Aeromonas salmonicida subsp. *achromogenes* and the effect of the autoinducer synthase AsaI on bacterial virulence

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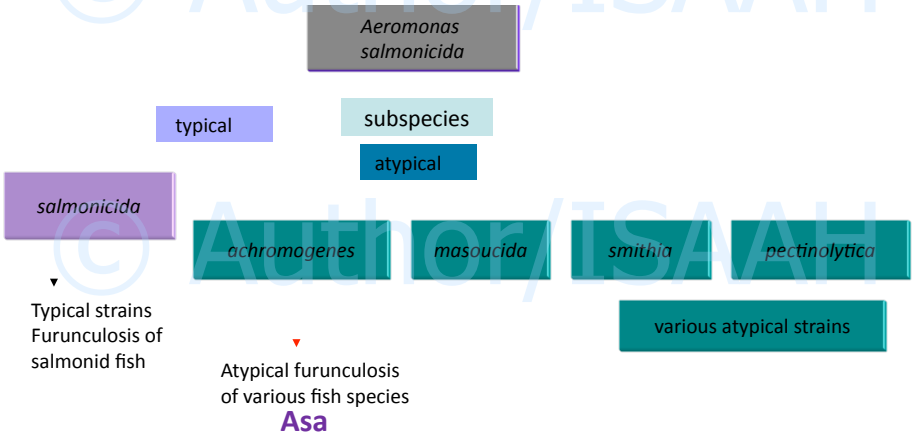
Quorum sensing (QS)

- Quorum sensing (QS) is a cell–cell communication system that enables bacteria to synchronize gene expression with population density
- Many bacterial phenotypes, such as expression of virulence factors, biofilm formation and more are under QS control
- LuxIR-type QS via N-acyl-homoserine lactones (AHLs) autoinducers (AI) are used by many Gram-negative bacteria, including bacteria in the genus *Aeromonas* for intraspecies QS
- LuxI is the autoinducer synthase and LuxR is an AHL-dependent transcriptional regulator

Taxonomy of *Aeromonas salmonicida*

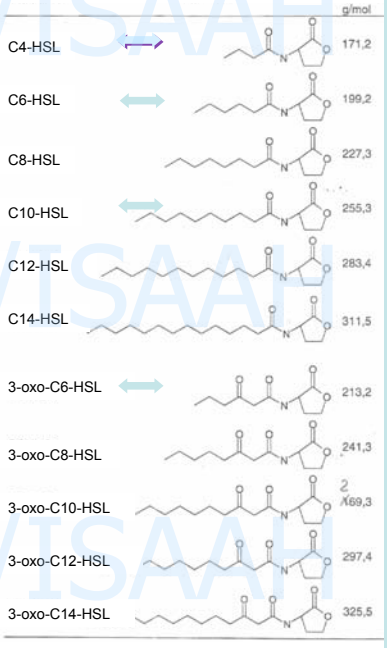


AHL molecules from G⁻ bacteria

In our studies only one type of AHL, **C4-HSL** has been detected from *A. salmonicida* subsp. *achromogenes*, **Asa**

Four types of AHLs have been characterized from ***A. salmonicida* subsp. *salmonicida*: C4-HSL,** C6-HSL, C10-HSL and 3-oxo-C6-HSL

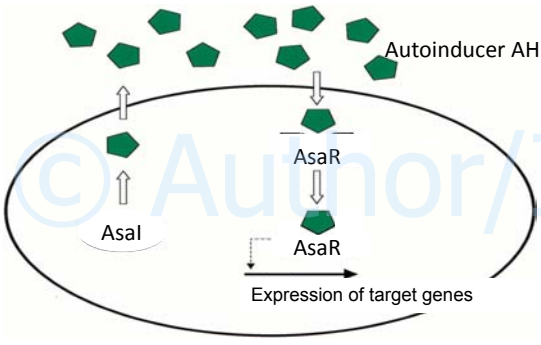
Acylated Homoserine Lactones



Subject matter

QS in *Aeromonas salmonicida* subsp. *achromogenes* and its relation to bacterial phenotypes and virulence in Arctic charr (*Salvelinus alpinus* L.) with emphasis on the role of the autoinducer synthase AsaI and the effect of synthetic QS inhibitors (QSI)

Construction of a Δ*asaI* mutant of **Asa** by allelic exchange

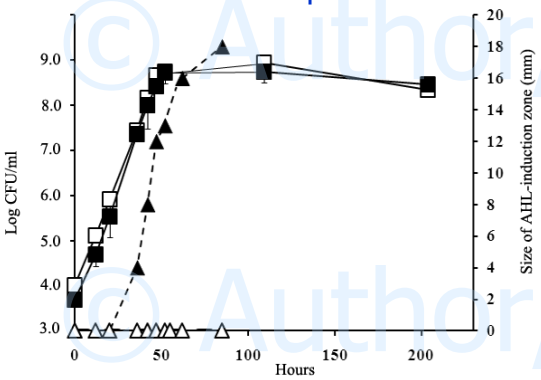


Johanna Schwanteit
PhD student

Schwanteit *et al.* Vet. Microbiol. (2010), doi:10.1016/ j.vetmic.2010.07.020

Analyses of the Δ*asaI* mutant compared to the (wt) **Asa**

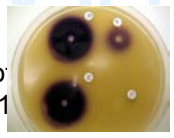
Growth and AHL production



Solid lines show bacterial growth and dashed lines AHL production

- The growth of the **Δ*asaI*** mutant did not differ from that o
  Asa wt strain in liquid cultures with initial cell density of 1

As grown in the presence of the AHL monitor bacterium *Chromobacterium violaceum*



- Knock out of the *asaI* gene resulted in a complete lack of AHL production

Virulence of **Asa** in Arctic charr is QS regulated

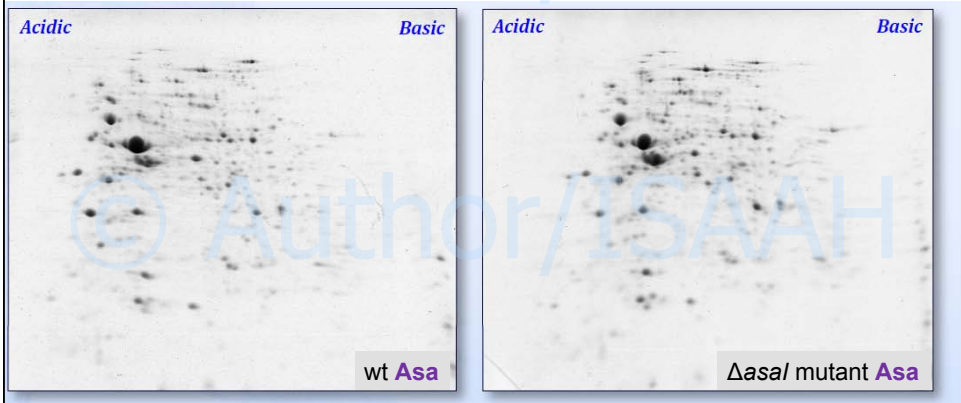
CFU/ fish	wt Keldur265-87		*ΔasaI* mutant Keldur265-87-3	
	Mortality %	MDD	Mortality %	MDD
10^8	100	3.3	100	5.1*
10^7	100	4.4	92	6.9*
10^6	100	6.5	88	9.0*
10^5	85	8.6	46	11.7*
10^4	71	9.7	0	-
10^3	8	14	0	-
LD_{50}	$5*10^3$ CFU/ fisk		$1*10^5$ CFU/ fisk	

LD_{50}, fifty percent lethal dose
MDD, mean day to death

The LD_{50} of the Δ*asaI* mutant of **Asa** is **20** times higher than that of its isogenic wt strain

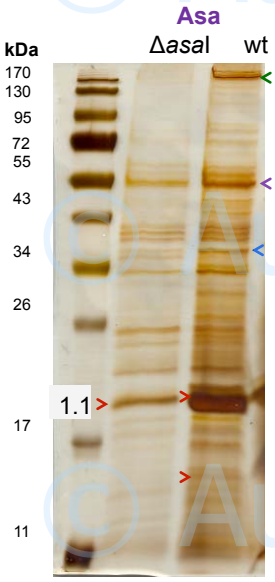
Proteomic detection of cell associated proteins- 2D-electrophoresis

Aberdeen proteomics



No significant differences in cell associated proteins were detected between the AsaI-deficient **Asa** mutant and its isogenic wild type strain

Extracellular products (ECP) after 96 h cultivation
MALDI-TOF mass spectrometry



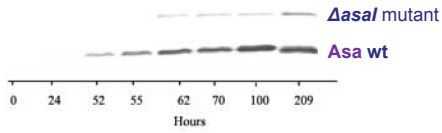
Mascot Search Results from Bruker Daltonics flex analysis

- 1.1 AsaP1, toxic extracellular endopeptidase
- 2.1 Repeat in toxin, RTX
- 2.2 N-acetylglucosamine binding protein
- 2.3 Leucyl aminopeptidase, peptidase M28 family
- 2.5 AsaP1, toxic extracellular endopeptidase
- 2.6 AsaP1, toxic extracellular endopeptidase

Total ECP protein concentration

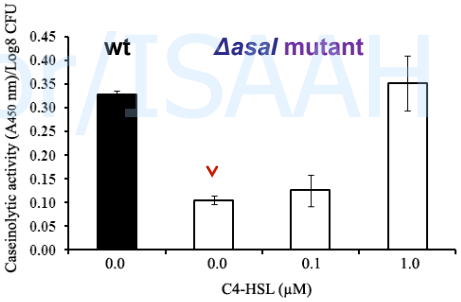
Δ*asal*	wt
40µg ml^{-1}	315 µg ml^{-1}

AsaP1 production is QS regulated



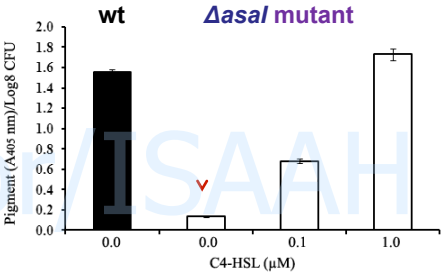
Immunostaining of AsaP1 in the extracellular products during growth with anti-AsaP1 antibodies

Complementation of AsaP1 production of the ***Δasal*** mutant by C4-HSL supplementation confirms that AsaP1 is under QS regulation



Pigment production is QS regulated

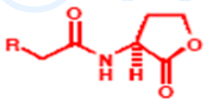
***Δasal* Asa** wt **Asa**



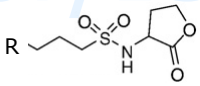
Complementation AsaP1 and pigment production of the ***ΔasaI*** mutant by C4-HSL supplementation confirms that both factors are under QS regulation

AHL inhibitors that compete with AHLs for binding to the transcriptional regulator AsaR

- 3-sulfide AHL analogues (a kind gift from Michael Givskov, Univ. Copenhagen)
- Used dissolved in DMSO to 10 μM in culture broth
  - ProS-AHL, N-(propylsulfanylacetyl)- L-homoserine lactone
  - PenS-AHL, N-(pentylsulfanylacetyl)- L-homoserine lactone
  - HepS-AHL, N-(heptylsulfanylacetyl)- L-homoserine lactone



Acylated homoserine Lactones-AHL

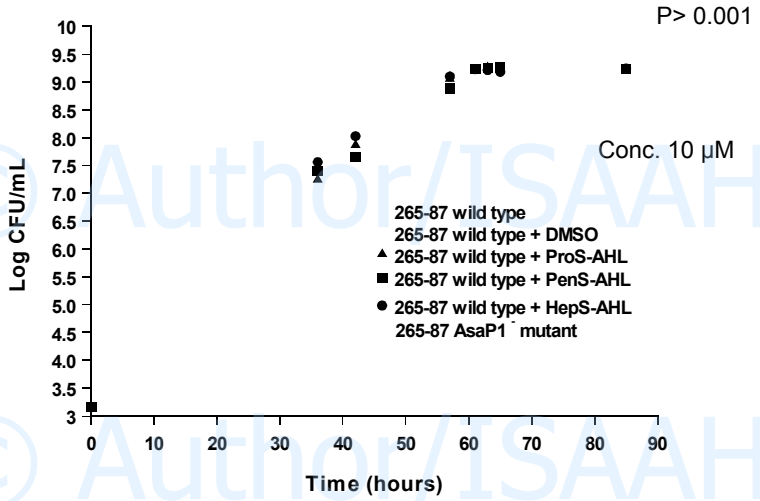


3-sulfide AHL analogues

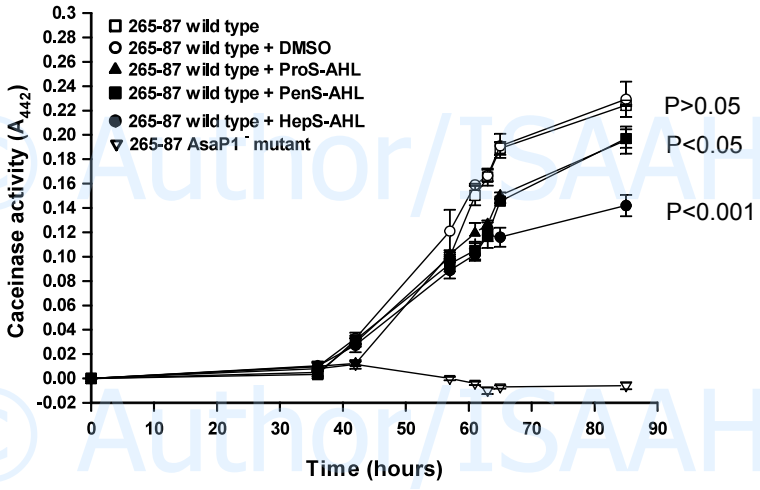
R for ProS-AHL= $(CH_2)_2CH_3$
R for PenS-AHL= $(CH_2)_4CH_3$
R for HepS-AHL= $(CH_2)_6CH_3$

Synthesised

AHL inhibitors (10 µM)do not affect bacterial growth



- **AsaP1** production was significantly inhibited by all three inhibitors ProS-AHL, PenS-AHL and HepS-AHL
- HepS-AHL was the most potent inhibitor



Summary

- Inactivation of the AHL synthase results in complete lack of AHL production
- Virulence of the bacterium in fish is QS regulated
- Expression of the virulence factors AsaP1, RTX, N-acetylglucosamine binding protein and leucyl aminopeptidase is QS regulated
- Expression of a dark brown pigment is QS regulated
- Quencing of QS can be applied to suppress expression of a major virulence factor, AsaP1, without affecting bacterial growth

Concluding remarks

- The observation that AHL-mediated QS is involved in virulence regulation of *A. salmonicida* subsp. *achromogenes,* the simplicity of its LuxIR-type QS system, and the ability of synthesized QS quenching molecules to inhibit an important virulence factor without affecting bacterial growth, makes *A. salmonicida* subsp. *achromogenes* an interesting target organism for further studies on the involvement of QS in disease and disease control

QS systems detected in G⁻ fish pathogenic bacteria

Species	QS system	Quorum sensing-regulated virulence	References
Aeromonas hydrophila	AI-1	biofilm formation, exoprotease production virulence	Swift et al. (1997), Swift et al. (1999); Lynch et al. (2002); Bi et al.(2007)
Aeromonas salmonicida	AI-1	serine protease production	Swift et al. (1997), Rash et al. (2007)
Vibrio anguillarum	AI-1, AI-2	QSI, furanone C-30, reduced mortality	Milton et al. (1997) Rash (2004), Milton et al. (2004)
Vibrio salmonicida	AI-1	virulence	Nelson et al (2007)
Vibrio harveyi	HAI-1, CAI-1, AI-2	siderophore production, production of type III secretion system components, extracellular	Bassler et al. (1993), Lilley and Bassler (2000), Manefield et al. (2000), Mok et al. (2003), Henke & Bassler (2004), Tinh et al.
Vibrio alginolyticus	AI-1, AI-2	virulence	Ye et al. (2007); Wang et al. (2008); Tian et al. (2008)
Vibrio parahaemolyticus	AI-1	opacity	McCarter (1998)
Vibrio vulnificus	AI-2	protease and haemolysin production, lethality to mice	McDougald et al. (2000), Kim et al. (2003)
Edwardsiella tarda	AI-1	55 kDa virulence factor	Morohoshi et al. (2004)
Yersinia ruckeri	AI-1	unknown	Kastbjerg et al. (2006)

Cytotoxic activity of Asa is QS regulated

Extracellular products	CFU/ml	Percent cytotoxicity
wt **Asa**	1.8×10^9 ($\pm$ 6.3×10^8)	11.1 ($\pm$ 1.3)
ΔasaI mutant **Asa**	1.3×10^9 ($\pm$ 1.1×10^8)	5.3 ($\pm$ 0.8)*

- Cytotoxicity was determined by calculating percent cell mortality by measuring the release of lactate dehydrogenase (LDH) in supernatants of *Epithelioma papulosum* cyprini (EPC) cells treated with ECPs
- The ΔasaI mutant **Asa** strain produced significantly less cytotoxicity than its isogenic wt strain (P=0.0004)