The AinS autoinducer synthase and LitR master regulator of quorum sensing regulate N-3-hydroxy-decanoyl-homoserine-lactone production and motility in Aliivibrio wodanis 06/09/139

CURRENT STATUS: POSTED

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DOI:
10.21203/rs.2.10994/v1

SUBJECT AREAS
General Microbiology

KEYWORDS
Aliivibrio wodanis, Quorum sensing, 3OHC10-HSL, Motility
Abstract
Background Quorum Sensing (QS) is a cell to cell communication system, in which bacteria synthesize and respond to signaling molecules called autoinducers (AI). QS is cell density dependent and known to be involved in regulating virulence, motility and secretion systems to interact with the host or other bacteria. *Aliivibrio wodanis* is frequently isolated together with *Moritella viscosa* from the infected Atlantic salmon during outbreaks of the winter ulcer disease. *M. viscosa* is the main causative agent of the disease while the presence of *A. wodanis* is still unclear. It is hypothesized that *A. wodanis* might influence the progression of winter ulcer. The genome of *A. wodanis* 06/09/139 encodes two autoinducer synthase genes (ainS and luxS) and a master regulator litR. LitR homologs in other allivibrios have been shown to regulate several phenotypes in a cell density dependent manner. Moreover, a previous study has shown that *A. wodanis* 06/09/139 produces only one AHL N-3-hydroxy-decanoyl-homoserine-lactone (3OHC10-HSL). Hence, in this work, we have studied the QS system in *A. wodanis* 06/09/139 by knocking out QS genes ainS and litR. The effects of the deletions were studied with regard to growth, AHL production and motility at different temperatures. Results By using HPLC-MS/MS, we found that the deletion of ainS in *A. wodanis* 06/09/139 resulted in the loss of 3OHC10-HSL production. The 3OHC10-HSL production in *A. wodanis* 06/09/139 increased with increase in cell density and more 3OHC10-HSL was produced at 6°C than at 12, 16 and 20°C. The litR mutant demonstrated a ~20% reduction in the production of 3OHC10-HSL relative to the wild type at the stationary phase. Compared to the wildtype and the ainS mutant strains, the litR mutant resulted in a strain with improved temperature tolerance. The motility in mutants (ΔlitR and ΔainS) were significantly higher than that of the wildtype. Conclusions Our study shows that AinS in *A. wodanis* 06/09/139 is the AHL synthase responsible for 3OHC10-HSL production, where the production is both cell density and temperature dependent. Our data also shows that LitR regulates 3OHC10-HSL production only to a minor extent and both LitR and AinS are negative regulators of motility.

Background
*Aliivibrio wodanis* is a Gram-negative gammaproteobacteria that belongs to the genus *Aliivibrio* and is closely related to other allivibrios such as *Aliivibrio salmonicida*, *Aliivibrio logei* and *Aliivibrio fischeri*
A. wodanis is repeatedly isolated together with Moritella viscosa from Atlantic salmon (Salmo salar) during a disease called winter ulcer [3-5]. Winter ulcer is a temperature dependent disease that occurs at low temperature below 8°C [3, 6]. M. viscosa is the main causative agent of winter ulcer disease while the presence of A. wodanis in the infected fish is not clear yet [6, 7]. However, A. wodanis 06/09/139 is cytotoxic to several salmon cell lines and is able to inhibit the growth of M. viscosa 06/09/139 by altering its gene expression [8, 9]. After bath challenge, A. wodanis 06/09/139 produces clinical symptoms such as septicemia, fin rot and other internal pathological signs; as well as mortality in the Atlantic salmon [8].

Bacteria exist as a community in nature and can use inter and intraspecies communication to cooperate or compete with each other [10]. This cell to cell communication can be regulated by secretion and accumulation of small diffusible signaling molecules called autoinducers (AI) in a mechanism known as Quorum Sensing (QS) [11-14]. QS is cell density dependent and the AI concentration increases with increase in cell density [15]. The QS system was first described in A. fischeri and later in many other bacteria [15-17]. Through QS, the bacterial population collectively regulates mechanisms like luminescence, motility, protease production, siderophore production, secretion systems, biofilm formation, hemolysis and virulent factor gene expression [15-17].

Several QS systems have been reported that can function either parallel as in Vibrio harveyi or in a hierarchical way as in A. fischeri [14, 18, 19]. The most conserved interspecies QS system in Gram-negative and positive bacteria is LuxS/LuxPQ that produces autoinducer-2 (AI-2) [13, 20-22]. On the other hand, in most of the Gram-negative bacteria, QS is driven by the autoinducer AI-1 called N-acyl homoserine lactone (AHL) that allows intraspecies communications [23-26]. In A. fischeri, there are two AHL based QS systems LuxI/R and AinS/R and a AI-2 based QS system LuxS/LuxPQ that controls the colonization factors and luminescence, where LuxI, AinS and LuxS are the autoinducer synthases [14, 18, 27]. LuxR in A. fischeri is the transcriptional regulator protein that binds the AHL produced by LuxI and regulates bioluminescence by inducing the lux operon genes luxCDABEG whereas AinR and LuxPQ acts as receptor proteins where the autoinducers produced by AinS and LuxS binds respectively [14, 28]. At low cell density, no signaling molecules are produced; therefore AinR and
LuxQ act as kinases and relay phosphatases to $\sigma^{54}$ dependent activator LuxO via phosphotransferase LuxU. Once LuxO is phosphorylated, it activates the expression of qrr that encodes small regulatory RNAs. Qrr sRNA, which together with RNA chaperone Hfq represses the mRNA that encodes the master regulator LitR and therefore luminescence is repressed [18, 29, 30]. At high cell density, the signaling molecules released by LuxS and AinS, accumulate into environment and bind to their respective receptors LuxPQ and AinR. This binding converts LuxPQ and AinR into phosphatase. Consequently, LuxO is dephosphorylated, qrr is repressed and litR is activated [29, 31]. LitR activates luxR and induces luminescence and colonization factors [17, 18, 29]. A. salmonicida and A. fischeri are closely related to A. wodanis and has QS systems similar to each other [32, 33]. In both A. fischeri and A. salmonicida, LitR activates the expression of ainS and luxR, and connects the QS systems AinS/R and LuxS/PQ to LuxI/R, as well as regulates other mechanisms such as motility, colonization, luminescence, and biofilm [18, 32]. There is also another QS autoinducer, AI-3, in different bacterial species, which is mainly involved in interkingdom communications [34, 35].

In bacterial AHL based QS systems, the number of autoinducer synthases, AHLs produced and LuxR type transcriptional regulator proteins involved differs between bacteria and also within same genus [14, 18, 36, 37]. For example, A. fischeri ES114 produces eleven AHLs whereas A. salmonicida LF11238 produces eight AHLs [32, 38, 39]. A. wodanis 06/09/139 was shown to produce one AHL N-3-hydroxy-decanoyl-homoserine-lactone (3OHC10-HSL) whereas other A. wodanis strains produce several AHLs as described in [38]. The A. wodanis 06/09/139 genome encodes two autoinducer synthase genes (ainS and luxS) and a LuxR type transcriptional regulator gene annotated as litR. No luxI homologs were identified in this strain. The proposed QS system of A. wodanis 06/09/139 is illustrated in Fig 1.

Since A. wodanis 06/09/139 is closely related to A. salmonicida and A. fischeri, it was proposed that the bacteria may have a similar QS system, where the LitR is regulated by ainS/R and luxS/PQ systems [32]. Moreover, the LitR of A. salmonicida and A. fischeri has been shown to regulate several activities in a cell density dependent manner [16, 17, 32, 40, 41]. As mentioned above, the A. wodanis 06/09/139 has been shown to produce only one AHL [38]. Thus, we wanted to investigate the
role of AinS and LitR in QS and whether AinS is responsible for the AHL production. Further, we also wanted to know if the transcriptional regulator LitR influences the production of the AHL. Since winter ulcer is a temperature dependent disease and A. wodanis 06/09/139 showed increased cytotoxic effects \textit{in vitro} at increased cell density [3, 7, 8], we performed AHL profiling at different temperatures and cell densities. Since LitR and AinS in other bacteria regulate different phenotypes [16, 17, 42, 43], we investigated their role in motility of A. wodanis 06/09/139 at different temperatures.

\textbf{Methods}

\textbf{Bacterial strains, plasmids and growth conditions}

The bacterial strains and plasmids used in this study are listed in the Table 1. A. wodanis 06/09/139 from glycerol stock was grown at 12°C for 3 days on Luria-Bertani Agar, LA2.5 (Difco BD Diagnostics Sparks, MD, USA) plates containing 1.0% peptone (Sigma-Aldrich, St. Louis, MO, USA), 0.5% (wt/vol) yeast extract (Merck, Darmstadt, Germany), 2.5% NaCl (wt/vol) (Sigma-Aldrich, St. Louis, MO, USA) and 1.5% agar (Sigma-Aldrich, St. Louis, MO, USA); and the pH of the media was adjusted to 7.5. Pre-culture of A. wodanis 06/09/139 was grown in 2 ml of Luria-Bertani Broth (LB2.5) overnight at 12°C at 220 rpm.

\textit{Escherichia coli} strains JM109 and S.17-1 were grown at 37°C overnight on LA supplemented with 1.0% NaCl (LA1). The \textit{E. coli} JM109 and the S.17-1 transformants were selected on LA1 with 100µg/ml ampicillin and 25µg/ml chloramphenicol respectively. The transconjugants of A. wodanis 06/09/139 were selected on LA2.5 with 2µg/ml chloramphenicol at 12°C.

\textbf{DNA extraction, PCR and sequencing}

Genomic DNA was purified using Masterpure™ complete DNA/RNA purification kit (Epicentre, Cambio Ltd., Cambridge) and plasmids were purified using E.Z.N.A® plasmid mini kit (Omega Bio-tek, Inc., Norcross, GA). The DNA concentration was measured in NanoDrop™ 2000c spectrophotometer (Thermoscientific, DE, USA). The primers were synthesized by Sigma-Aldrich (Norway) and are listed in Table 1. The amplification PCR program was used as recommended by Taq polymerase (Sigma, St. Louis, MO, USA) and Phusion® polymerase (Thermo Fisher Scientific, Waltham, MA, USA) in a Arktik™
thermal cycler (Thermo Fisher Scientific), and the products were separated using agarose gel electrophoresis and extracted using Montage® gel extraction kit (Millipore, Norway). Restriction enzymes XhoI and SpeI and T4 ligase were obtained from New England Biolabs (Ipswich, MA, USA). Transformation and conjugation were performed as reported by others [44, 45].

**Construction of litR and ainS mutants**

The *litR* (AWOD_I_0419) and *ainS* (AWOD_I_1040) genes were in-frame deleted by allelic exchange using the suicide vector pDM4 (accession number KC795686) and *E. coli* S.17 as donor cells in conjugation with recipient as described by others [45]. Briefly, *litR* and *ainS* genes were deleted by amplifying and fusing regions flanking these genes. The upstream (280bp) and downstream (263bp) flanking regions of *litR* gene were amplified by primer pairs LitRA/LitRB and LitR C/LitRD respectively. The upstream region comprises the start codon (ATG) and the downstream region comprises the last three codons (TAA) at the C-terminal end of *litR* gene. The upstream (253bp) and downstream (271bp) flanking regions of *ainS* were amplified using primer pairs AinSA/AinSB, and AinSC/AinSD, respectively. The upstream PCR product ended just before the start codon (ATG) and the downstream PCR product contained the last 149bp of the *ainS* gene. The upstream and downstream PCR products of *litR* and *ainS* were fused by overlap extension PCR by primer pairs LitRA/D and AinSA/D respectively as described by others [16]. The primers LitRA, LitRD and AinSA, AinSD contain restriction sites to enable ligation of the products into the suicide vector pDM4 after digestion with restriction enzymes SpeI and XhoI. Before cloning into pDM4, TA cloning was performed using pGEM-T Easy vector (Promega) [40]. The inserts from the pGEM vector were excised and ligated into pDM4. The resulting plasmids were designated as pDM4∆litR and pDM4∆ainS.

The pDM4∆litR and pDM4∆ainS constructs were transferred into wild type *A. wodanis* 06/09/139 by bacterial conjugation as described previously [16, 45]. Briefly, the donor strain S.17-1 λpir was grown until optical density at 600nm (OD\(_{600}\)) 0.5 to 1.0 and the recipient strain was grown until OD\(_{600}\) 2.0.

One ml from each culture was centrifuged separately at room temperature. The pellets were separately washed twice in LB1, mixed and resuspended in 10 µl. The resuspended pellet was spotted onto LA1. The plates were incubated at 19°C for 6 hours and then for 48 hours at 12°C. The spots
were then resuspended in 2 ml LB2.5 and incubated overnight before plating (20, 40, 60, 80, and 100 µl) on LA2.5 plates with 2µg/ml chloramphenicol. Potential transconjugants were selected after 3 to 5 days and confirmed using colony PCR. The potential transconjugants were plated on LA2.5 plates with 5% sucrose to allow the second cross over to take place. After sucrose selection, the potential transconjugants with deleted gene were confirmed by colony PCR and further verified by sequencing.

**Construction of ΔlitR complementary strain**

The complementary strain (litR+) of the ΔlitR mutant was constructed by amplifying the full-length wild type litR gene using primers LitRA and LitRD. The PCR products were then cloned into pDM4 using restriction digestion as described above. The resulting plasmid pDM4litR+ was conjugated to the ΔlitR mutant. The selection and verification of the potential complementary strain was performed as described above.

**Sample preparation and AHL measurements using HPLC-MS/MS**

The A. wodanis 06/09/139 wild type, ΔlitR, litR+ and ΔainS were cultivated in parallel at four different temperatures (6, 12, 16 and 20°C). The cultures were diluted to a start OD$_{600}$ of 0.001 in LB2.5 with a final volume of 60 ml in baffled flasks. Cultures of 1 ml were collected at eight different cell densities in total, six in the log phase (OD$_{600}$ 0.5, 1.0, 2.0, 3.0, 4.0, 5.0), one in the stationary phase (8.0) and one at the end where the growth started to decline. For AHL measurements in ΔainS, samples were only harvested at the late log phase OD$_{600}$ 6.0. The cultures were centrifuged at 13,000xg for 2 min at 4°C (Heraeus fresco 21, Thermo Scientific). Three technical replicates, each of 75 µl supernatant were acidified with 4 µl of 1 M HCl each and stored at -20°C before measuring AHL. The commercial 3OHC10-HSL standard (Sigma-Aldrich, St. Louis, MO, USA) with known concentration 230ng/ml of LB2.5 was extracted from LB2.5 using the same protocol as above. The sample preparation for HPLC-MS/MS were done as described by others [32, 38]. Briefly, the acidified supernatants were mixed with three volumes of ethyl acetate (225 µl) and vortexed. The ethyl acetate phase of the three technical replicates were pooled together into a 1 ml 96 well plate and dried in a rotary vacuum centrifuge (SpeedVac Savant™ concentrator, Thermo Scientific™). The dried samples were dissolved in 150 µl of
20% acetonitrile containing 0.1% formic acid and 660 ng/ml of internal standard 3O-C12-HSL (Sigma-Aldrich, St. Louis, MO, USA).

The HPLC-MS/MS analysis was performed as described in [32]. Briefly HPLC-MS/MS was performed using Ascentis Express C18, reversed phase column (50x2.1mm, 2.7µm particle size, Sigma). A sample of 20µl was injected into the column and eluted using 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 200 µl/min. The elution profile obtained was 5% acetonitrile in 30 s, 90% in 300 s and 5% in the next 60 s. The separated compounds were detected by Linear Ion Trap Quadrupole (LTQ) part of the LTQ-Orbitrap (Thermo Fisher Scientific). The LTQ was used in selected reaction monitoring (SRM) mode and the SRM was divided into two segments. Segment 1 scanned AHL 3OHC10-HSL and segment 2 scanned the internal standard 3O-C12-HSL with a retention time of 0-3.15 mins and 3.15-6.00 mins respectively. The ion trap parameters chosen for MS/MS were, maximum injection time 50 ms, isowidth 1.0 m/z, collision energy 35, act Q 0.25 and act time 30 ms. The measured AHLs are presented in ng/ml. The AHL measurements at different temperatures were performed twice.

**Motility assay**

Motility assay was performed in LB2.5 soft agar plates with 0.25% agar. Overnight cultures of A. wodanis 06/09/139 wild type, ΔlitR, litR+ and ΔainS were diluted 1:100 and grown overnight to OD₆₀₀ 1.0 in LB2.5. Cultures of 2 µl were spotted onto the soft agar plates, incubated at 6°C and 12°C and the motility zones were measured every 24 hours.

**Results**

**AinS autoinducer synthase is responsible for the production of 3OHC10-HSL**

AinS of A. salmonicida has been shown to be responsible for production of 3OHC10-HSL [32]. In order to understand the QS of A. wodanis 06/09/139, a complete deletion mutant (ΔainS) was constructed by removing 347 of 397 codons of ainS gene through allelic exchange. The ΔainS and the wild type were grown at 12°C and the supernatants were harvested at late log phase of OD₆₀₀ 6.0. The growth rates were similar in the ΔainS and the wild type (Additional file 1: Figure S1). As expected, the ΔainS did not produce AHL as shown in Fig. 2 and the wild type produced only one (3OHC10-HSL) confirming
our previous results where A. wodanis 06/09/139 produces only one AHL, 3OHC10-HSL [38].

LitR inhibits growth at 20°C in A. wodanis 06/09/139

LitR homologs have been shown to regulate different phenotypes in other aliivibrios [16, 17, 40]. To investigate the function of LitR in A. wodanis 06/09/139, a complete deletion of \textit{litR} gene and a complementary strain (\textit{litR}+) was constructed through allelic exchange. The mutant \textit{\Delta}\textit{litR} was constructed by deleting 200 codons of the \textit{litR} gene leaving only the start codon and the last three codons of the C-terminal end of \textit{litR} open reading frame. The \textit{litR}+ was made by inserting a full length copy of the wild type \textit{litR} gene into the \textit{litR} mutant.

Since winter ulcer is a cold water disease that occurs at temperatures below 8°C [3], the wild type, \textit{\Delta}\textit{litR}, \textit{\Delta}\textit{ainS} and \textit{litR}+ strains were analyzed with respect to growth at different temperatures (6, 12, 16 and 20°C). As shown in Fig. 3 and 4, the growth rate of the different strains increased with increasing temperature, where the duration of log phase (OD\textsubscript{600} 0.5 to 8.0) was 50-100 hours at 6°C, 20-40 hours at 12°C, 10-20 hours at 16°C and 5-10 hours at 20°C. At 20°C, the \textit{\Delta}\textit{litR} reached the maximum OD\textsubscript{600} ranging from 8.0 to 8.5, whereas the wild type, \textit{\Delta}\textit{ainS} and \textit{litR}+ reached OD\textsubscript{600} of 5.0 to 6.0 as shown in Fig. 3. There was a significant difference between the maximum cell densities of \textit{\Delta}\textit{litR} and wild type A. wodanis 06/09/139 (p-value < 0.05) at 20°C. No such differences in growth were seen at 6, 12 and 16°C. Therefore, LitR seems to repress growth only at 20°C.

The 3OHC10-HSL production is influenced by cell density and temperature

The production of AHLs in A. salmonicida was shown to be both temperature and cell density dependent [32]. In order to explore the role of temperature and cell density in the production of AHL (3OHC10-HSL), the wild type A. wodanis 06/09/139 was grown at 6, 12, 16 and 20°C and the supernatants were harvested at different cell densities. The samples were analyzed for the presence of 3OHC10-HSL using HPLC-MS/MS. Due to the limited growth at 20°C, the supernatant of stationary phase was harvested at OD\textsubscript{600} 6.0 (the highest OD\textsubscript{600} observed).

As shown in Fig. 4, the 3OHC10-HSL production started at OD\textsubscript{600} 0.5 and increased significantly (p < 0.05) along the growth curve in a cell density dependent manner. The 3OHC10-HSL reached its
highest concentration at the stationary phase OD$_{600}$ 8.0. In the wild type, during the stationary phase, the highest 3OHC10-HSL production was observed at 6°C (154 ng/ml) and the lowest at 20°C (71 ng/ml, 46% of 6°C). When the 3OHC10-HSL production at 16°C (125 ng/ml, 81% of 6°C) was compared with 12°C (119 ng/ml, 77% of 6°C), no significant difference was observed. The 3OHC10-HSL production at 6°C was significantly higher when compared to other temperatures 16°C, 12°C and 20°C (p < 0.05). When the growth curve started to decline, the 3OHC10-HSL was steadily produced at 6°C but declined at other high temperatures.

**LitR is a positive regulator of 3OHC10-HSL production**

LitR has been shown to be a positive regulator of AinS by influencing the production of 3OHC10-HSL in *A. salmonicida* [32]. To investigate the role of LitR in QS and its potential regulation on AinS, the *litR* mutant was screened for 3OHC10-HSL production along the growth curve as described above and compared to the wild type profile. There was a significant difference between ∆*litR* and wild type from OD$_{600}$ 4.0 and onwards at temperatures 6, 12 and 16°C and from OD$_{600}$ 3.0 and onwards at 20°C. When the maximum concentrations of wild type and ∆*litR* were compared, ∆*litR* produced 82% of the wild type 3OHC10-HSL at 6°C (p < 0.05), 80% at 12°C (p < 0.05) and 82% at 16°C (p < 0.05) as shown in Fig. 4. At 20°C, a comparison was not possible due to the difference in both growth rate and maximum cell density. Therefore, in the absence of LitR, AHL production is only affected to a small extent at the examined temperatures.

Similar to the wild type, the complementary mutant *litR*+ produced significantly higher 3OHC10-HSL than the ∆*litR* at all temperatures except at 12°C, where no significant difference in 3OHC10-HSL concentration was observed during the stationary phase between the *litR*+ and ∆*litR*. Due to this unexpected result, the AHL profiling was repeated in three replicates to verify the results. In the second experiment, *litR*+ produced significantly more 3OHC10-HSL than the ∆*litR* at all temperatures (data not shown).

**LitR and AinS are negative regulators of motility**

LitR and AinS regulate motility in several members of *Vibrionaceae* family [16, 17, 42, 43]. Hence, the
swimming motility of *A. wodanis* 06/09/139, the ΔlitR and the ΔainS mutants were examined using soft agar plates at 6 and 12°C. As shown in Fig. 5 and Additional file 2: Table S1, the ΔlitR and ΔainS strains were more motile than the wild type (p < 0.05) at all tested temperatures. The complementary mutant litR+ behaved similar to the wild type.

**Discussion**

Although *A. wodanis* is not the main pathogen of winter ulcer in Atlantic salmon, it has been isolated together with *M. viscosa* from the diseased fish [4, 7, 46]. Winter ulcer is a temperature dependent disease, therefore understanding QS in *A. wodanis* and its relation to temperature could improve the treatment of winter ulcer.

*A. wodanis* is a psychrotrophic bacteria that can grow in a range of 4 to 25°C [7, 47]. In our study, *A. wodanis* 06/09/139 and ainS mutant do not grow at temperatures higher than 24°C on blood agar plates whereas litR mutant grew at 25°C as small colonies (data not shown). LitR influenced growth at higher temperatures in *A. fischeri* and *A. salmonicida*, where litR mutant has been shown to grow slower than the wild type[16, 17, 32]. Contrary to what was shown in *A. fischeri* and *A. salmonicida*, LitR influenced growth oppositely at high temperature in *A. wodanis*. Our results indicate that the litR mutant resulted in strains that is able to grow faster and to a higher cell density compared to the wild type and ΔainS at high temperature 20°C. This is similar to the negative regulation of cell viability exhibited by QS transcriptional regulators of *Pseudomonas aeruginosa* where the inactivation of lasR and rhl, resulted in strains with better survival than the wildtype during alkaline stress [48, 49].

In the environment, bacteria experience fluctuations related to osmotic stress, nutrient limitation, oxygen free radicals and temperature stress. During such stressful situations, bacteria use sigma factor RpoS, a master regulator of general stress response for survival and environmental adaptation [50]. In *E. coli*, stress can induce and accumulate RpoS and is always connected with decrease in growth rate. This decline in growth is due to the competition between RpoS and vegetative sigma factor σ70 for binding to the RNA polymerase [51]. The *A. wodanis* 06/09/139 genome encodes RpoS and RpoD and the stress response mechanism may work in a similar way. Thus, LitR in *A. wodanis* may have a positive effect in inducing RpoS and thereby stress response genes.
Several studies have shown that vibrios and aliviibrios can produce many different types of AHL, and the AHLs were shown to play an important role in QS based virulence [32, 38].

The AHL molecules bind to their respective receptors, activate the QS circuit and regulate the expression of the virulent phenotypes [26]. In the pathogen V. harveyi, which can affect a wide range of marine organisms, the luxM (autoinducer synthase) mutant with no 3-OH-C4-HSL production was found to negatively affect the important virulent factor flagellar motility [52]. In the fish pathogen A. salmonicida, the deletion of AHL autoinducer synthase genes ainS and luxI simultaneously, led to biofilm formation and colony rugosity [32, 43]. The V. cholerae strains with inactivated autoinducer synthase genes (cqsA and luxS), resulted in strains more susceptible to phage, suggesting that autoinducers are important for resistance towards phage [53]. Only one AHL 3OHC10-HSL was identified in A. wodanis 06/09/139 [38]. Similar to A. salmonicida which produces only one AHL by its AinS [32], the ainS gene of A. wodanis 06/09/139 was responsible for producing 3OHC10-HSL. Therefore, the presence of only one AHL may play a vital role in different cellular processes that occurs inside and outside of the hosts.

Winter ulcer is a temperature dependent disease that occurs at temperature below 8°C [3]. It has also been shown that wound healing in Atlantic salmon occurs faster at 12°C than at 6°C [54]. Similarly, as shown in our present study, the growth temperatures of A. wodanis had a significant effect on AHL production. The highest AHL concentration was detected at 6°C and it decreased ~20% at 12 and 16°C and 50% at 20°C. The differences in AHL concentrations of wild type between 6°C and 20°C could be due to the difference in growth, suggesting that the variation in metabolic processes could have affected the AHL production. AHLs are known to be sensitive to high pH in the medium and also by some hydrolytic enzymes [55]. We therefore monitored the pH in the medium during growth. At all four temperatures, the pH increased from 7.5 in the beginning of the growth curve to 8.5 during the stationary phase. Hence, the differences in 3OHC10-HSL concentrations are not due to changes in pH. Unlike in A. salmonicida, the 3OHC10-HSL concentration in A. wodanis is not drastically reduced when the growth temperature is increased from 6 to 12°C [32]. However, highest AHL production was found when A. wodanis was grown at 6°C indicating that low temperature may have some impact during
winter ulcer disease.

In a study by Karlsen et al. [8], A. wodanis 06/09/139 supernatants harvested at different cell densities were tested on fish cell lines and the cytotoxicity was found to be increased with increasing cell densities. Our study shows that the 3OHC10-HSL production in A. wodanis 06/09/139 is cell density dependent with highest 3OHC10-HSL concentrations measured in stationary phase. Therefore, the higher 3OHC10-HSL production at higher cell densities may influence the winter ulcer or other bacteria present in the diseased fish by expressing the QS based virulent genes.

Similar to A. salmonicida and A. fischeri, LitR in this study is a positive regulator of AinS produced AHL 3OHC10-HSL [16, 28]. However, unlike A. salmonicida and A. fischeri, where litR deletion reduced ~ 90% of AinS produced AHL [32, 56], deletion of litR in A. wodanis only has a small impact on AHL production. The litR mutant of A. wodanis produced ~ 80% of the wild type AHL, which proposes that either ainS is autoregulated [28] or a LitR independent AHL production occurs. LitR independent AHL production has been shown in A. fischeri, where ainS was regulated by global regulator cyclic adenosine monophosphate (cAMP)- cAMP receptor protein (CRP) [57]. Similar to LitR, CRP is a global regulator in V. vulnificus where upon its absence the AI-2 production is reduced [58]. A. wodanis 06/09/139 genome encodes a CRP homolog (AWOD_I_0320), suggesting that the LitR independent AHL production in A. wodanis could be regulated by the cAMP-CRP dependent mechanism.

Motility is an important virulent factor in many bacteria [45, 52, 59]. For some bacteria, the flagella mediated motility is required for only aggregation and initial colonization of host [60, 61] while in others, flagella is required for persisted systemic infection [45]. Since flagellar-based motility is an expensive process for the bacteria and a target for the immune system of the host, the bacteria must strictly regulate motility in response to environmental factors and during bacterial-host interactions [52, 62]. Motility and QS has been studied for numerous vibrios and aliivibrios. For example in V. harveyi, the transcriptional regulator luxR and the other genes connected to the QS positively regulates motility whereas in A. salmonicida, A. fischeri, V. cholera, V. parahaemolyticus, V. alginolyticus and V. cholera, QS downregulates motility [16, 52, 61, 63-66]. Similar to other members of Vibrionaceae family, LitR and AinS of A. wodanis 06/09/139 downregulated motility. Motility has
been shown to be linked to colonization of the host in *A. fischeri*, where hypermotility led to poor colonization of squid light organ [61]. Like *A. fischeri* and *A. salmonicida*, in this study, the hypermotilite ΔlitR and ΔainS strains may behave like planktonic cells and result in poor colonization and virulence in the host.

Conclusions
This study shows that ainS is responsible for AHL 3OHC10-HSL production in *A. wodanis* 06/09/139, where the AHL production is both cell density and temperature dependent. The AHL production is more efficient at 6°C than at higher temperatures. Our data shows that, LitR has only a small effect on 3OHC10-HSL production. Therefore in addition to LitR, some other systems like CAMP-CRP may regulate the AHL production. The litR mutant in this study showed an improved temperature tolerance than the wildtype. We also found that LitR and AinS are negative regulators of motility in *A. wodanis*.

Abbreviations
AA: Peak area count
AHL: N-acyl-homoserine-lactone
3OHC10-HSL N-3-hydroxy-decanoyl-homoserine-lactone
AI: Autoinducers
cAMP: cyclic adenosine monophosphate
CRP: cAMP receptor protein
HPLC-MS/MS: High performance liquid chromatography mass spectrometry
LB: Luria-Bertani broth
OD_{600}: Optical density measured at 600nm
PCR: Polymerase chain reaction
QS: Quorum Sensing
RT: Retention Time
WT: Wild type

Declarations
Acknowledgement
We thank Dr. Debra Milton (Umeå University) for the pDM4 plasmid used in this study. We thank
Jostein A. Johansen from The Department of Chemistry, UiT- The Arctic University of Tromsø for helping us with HPLC-MS/MS.

Funding

This work was funded by The Research council of Norway and UiT-The Arctic University of Tromsø. The publication charges for this article have been funded by UiT-The Arctic University of Tromsø.

Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author upon request.

Author’s contribution

AM, HH and NPW conceived and designed the experiments. AM and HH constructed the mutants. AM performed the growth cultivation, AHL measurements and motility assays. AM and HH wrote the paper. All authors read and approved the final manuscript.

Ethical statement

We do not have any subjects that cause ethical issues.

Consent for publication

Not Applicable

Competing interests

The authors declare that they have no competing interest.

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Tables

Table 1. Bacterial strains and plasmids used in this study

| Strains, plasmids and primers | Description | Source reference |
|-------------------------------|-------------|-----------------|
| **A. wodanis**                |             |                 |
| A. wodanis 06/09/139          | Wild type from head kidney of Atlantic salmon from west coast of Norway | [8] |
| ΔlitR                         | A. wodanis 06/09/139 with a complete deletion of *litR* gene | This study |
| *litR*                        | A. wodanis 06/09/139 with a complete deletion of *ains* gene | This study |
| *ains*                        | Δ*litR* complemented with the wild type *litR* gene | This study |
| **E. coli**                   |             |                 |
| JM109                         | Competent strain for transformation of pGEM vector with insert | [67] |
| SY327                         | Strain for replicating suicide vector, λpir | [68] |
| S.17-1                        | Donor strain used for conjugation, λpir | [69] |

| Plasmids                      | Description | Source reference |
|-------------------------------|-------------|-----------------|
| pDM4                          | Suicide vector with sacB, cm<sup>R</sup>, R6K origin (λpir) | [45] |
| pGEM®-T Easy vector           | Cloning vector with β-galactosidase, Amp<sup>+</sup>, lacZ, 3' T overhangs, blue/white screening | Promega |
| pGEM*Δ*litR                   | pGEM®-T Easy vector with Δ*litR* | This study |
| pDM4Δ*litR                    | pDM4 with regions flanking the deleted *litR* gene | This study |
| pDM4*litR*                    | pDM4 with flanking regions and full length *litR* gene | This study |
| pDM4Δ*ains*                   | pDM4 with regions flanking the deleted *ains* gene | This study |

| Primers                       | Description | Source reference |
|-------------------------------|-------------|-----------------|
| LitRA-F                       | ATATACTCGAGTTTACAACAAAAAGCGCACCCTG | This study |
| LitRB-R                       | CATATTTATTTATATCCTTGGCCCAAACAA | This study |
| LitRC-F                       | GATATAAAATAATGTAATATTCGAAACCTCAGAAAAGTAGATA | This study |
| LitRD-R                       | TATAACTAGTGAGCCTTCTCTGGTAAAATTGG | This study |
| LitRG-F                       | GAGCCACGTAATAACCAATCATC | This study |
| LitRH-R                       | CGTTTTATCGGTGGTGCTATT | This study |
| AinsA-F                       | AATAACTCGAGGTGATTGTAATATATTTACACATCAGGAAG | This study |
| AinsB-R                       | CTAGATGGTTTAGATCAAATTTGTAATTGATA | This study |
| AinsC-F                       | GATCTAAACAAATCTAGACGAGACCACCAAGATTACATA | This study |
| AinsD-R                       | TATAACTAGTCACACCCATCCCGATCTTATA | This study |
| AinsG-F                       | TCACGACGAGAACCAAG | This study |
| AinsH-R                       | TTAGGTGTAGACGGAGAAG | This study |
| NQCAT                         | TAACGGCAAAGACCGCAGCCGACATCA | [45] |
| NQREV                         | TGTACACTTTACACTCGCCTATTGTT | [45] |
The proposed model of QS systems AinS/R and LuxS/PQ in A. wodanis 06/09/139. The AinS and LuxS autoinducer synthases synthesize AHL 3OHC10-HSL and AI-2 respectively. These autoinducers are believed to be detected by their respective receptor proteins AinR and LuxPQ. At low cell density, AinR and LuxPQ may act as kinases and a phosphorylation is believed to occur from AinR and LuxPQ to LuxO via LuxU and activate qrr that destabilizes mRNA encoding LitR. At high cell density, the receptor proteins may act as phosphatases to repress qrr and litR is expressed. LitR is the master transcriptional regulator that probably
regulates the expression of different target genes and activates ainS.

Figure 2

Effect of AinS on AHL 3OHC10-HSL production. HPLC-MS/MS chromatograms showing the 3OHC10-HSL measurements in supernatants harvested from the wild type A. wodanis 06/09/139 and the ΔainS mutant at OD600 6.0 grown at 12°C. LB2.5 was used as a blank.

RT: Retention Time, AA: Peak area count.
Figure 3

Growth curves of wild type A. wodanis 06/09/139 and ΔlitR, ΔainS and litR+ at 20°C. The strains were grown in LB2.5 at 20°C, 220 rpm. The error bars indicate the standard deviation of three replicates.
3OHC10-HSL profiling during growth of wild type A. wodanis 06/09/139, ΔlitR and litR+ at different temperatures. The 3OHC10-HSL concentrations of different strains were measured by HPLC-MS/MS. The black line indicates the growth curve (OD600) and the red line indicates the 3OHC10-HSL concentrations (ng/ml). The error bars indicate the standard deviation of three replicates.
Motility of wild type A. wodanis and mutants on soft agar. A) Soft agar plates showing the motility zones of A. wodanis 06/09/139, ΔlitR, ΔainS and litR+ after two days of incubation at 6°C and 12°C. The diameter of petri plate is 90mm. B) The bar chart represents the motility zones measurements (mm) in diameter for different strains. The error bars indicate the standard deviation of three biological replicates.

Supplementary Files
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