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Exploring the transcriptome of *luxI*$^{-}$ and *ΔainS* mutants and the impact of N-3-oxo-hexanoyl-L- and N-3-hydroxy-decanoyl-L-homoserine lactones on biofilm formation in *Aliivibrio salmonicida*

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Background. The marine bacterium *A. salmonicida* uses the quorum sensing (QS) systems, AinS/R and LuxI/R to produce eight acyl-homoserine lactones (AHLs) in a cell density dependent manner. Biofilm formation is one of the QS regulated phenotypes, which requires the expression of exopolysaccharides (EPS).We previously demonstrated that inactivation of LitR, the master regulator of QS in *A. salmonicida* resulted in biofilm formation, which was, similar to the biofilm formed by the AHL deficient mutant *ΔainSluxI*$^{-}$.In this work, we have identified genes regulated by AinS and LuxI using RNA sequensing (RNA-Seq), and studied their role in biofilm formation, colony morphology and motility. We have also studied the effect of two AHLs on the biofilm formation.

Results. The transcriptome profiling of *ΔainS* and *luxI*$^{-}$ mutants allowed us to identify essential genes regulated by QS in *A. salmonicida*. Relative to the wild-type, the *ΔainS* and *luxI*$^{-}$ mutants revealed 40 and 500 differentially expressed genes (DEGs), respectively. The functional analysis demonstrated that the most pronounced DEGs were involved in bacterial motility and chemotaxis, exopolysaccharide production, and surface structures related to adhesion. Inactivation of *luxI* but not *ainS* genes resulted in wrinkled colony morphology. While inactivation of both genes (*ΔainSluxI*$^{-}$) resulted in strains able to form wrinkled colonies and mushroom structured biofilm. Moreover, when the *ΔainSluxI*$^{-}$ mutant was supplemented with N-3-oxo-hexanoyl-L- homoserine lactone (3OC6-HSL) and N-3-hydroxy-decanoyl-L-homoserine lactone(3OHC10-HSL), the biofilm did not develop. We also show that LuxI is needed for motility and repression for EPS production, where repression of EPS is likely operated through the RpoQ-sigma factor.

Conclusion. These findings imply that LuxI and AinS synthases have a critical contribution to the QS-dependent regulation on gene expression and the phenotypic traits related to it.

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16 Abstract

17 **Background.** The marine bacterium *A. salmonicida* uses the quorum sensing (QS) systems,
18 AinS/R and LuxI/R to produce eight acyl-homoserine lactones (AHLs) in a cell density
19 dependent manner. Biofilm formation is one of the QS regulated phenotypes, which requires the
20 expression of exopolysaccharides (EPS). We previously demonstrated that inactivation of LitR,
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22 the biofilm formed by the AHL deficient mutant *ΔainSluxI*$^{-}$. In this work, we have identified
23 genes regulated by AinS and LuxI using RNA sequencing (RNA-Seq), and studied their role in
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26 **Results.** The transcriptome profiling of *ΔainS* and *luxI*$^{-}$ mutants allowed us to identify essential
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motility and repression for EPS production, where repression of EPS is likely operated through the RpoQ-sigma factor.

Conclusion. These findings imply that LuxI and AinS synthases have a critical contribution to the QS-dependent regulation on gene expression and the phenotypic traits related to it.

Introduction

Quorum sensing (QS) is a widespread mechanism in bacteria, which employs autoinducing chemical signals in response to cell density to coordinate several traits as biofilm formation, motility, bioluminescence and virulence (Whitehead et al., 2001). A variety of classes of QS chemical signals have been identified in different bacteria. Gram-negative bacteria usually employ N-acyl homoserine lactones (AHLs) which contain a conserved homoserine lactone (HSL) ring and an amide (N)-linked acyl side chain. The acyl groups identified to date, range from 4 to18 carbons in length (Fuqua, Parsek, & Greenberg, 2001; Swift et al., 2001; Whitehead et al., 2001). AHL- mediated QS was originally discovered in the marine bacterium *Aliivibrio* (*vibrio*) *fischeri*, which was found to regulate bioluminescence in a cell-density dependent manner (Eberhard et al., 1981; Ruby & Lee, 1998). *A. fischeri* controls luminescence by the QS systems LuxS/LuxPQ, LuxI/LuxR and AinS/AinR, where LuxS, LuxI and AinS are the autoinducer synthases (Lupp & Ruby, 2005, 2004; Lupp et al., 2003). LuxI synthesizes a diffusible molecule, N-(3-oxohexanoyl)-L-homoserine lactone (3OC6-HSL), which increases in concentration with cell density. 3OC6-HSL then binds to LuxR, and this complex activate light production from *lux* operon (Verma & Miyashiro, 2013).

The marine bacterium *Aliivibrio salmonicida*, is known to cause cold-water vibriosis in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and captive Atlantic cod (*Gadus morhua*) (Egidius et al., 1981; Egidius et al., 1986; Holm et al., 1985). The genome sequence of *A. salmonicida* revealed five QS systems, where three are similar to those of *A. fischeri*, the LuxS/PQ, LuxI/P and AinS/R (Hjerde et al., 2008). *A. salmonicida* produces eight AHLs, where the LuxI/R system is responsible for seven AHLs (3OC4-HSL, C4-HSL, 3OC6-HSL, C6-HSL, C8-HSL, 3OC8-HSL and 3OC10-HSL) while the AinS/R system synthesizes only one autoinducer, 3OHC10-HSL (Hansen et al., 2015). Although, *A. salmonicida* encodes the *lux* operon (*luxCDABEG*) (Nelson et al., 2007), the bacteria is only able to produce bioluminescence after addition of decyl aldehyde (Fidopiastis, Sørum & Ruby, 1999). LitR, the master regulator of QS is a positive regulator of AHL production and hence, cryptic bioluminescence in *A. salmonicida* (Bjelland et al., 2012).

In addition to regulating bioluminescence, AHLs are also involved in multiple other physiological processes in bacteria such as production of virulence factors, drug resistance and biofilm formation (Abisado et al., 2018). AHL-mediated QS affects biofilm formation in a number of bacterial species (Fazli et al., 2014; Yildiz & Visick 2009; Hmelo, 2017), and is associated with almost all stages, such as initial surface attachment, bacterial growth, maturation, and detachment of cells. For some species, QS regulates flagellar activity, which in turn influences the attachment of bacteria to surface (Guvener & McCarter 2003; Pratt & Kolter 1998). In *Pseudomonas aeruginosa* PAO1 and *Burkholderia cepacian*, QS regulates other aspects of biofilm formation, including biofilm structure and maturation (Huber et al., 2001; Whitehead et al., 2001). QS further increases dispersal of detached bacteria from mature biofilm to trigger a new cycle of biofilm formation (Emerenini et al., 2015). In many *Vibrio* species

development of rugose colony morphology and biofilm formation correlates with exopolysaccharide production. For example, in *Vibrio parahaemolyticus* and *Vibrio vulnificus*, QS activate formation of biofilm and opaque colonies at high cell density. Mutation in the QS regulators OpaR and SmcR in *V. parahaemolyticus* and *V. vulnificus*, respectively results in translucent colonies indicating a decrease in exopolysaccharide production (Lee et al., 2013; McCarter, 1998). In contrast to the two species mentioned above, *Vibrio cholerae* presents a different effect of QS regulation on biofilm formation. A mutation in the master regulator HapR, results in a state mimicking low cell density conditions, where the mutant produces more exopolysaccharides compared to wild-type (Zhu & Mekalanos, 2003). *A. salmonicida* behaves in a similar fashion to *V. cholerae*, where deletion of *litR* leads to increase exopolysaccharide production and formation of three-dimensional biofilm structure (Bjelland et al., 2012; Hansen et al., 2014).

We have previously shown that AinS and LuxI in *A. salmonicida* are responsible for the production of eight AHLs and that both these AHL synthases are needed for downregulation of biofilm formation (Hansen et al., 2015). In the work presented here, we show that the AHLs 3OC6-HSL (LuxI product) and 3OHC10-HSL (AinS product) are biologically active and downregulate biofilm formation in *A. salmonicida*. RNA-Seq was performed to identify genes regulated by AinS/R and LuxI/R QS systems. At high cell density, inactivation of *luxI* had a global effect on the transcriptome and resulted in nearly 500 differently expressed genes (DEGs), whereas deletion of *ainS* only resulted in 29 DEGs at the same condition. Genes involved in motility and EPS production were among the DEGs in the *luxI*$^{-}$ mutant, which explains the finding that this mutant lacks flagella, is non-motile and produces rugose colonies.

Materials and methods

Bacterial strains, culture conditions and supplements

Bacterial strains used in this study are listed in Table 1. *A. salmonicida* LFI1238 strain and the *A. salmonicida* mutants were grown from a frozen glycerol stock on blood agar base no. 2 (oxoid, Cambridge, UK) with a total concentration of 5% blood and 2.5% NaCl (BA2.5) or in Luria Berthani broth (Difco, BD Diagnostics, Sparks, MD) with a total concentration of 2.5% NaCl (LB2.5). *A. salmonicida* strains were cultivated from a single colony in 2 ml (LB2.5) at 12°C, 220 rpm for 2 days (primary culture). The primary cultures were diluted 1:20 and grown at 12°C, 220 rpm for an additional day (secondary cultures).

The GFP (green fluorescence protein) constitutive plasmid pVSV102 and helper plasmid pEVS104 propagated in *Escherichia coli* (*E. coli*), DH5αλpir and CC118λpir, respectively. The *E. coli* strains were cultivated in LB or LA containing 1% NaCl (LB1 and LA1 respectively) and incubated at 12°C and 220 rpm. The potential tagged strains were selected on BA2.5 supplemented 150 µl/ml kanamycin.

A seawater-based medium (SWT) was used for the HPLC-MS/MS, transcriptomics, biofilm and morphology assay. The medium consists of 5 g/L of bacto peptone (BD), 3 g/L of yeast extract (Sigma) and 28 g/L of a synthetic sea salt (Instant Ocean, Aquarium Systems).

Transcriptomics

Sample collection

Three biological replicates were used for *ΔainS*, *luxI*$^{-}$ and *A. salmonicida* LFI1238 wild-type strains. The overnight secondary cultures were diluted to $OD_{600} = 0.05$ (optical density measured at 600 nm) in a total volume of 70 ml SWT media supplemented with 2.5% sea salt. The cultures were grown further at 8°C and 220 rpm in 250 ml baffled flask. Samples (10 ml) at low cell density $OD_{600} = 0.30$ and (2.5 ml) at high cell density $OD_{600} = 1.20$ were harvested (13000 x g, 2 minutes, 4°C) (Heraeus 3XR, Thermo Scientific). Samples were persevered in RNA*later* and stored at -80°C until RNA extraction.

Total RNA isolation and rRNA depletion

The total RNA was extracted from the cell pellets following the standard protocols by manufactures (Masterpure DNA & RNA purification kit, Epicenter). The quality of total RNA was determined using a Bioanalyzer and Total RNA nano chip (Agilent Technologies). The ribosomal rRNA was removed from the samples using Ribo-Zero rRNA Removal kit for bacteria (Illumina) following manufactures instructions. The quality of RNA after depletion was determined using Bioanalyzer and Total RNA pico chip (Agilent Technologies).

RNA sequencing and data analysis

The rRNA depleted samples were used to generate RNA-sequencing libraries using TruSeq strandard mRNA library prep kit (Illumina), and sequenced at the Norwegian Sequencing Center

using the Illumina NextSeq 500 with mid output reagents with 75 bp. read length and paired end reads.

The sequencing quality of FASTQ files was assessed using FastQC (Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Further analysis of the RNA-Seq data was performed using EDGE-pro v1.0.1 (Magoc, Wood, & Salzberg, 2013) and DESeq2 (Love, Huber & Anders, 2014). EDGE-pro was used to align the reads to the *A. salmonicida* LFI1238 genome (Hjerde et al., 2008), and to estimate gene expression levels. Differences in gene expression between wild-type and *ΔlitR* and *ΔrpoQ* mutants were determined using DESeq2. Log2 fold changes of the genes were recalculated to × differential expression values (i.e., Δ*ainS*/wt) and genes were defined as significantly differentially expressed genes based on a p-value ≤ 0.05 and differentially expression values (fold change values) of ≥ 2 × and ≤ -2 ×. tRNA and rRNA reads were filtered out before analysis.

The sequences of *ΔainS*, *luxI*$^{-}$ and *A. salmoncida* LFI1238 have been deposited in the European Nucleotide Archive (www.ebi.ac.uk/ena) under study accession numbers PRJEB29457 and PRJEB28385, respectively.

High-Performance Liquid Chromatography Tandem Mass Spectrometry (HPL-MS/MS) assay

AHL standards

AHL standards purchased from University of Nottingham, UK were: N-3-oxo-butyryl-L-homoserine lactone (3OC4-HSL), N-3-hydroxy-butyryl-L-homoserine lactone (3OHC4-HSL), N-3-hydroxy-hexanoyl-L-homoserine lactone (3OHC6-HSL), N-3-hydroxy-octanoyl-L-homoserine lactone (3OHC8-HSL), N-3-hydroxy-decanoyl-L-homoserine lactone (3OHC10-HSL). Standards purchased from Sigma-Aldrich were: N-butyryl-DL-homoserine lactone (C4-HSL), N-hexanoyl-L-homoserine lactone (C6-HSL), N-3-oxo-hexanoyl-L- homoserine lactone (3OC6-HSL), N-octanoyl-L-homoserine lactone (C8-HSL), N-3-oxo-octanoyl-L-homoserine lactone (3OC8-HSL), N-decanoyl-DL-homoserine lactone (C10-HSL), N-3-oxo-decanoyl-L-homoserine lactone (3OC10-HSL), N-dodecanoyl-DL-homoserine lactone (C12-HSL), N-3-oxo-dodecanoyl-L-homoserine lactone (3OC12-HSL), and N-3-hydroxy-dodecanoyl-DL-homoserine lactone (3OHC12-HSL). Acetonitrile and formic acid for HPLC were purchased from Sigma.

Preparation of bacterial supernatants for AHL measurements

Two biological replicates were used for all *A. salmonicida* strains. The overnight secondary cultures were diluted to an $OD_{600} = 0.05$ in a total volume of 60 ml SWT media supplemented with 2.5% sea salt. The cultures were grown further at 8°C and 220 rpm in 250 ml baffled flask for 50 h. 1 ml was harvested from each culture at 13000 x g (Heraeus Fresco 21, Thermo Scientific), 4°C for 2 min. The supernatants were acidified before threefold ethyl acetate extraction as previously described (Purohit et al., 2013). The ethyl acetate phase was dried using rotary vacuum centrifuge (CentriVap, Labconco) at 40°C for 15 min, and then redissolved in 150 µl of 20% acetonitrite containing 0.1% formic acid and 775 nM of the internal standard 3OC12-HSL.

Detection of AHL profiles using a mix of HPLC-MS/MS and full scan HR-MS analysis

The detection of AHL was adapted from the methods described previously (Hansen et al., 2015). Briefly: the samples (20 µl) were injected onto an Ascentis Express C185 cm x 2.1 mm, 2.7 µm reverse phase column (Supelco) using an Accela autosampler (Thermo scientific). The elution was performed using an Accela pump (Thermo scientific) with an acetonitrile gradient in 0.1% formic acid, and consisted of 5% acetonitrile for 18 seconds, followed by a linear gradient up to 90% acetonitrile over 222 seconds, and finally 90% acetonitrile for 60 seconds. The column was re-equilibrated for 60 seconds with 5% acetonitrile in 0.1% formic acid before the next sample was injected. Flow rate was 500 µl/min for all steps.

The separated compounds were ionized in positive ion electrospray using the following settings: sheath gas flow rate 70, auxiliary gas flow rate 10, sweep gas flow rate 10, spray voltage +4.50 kV, capillary temperature 330°C, capillary voltage 37 V, and tube lens 80 V.

The ionized components where detected using an LTQ Orbitrap XL (Thermo scientific) run in either ms/ms low resolution mode or full scan HRMS mode. C4 AHL's are difficult to detect using full scan HR-MS analysis due to co-eluting isobaric compounds seen in some samples, so these components together with 3OC6 and 3OHC6 where measured using HPLC MS/MS using the LTQ part of the LTQ orbitrap XL. The rest of the compounds where measured using Full Scan HR-MS analysis. The C4's, 3OHC6 and 3OC6 elute early in the chromatogram, and where measured in 2 segments each with 3 scan events. Segment 1 ran from 0 min to 0.88 min, with the following scan events. m/z 172.10 -> (101.2-103.2) (C4-HSL), m/z 186.10-> (101.2-103.2) (3OC4-HSL) and 188.10-> (101.2-103.2) (3OHC4-HSL). Segment 2 ran from 0.88 min to 1.76

min with the following scan events: 172.10-> (101.2-102.3) (C4-HSL), 214.10-> (101.2-102.3) (3OC6-HSL), 216.12-> (101.2-102.3) (3OHC6-HSL). Segment 3 ran from 1.76 min to 5 min in which the rest of the compounds where measured using only one scan event, FTMS (165-450) resolution 15000. Target setting was 5x10^5 ions per scan, and maximum injection time was 250 ms. Lock mass was enabled for correction of background ions from caffeine (m/z 195.0877) and diisooctyl phthalate (m/z 391.2843 and m/z 413.2662). The system was calibrated with a mixture of 15 AHLs including the internal standard 3OC12-HSL, and the ion chromatograms were analyzed using the Xcalibur v. 2.18 software package. The mass window was set to 8 parts per million. The limit of detection (LOD) and the limit of quantification (LOQ) for the different AHLs were calculated as previously described (Purohit et al., 2013).

Construction of GFP tagged *A. salmonicida* strains

A. salmonicida mutants (*ΔainS, luxI⁻, ΔainSluxI⁻* and *ΔlitR)* used in this study were constructed previously (Bjelland et al., 2012; Hansen et al., 2015). The mutants were tagged with GFP using tri-parental mating as described by others. Briefly; the pVSV102 plasmid carrying the gene coding for GFP and kanamycin was transferred from *E. coli* DH5αλpir to the mutant strains using the conjugative helper strain CC118λpir harboring pEVS104 helper plasmid. Donor and helper cells were grown to mid-log phase (OD_{600} = 0.7) in LB1. Recipient strains (*A. salmonicida*) were grown to early stationary phase (OD_{600} = 1.2) in LB2.5. The donor, helper and recipient strains were harvested (13000 x g, 1 min) and washed twice with LB1 before they were mixed in 1 to 1 ratio and spotted onto BA2.5 plates, followed by overnight incubation at 16°C. The spotted cells were resuspended in LB2.5 and incubated for 24 h. at 12°C with

agitation (220 rpm). The potential tagged strains were selected on BA2.5 after 5 days. The tagged strains were confirmed microscopically with Nikon Eclipse TS100.

Static biofilm assay

The biofilm assay was performed as described previously (Hansen et al., 2014; Khider, Willassen & Hansen, 2018). Briefly; the overnight secondary cultures were grown to an OD_{600} of 1.3. The secondary cultures were further diluted 1:10 in SWT and a total volume of 300 µl was added to each well in flat-bottom, non-tissue culture-treated Falcon 24-well plates (BD Bioscience). A final concentration of 1400 ng/ml of 3OC6-HSL, 100 ng/ml of 3OHC10-HSL, 197 ng/ml of 3OC8, 100 ng/ml of C8 and 400 ng/ml of C6 were added to each well. The plates were incubated statically at 8°C, for 72 h. and the biofilm was visualized using Nikon Eclipse TS100 microscope at 10x magnification and photographed with Nikon DS-5Mc. The biomasses of the biofilms were quantified using crystal violet. The medium was removed and 300 µl of 0.1% (wt/vol) crystal violet in H_2O was added. The plates were incubated at room temperature for 30 min. The crystal violet stain was removed by flipping the plates gently, and the wells were washed twice with 0.5 ml of H_2O. The wells were air dried overnight and the biofilm was dissolved in 0.5 ml of 96% ethanol with agitation (250 rpm) overnight. The dissolved biofilm was diluted 1:10 in 96% ethanol and transferred to a 96-well plate (100 µl/well). The absorbance was measured at 590 nm (Vmax kinetic microplate reader; Molecular Devices).

Soft agar motility assay

The motility assay was performed using soft agar plates containing 0.25% agar and 2.5% NaCl as previously described (Khider, Willassen & Hansen, 2018). Briefly; the secondary overnight cultures were diluted to an OD_{600} of 0.4. Then 3 µl of each culture was spotted on the soft agar plates and incubated at 8°C for 5 days. The degree of motility for each strain was monitored every 24 hours for 5 days by measuring the diameters of spreading halos on the soft agar plate.

Colony morphology assay

The colony morphology assay was carried mainly as described before (Hansen et al., 2014; Khider, Willassen & Hansen, 2018). From each secondary overnight culture, a 250 µl was harvested by centrifugation, and the pellet was re-suspended in 250 µl SWT. Then, 2 µl of each culture was spotted onto SWT agar plates, and incubated at 8°C for 14 days. The colonies were viewed microscopically with Zeiss Primo Vert and photographed with AxioCam ERc5s at 4x magnification.

Scanning Electron Microscopy (SEM)

Secondary cultures of *A. salmonicida* strains were grown overnight and fixed with 2.5% (wt/vol) glutaraldehyde and 4% formaldehyde in PHEM-buffer and incubated for one day at 4°C. 100 µL of each sample were mounted on a poly-L-lycine coated coverslip for 5 min. Coverslips were washed three times with PHEM buffer before they were postfixed in 1% (wt/vol) Osmiumtetroxide (OsO4). Samples were further washed for three times with PHEM buffer, all samples were dehydrated with a graded series of ethanol at room temperature for 5 min. The

samples were dried using hexamethyldisilazane (HMDS) as a drying agent and further left to dry in a desiccator overnight before being mounted on aluminum stubs, using carbon tape and silver paint. The samples were coated with gold-palladium using a Polaron Range Sputter Coater. Picture were taken with Ziess Zigma SEM.

Results

AHL profiling of *A. salmonicida* in SWT medium

In our previous studies, AHL profiling of *A. salmonicida* LFI1238 and mutants thereof were performed after growth in LB2.5 medium. The autoinducer synthase, AinS produced one AHL (3OHC10-HSL), and the LuxI synthase produced seven AHLs, where the most abundant AHL signal was 3OC6-HSL (Hansen et al., 2015; Purohit et al., 2013). Consequently, when both *luxI* and *ainS* synthases were inactivated, no AHL production was observed in *A. salmonicida* and the double mutant (*ΔainSluxI*$^-$) produced a biofilm similar to the biofilm of *ΔlitR* mutant (Hansen et al., 2015). In the work presented here, we wanted to analyze if addition of AHLs could interfere with the biofilm formation of the *ΔainSluxI*$^-$ double mutant. However, since SWT medium is required for biofilm formation we first wanted to know whether a change of medium would affect the AHLs profiles of *A. salmonicida* wild-type and the mutants.

The different *A. salmonicida* strains (LFI1238, *ΔlitR, ΔainS, luxI*$^-$ and *ΔainSluxI*$^-$) were grown in SWT medium at 8°C for 50 h. (OD_{600} ~ 2.0) before samples were harvested and analyzed using HPLC-MS/MS. The *A. salmonicida* wild-type and mutants showed AHL profiles (Table 2) similar to what have been shown after growth in LB (Hansen et al., 2015) with the exception of C4 and 3OC4. Thus, the wild-type and the *ΔlitR* AHL profiles consisted of 6 AHLs, where the

278 3OC6-HSL was the most abundant. No AHLs were detected in the *ΔainSluxI*$^{-}$ supernatant, the
279 *luxI*$^{-}$ mutant produced only 3OHC10-HSL, and the *ΔainS* mutant produced the remaining five
280 AHLs. Compared to the wild-type, the *ΔlitR* mutant produced lower concentrations of the 3OC6-
281 HSL and 3OHC10-HSL confirming that LitR is a positive regulator of these two AHLs also after
282 growth in SWT medium.
283
284 **N-acyl homoserine 3OHC10 and 3OC6 downregulate biofilm formation in *A. salmonicida***

285 To investigate possible effects of 3OC6-HSL (LuxI product) and 3OHC10-HSL (AinS product)
286 on biofilm formation the different AHLs were added to the SWT medium, and *A. salmonicida*
287 strains were allowed to form biofilm at 8°C for 72 h. As shown in Figure 1A, the biofilm
288 formation of *ΔainSluxI*$^{-}$ was totally inhibited when supplemented with either 3OHC10-HSL or
289 3OC6-HSL. The *ΔainS, luxI*$^{-}$ and the wild-type do not form a biofilm (Hansen et al., 2015), and
290 no difference in biofilm formation was found when treated with 3OHC10-HSL or 3OC6-HSL
291 (Figure 1A). The mushroom structured *ΔlitR* biofilm remained unchanged after the addition of
292 AHLs. This shows that LuxI-3OC6-HSL and AinS-3OHC10-HSL functions through LitR, and
293 downregulation on the biofilm formation can not be achieved when *litR* is inactivated (Figure.
294 1A). The addition of C6, C8, and 3OC8 AHLs did not interfere with the biofilm formation of any
295 of the *A. salmonicida* strains (data not shown). Next, the biomasses of treated and untreated
296 biofilms were quantified using crystal violet. Relative to the untreated control samples, the
297 addition of either 3OHC10-HSL or 3OC6-HSL, significantly decreased the biomass of
298 *ΔainSluxI*$^{-}$ biofilm (p-value < 0.05). Quantitation of treated and untreated *ΔlitR*, LFI1238, *ΔainS*
299 and *luxI*$^{-}$ had no significant differences (Figure 1B). These observations suggest that these two

AHLs (3OHC10-HSL and 3OC6-HSL) inhibit biofilm formation in *A. salmonicida* and are operated *via* LitR.

luxI$^-$ mutant forms wrinkled colonies in *A. salmonicida*

To determine whether any of the *A. salmonicida* QS systems (*lux* or/and *ain*) are involved in wrinkled colony formation, the *luxI*$^-$, *ΔainS* and the double mutant *ΔainSluxI*$^-$ were allowed to form colonies on SWT plates at 8°C. As shown in Figure 2, the *ΔainS* mutant formed smooth colonies indistinguishable from those formed by the wild-type. This indicates that *ainS* is not required for formation of rugosity. Whereas the *luxI*$^-$ and *ΔainSluxI*$^-$ mutants formed wrinkled colonies similar to the *ΔlitR* after 14 days of incubation.

Expression profiles of *A. salmonicida luxI*$^-$ and *ΔainS* mutants revealed genes related to QS

In order to gain a better understanding of how LuxI and AinS work in the QS system, the transcriptome expression profiles of *luxI*$^-$ and *ΔainS* mutants were compared to the *A. salmonicida* LFI1238 wild-type. The expression profiling of *luxI*$^-$ mutant relative to the wild-type LFI1238 reveled 494 and 446 DEGs at low and high cell densities, respectively, that fell into various functional gene classes adapted from MultiFun (Serres & Riley, 2000) (Figure 3). Among the DEGs at low cell density (LCD) 366 were downregulated and 128 were upregulated (Table S1). Whereas, at high cell density (HCD) 224 genes were downregulated and 222 genes were upregulated (Table S2). Among the upregualted genes that fell into *surface structures* functional group we identified genes of the *tad* operon, which is believed to be associated with adhesion, *VSAL_II0366* (83.8-fold change at LCD and 151.5-fold change at HCD) and

320 *VSAL_II0377* (57.3-fold change at LCD and 39.5-fold change at HCD) coding for fimbrial
321 proteins, Flp/Fap pilin component and type IV leader peptidase, respectively. The remaining
322 genes of the *tad* operon were also upregulated in the *luxI*$^-$ mutant relative to the wild-type at both
323 cell densities, and are listed in details in the supplementary material (Table S1 and Table S2).

324 As described in our results presented above, the *luxI*$^-$ mutant formed wrinkled colony
325 morphology on SWT plates. The rugosity is associated with the enhanced production of
326 exopolysaccharides (EPS), which requires the expression of *syp* operon (18 genes) in *A.*
327 *salmonicida* (Hansen et al., 2014; Khider, Willassen & Hansen, 2018). Consistently, our RNA-
328 Seq data at HCD demonstrated 11 significantly upregulated genes of the *syp* operon with fold
329 change values ranging from 9.55 to 2.04.

330 For several bacteria, QS regulates motility and flagellar synthesis (Kim et al., 2007; Ng &
331 Bassler, 2009). Within the *cell processes* functional group, the transcriptome of *luxI*$^-$ mutant
332 revealed genes associated with motility and chemotaxis (59 DEGs at LCD and 57 DEGs at
333 HCD) (Table S1 and Table S2). The greatest transcript abundance at LCD and HCD were genes
334 encoding sigma 54-dependent transcription regulator, *flrA* and *flrC* in addition to the two-
335 component system *flrB*. Other genes coding for flagellin subunits and flagellar basal body rod,
336 ring, hook and cap proteins, were also downregulated in the *luxI*$^-$ mutant relative to the wild-type
337 at both cell densities. Additionally, genes coding for methyl-accepting chemotaxis proteins and
338 motor proteins as MotA and MotB were downregulated in the *luxI*$^-$ mutant.

339 The transcriptome of *ΔainS* revealed fewer genes compared to the *luxI*$^-$. Our results presented
340 here show that at LCD we were able to determine a total of 20 DEGs (8 up- and 12

downregulated) and at HCD we were able to identify 29 DEGs where 8 were upregulated and 21 genes were downregulated (Table S3 and Table S4). The DEGs fell into 10 functional groups (Figure S1). At LCD and in the absence of AHLs, *ain* system act as kinase and serve as phosphoryl-donors to LuxU, which in turn phosphorylates LuxO (Freeman & Bassler, 1999). The *ΔainS* transcriptome demonstrates an upregulation in genes responsible for phosphorylation. The DEGs with high expression level relative to the wild-type was phosphorelay prorein LuxU (*VSAL_I1875*) with a fold change values of 2.22 and 2.37 at low and high cell densities, respectively. Among the upregulated genes that fell into the *surface structures* functional group we were able to identify genes of the *tad* operon, *VSAL_II0366* gene coding for fimbrial protein with a fold change values of 2.82 and 4.24 at LCD and HCD, respectively. *VSAL_II0367* coding for Flp/Fap pilin component and type IV leader peptidase was identified among upregulated genes at LCD only (Table S3). Among the 21 downregulated genes at HCD our data revealed DEGs with highest fold change to be allocated into *amino acid biosynthesis* as sulfate adenyltransferase subunit 1 and 2 encoded by *VSAL_I0421* and *VSAL_I0420*, respectively (Table S4).

LuxI controls motility in *A. salmonicida* LFI1238

The flagellum is required for motility of bacteria, mediating their movements towards favorable environments and avoiding unfavorable conditions (Utada et al. 2014; Zhu, Kojima & Homma, 2013). Because the transcriptome results demonstrated that a large panel of flagellar biosynthesis and assembly genes are regulated by *lux* system, we wished to analyze the motility behavior of QS mutants (*luxI*$^-$, *ΔainS* and *ΔainSluxI*$^-$), using soft motility assay.

We show that inactivation of *luxI* resulted in a non-motile strain, where the size of the spotted colony (2.0 mm) did not change, indicating no migration from the site of inoculation (Figure 4AB). AinS was shown to negatively regulate motility in *A. fischeri* (Lupp & Ruby, 2004), and similarly, we assessed the impact of *ainS* deletion on motility of *A. salmonicida.* Compared to the wild-type, which showed motility zones of 26.6 ± 0.57 mm, the *ΔainS* showed an increased motility, where migration through the soft agar resulted in motility zones of 30.3 ± 0.57 mm. The *ΔainSluxI*$^{-}$ double mutant also demonstrated an increase motility compared to the wild-type with motility zones of 31.3 ± 1.15 mm. (Table S5). In order to determine whether the strains analyzed by soft motility assay possess or lack flagella, the wild-type and the constructed mutants were visualized by SEM. The *ΔainS* and *ΔainSluxI*$^{-}$ mutants produced several flagella similar to the wild-type. As expected the *luxI*$^{-}$ mutant that showed a motility defect, lack flagella in all replicates (Figure 4C).

Discussion

AHLs have been identified in many vibrio and aliivibrio species including *A. salmonicida* (Buchholtz et al., 2006; Garcia-Aljaro et al., 2008; Purohit et al., 2013; Valiente et al., 2009), which showed to produce a broad range of AHLs through LuxI and AinS synthases (Hansen et al., 2015). However, there is still limited understanding of the biological advantages of this AHL diversity in the QS mechanism. In this study, we have demonstrated the influence of *luxI* and *ainS* on the global gene regulation and the impact of AHLs on several phenotypic traits related to QS in order to reveal some answers on the complex network of signal production and regulation in *A. salmonicida*.

383 The ability to form rugose colonies and biofilm are often correlated features in vibrios (Casper-
384 Lindley & Yildiz, 2004; Yildiz & Schoolnik, 1999; Yildiz et al., 2004), where wrinkled colony
385 phenotype is generally associated with enhanced exopolysaccharide production (Yildiz &
386 Schoolnik, 1999). Likewise, in *A. salmonicida* colony wrinkling (rugosity) and biofilm formation
387 requires the expression of *syp* genes responsible for the production of EPS (Hansen et al., 2014;
388 Khider, Willassen & Hansen, 2018). In the study presented here we show that *luxI*$^{-}$ mutant
389 exhibited a strong wrinkling colony morphology, indicating an enhanced polysaccharide
390 production. This finding is further confirmed by the transcription analysis, which revealed
391 upregulation of 11 *syp* genes and downregulation of the in the *rpoQ* gene in the *luxI*$^{-}$ mutant
392 relative to the wild-type. The sigma factor RpoQ is known to be a strong repressor of *syp* in *A.*
393 *salmonicida* (Khider, Willassen & Hansen, 2018). Earlier studies demonstrated that inactivation
394 of the AHL synthase (e.g *luxI* homologous) in several bacteria caused a reduction in both AHL
395 and EPS production (Koutsoudis et al., 2006; Molina et al., 2005; Von Bodman, Bauer & Coplin,
396 2003). However, here we show that inactivation of *luxI* in *A. salmonicida,* enhanced the EPS
397 production and resulted in wrinkled colonies. Unlike the *luxI*$^{-}$ mutants, the *ΔainS* mutant formed
398 smooth colonies, similar to the wild-type and did not show any differentially expressed genes
399 associated with EPS production in the transcriptomics profiling. This indicates that LuxI-derived
400 AHLs are involved in the repression of *syp* genes, where this repression is most likely operated
401 through RpoQ, independent of AinS and the 3OHC10-HSL production.

402 LitR was suggested to link AinS/R and LuxS/PQ systems to LuxI/R systems in *A. salmonicida,*
403 where its deletion influenced the production of AinS and LuxI AHLs. When both *luxI* and *ainS*
404 were inactivated simultaneously, biofilm and colony morphology similar to *ΔlitR* was formed
405 (Hansen et al., 2015). A simple explanation for this observation is the deficiency in AHL

406 production, leading to *litR* inactivation (also *litR* knockout) (Hansen et al., 2014; Bjelland et al.,
407 2012), and thereby no repression on biofilm or colony rugosity is achieved. Furthermore, the
408 exogenous addition of either 3OHC10-HSL (AinS signal) or 3OC6-HSL (LuxI signal) to
409 *ΔainSluxI*$^{-}$, completely inhibited biofilm formation. We have previously shown that the
410 disruption of either EPS or other matrix components (e.g proteins, lipoproteins and eDNA),
411 disrupts the mature biofilm formation in *A. salmonicida* (Hansen et al., 2014; Khider, Willassen
412 & Hansen, 2018). While *ΔainS* mutant did not produce neither mature biofilm nor wrinkled
413 colonies, introduction of *luxI* mutation into *ΔainS,* resulted in strains (*ΔainSluxI*$^{-}$) with three-
414 dimensional biofilm architecture and wrinkled colonies. These data suggest that these two
415 systems regulate biofilm formation synergistically, where the effect of AinS and LuxI AHLs is
416 operated through a common pathway as previously reported (Hansen et al., 2015). The results
417 presented here show that both systems function to allow or repress production of EPS and other
418 matrix components. However, the *lux* system is believed to be essential for the production of
419 EPS rather than the *ain* system (discussed above). Studies showed that one key function of EPS
420 involves the attachment of cells to different substratum, which is the initial step in biofilm
421 formation (Vu et al., 2009). For example in *V. cholera,* EPS production is the first step in biofilm
422 formation as cells switch from motile planktonic state to being non-motile and surface attached
423 (Silva & Benitez, 2016). Likewise, we suggest that the non-motile *luxI*$^{-}$ mutant, increases EPS
424 production to mediate the initial steps in biofilm formation, whereas *ainS* is neither fully
425 activated nor required at this time. This suggests that *lux* system may operate at a lower threshold
426 cell density than *ain* system, which is more essential at later stages of biofilm development,
427 mainly the maturation into three-dimensional mushroom structure. With our results we expand
428 the previously suggested model, to include *luxI* and *ainS* and their proposed role in regulating

biofilm formation and colony rugosity. In the model presented in Figure 5, we propose that as cell density rises 3OHC10-HSL binds AinR receptor, resulting in activation of LitR, which in turn regulates the production of AinS AHL. The activated LitR leads to a repression on other matrix components required for building a mature biofilm as well as activating *rpoQ* resulting in repression of *syp* genes. The *luxI* was proposed to be activated by both LitR and LuxRs. The active LuxI synthesizes seven AHLs and represses *syp* operon via RpoQ. In summary, our results provide clear evidence that the biofilm formation is a low cell density dependent phenotype, when neither LuxI nor AinS AHLs are present. As AHLs accumulate at high cell densities the biofilm is dispersed, indicating that AHL-mediated QS in *A. salmonicida* is involved in the dispersal step of the biofilm cycle.

Several bacteria are known to regulate motility via QS, where motility is either activated or inhibited by AHLs (Atkinson et al., 2006; Hoang, Gurich & Gonzalez, 2008; Hussain et al., 2008; Kim et al., 2007; Quinones, Dulla & Lindow, 2005). In the sponge-associated bacteria KLH11 flagellar motility was abolished in the *ssaI* and *ssaR* mutants homologous of *luxI* and *luxR*, respectively (Zan et al., 2012). Similarly, inactivation of *luxI* in *A. salmonicida* led to loss of flagella and motility under our experimental conditions (Figure 4). Consistent with these results, the most pronounced regulation in the *luxI*$^-$ transcriptome data was observed for genes involved in motility and chemotaxis, exhibiting a significantly low expression level. The motility regulatory cascade has not been well elucidated in *A. salmonicida*, however the motility genes are organized in a similar fashion to *A. fischeri* (Karlsen et al., 2008). Flagellar genes are often grouped into different hierarchal classes; early, middle and late genes, where each class encode genes responsible for entire motility regulon, structural components of the hook-basal body, flagellar filaments, chemotaxis and motor force, respectively (Aldridge & Hughes, 2002). Our

results demonstrate DEGs that fell into all classes and thus it is unclear into which regulatory level LuxI affects motility genes. This defect in motility, observed by *luxI*$^{-}$, can not be explained by differences in growth rate, as cultures for different mutants reached the stationary phase (Figure S2). Hence, our observations indicate that LuxI is a positive regulator and required for full motility in *A. salmonicida*, suggesting that the defect in motility is most likely due to impact of LuxI-derived AHLs on the flagellar apparatus. LitR has been shown to be a positive regulator of *ainS* (Hansen et al., 2015). Thus, not surprisingly, we found that *ΔainS* displayed an increased motility compared to the wild-type, similar to what was reported for *ΔlitR* (Bjelland et al., 2012). However, the regulation of motility in *A. salmonicida* and the target of these regulators remain to be determined.

We have previously shown that changes in media composition altered biological traits as biofilm formation and colony rugosity in *A. salmonicida* (Hansen et al., 2014). Contrary to what was previously reported (Hansen et al., 2015; Purohit et al., 2013) neither C4-HSL nor 3OC4-HSL were detected in the present work, suggesting that the concentration of these AHLs are either below the detectable limit or not produced due to different culturing temperatures and/or media. However, the profile for the remaining AHLs was unaffected.

Conclusion

In this study we have shown that *luxI* but not *ainS* is essential for formation of wrinkled colonies at low cell density, whereas both systems are required to form a three-dimensional mature biofilm in *A. salmonicida* LFI1238. We also demonstrated that addition of either LuxI-3OC6-HSL or AinS-3OHC10-HSL is able to inhibit the biofilm formation. Our results show that *lux*

and *ain* systems regulates biofilm formation through a common pathway, where LuxI acts mainly as a repressor of EPS production (*syp* operon) via RpoQ. While AinS is probably involved in the repression of other matrix components required to build the mature biofilm. Furthermore, we identified differentially expressed genes associated with motility to be regulated by LuxI. These results add a new knowledge to the QS mechanism of *A. salmonicida*, however further investigations are needed to understand the regulation and complexity of this mechanism.

Abbreviation

AHL: Acyl homoserine lactone; **GFP:** Green fluorescent protein; **rpm:** rounds per minute; **RNA:** Ribonucleic acid; **tRNA:** transfer RNA; **rRNA:** ribosomal RNA; **h:** hours; **OD:** optical density; **RNA-Seq:** RNA sequencing.

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Table 1(on next page)

Bacterial strains and plasmids used in this study

1

Bacterial strains or plasmids	Description	Source
A. salmonicida		
LFI1238	Wild-type, isolated from Atlantic cod	(Hjerde et al., 2008)
ΔlitR	LFI1238 containing an in-frame deletion in *litR*	(Bjelland et al., 2012)
ΔainS	LFI1238 containing an in-frame deletion in *ainS*	(Hansen et al., 2015)
luxI$^-$	LFI1238 containing an insertional disruption in *luxI*, Cm^r	(Hansen et al., 2015)
ΔainSluxI$^-$	*ΔainS* containing an insertional disruption in *luxI*, Cm^r	(Hansen et al., 2015)
LFI1238-pVSV102	*A. salmonicida* LFI238 carrying pVSV102, Kn^r	(Khider, Willassen & Hansen, 2018)
ΔlitR-pVSV102	*ΔlitR* carrying pVSV102, Kn^r	(Khider, Willassen & Hansen, 2018)
ΔainS-pVSV102	*ΔainS* carrying pVSV102, Kn^r	This study
luxI$^-$-pVSV102	*luxI*$^-$ carrying pVSV102, Kn^r	This study
ΔainSluxI$^-$-pVSV102	*ΔainS luxI*$^-$ carrying pVSV102, Kn^r	This study
E. coli		
C118λpir	Helper strain containing pEVS104	(Dunn et al., 2006)
DH5αλpir	*E. coli* strain containing GFP plasmid pVSV102	(Dunn et al., 2006)
Plasmids		
pVSV102	pES213, constitutive GFP, Kn^r	(Dunn et al., 2006)
pEVS104	R6Korigin, RP4, *oriT, trb tra* and Kn^r	(Stabb & Ruby, 2002)

2
3

Table 2 (on next page)

AHL production in *A. salmonicida* LFI1238, *ΔlitR*, *luxI*$^{-}$, *ΔainS* and *ΔainSluxI*$^{-}$.

The values represent the mean of two biological replicates ± standard deviation.

1

Strains	3OC6 (nM)	C6 (nM)	3OC8 (nM)	3OC10 (nM)	3OHC10 (nM)	C8 (nM)
LFI1238	8403 ± 279.3	606 ± 3.5	366 ± 27	67 ± 5.9	161 ± 2.1	28 ± 3.0
ΔlitR	5173 ± 113.6	593 ± 82.3	330 ± 42.1	72 ± 4.7	11 ± 1.70	25 ± 3.4
luxI⁻	NF	NF	NF	NF	105 ± 6.7	NF
ΔainS	8691 ± 0.0	709 ± 54.6	382 ± 42.5	89 ± 16.9	NF	30 ± 0.0
ΔainSluxI⁻	NF	NF	NF	NF	NF	NF

2

3 • C4-HSL and 3OC4 were not detected in this analysis
4 • NF: not found

5

Figure 1

The effect of 3OC6-HSL and 3OHC10-HSL on biofilm formation of LFI1238, *ΔlitR*, *luxI*$^{-}$, *ΔainS* and *ΔainSluxI*$^{-}$.

(A) The strains (LFI1238, *ΔlitR*, *luxI*$^{-}$, *ΔainS* and *ΔainSluxI*$^{-}$) were allowed to form biofilm in SWT media supplemented with 1400 ng/ml 3OC6-HSL or 100 ng/ml 3OHC10-HSL at 8°C for 72 h. The biofilms were viewed in a Nikon Eclipse TS100 microscope at 10x magnification and photographed with Nikon DS-5Mc. **(B)** The formed biofilms were staining with crystal violet and quantified by measuring the absorbance at 590 nm. The error bars represent the standard deviation of biological triplicates. (*) represents p-value < 0.05.

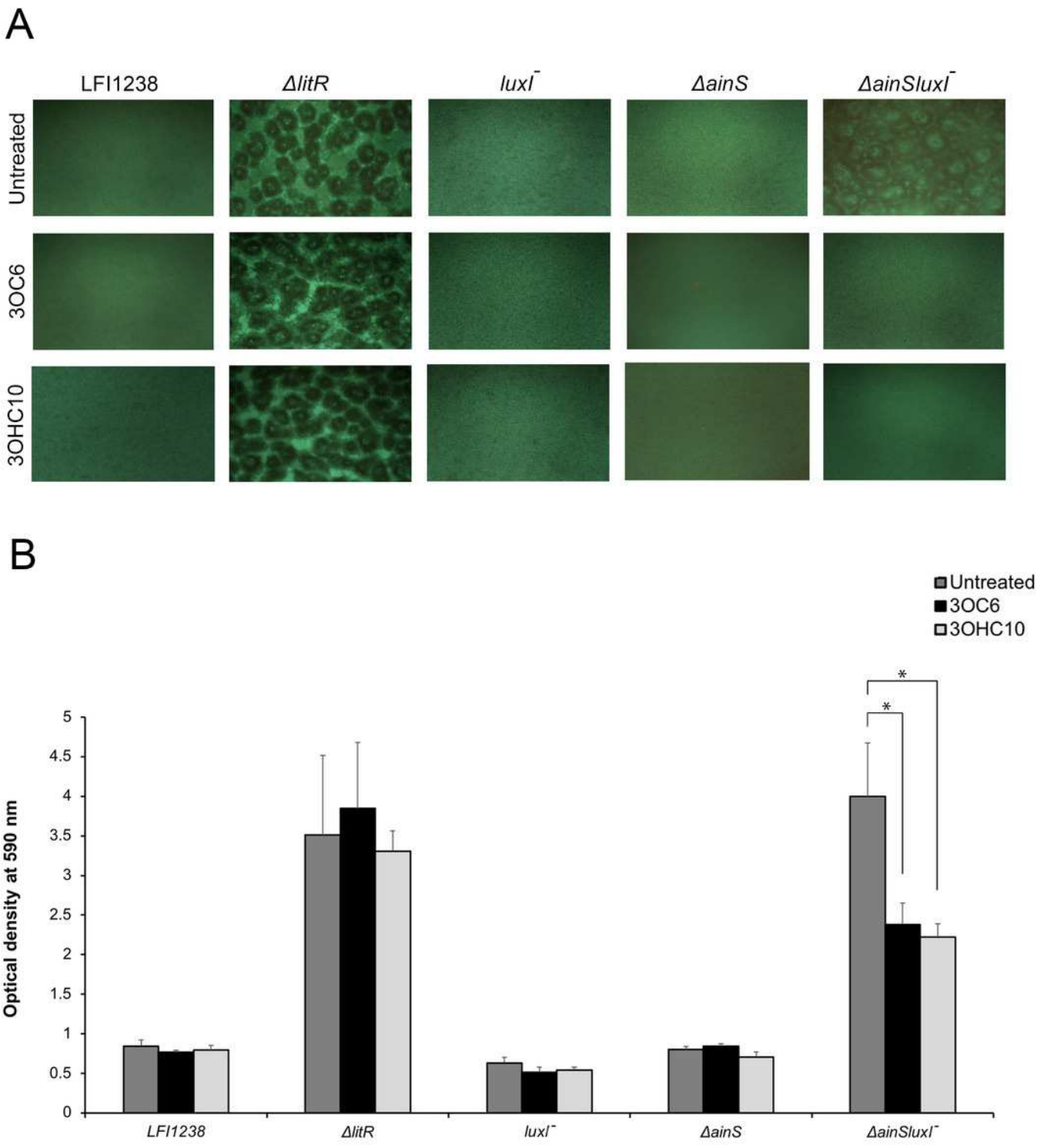

Figure 2

Colony morphology of LFI1238, *ΔlitR*, *luxI*$^{-}$, *ΔainS* and *ΔainSluxI*$^{-}$.

The colonies of different strains were allowed to form on SWT plates at 8°C for 14 days. The colonies were viewed in a Zeiss Primo Vert microscope at 4x magnification and photographed with AxioCam ERc5s. Scale bars represent 0.5 mm.

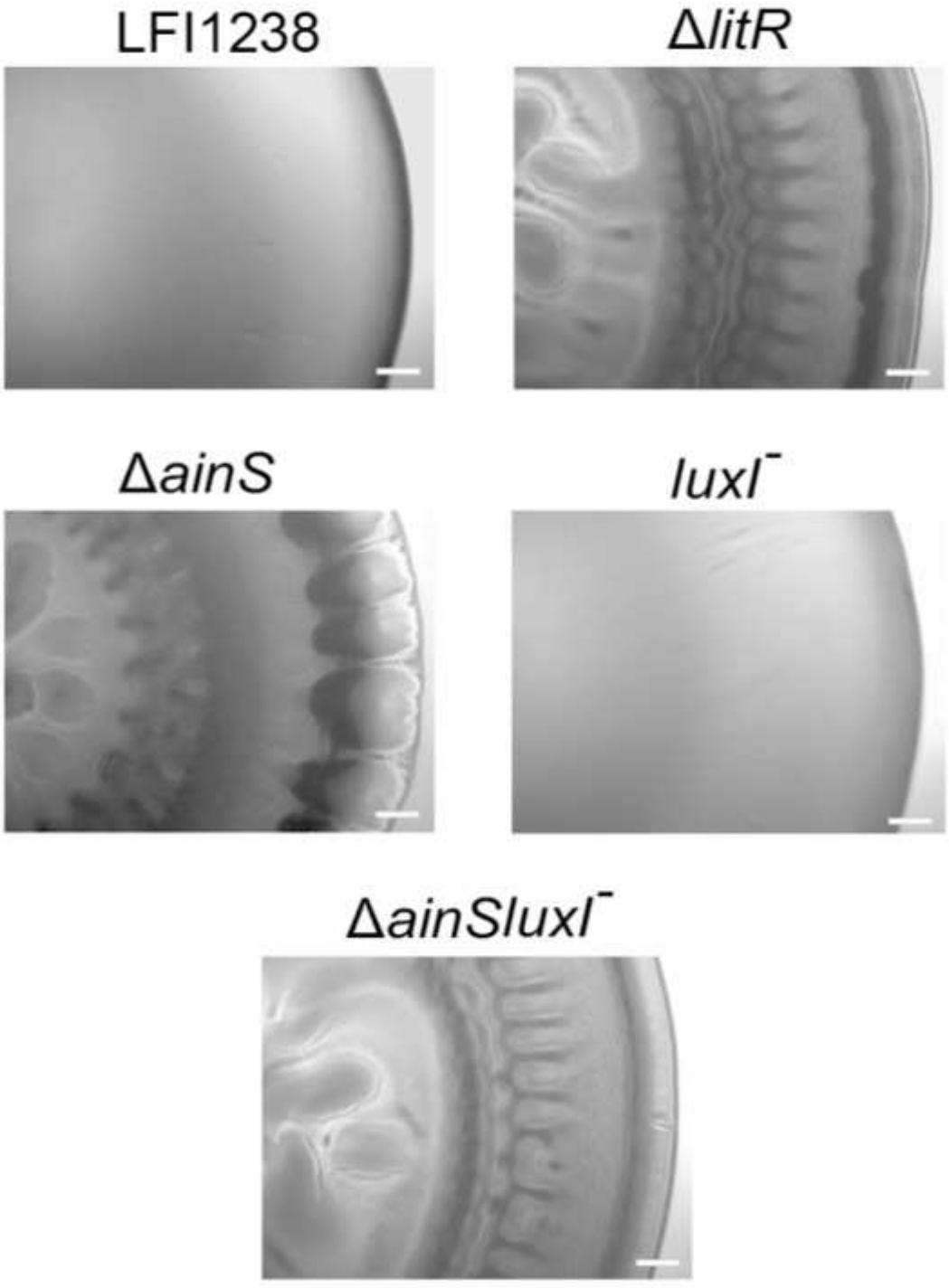

Figure 3

Functional distribution of genes between *A. salmonicida* wild-type and *luxI*$^{-}$mutant at HCD and LCD that are ≥ 2 × differentially expressed.

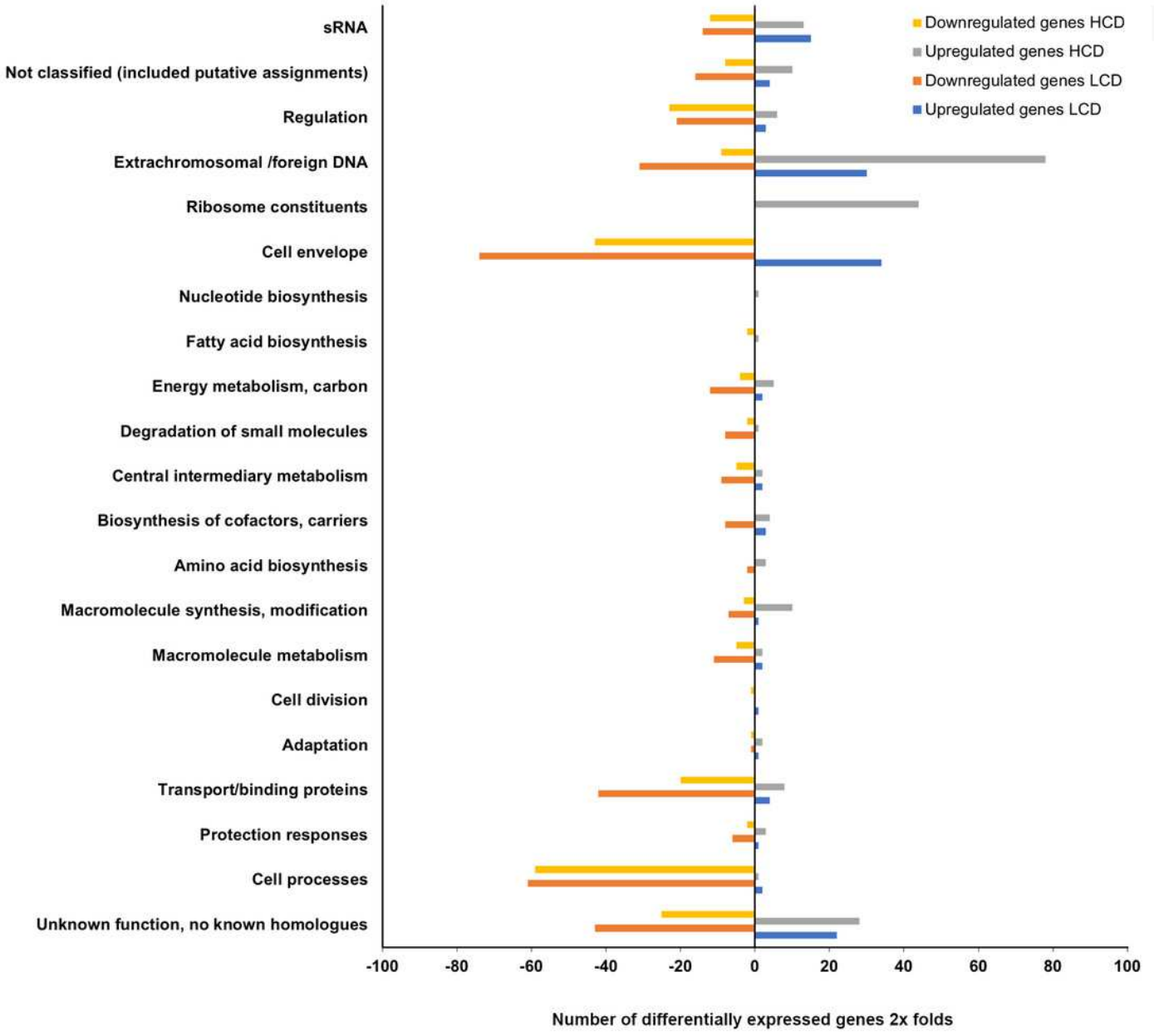

Figure 4

Motility of LFI1238, *luxI*$^{-}$, *ΔainS* and *ΔainSluxI*$^{-}$.

(A) Motility zones on soft agar plates after 5 days on incubation at 8°C.**(B)** Measurement of motility zones (mm) of LFI1238, *luxI*$^{-}$, *ΔainS* and *ΔainSluxI*$^{-}$ after 5 days, error bars are standard deviation of biological triplicates. **(C)** SEM images for flagellum observation ofLFI1238, *luxI*$^{-}$,*ΔainS* and *ΔainSluxI*$^{-}$ taken with Ziess Zigma at 2kV with an in-lens detector. Scale bars represent 1 mm.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

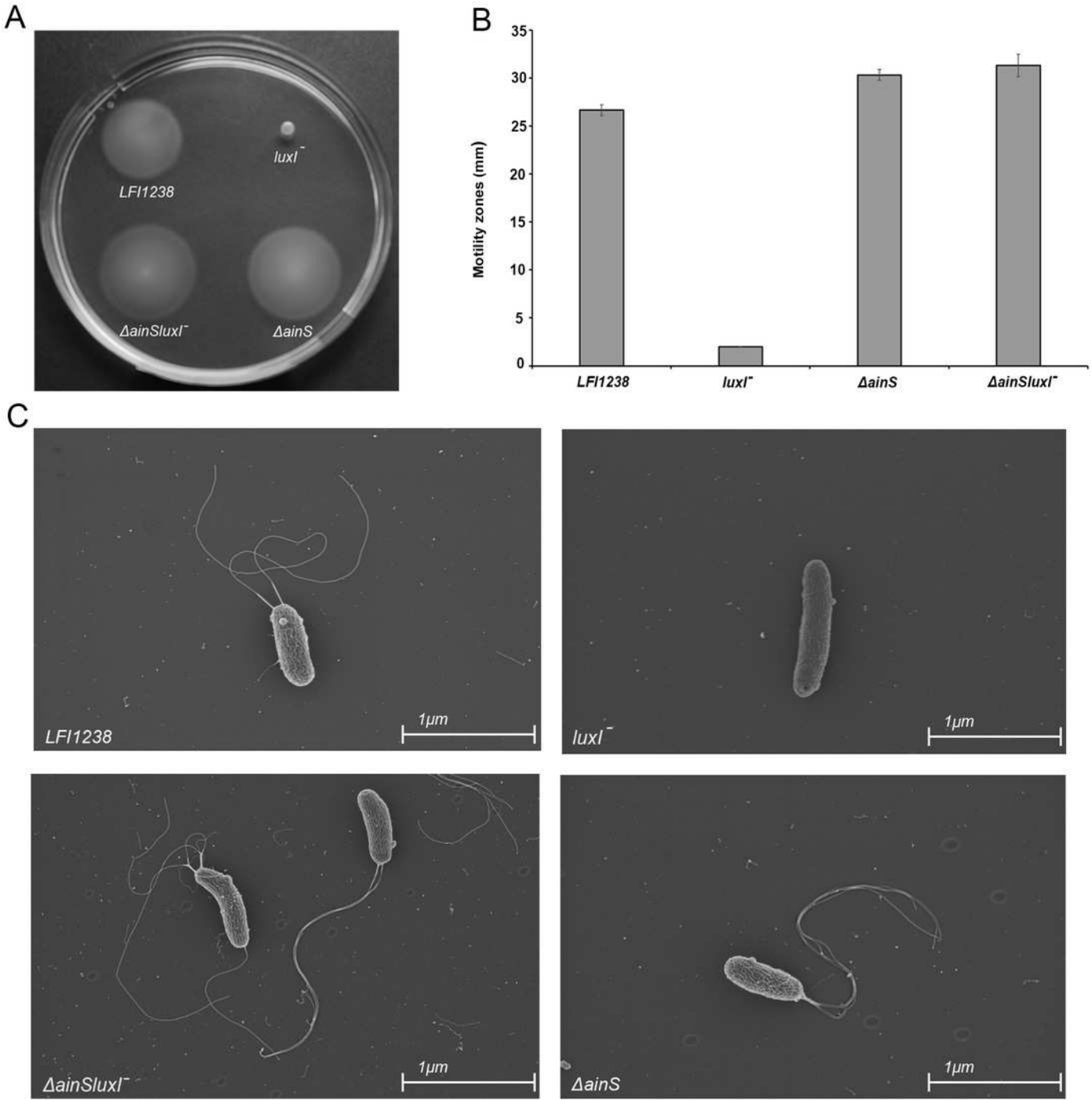

Figure 5

The proposed model of QS system in *A. salmonicida* LFI1238.

The autoinducer synthases LuxI and AinS produce eight AHLs that are transported across the outer (OM) and inner membrane (IM) (Hansen et al., 2015). At high cell density these AHLs are accumulated to reach a critical concentration to be sensed by their receptors (AinR or LuxRs). The AinS-3OHC10-HSL binds AinR, which in turn induces a dephosphorylation cascade, resulting in LitR activation. The expressed LitR, activates the production of the AinS AHL (3OHC10) and the expression of downstream *rpoQ* gene. The increased RpoQ levels represses the *syp* operon leading to biofilm disruption and inhibition of colony rugosity. Moreover, LitR represses other matrix components, through a pathway that remain unknown (Khider, Willassen & Hansen, 2018). LitR together with LuxRs are proposed to regulate *luxI*. The expressed LuxI mediate the production of seven AHLs and represses the *syp* genes via RpoQ. Blue arrows and red lines with bar end indicate pathways of positive and negative regulation, respectively, and may consist of several steps. The thicker, empty arrows indicate the resulting phenotypes.

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