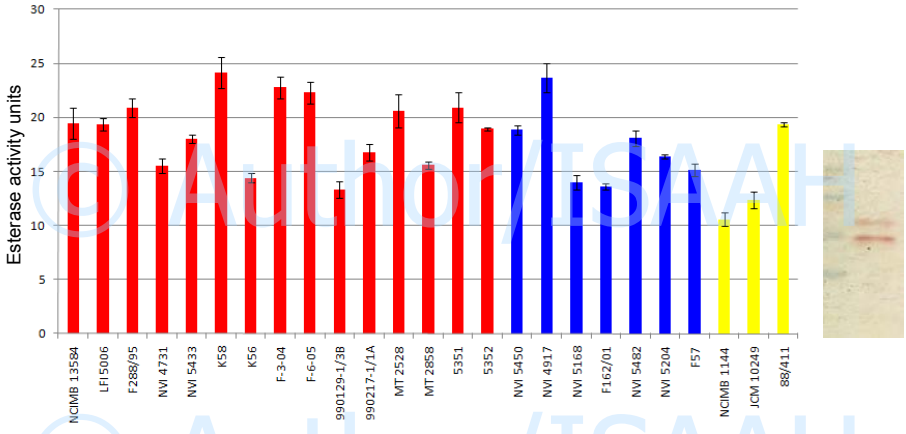
Caseinase activity



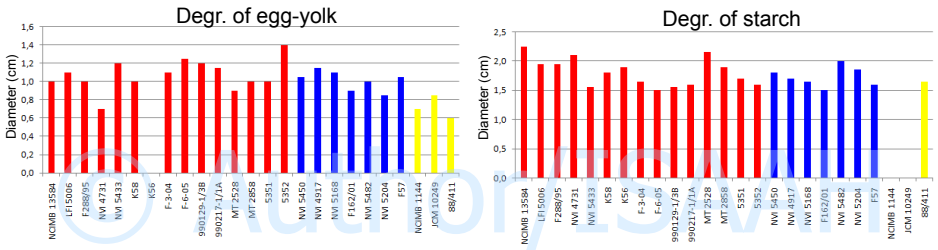

Veterinary Microbiology

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

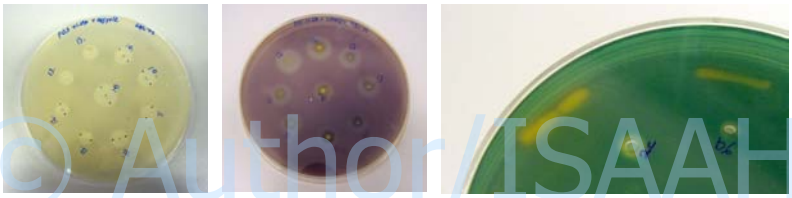
Esterase activity



Agar diffusion assays and siderophore production



All *M. viscosa* ECPs positive for siderophore production



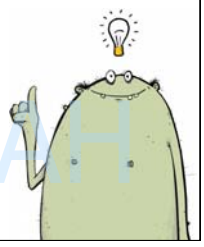
Degr. of egg-yolk (putative lecithinase activity)

Degr. of starch (putative amylase activity)

Yellow halos on CAS medium indicate siderophore production

Discussion and conclusions

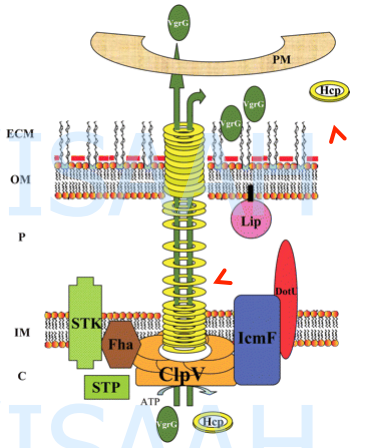
- *M. viscosa* secretes a lethal toxic factor of unknown nature
  - ECP virulence was not linked to enzymatic activity or siderophore production
- ECPs of salmon strains less cytotoxic/hemolytic than ECPs of non-salmon strains
  - Cytotoxin production may reflect host adaptation
  - Membrane disruption of cell-cultures and salmon erythrocytes caused by the same product
    - Consistency between cytotoxic and hemolytic activities
  - Membrane damaging factor is not the lethal factor
    - Poor correlation between cytotoxic/hemolytic activities and lethality of ECPs
- Non-virulent strains produced non-lethal ECPs
  - Indicates the importance ECP may have on winter ulcer disease
  - Useful tools for virulence studies
- Cell-culture models may not be optimal for determining *M. viscosa* virulence
  - Cytotoxicity did not correlate well with strain virulence



Type VI secretion (T6SS)

- Recently identified protein secretion system in proteobacteria
  - pathogenic and non-pathogenic
- Key virulence factor in some important pathogenic bacteria
- Injects effector molecules directly into host cells through a channel
- Core set of 13 proteins
  - Hcp (haemolysin co-regulated protein)
  - VgrG (valine-glycine repeat protein G)

    secreted through T6SS and found in culture supernatants
- Many bacteria contain more than one T6SS
  - Several T6SS subgroups exist
  - Indication of different functions



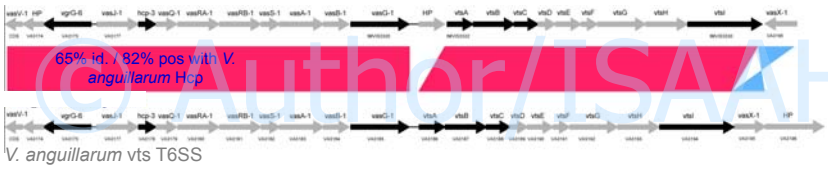
Schematic representation of a T6SS.
Microbiology **154** (2008), 1570-1583. Filloux, Hachani & Bleves

Two T6SS genetic systems in *M. viscosa*

Genomic analysis

Two Hcp encoding genes

mts-1 (*Moritella* type six secretion – 1)



V. anguillarum vts T6SS

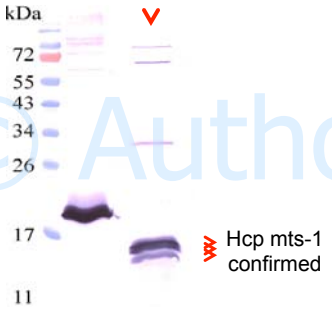
mts-2 (*Moritella* type six secretion – 2)

V. harveyi T6SS

T6SS in *M. viscosa*

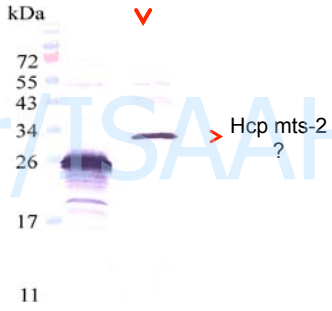
Hcp protein detection in ECP

α-Hcp from *V. anguillarum* vts T6SS



V. ang *Mv*

α-Hcp from *V. cholerae* vas T6SS



V. ang *Mv*

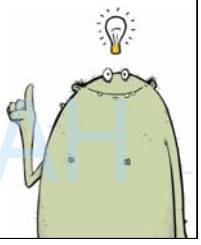
11 *M. viscosa* strains positive

- virulent and non-virulent

Cell pellets also positive

Discussion and conclusions

- The *M. viscosa* genome contains two T6SS
  - Both appear to be active (secrete Hcp)
  - Different regulatory genes indicate different functions
  - Possible role in virulence?
- All tested *M. viscosa* strains positive for Hcp secretion
  - Both virulent and avirulent strains



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  - Debra Milton
  - Sun Nyunt Wai
- **Funding**
  - AVS research fund
  - The univeristy fund of Eimskip
  - RANNIS research fund
- **American Fisheries Society**
  Fish Health Section Awards Committee
  - Snieszko Student Travel Award

Production of ECP

- Two production methods:
  - Cellophane overlay method; **C-ECP**
    - Cultivation on cellophace covered agar (15°C, 72 h)
  - Batch culture supernatant; **B-ECP**
    - Collection of spent medium from batch cultures (15°C, 48 h, 200 rpm)
- Cultures centrifuged and sterile filtered

C-ECP

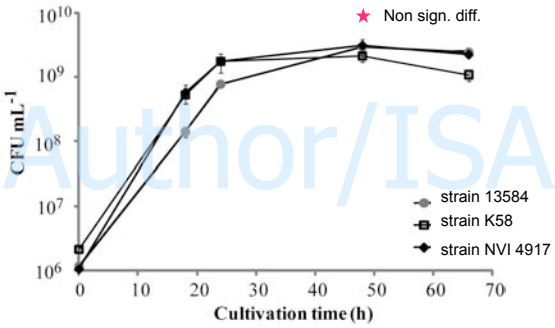
B-ECP

B-ECP versus C-ECP (strain K58)

		B-ECP	C-ECP
	Total protein (µg/ml)	135	617
Virulence assays	Min. lethal dose (µg protein/fish)	3	62
	Cytotoxicity (%)	31	2
	Hemolysis (dil. of ≤50% hemol.)	1:512	non hemol.
Enzymatic assays	Caseinase activity (units)	8.6	117.3
	Esterase activity (units)	24.1	479.6

Coparison of Mv strain growth

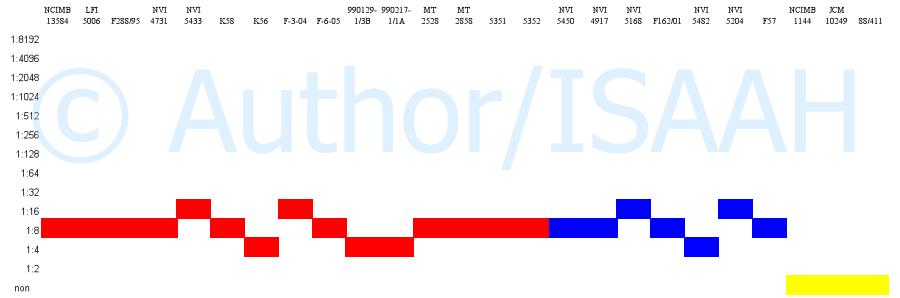
Triplicate broth cultures at 15 °C



ECPs of all strains collected after 48 h of cultivation (OD_{600} 0.87 – 1.08)

Hemolytic activity

of ECP against sheep erythrocytes



salmon origin

non-salmon origin

non-*M. viscosa*

Determination of the ECP dilution which gave ≤ 50% hemolysis

Detection of a T6SS in *M. viscosa*

- Genome analysis
- WB using anti Hcp-antibodies
- N-terminal sequencing

11 *M. viscosa* strains screened for Hcp

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JCM 10249	*Moritella japonica*	seawater sediment	Japan
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M. viscosa Hcp genes

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mts-1   -MASIYMRIDGVK---VDGGATAEGLPGEGWFALNSYSWGAVRSVAMDIGNGTNSDSGMV 56
mts-2   MPTPAYMSINGETQGHITKDAYSADSVGNTWQEAHVDEF-LVQELDHVLTVPRDPQSGQP 59
          :. ** *:* .   :  .* : .  *: *   :  .:  *:.:   :    :.:**

mts-1   AMSEVNISKEVDGSSEDLLSFLFSPGPEGKNVEIVFTKPARD--GSGADIYFQVKLEKAR 114
mts-2   TGQRVHRPIIVTKQQDRCSPLLFNSLVSGEKLPECYINFYRTSIQGKQEHYYTIKLIDAL 119
        : ..*:.  *  ..:   .:**..  .*:::   : :  *    . : *: :** .*

mts-1   LVSYNVSGSDGGQPFESIALSYIQLDQLHWHEASGGKLE-KGGLVSYNVPQGKMLSGSK 172
mts-2   IVDIETRMAHCQDDATSTRVVEEVLQLTYRAIEVTHEVCGTAGNDDWRAPREA------ 172
        :*. :.  :. :  * :   *:  :      ::  ..*  .:..*:
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ASIYMRID: N-terminal end

mts-1 Hcp: 78% id. / 93% pos. *Moritella* sp. PE36
mts-2 Hcp: 90% id. / 94% pos. *Aliivibrio salmonicida* LFI1238