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AHL-mediated quorum sensing regulation: Role in controlling cytotoxicity, T6SSs and CRISPR-Cas systems in *Aliivibrio wodanis*.

Quorum sensing, Cytotoxicity, T6SS, CRISPR-Cas and Pan-genome

Amudha Deepalakshmi Maharajan

A dissertation for the degree of Philosophiae Doctor (November 2022)

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SUMMARY

Aliivibrio wodanis has been associated with winter ulcer disease outbreaks. It has been reported that *A. wodanis* may act as a secondary pathogen in disease outbreaks and pathogenesis. The culture supernatant of *A. wodanis* causes a cytopathogenic effect (CPE) in various salmon cell lines. Moreover, in an Atlantic salmon bath challenge experiment, *A. wodanis* alone produces clinical symptoms in the fish. However, the contribution of *A. wodanis* to winter ulcer disease is not clear. Despite the knowledge to date and the research done in the field, there are still unanswered questions and a knowledge gap. Several *Vibrio* and *Aliivibrio* species use quorum sensing (QS) to regulate genes connected to host-pathogen interaction, virulence, survival, and adaptation mechanisms. In other aliivibrios, several QS systems, including the AinS/AinR and a transcriptional regulator LitR (LuxR homologs), have been reported to regulate various phenotypic traits.

Moreover, temperature is an essential factor that governs the prevalence of bacteria in the environment and host and is a potent regulator of pathogenesis in many bacteria. Hence the thesis aimed to understand better the QS`s role and the effect of temperature in *A. wodanis*. Our study reveals that AinS autoinducer synthase is required to produce the Acyl-Homoserine Lactone (AHL) 3OHC10-HSL in *A. wodanis*.

Furthermore, we found that the 3OHC10-HSL production is cell density and temperature-dependent. The 3OHC10-HSL concentration was higher at 6°C, the temperature below the threshold temperature at which winter ulcer occurs, compared to 12°C. The results also showed that QS and temperature regulate various functions such as AHL production, motility, and production of proteases, hemolysin and siderophores. The cell culture study further revealed that cell density, QS and temperature influence the cytotoxicity in CHSE salmon cell lines. This suggests that *A. wodanis* produces cytolysins and cytotoxins that are implicated in cytotoxicity.

Bacteria use the Type VI secretion system (T6SS) for multiple functions like iron transport, interspecies competition, virulence, and niche adaptations. *A. wodanis* co-exists with the main pathogen *M. viscosa* in the infected fish during the winter ulcer disease outbreaks. In addition, the bacterium has been shown to hinder the growth and virulence of *M. viscosa*. The thesis further investigated the mechanisms by which *A. wodanis* may survive together with *M. viscosa* in skin ulcers and during winter ulcer outbreaks. We found that the *A. wodanis* genome encodes three T6SSs (T6SS1-T6SS3) and auxiliary clusters (Aux1-4); and several potential Type VI secretion system effectors (T6SEs). In addition, the *A. wodanis* genome is found to contain a type IF CRISPR-Cas system. This suggests that these two mechanisms may play a role during the survival,

adaptation, and immune function of *A. wodanis* in its natural environment, inside the host or during its co-existence with *M. viscosa*. Next, we analyzed the genome-wide transcriptomics of *A. wodanis* and QS mutants *litR* and *ainS* mutants grown at different temperatures and cell densities. We found that the genes involved in T6SSs, and CRISPR-Cas systems are regulated by cell density, temperature, and QS. In this study, the transcriptome analysis showed that the complete T6SS2 apparatus was less expressed in the *litR*/WT, suggesting LitR regulates T6SS2 in *A. wodanis.* The transcriptome analysis also demonstrated that deletion of *litR* decreased the *hcp1* expression, *a* gene involved in bacterial competition and virulence in other bacteria. In addition to LitR dependent expression, expression value of *hcp* decreased three times in *litR*/WT, HCD at 12°C when compared to 6°C and HCD. Our observation suggests that temperature 6°C and LitR are crucial for expressing the genes related to virulence.

Understanding the strain diversity of the same species is important in exploring strategies to survive in a changing environment. Finally, we wanted to study the genomic similarity and variation between *A. wodanis* isolates by performing a pan-genome analysis of twenty-two *A. wodanis* isolates collected from various locations across Norway. The analysis revealed an open pan-genome with a wide inter-species diversity in *A. wodanis* genomes. We examined the phylogenetic relatedness between the isolates using single nucleotide variants (SNVs) and core genes. The phylogenetic trees were distributed into five groups, where Group 2, 4 and 5 encompassed conserved isolates. The accessory genomes (shell, cloud and unique) accounted for about 73% of the total pan-genome suggesting the genomes have acquired most of the genes through horizontal gene transfer (HGT). Whole and cloud genome functional annotation revealed a larger number of genes related to functional families such as *metabolism, signaling and cellular processes* and *genetic information processing,* suggesting they are involved in energy metabolism and environmental interactions. By further analyzing the twenty-two *A. wodanis* genomes, we identified diverse CRISPR-Cas systems, spacers, and prophages. About 60% of isolates encoded a different CRISPR-Cas system compared to the reference strain. Like the reference strain, the other twenty-one *A. wodanis* isolates also encoded multiple T6SSs where the T6SS2 and Aux-2 are either absent or showed differences in about 80% (18 out of 22) of isolates. In addition to the T6SSs and CRISPR-Cas, other elements relevant for adaptation, virulence, and survival potentials, such as virulence factors (VFs) and biosynthetic gene clusters (BGCs), were explored. We have found that the predicted VFs and BGCs were conserved between the *A. wodanis* isolates.

The results presented in this work yield knowledge about QS regulation of virulence, survival, and adaptation mechanisms in *A. wodanis*. This aids in understanding the mechanisms connected to co-existence of *A. wodanis* with *M. viscosa* and winter ulcer disease development and treatment.

LIST OF PAPERS

Paper 1

Amudha Deepalakshmi Maharajan, Hilde Hansen, Miriam Khider, Nils Peder Willassen (2021). Quorum sensing in *Aliivibrio wodanis* 06/09/139 and its role in controlling various phenotypic traits. Published in PeerJ. doi: 10.7717/peerj.11980

Paper 2

Amudha Deepalakshmi Maharajan, **Erik Hjerde, Hilde Hansen, and Nils Peder Willassen** (2022). Quorum Sensing Controls the CRISPR and Type VI Secretion Systems in *Aliivibrio wodanis* 06/09/139. Published in Frontiers in Veterinary Science. https://doi.org/10.3389/fvets.2022.799414

Paper 3

Amudha Deepalakshmi Maharajan, Terje Klemetsen and Nils Peder Willassen (2022). Pan-genome analysis of *Aliivibrio wodanis* provides insight into the genetic diversity of the CRISPR-Cas system, T6SS2 and phages present (Manuscript).

ABBREVIATIONS

AHL: N-acyl homoserine lactone

AI: Autoinducer

Aux: Auxiliary cluster

BLAST: Basic local alignment search tool

BGCs: Biosynthetic gene clusters

CHSE: Chinook salmon embryo cells

CRISPR-Cas: Clustered Regularly Interspaced Palindromic Repeats-CRISPR associated protein

Cytopathogenic effect: CPE

DB: Database

DEGs: Differentially expressed genes

DR: Direct Repeats

DNA: Deoxyribonucleic acid

FC: Fold change

HCD: High cell density

HGT: Horizontal gene transfer

LCD: Low cell density

MTase: methyltransferase

OD_{600}: Optical density measured at 600 nm

Qrr sRNAs: Quorum regulatory small RNA

QS: Quorum sensing

REase: restriction endonuclease

RM: Restriction modification

RNA: ribonucleic acid

RNA-seq: RNA sequencing

SNVs: Single nucleotide variants

sRNA: small regulatory RNA

T6SS: Type VI secretion system

T6SEs: Type VI secretion system effectors

tp: Transcriptomes profile

VFDB: Virulence factor database

VFs: Virulence factors

WT: Wild type

BACKGROUND

Vibrionaceae members

Vibrionaceae strains are Gram-negative, Gammaproteobacteria, motile rods or curved and typically easy to cultivate (Levanova & Blokhina 1976; Thompson et al. 2005; Dryselius et al. 2007; Thompson et al. 2009). They are found in aquatic environments ranging from marine, brackish and freshwater environments (Thompson et al. 2004; Thompson et al. 2009). The *Vibrionaceae* family consists of a genetically and metabolically diverse species of heterotrophic bacteria that commonly live as either free living, symbionts, and pathogens (Urbanczyk et al. 2007). *Vibrionaceae* includes genera *Aliivibrio*, *Vibrio*, *Photobacterium*, *Beneckea*, *Echinimonas*, *Lucibacterium*, *Grimontia*, *Candidatus*, *Listonella*, *Enhydrobacter*, *Salinivibrio*, *Enterovibrio*, *Allomonas* and *Catenococcus*, of which the genus *Vibrio* has over 100 species (Sawabe et al. 2007; Urbanczyk et al. 2007; Onohuean et al. 2022).

An Italian physician Filippo Pacini identified the first *Vibrio* species, *Vibrio cholerae*, the causative agent of cholera in 1854 (Craster 1914). Later first non-pathogenic *Vibrio* species, *Vibrio fischeri* (now known as *Aliivibrio fischeri*) and *V. splendidus* were discovered in the late 1880s (Thompson et al. 2004; Sawabe et al. 2007). All *Vibrionaceae* species have two unequally sized chromosomes, and this seems to be evolutionarily stable (Trucksis et al. 1998; Okada et al. 2005). The large chromosome contains all the essential genes required for survival and the small chromosome contains the species-specific traits for adaptation (Dryselius et al. 2007).

The genus *Aliivibrio* contains several species such as *Aliivibrio wodanis, Aliivibrio finisterrensis*, *A. fischeri*, *Aliivibrio logei*, *Aliivibrio salmonicida*, *Aliivibrio sifae*, *Aliivibrio* sp. *"*thorii*"*, *Aliivibrio* sp. *"*magni*"*, *Aliivibrio* sp. *"*vili*"*, *Aliivibrio* sp. *"*bragi*" Aliivibrio* sp. *"*thrudae*"* and Aliivibrio sp. *"*friggae*"* (Ast et al. 2009; Beaz-Hidalgo et al. 2010; Yoshizawa et al. 2010; Klemetsen et al. 2021). Most luminous species are members of *Vibrionaceae* and belong to the genera *Aliivibrio*, *Vibrio* and *Photobacterium*, however some species of these genera lack the *lux* operon (Ast et al. 2009). In the genera *Aliivibrio* and *Vibrio*, many non-pathogenic species are commensals or symbionts that have been associated to aquatic eukaryotic organisms and marine environments. Members of the genus *Vibrio* are isolated as normal flora from a wide variety of living organisms. Some examples include *Vibrio hemicentroti* from sea urchin, *Vibrio hippocampi*

from sea horses, *Vibrio rotiferianus* from rotifers, *Vibrio pacinii* from crustaceans, *Vibrio tasmaniensis* and *Vibrio alfacsensis* from fish (Gomez-Gil et al. 2003; Thompson et al. 2003a; Thompson et al. 2003b; Balcazar et al. 2010; Gomez-Gil et al. 2012; Kim et al. 2013).

A. fischeri is a well-studied luminous bacteria found in symbiotic association with squid of the genus *Euprymna* (Engebrecht et al. 1983; Fidopiastis et al. 1998). In addition, several other *Aliivibrio* species such as *Aliivibrio* sp. "thorii" and *A. logei* have been reported to form a bioluminescent symbiosis with marine hosts (Benediktsdottir et al. 1998; Ast et al. 2009). Luminous bacteria contain the *lux* operon *luxCDABEG* that code for proteins involved in light production (Engebrecht et al. 1983). The *luxA* and *luxB* genes encode two subunits α and β of enzyme luciferase and light is released during the oxidative reaction of a long chain aldehyde (RCOH) and reduced flavin mononucleotide ($FMNH_2$). The *luxCDE* encodes reductase complex while *luxG* reduces FMN to $FMNH_2$ (Nijvipakul et al. 2008). In symbiosis, the host provides nutrients and oxygen for bacterial reproduction while the host uses luminescence to escape from predators or to attract prey (Ruby & Lee 1998; Visick & Ruby 2006).

Pathogenic *Vibrionaceae*

Various members of *Vibrionaceae* are pathogenic to human and marine organisms, where the severity of illness varies depending on the species and type of disease. The human pathogens include *V. cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* while *Vibrio anguillarum*, *A. salmonicida and Vibrio harveyi* are among the main bacterial pathogens of several fish and shellfish reared in aquaculture (Enger et al. 1989; Alvarez et al. 1998; Frans et al. 2011). Some *Vibrio* species (*Vibrio alginolyticus*, *V. parahaemolyticus* and *V. vulnificus*) are common pathogens of both humans and marine organisms (Horre et al. 1996; Chavez-Dozal et al. 2012; Damir et al. 2013; Raszl et al. 2016). Several coral pathogenic vibrios (*Vibrio shilonii, Vibrio coralliilyticus, Vibrio fortis, Vibrio campbellii* and *Vibrio rotiferianus*) are also associated with coral bleaching (Grottoli et al. 2014).

Vibrio cholerae

V. cholerae is a curved rod-shaped, Gram-negative, facultative anaerobe, non-halophilic waterborne human pathogen that causes an epidemic diarrheal disease called cholera (Garay et al. 1985). The organism is widely spread in aquatic environments as a free-living organism (Garay et

al. 1985). Cholera is transmitted through contaminated water and prevails as a persistent cause of mortality and morbidity in Africa and Asia (Faruque et al. 1998; Yoon & Waters 2019). Only two serogroups *V. cholera* O1 and O139 have been known to cause epidemic cholera, and others are environmental strains (Faruque et al. 1998). The virulent *V. cholerae* adheres to, colonizes the small intestine, and produces cholera symptoms using key virulence factors (VFs), cholera toxins and toxin-coregulated pilus (Reidl & Klose 2002). *V. cholera* is also able to form biofilms on solid surfaces, and it is known to be important for their environmental survival (Watnick & Kolter 1999).

Vibrio anguillarum

V. anguillarum is a Gram-negative halophilic bacterium that causes vibriosis in warm and cold water cultured and wild fish species (Naka & Crosa 2012). *V. anguillarum* causes disease outbreaks in a wide range of temperatures (Frans et al. 2011; Lages et al. 2019). *V. anguillarum* is the most important pathogen in Danish farms (Pedersen et al. 1997). Vibriosis is characterized by hemorrhagic septicemia and has been reported as a major problem in the fish culture industry (Toranzo et al. 2005). Out of 23 different serotypes, the serotypes O1, O2 and O3 have been reported as the main causative agents of vibriosis and the remaining are environmental strains (Myhr et al. 1991). Some of the identified VFs include multiple iron transport systems, hemolysins, metalloproteases, motility and exopolysaccharides (Naka & Crosa 2012). The expression of VFs have been regulated by temperature and iron levels (Lages et al. 2019).

Aliivibrio salmonicida

A. salmonicida is the causative agent of cold-water vibriosis or Hitra disease in various fish hosts (Nicolson & Dodge 1995; Southgate & Jones 1995). This bacterium is Gram-negative, halophilic, curved rod-shaped, motile with multiple flagella and cryptically luminescent (Enger et al. 1989; Fidopiastis et al. 1998). *A. salmonicida* is psychrophilic, and the growth occurs between 1-22°C (Enger et al. 1989). The Hitra disease is characterized by necrosis in the gills, kidney, spleen and muscles, and external haemorrhaging in the skin and gills (Egidius 1987). The disease outbreaks occur when the water temperature is below 10°C (Eggset et al. 1997). Vaccinations give complete protection against infection with *A. salmonicida* (Eggset et al. 1997). However, only a few VFs have been characterized in *A. salmonicida.* Surface antigen VS-P1 in *A. salmonicida* is a potential virulence factor involved in escaping the host immune response (Hjelmeland et al. 1988). In *A. salmonicida*, motility is dependent on salinity and temperature (Bjelland et al. 2012b). Like other

vibrios, motility in *A. salmonicida* is linked to virulence (Norstebo et al. 2017). *A. salmonicida* harbors *syp* operon and deletion of QS regulator, *litR* in *A. salmonicida* resulted in biofilm formation and rugose colony morphology (Hansen et al. 2014). *A. salmonicida* contains several iron acquisition systems that may be involved in pathogenesis, and the siderophore production is temperature dependent (Colquhoun & Sorum 2001). Further genome analysis identified genes encoding proteases, hemolysins, and secretion systems (Hjerde et al. 2008; Bjelland et al. 2013; Huang et al. 2018).

Aliivibrio wodanis

A. wodanis is a psychrotrophic Gram-negative, motile, rod-shaped, non-luminescent bacterium that grows in a temperature range of 4-25°C and a salt concentration range of 1-4% (Lunder et al. 2000). *A. wodanis* has been repeatedly isolated together with the main pathogen *M. viscosa* from the infected Atlantic salmon during the winter ulcer outbreaks (Lunder et al. 1995a; Benediktsdottir et al. 1998). Other environmental pathogenic *Tenacibaculum* sp. have also been identified from the skin lesion together with *M. viscosa* (Olsen et al. 2011). However, *Tenacibaculum* sp. have been later identified as a solo causative agent of "tenacibaculosis", a disease that infects the fish at warmer temperatures above 8°C (Smage et al. 2018). Preliminary challenge experiments and intramuscular injection of *A. wodanis* in Atlantic salmon showed neither ulceration nor mortality, indicating that *A. wodanis* is not the primary pathogen (Lunder et al. 1995a; Benediktsdottir et al. 1998; Bruno et al. 1998; Greger & Goodrich 1999). Winter ulcer was first identified during the early 1980s in the Norwegian farmed Atlantic salmon and later in the 1990s, it was reported in about 50 Norwegian fish farms (Salte et al. 1994). Winter ulcer is characterized by several internal and external pathological symptoms such as lesions in skin and muscles, gill pallor and mortality (Lunder et al. 1995a; Benediktsdottir et al. 1998; Bruno et al. 1998). The disease occurs at temperatures lower than 8°C and the fish recovers when the temperature increases above 8°C (Lunder et al. 1995a). Although *A. wodanis* is not the main pathogen, the reason for its co-existence with *M. viscosa* and its presence during winter ulcer outbreaks are not clear. In an experimental study reproducing field observation, *A. wodanis* colonizes well in the predisposed salmon tissue (Karlsen et al. 2014b). Furthermore, in a cell culture study, *A. wodanis* adheres to Atlantic salmon head kidney cells, and the supernatants cause cytopathogenic effect (CPE) on various cell lines of salmonoid origin including Chinook salmon embryo (CHSE) cell line (Karlsen et al. 2014b). *A. wodanis* genome contains two chromosomes

and four plasmids (Hjerde et al. 2015). The genome encompasses 54 insertion sequence elements on both chromosomes and one plasmid pAWOD920 and 4079 coding domain sequences (Hjerde et al. 2015). The plasmid pAWOD920 contains a bacteriocin gene cluster, which was upregulated when implanted in fish together with *M. viscosa* (Hjerde et al. 2015). In a co-cultivation experiment, *A. wodanis* negatively affects the growth and virulence of *M. viscosa* (Hjerde et al. 2015). Since *A. wodanis* has been present in the outbreak environment, it is likely that it is directly or indirectly involved in the development of winter ulcer disease.

The significance of *A. wodanis* during the winter ulcer outbreaks have been speculated by others (Karlsen et al. 2014b; Hjerde et al. 2015), as follows: (i) *A. wodanis* may exploit the impaired host's defense mechanism to develop disease as a secondary pathogen. (ii) *A. wodanis* is fighting for the same niche as *M. viscosa*. (iii) Interplay between the two bacteria. (iv) Both are competitors and (v) *A. wodanis* influences the infection caused by *M. viscosa*. Hence, it is important to explore the genomes and phenotypes of *A. wodanis* to get a better understanding of their survival and virulence potential, which is the focus of this thesis.

Quorum sensing

Quorum sensing signaling mechanism

Quorum sensing (QS) is a signaling mechanism used by bacteria to sense the cell density and regulate various functions required for the host-pathogen interaction and environmental adaptation (Bassler 1999; Egland & Greenberg 1999; Parsek et al. 1999). Some bacterial species can produce signaling molecules called autoinducers (AIs), that when they reach a threshold concentration initiates the signal transduction cascade **(Figure 1)** (Fuqua et al. 1994; Hastings & Greenberg 1999). This induction changes the behavior of the population and favors the inter- and intra-species relationships (Schauder et al. 2001; Bassler 2002). Both Gram-positive and Gram-negative bacteria use QS for cell-to-cell communication, but they produce different AIs. Gram-negative bacteria use autoinducer-1 (AI-1), N-acyl homoserine lactone (AHL) whereas Gram-positive bacteria depend on oligopeptides (Bassler 1999; Federle & Bassler 2003). LuxS, an autoinducer synthase that produces autoinducer-2 (AL-2) is conserved in both Gram-positive and Gram-negative bacteria (Kozlova et al. 2008). Therefore, AI-1 and AI-2 mediate intra- and inter-species communication respectively (Bassler 2002; Federle & Bassler 2003). Like AI-1, AL-2 also controls several phenotypes such as virulence, drug resistance, bioluminescence, biofilm

formation, motility, and toxin production (Schauder et al. 2001; Xu et al. 2006; Duanis-Assaf et al. 2015). In addition to AI-2 production, LuxS also participates in activating the methyl cycle of bacterial metabolism and plays a key role in vitamin synthesis (Hu et al. 2018; Wang et al. 2019b). Bacteria producing similar AHLs are known to have an evolutionary relationship when mapped to 16S rRNA gene sequence (Purohit et al. 2013). In addition to AHL, there are many signaling molecules that in coordination with AHLs regulate target genes (Flavier et al. 1997). Some of the known AI-2 molecules are (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (S-THMF-borate) and (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (R-THMF) (Cloak et al. 2002; Federle & Bassler 2003; Xavier & Bassler 2005). Some bacteria such as *M. viscosa*, *Photobacterium damselae* and *Flavobacterium psychrophilum* do not produce AHL but encode receptors to benefit from other AHL-producing bacteria (Bruhn et al. 2005). Though the QS system components are quite similar, the QS mechanisms differ in complexity and cellular output (Milton 2006).

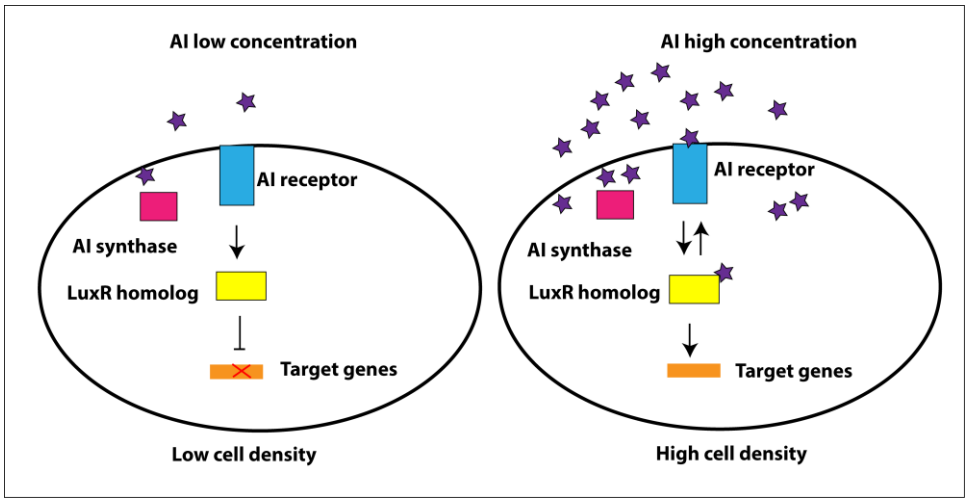

Figure 1. Quorum sensing system in bacteria. QS mechanism at low cell density (left) and high cell density (right). Bacteria secrete AHL (purple stars) produced by AI synthase (pink box), which when reaches a threshold concentration binds the cognate AHL receptor (blue box) to activate the QS regulated target gene expression (orange box) through master QS regulator LuxR homologs (yellow box).

Quorum sensing in *V. harveyi*

The bioluminescent bacterium *V. harveyi* produces three autoinducers. The first AI-1 *N*-(3-hydroxybutyryl)-homoserine lactone (3OH-C4-HSL) is produced by the AI synthase LuxM while the second AI-2 furanosyl borate diester is synthesized by LuxS AI synthase (Bassler et al. 1993). The third AI is a long chain amino ketone *(Z)* 3-aminoundec-2-en-4-one (Ea-C8-CAI-1) produced

by AI synthase CqsA, which is specific to several vibrios and enteropathogenic *Escherichia coli* (Ng et al. 2011; Gorelik et al. 2019). These three AIs are perceived by their corresponding receptors LuxN, LuxPQ and CqsS. At low cell density and low AIs concentration, the receptors act as kinases and the conserved histidine residue of the receptors gets autophosphorylated. This subsequently transfers the phosphoryl group to the histidine phosphotransfer protein (HPt) LuxU, which in turn phosphorylates the conserved aspartate residue of the response regulator LuxO (Freeman et al. 2000). Phosphorylated LuxO together with the sigma factor σ^{54} promotes the transcription of Quorum regulatory small RNAs (Qrr sRNAs) (1-5) (Lenz et al. 2004). These sRNAs function together with RNA chaperone Hfq to destabilize the mRNA of the master regulator *luxR* and activate the LCD master regulator AphA (Feng et al. 2015). The AphA induces the expression of Qrr sRNAs (Rutherford et al. 2011; Feng et al. 2015). At high cell density, the AIs bind to their cognate receptors, which prevent their kinase activities. This in turn dephosphorylates LuxU and inactivates LuxO. Thus, the downstream cascade lacks the phosphoryl groups, which in turn inhibits Qrr sRNAs and promotes the translation of the master regulator, LuxR **(Figure 2)** (Bassler et al. 1993). Furthermore, the QS cascade in *V. harveyi* comprises several feedback loops (Tu et al. 2010). The Qrr sRNAs control the *luxO* mRNA and negatively regulate the translation of LuxMN whereas the LuxR directly activates the sRNAs (Tu et al. 2008; Feng et al. 2015). Moreover, the LuxR and LuxO negatively regulate their own transcription by binding to their promoters (Tu et al. 2010).

Quorum sensing in *V. cholerae*

Four QS systems have been identified in *V. cholerae* (Jung et al. 2015). The QS systems rely on histidine kinase phosphorelay and work in parallel to regulate downstream genes (Jung et al. 2015). At LCD, the two QS systems CqsA/CqsS and LuxS/LuxPQ and two other histidine kinases CqsR and VpsS act in parallel to phosphorylate LuxO through LuxU. This activates the transcription of Qrr sRNAs (1-4) to promote the translation of the master regulator, AphA (Rutherford et al. 2011). Conversely, at high cell density, when the signals bind to their cognate receptors, LuxO is dephosphorylated. This subsequently inhibits the transcription of *qrrs* to repress AphA and promote HapR translation **(Figure 2)** (Rutherford et al. 2011). The autoinducer synthases CqsA and LuxS catalyze the production of autoinducers AI-1 (*S*-3-hydroxytridecan-4-one) and AI-2 (*S*-TMHF-borate) respectively (Wei et al. 2012). The autoinducers that bind the receptors CqsR and VpsS have not been identified yet (Watve et al. 2020).

Quorum sensing in *A. fischeri*

The first QS system was characterized in *A. fischeri*. The QS network in aliivibrios differs from other vibrios in various aspects: The LuxI/LuxR QS system is exclusively present in aliivibrios. Therefore, in aliivibrios, the target genes are regulated by both LuxI/LuxR QS system and a LitR homologue (Nelson et al. 2007). Moreover, unlike the QS systems in other vibrios that act in parallel, the QS cascades in aliivibrios function in a parallel and hierarchical manner (Verma & Miyashiro 2013). Furthermore, the numbers of Qrr sRNAs present are not as high as in non-*Aliivibrio Vibrionaceae* (Miyashiro et al. 2010).

In *A. fischeri*, three QS systems LuxI/LuxR, AinS/AinR and LuxS/LuxPQ and a LitR have been reported (Lupp & Ruby 2005). LitR is a transcriptional regulator protein essential for the regulation of gene expression (Fidopiastis et al. 2002). LitR either functions alone or binds with the activator (promoter) or repressor to block the RNA polymerase (Fidopiastis et al. 2002). LitR in *A. fischeri* controls phenotypes like motility, biofilm, host colonization and siderophore production (Fidopiastis et al. 2002; Chavez-Dozal et al. 2012). AHL is synthesized by two AHL synthases LuxI and AinS while the AI-2 synthase LuxS produces the AI-2 (Fuqua et al. 1994). LuxI/LuxR in *A. fischeri* is the QS system responsible for producing 3-oxo-C6-HSL where the AI binds to transcriptional regulator LuxR and activates bioluminescence (Fuqua et al. 1994). The AinS synthesizes AI C8-HSL, which can bind to LuxR and activate luminescence (Lupp & Ruby 2004). At low cell density, the receptors AinR and LuxPQ act as kinases to autophosphorylate and transfer the phosphoryl group to LuxO through LuxU. When LuxO is phosphorylated, the expression of *qrr* is activated, which prevents the translation of *litR* mRNA. Since LitR is a direct activator of *luxR*, bioluminescence production is inhibited in the absence of LitR. At high cell density when the LuxO is dephosphorylated, *qrr* expression is repressed to translate LitR **(Figure 2)** (Miyashiro et al. 2010). Thus, *luxR* is transcribed to activate the *lux* operon responsible for bioluminescence production. LitR activates the *ainS* expression in *A. fischeri* (Lupp & Ruby 2004). Moreover, AinS and LuxI are also required for normal growth yield and colonization of the squid (Studer et al. 2008).

Quorum sensing in *A. salmonicida*

A. salmonicida genome encodes five QS systems. In addition to LuxI/LuxR, AinS/AinR and LuxS/LuxPQ QS systems, the bacterium encodes QS systems LuxM/LuxN and VarS/VarA, however the latter two QS systems are believed to be non-functional (Hjerde et al. 2008). The AI synthase LuxI in *A. salmonicida* produces seven AHLs while the AinS produces one AHL 3OHC10-HSL (Hansen et al. 2015). The LuxI/LuxR and *lux* operon in *A. salmonicida* are different from in *A. fischeri* (Hansen et al. 2015). Like *A. logei*, *A. salmonicida* contains two copies of *luxR* and inactivation of these *luxR* genes results in loss of LuxI produced AHLs (Hansen et al. 2015). Unlike the bioluminescence function of *lux* operon in *A. fischeri*, *lux* operon in *A. salmonicida* is only cryptically luminescent (Makarova et al. 2011). In *A. salmonicida*, multiple QS systems control various phenotypes such as biofilm formation, colony rugosity and motility (Khider et al. 2019). At low cell density, the AinS/AinR and LuxS/LuxPQ QS systems produce less AIs and therefore the receptors AinR and LuxPQ are believed to work as kinases to get autophosphorylated. As a result, the phosphoryl group is transferred to LuxO through LuxU. Once LuxO is phosphorylated, the *qrr* expression is activated, and translation of *litR* mRNA is inhibited. At high cell density, the LuxO is dephosphorylated, and the *qrr* expression is repressed to induce the LitR translation **(Figure 2)**. In *A. salmonicida*, *litR* mutant reduces the mortality rate in fish suggesting the importance of the QS system in pathogenesis (Bjelland et al. 2012b). Furthermore, deletion of *litR* reduces AinS-produced AHL, and the effect was more noticeable at low temperatures (Hansen et al. 2015).

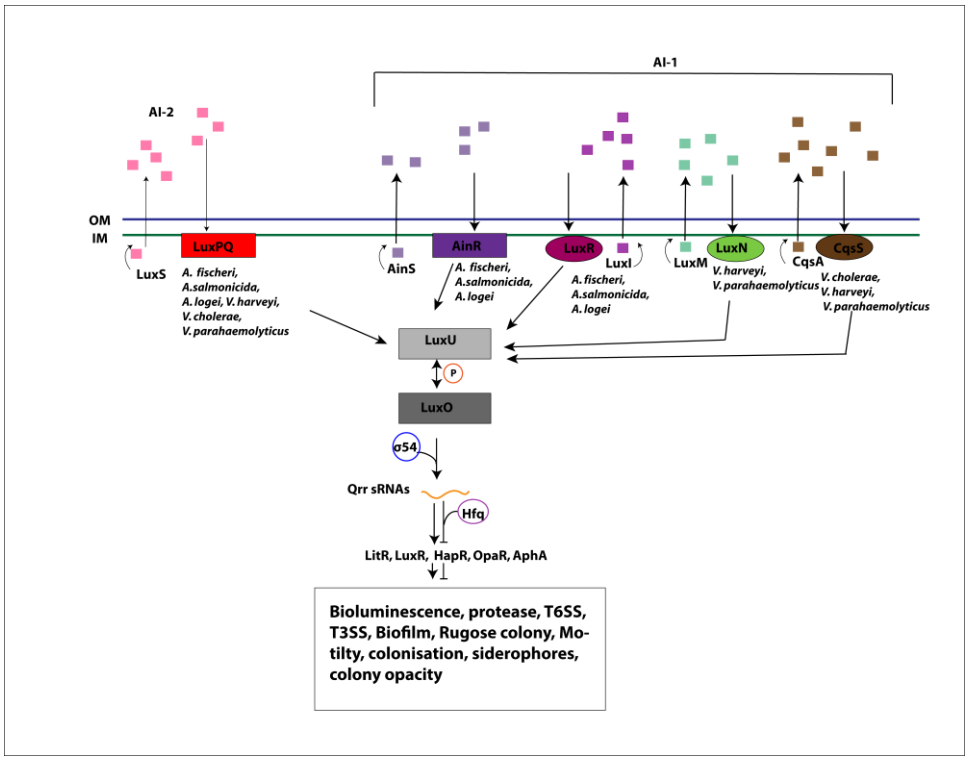

Figure 2. QS signaling pathway in *Vibrionaceae* members. AIs (colored squares) are produced by autoinducer synthases AinS, LuxI, LuxM, CqsS and LuxS. In the absence of AIs, LuxU and LuxO are phosphorylated leading to the expression of AphA in *V. cholerae* and inhibition of LitR, LuxR, HapR, OpaR production. Thus, the QS-regulated genes are not expressed. Once the AIs concentration reaches a threshold level, dephosphorylation of LuxU drives the production of QS regulators LitR (*A. fischeri* and *A. salmonicida*), LuxR (*V. harveyi*), HapR (*V. cholerae*), OpaR (*V. parahaemolyticus*). These QS regulators regulate the expression of genes involved in bioluminescence, protease, T6SS, T3SS, biofilm, rugose colony, motility, host colonization, siderophores and colony opacity (Jobling & Holmes 1997; McCarter 1998; Fidopiastis et al. 2002; Henke & Bassler 2004; Yildiz & Visick 2009; Zheng et al. 2010; Bjelland et al. 2012b; Khider et al. 2018).

Quorum sensing in *A. wodanis*

Despite the close phylogenetic relatedness between *A. salmonicida*, *A. logei* and *A. fischeri*, *A. wodanis* do not encode a LuxI/LuxR QS system and a *lux* operon (Manukhov et al. 2011; Hjerde et al. 2015; Khrulnova et al. 2016; Konopleva et al. 2016; Melkina et al. 2019; Bazhenov et al. 2021). However, like *A. salmonicida*, *A. logei* and *A. fischeri*, *A. wodanis* genome encodes AinS/AinR and LuxS/LuxPQ QS systems and a master regulator LitR (Hjerde et al. 2015). In a previous study, seventeen *A. wodanis* isolates were tested for AHLs production using HPLC-MS/MS analysis (Purohit et al. 2013). The analysis showed that out of seventeen, fourteen isolates, including *A. wodanis* reference strain (06/09/139) produce only one AHL 3OHC10-HSL whereas *A. wodanis* 01/09/401 (Vw11) is the only isolate produce an additional AHL C8-HSL (Purohit et

al. 2013). Furthermore, two *A. wodanis* isolates SA12 and SR6 have been shown to produce several AHLs, however, later these isolates were classified as *Aliivibrio* sp. "friggae" and not *A. wodanis* (Purohit et al. 2013; Klemetsen et al. 2021). Other than the AHLs screening and genome analysis, the QS pathway, and regulatory mechanisms in *A. wodanis* have not been studied yet. QS plays a significant role in the symbiosis and pathogenesis of other aliivibrios such as symbionts *A. logei* and *A. fischeri* and pathogen *A. salmonicida* (Callahan & Dunlap 2000; Bjelland et al. 2012b; Bazhenov et al. 2021). Since *A. wodanis* is closely related to other aliivibrios, it is likely that the QS systems may play a vital role in *A. wodanis* as well. Moreover, it is also likely that the AinS/AinR and LuxS/LuxPQ QS systems and a master regulator *litR* will function similarly to other aliivibrios.

Quorum sensing-dependent regulation

Aquatic environments are highly variable with many bacterial species, phages, and environmental cues such as temperature, pH, osmotic stress, salinity, antimicrobials, immune system components and nutrient limitation. Microbes have evolved various phenotypic traits to compete against other bacteria, and attack the host (Lee et al. 2008; Frans et al. 2011; Diard & Hardt 2017; Balado et al. 2018). To be able to efficiently express these phenotypic traits, bacteria depend on tight regulatory mechanisms (Heilmann et al. 2015). QS is an important cell density-dependent regulatory mechanism which regulates several community behaviors in other *Vibrionaceae* members (Freeman et al. 2000; Henke & Bassler 2004; Defoirdt et al. 2010; Tsou & Zhu 2010; Sheng et al. 2012). Many studies have shown an association between gene expression and regulation by QS (Nielsen et al. 2006; Chang & Lee 2018; Khider et al. 2018; Lages et al. 2019). QS regulation of phenotypic traits have been described in numerous bacteria where most of these collective traits are mediated by intracellular and extracellular products (Schauder et al. 2001; Defoirdt et al. 2010; Leung et al. 2011; Yang & Defoirdt 2015; Hoque et al. 2016; Schuster et al. 2017; McRose et al. 2018; Khider et al. 2019).

Phenotypic traits regulated by QS

Motility is an important virulence factor, which has been shown to influence the nutrient uptake mechanisms and colonization in some bacteria (Klausen et al. 2003; Ghoul & Mitri 2016). Motile strains have more access to nutrient-rich regions and hosts when compared to non-motile strains

(Ghoul & Mitri 2016). Additionally, in some bacteria, motility has been used to escape the prey bacteria and other unfavorable conditions (Pham et al. 2005). This process has been accomplished by thin, long, propellers called flagella that protrude from the bacterial cell body and rotate (Eisenbach et al. 1990; Milton 2006). Flagellar biosynthesis is a complex process, where more than 50 genes are expressed and regulated by various regulatory networks (Doll & Frankel 1993; Herrgard & Palsson 2004). The flagellum, a complex nano-machine, consists of the basal body, the hook, and the helical filament (Eisenbach 1990; Eisenbach et al. 1990). The core of the basal body is composed of trans-membrane proteins FlhAB and FliFOPQR, which play a crucial role in the assembly and export of flagellar apparatus through the flagellar type III secretion system. The MotAB and FliGMN are flagellar rotor proteins that determine the rotational direction. FliMN are the flagellar switch proteins that sense the regulatory protein CheY and pass the signal to FliG, which together with MotA generates a torque (Nishikino et al. 2018). The extracellular hook (FlgE) connects the helical filament to the membrane and cell wall embedded flagellum and the protein FliK controls the hook length (Erhardt et al. 2011). Besides its primary role in motility, it plays a role in chemotaxis, adhesion, invasion, aggregation, and initial colonization of the host (Gardel & Mekalanos 1996; Lee et al. 2004; Milton 2006; Defoirdt et al. 2010; Frans et al. 2011; Yang & Defoirdt 2015). Since flagellar-based motility is accompanied by higher metabolic and energetic costs and is a target for the hosts' immune system, bacteria use QS to regulate motility (Defoirdt et al. 2010; Yang & Defoirdt 2015; Khider et al. 2019). Several studies have demonstrated the QS regulation of motility in *Vibrionaceae*, where the contribution of QS varies between bacteria (Yang & Defoirdt 2015). In *A. salmonicida, A. fischeri, V. parahaemolyticus* and *V. alginolyticus*, QS negatively control motility whereas in some bacteria like *V. cholerae* and *V. harveyi* it positively regulates motility (Lupp & Ruby 2004; Rui et al. 2008; Bjelland et al. 2012b; Yang & Defoirdt 2015). All bacteria require iron as a cofactor to catalyze redox reactions and participate in cellular processes such as respiration, reactive oxygen species and DNA synthesis (Andrews et al. 2003). Besides, iron sequestration systems are an important virulence mechanism in some fish pathogens (Frans et al. 2011; Thode et al. 2015; Thode et al. 2018). Several iron sequestration systems have been characterized including siderophores synthesis and transport system, and heme transport and utilization system (Frans et al. 2011; Thode et al. 2015; Thode et al. 2018). Siderophores are secondary metabolite chelator compounds produced under low iron conditions to scavenge iron from the environment and take it back to the bacterial cells through siderophore receptors (Miethke & Marahiel 2007; Sandy & Butler 2009; Ahmed & Holmstrom 2014). Different vibrios and aliivibrios produce different siderophores where *V. anguillarum*

produces three siderophores piscibactin, vanchrobactin and anguibactin while *V. cholerae* produces vibriobactin and *A. salmonicida* produces bisucaberin (Butterton et al. 1992; Thode et al. 2015; Balado et al. 2018). Moreover, in addition to producing their own siderophores, some bacteria like *V. anguillarum* and *V. cholerae* utilize the siderophores ferrichrome and enterobactin produced by other organisms (Mey et al. 2002; Naka & Crosa 2012). Some bacteria do not produce siderophores at all but comprise receptors to bind the siderophores produced by other bacteria (Payne et al. 2016). As the secreted siderophores can potentially benefit other bacteria, its production is more often under QS control (Thode et al. 2015; McRose et al. 2018). QS represses siderophore production in *V. harveyi* and *V. vulnificus* whereas it stimulates the production in *Pseudomonas aeruginosa* (Stintzi et al. 1998; Lewenza & Sokol 2001; Griffin et al. 2004; Wen et al. 2012; Heilmann et al. 2015; McRose et al. 2018).

Vibrios produce numerous hydrolytic enzymes including chitinases that can be used as a sole source of carbon and nitrogen (Svitil et al. 1997; Nahar et al. 2011). Chitin is a linear polymer of β-1, 4-N-acetylglucosamine (GlcNAC) (Hamid et al. 2013). It is present in the cell wall of fungi, the exoskeleton of crabs, shrimps, lobsters, insects, and other crustaceans (Rathore & Gupta 2015). Crabs and shrimps make up 90% of the chitin waste (Shahidi & Abuzaytoun 2005). Several organisms, including bacteria, plants, fungi, and animals, produce chitinases to break down the chitin. Out of the three forms of chitin (α, β and ɣ), α-chitin is the most abundant one (Hamid et al. 2013). Chitinases hydrolyze chitin into disaccharides and oligosaccharides (Rathore & Gupta 2015). Furthermore, chitinases play a vital role in bacterial invasion and survival inside the host cells, indicating a role in host-pathogen interaction (Tran et al. 2011). For example, the food pathogen *Listeria monocytogenesis* produces chitinase to enhance the infection in human intestinal cells (Larsen et al. 2011). QS negatively regulates chitinases in *V. harveyi* and in some bacteria like *A. fischeri* it has no effect on chitinases (Defoirdt et al. 2010; Cao et al. 2012).

Commonly bacteria live in a dense biofilm, an aggregate of cells characterized by extracellular polymeric substances often composed of DNA, proteins and carbohydrates (Vu et al. 2009; Ray et al. 2012). Several vibrios produce biofilm during the environmental adaptation and pathogenesis processes (Yildiz & Visick 2009). Biofilm formation also develops resistance to several environmental stresses such as host immune responses, antibiotics, nutrient limitations, predation, and bacteriophages (Elias & Banin 2012; Rendueles & Ghigo 2012; Teschler et al. 2015). Moreover, in *A. fischeri* biofilm formation enhances its colonization within the squid host (Ray et al. 2012). Rugose colony formation is characterized by excess secretion of extracellular polysaccharides and serves as an indicator of biofilm formation (Teschler et al. 2015). QS

regulation of biofilm formation and rugose colony formation have been described in other studies (Sakuragi & Kolter 2007; Khider et al. 2019).

Competitive phenotypes regulated by QS

Nutrients and space are the two main resources for microbial survival and competition. A strain is considered as a competitor if it decreases the fitness of another strain (Ghoul & Mitri 2016). Microbes compete either indirectly through exploitation competition or by directly attacking the competing cells through interference competition. In many *Vibrio* species, QS regulates competitive phenotypes related to virulence functions and pathogenesis such as antibiotics, bacteriocin, secretion systems, hemolysin and proteases (Miller et al. 2002; Zhu et al. 2002; Federle & Bassler 2003; Duerkop et al. 2009; Elgaml & Miyoshi 2017; Shanker & Federle 2017; Pena et al. 2019). In a previous study, *A. wodanis* is cytotoxic to salmon cell lines and it impedes the growth of *M. viscosa* in a co-cultivation experiment (Karlsen et al. 2014b; Hjerde et al. 2015). Competitive phenotypes that directly target the competitors through attack mechanisms include proteases, hemolysins, cytolytic toxins, bacteriocins, antibiotics and secretion systems (Wright et al. 2013; Peng et al. 2016; Gao et al. 2018; Osei-Adjei et al. 2018; Jang et al. 2020). In *V. vulnificus* and *V. parahaemolyticus*, the VFs metalloprotease, serine protease and hemolysin play a leading role in infecting the hosts where they exhibit various toxic activities such as hemolytic, cytolytic and edema forming activities (Kim et al. 1993; Ishihara et al. 2002). Bacteriocins are strain specific toxins that mediate competition between same or closely related species (Cotter et al. 2005). Antimicrobial toxins such as antibiotics are broad-spectrum killers that mediate competition between distinct species (Chao & Levin 1981).

T6SS is a contractile protein nanomachine that secretes toxic effector molecules into a eukaryotic host or other bacteria in the environment **(Figure 3)** (Leung et al. 2011; Ho et al. 2014; Church et al. 2016). For example, virulent *V. cholerae* can kill both host and bacteria using T6SS (Pukatzki et al. 2006). T6SSs are common in bacterial symbionts, pathogens and commensals and can affect the diversity of the host-associated communities (Church et al. 2016; Marden et al. 2016; Speare et al. 2018; Kempnich & Sison-Mangus 2020). Some bacteria also escape the immune system of the host using T6SS (Yu & Lai 2017). The T6SS core apparatus consists of at least 13 subunits that resemble a bacteriophage-like structure along with other regulatory and accessory proteins (Pukatzki et al. 2007a; Wang et al. 2019a). The structural, regulatory and effectors components are present in the main cluster while additional effectors and structural components are distributed in the auxiliary cluster (Pukatzki et al. 2007a). In *V. cholerae*, the assemblage of the T6SS

apparatus comprises a membrane complex (VasDFK) which is made up of lipoprotein and gives structural support to the T6SS, a baseplate complex (HsiF and VasABE) formed by cytoplasmic proteins and a central spike valine-glycine repeat protein G (VgrG) tethered to the membrane complex (Joshi et al. 2017). After sheath contraction and translocation of effector molecules into the target cells, the ATPase (ClpV) disassembles and recycles the outer sheath components VipA/B (Leung et al. 2011; Silverman et al. 2012). To sharpen the spike and facilitate the puncturing of the target membrane, a proline-alanine-alanine-arginine repeats (PAAR) motif is located at the tip of VgrG (Shneider et al. 2013). This structure serves as a platform for the assembly of VipA/B with a rigid inner tube Hemolysin Coregulated protein (Hcp), allowing effectors to pass through the centers (Cascales & Cambillau 2012). VgrG is like the T4 phage, and it pierces the host cell and passes the proteins to the host cells (Spinola-Amilibia et al. 2016). The Hcp is an essential abundant protein of the T6SS apparatus, which forms a ring-shaped hexamer (Bartonickova et al. 2013). The number of T6SSs differs between different bacteria, where each T6SS may perform distinct functions. Additionally, T6SS also plays a role in stress responses against temperature, pH, and reactive oxygen species during environmental survival and host adaptation (Yu et al. 2021). The winter ulcer bacterium *M. viscosa* 06/09/139 with which *A. wodanis* co-exists encodes two putative secretion systems mts1 and mts2, however, the functions are not known yet (Bjornsdottir et al. 2012). Bacteria also possess multiple copies of *PAAR*, *hcp* and *vgrG* located outside of the main T6SS cluster (Barret et al. 2011). Besides the structural role of VgrG, PAAR and Hcp, they also act as an effector or chaperones of effectors towards the target cells (Pukatzki et al. 2007a; Zheng et al. 2011).

Genes encoding the effectors are located close to their cognate immunity proteins encoding genes in either the main or the auxiliary T6SS gene clusters (Dong et al. 2013). The self-protection mechanisms use the cognate immunity proteins to neutralize the effectors thus differentiating self from non-self and sibling intoxication (Dong et al. 2013). There are adaptor proteins with conserved domain (DUF4123) that bind to VgrG and effectors and are essential for loading and delivery of toxins (Liang et al. 2015). Genes of adaptor proteins are often encoded upstream of their cognate effector genes and downstream of *vgrG*s (Jana & Salomon 2019). Effectors that are involved in inter-bacterial competition include amidases, hydrolases, nucleases, pore forming toxins and phospholipases (Dong et al. 2013). The anti-eukaryotic effectors include EvpP in *Edwardsiella* sp., VgrG-1, and VasX in *V. cholerae* (Monjaras Feria & Valvano 2020). The VgrG effector contains a C-terminal extension of about 395 amino acids homologous to actin cross-linking domain of the RtxA toxin, a MARTX family member (Durand et al. 2012). VasX carries

a marker for type VI effector motif and a C-terminal colicin that guides T6SS assembly and a N-terminal Pleckstrin homology domain that binds to the membrane lipids and forms pores in the lipid bilayers of the host (Miyata et al. 2011; Salomon et al. 2014; Liang et al. 2019). QS regulation of T6SSs have been widely described in several pathogenic, non-pathogenic, and symbiotic bacteria (Jani & Cotter 2010; Schwarz et al. 2010). QS master regulators AphA and OpaR in *V. parahaemolyticus*, LasR and RhlR in *P. aerugniosa*, HapR in *V. cholerae*, LuxR in *V. alginolyticus*, VanT in *V. anguillarum* and HapR in *V. fluvialis* regulate T6SSs (Weber et al. 2009; Sana et al. 2012; Salomon et al. 2013; Shao & Bassler 2014; Majerczyk et al. 2016; Joshi et al. 2017; Zhang et al. 2020; Liu et al. 2021).

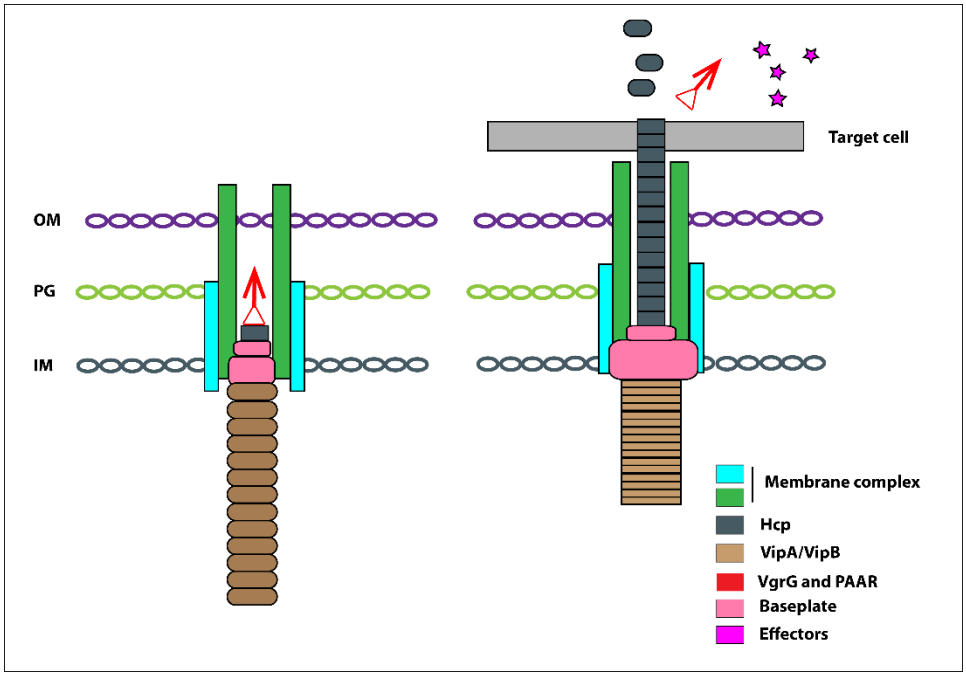

Figure 3: Schematic representation of structural components and functioning of T6SS. The figure has been adapted from (Zoued et al. 2014). T6SS consists of a membrane complex, baseplate, and tail tube sheath complex. After reaching the target cell, the T6SS sheath contracts to deliver the effector into the target cell.

QS regulation of Prokaryotic defense mechanisms

One of the important attributes of microbial evolution is the ability of bacteria to identify the difference between self-DNA and foreign DNA. Bacteriophages are DNA or RNA viruses that infect bacteria by injecting viral DNA or RNA into the bacterial host (Fuhrman & Noble 1995; Clokie et al. 2011). Studies on phage abundances revealed that about ten viruses exist for each bacterial or archaeal cell and they play a crucial role in ecology as a predator and mediator of

genetic exchange (Suttle 2005). The hosts have thus developed various immune systems to limit their exposure to foreign genetic elements and to fight virus infections. The immune systems have been grouped into innate and adaptive immune systems to protect against viruses and other foreign elements. Some of the defense mechanisms described are the Restriction modification (R-M) system, Toxin/antitoxin (TA) systems and Clustered regularly interspaced short palindromic repeats-CRISPR associated proteins (CRISPR-Cas) (Makarova et al. 2011; Loenen & Raleigh 2014; Kedzierska & Hayes 2016). R-M system is a defense mechanism against bacteriophage infection that targets the unmethylated DNA sequences (Vasu & Nagaraja 2013). The R-M system performs two enzymatic activities such as restriction endonuclease (REase) and modification methyltransferase (MTase). The REase degrades the non-self-unmethylated target sequence while the MTase methylates and protects from degradation (Tock & Dryden 2005). There are four types of R-M systems namely type I, II, III and IV (Bourniquel & Bickle 2002; Loenen & Raleigh 2014). Type I enzymes are hetero-oligomeric protein complex which performs both restriction and modification activities. Examples include: EcoKI and EcoKR124I. Type II enzymes are a well-studied system with separate REase and MTase enzymes and are utilized extensively in genetic engineering. Type III are the heterotrimers or heterotetramers encompassing restriction, methylation and DNA-dependent NTPase activities that compete within themselves for restriction and modification. For example, EcoP1I and EcoP5I are typical Type III enzymes. Type IV is the only R-M that lacks the MTase and it targets only the modified DNA with glycosylated bases or methylated at the adenine/cytosine residues (Loenen & Raleigh 2014). Several phages are also known to encode MTases and thus protect their own genome from REs (Tock & Dryden 2005). TA systems are widespread in bacteria and archaea (Yamaguchi et al. 2011). The first TA module was identified in the plasmid of *E. coli* (Ogura & Hiraga 1983). TA modules are associated with pathogenesis, phage inhibition, biofilm formation, growth arrest, gene regulation and survival (Jayaraman 2008; Kim et al. 2009). Today, eight different classes of TA modules have been described where the type I-VII toxins are proteins whereas the type VIII toxin is a sRNA (Song & Wood 2020; Singh et al. 2021). Pathogenic bacteria possess type II compared to non-pathogenic bacteria whereas type III is abundant in the intestinal microbiome (Kang et al. 2018).

CRISPR locus was first identified in *E. coli* (Ishino et al. 1987). It is an adaptive immune system widely distributed within bacteria and archaea that protects against phages and other foreign genetic elements (Barrangou et al. 2007). A previous study in vibrios has shown that the CRISPR-Cas systems are present on a mobile genetic element acquired through Horizontal gene transfer (HGT) (McDonald et al. 2019). The CRISPR is a short array of repeated sequences separated by

unique spacers that are derived from viruses and plasmids. The *cas* genes are usually present adjacent to the CRISPR array(s) (Barrangou et al. 2007). Another feature is the presence of a leader sequence upstream of the CRISPR array (Barrangou et al. 2007). CRISPR-Cas systems have been classified into two classes such as Class 1 and Class 2. Class 1 includes type I, III and IV systems and Class 2 includes II, V and VI **(Figure 4)**. Class 1 type I systems make up to 60% in bacteria and archaea whereas class 2 makes up to 10% (Hidalgo-Cantabrana et al. 2019). Cas1 protein is present in most of the CRISPR-Cas systems and is the most conserved protein compared to other Cas proteins (Takeuchi et al. 2012). Type IV systems are encoded by plasmids or prophage genomes, and they lack highly conserved adaptation modules and an effector nuclease (Makarova et al. 2011).

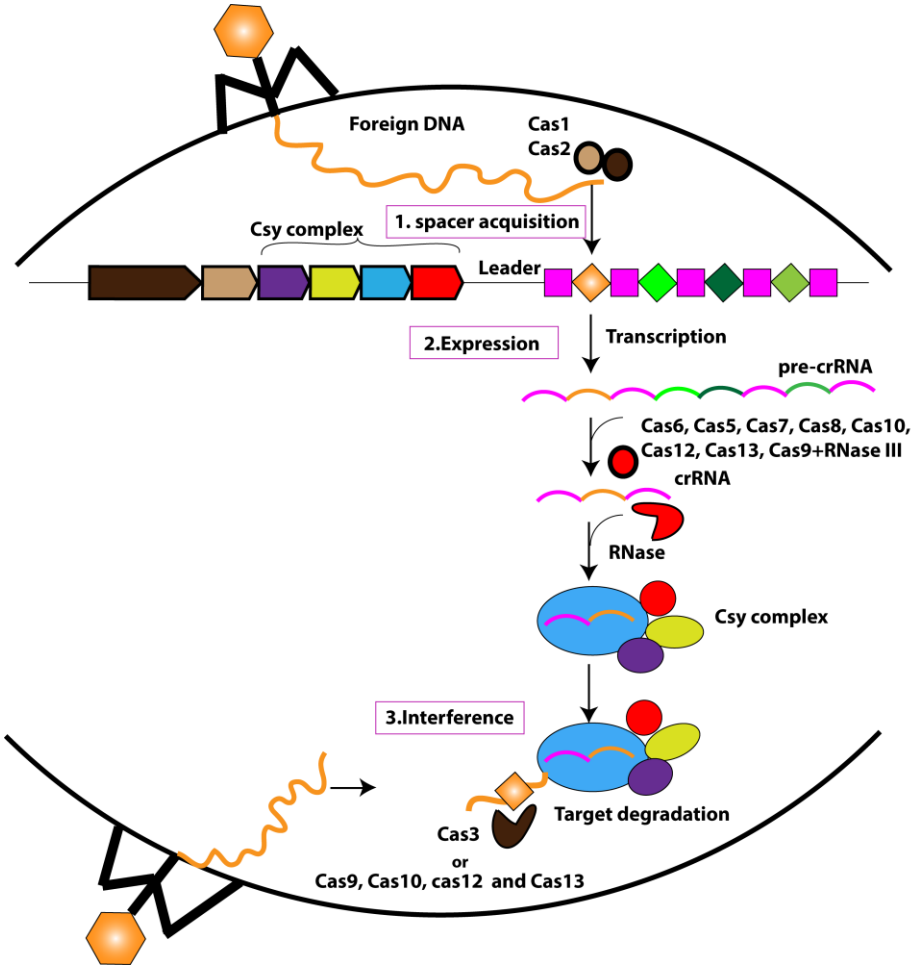

Figure 4: Schematic representation of CRISPR-Cas mechanism. The mechanism of the CRISPR-Cas system involves three stages: spacer acquisition, expression, and interference. The CRISPR-Cas system contains a *cas* operon, and a CRISPR array composed of identical repeats (pink squares) intervened by phage-derived spacers (colored diamonds). Upon phage infection, Cas1 and Cas2 complex (Cas2 is merged with Cas3 protein in type IF) incorporate the protospacers into the CRISPR array. Further, the CRISPR array is transcribed into pre-cRNA and then processed by CRISPR associated nucleases (Cas6 in Class 1 systems, Cas9 together with RNase

III in Class 2 systems and Cas5, Cas7, Cas8, Cas10, Cas12 and Cas13 for other systems) (Koo et al. 2013; Staals et al. 2013; Cass et al. 2015; Wang et al. 2019a). In the type I CRISPR-Cas system, Cas3, a signature protein of Type I degrades the foreign DNA with the guidance of cRNA. Cas9 is a signature protein of Type II for interference of target DNA. Cas10, 12, 13 are signature proteins for crRNA expression and interference of target DNA in type III, V and VI respectively (Yan et al. 2019).

CRISPR-Cas system functions in three steps as follows: (i) spacers acquisition, where the new spacer sequences are derived as protospacers from previous phage infection through a Cas1-2 complex and are integrated between the leader sequence and the first repeat of CRISPR array through a cleavage-ligation and a repeat duplication process **(Figure 4)** (McGinn & Marraffini 2019). In type IF systems, Cas3 is fused to Cas2, linking the adaptation and interference processes (Koonin et al. 2017; Hidalgo-Cantabrana et al. 2019). (ii) expression of CRISPR RNAs (crRNAs) and *cas* genes (Brouns et al. 2008; Koonin et al. 2017), and (iii) interference with the foreign genome, where the crRNAs together with Cas proteins recognize the protospacers adjacent motif (PAM), a short DNA sequence (2-6 base pairs) that immediately follows the target DNA and bind to the complementary foreign DNA or RNA to cleave the nucleic acid complex (Brouns et al. 2008; Makarova et al. 2011; Koonin et al. 2017). In addition to the immune function, the CRISPR-Cas systems are also known to be involved in other functions like virulence, DNA repair and evolution (Sampson et al. 2019). Moreover, CRISPR inhibits biofilm formation in *P. aeuroginosa* (Zegans et al. 2009).

QS regulation of CRISPR systems have been reported in several bacteria, such as *P. aeruginosa*, *Serratia sp.* and *Chromobacterium violaceum* (Patterson et al. 2016; Hoyland-Kroghsbo et al. 2018; Broniewski et al. 2021). In *P. aeruginosa,* the expression of CRISPR-Cas system is expensive, due to autoimmunity or deployment of nutrients that could be used for growth (Patterson et al. 2016). Therefore, to reduce the fitness cost, CRISPR-Cas systems are activated by QS only upon phage infection and with an increase in cell density (Patterson et al. 2016; Hoyland-Kroghsbo et al. 2018; Broniewski et al. 2021). Even though multiple CRISPR systems have been characterized in *Vibrionaceae* species, QS regulation of CRISPR systems has not been reported yet.

Transcriptomics

The central dogma of molecular biology is to pass the genome information from DNA to RNA and then to protein (Crick 1958; Gerstein et al. 2007). RNAs are important molecules produced during the process of transcription (Crick 1958). A genome contains both protein-coding and non-coding RNA genes. The protein-coding genes are transcribed to messenger RNAs (mRNAs) through transcription, which further becomes translated to protein through a process called translation. On the other hand, the non-coding RNA genes are only transcribed but not assembled into a protein. The non-coding RNAs include transfer RNAs, ribosomal RNA (rRNAs), micro-RNA, small RNAs (sRNAs), pseudogenes and long non-coding RNAs (Higgs 2000; Lee & Gutell 2004; Lee et al. 2004; Hung & Chang 2010; Hung & Stumph 2011; Chen et al. 2016). Transcriptomics is the study of a transcriptome that encompasses a complete set of RNAs expressed in cells, tissues, and organisms (Okazaki et al. 2002). High-throughput methods are used in modern transcriptomics to analyze the expression profiling in different physiological and pathological conditions or at different developmental stages (Wang et al. 2009). Genome-wide transcriptomics provides a better understanding of the relationships between the transcriptome and the phenotypic traits. Moreover, transcriptomics explores the fundamental mechanisms behind phenotypes. In human clinical studies, transcriptomics has been used as a guide to understand the disease mechanisms by comparing the differentially expressed genes between healthy controls and disease patients. Frequently used techniques for transcriptomics are Serial analysis of gene expression (SAGE), real-time PCR, Expressed sequence tag (EST), RNA sequencing (RNA-seq) and microarray analysis (Adams et al. 1991; Blackshaw et al. 2007; Slonim & Yanai 2009). Microarray technology is a chip-based method that elucidates the transcript abundances between a test sample and a normal sample at the same time point. In this method, the cDNA is transcribed from RNA and tagged with dyes to assess the transcripts. RNA-seq is a high-throughput system that utilizes next-generation sequencing (NGS) tools (Wang et al. 2009).

Comparative genomics

High throughput NGS technologies have paved the way for studying from single genome to multiple pan-genomes (Mardis 2008). The genome of *haemophilus influenza* was the first sequenced bacterial genome in 1995 (Fleischmann et al. 1995). With the increasing number of bacterial genomes available in public repositories, multiple genomes within the same species can be compared. To develop complex lifestyles, the genomes of *Vibrionaceae* species have evolved

remarkably from HGT events such as transformation, conjugation, and transduction (Reen et al. 2006). HGT is a major driving force in the evolution of bacterial genomes (Vogan & Higgs 2011). The association between genetic features and adaptation strategies are important to understand the ecological functions of *Vibrionaceae* in the marine environment (Thompson et al. 2004; Paparini et al. 2006; Reen et al. 2006). The plasticity in bacteria has been maintained by bacteriophages, transposons, plasmids, and other mobile genetic elements (Medini et al. 2005). However, some bacterial species like *Bacillus anthracis* have been found to have closed genomes (Medini et al. 2005). Multiple approaches have been developed to compare the genomes of same or different bacterial species to explore microbial diversity, pathogenicity, and environmental adaptation strategies. The concept "pan-genome" was first introduced by Tettelin *et al*., 2005 as a collection of genetic sequences found in a defined bacterial species (Medini et al. 2005; Tettelin et al. 2005). The pan-genome analysis provides an insight into the ability of a strain to lose or acquire genes (Medini et al. 2005). The pan-genome consists of core genes that are shared among all the genomes; and accessory genes, which can be present in several strains or specific to a single organism **(Figure 5)** (Medini et al. 2005). The main role of core genes is to maintain the housekeeping functions such as DNA replication, transcription, and translation (Tettelin et al. 2005). The accessory genes which include the shell, cloud and unique genes are believed to be acquired through HGT, which provides new traits that confer selective advantages in species (Segerman 2012). Identification of homologous sequences between species is essential in understanding evolutionary processes. The homologous sequences are categorized into orthologs and paralogs. Orthologs are the genes diverged through speciation events from a common ancestor whereas paralogs are the genes diverged through duplication events (Fouts et al. 2012). Various methods are used to identify the homology between sequences such as tree-based, pairwise alignment and structural prediction of conserved domains (Terrapon et al. 2014). A pairwise global alignment was started by aligning the entire sequences whereas the pairwise local alignment performs alignment of the sub-sequences (Needleman & Wunsch 1970; Smith et al. 1981). The best example of a pairwise local alignment tool is the basic local alignment search tool (BLAST). Some of the pan genome tools available are BGDMdocker, ClustAGE, GET_HOMOLOGUES, PanGeT, panX and Roary (Contreras-Moreira & Vinuesa 2013; Page et al. 2015; Vinuesa & Contreras-Moreira 2015; Cheng et al. 2017; Contreras-Moreira et al. 2017; Yuvaraj et al. 2017; Ding et al. 2018; Ozer 2018).

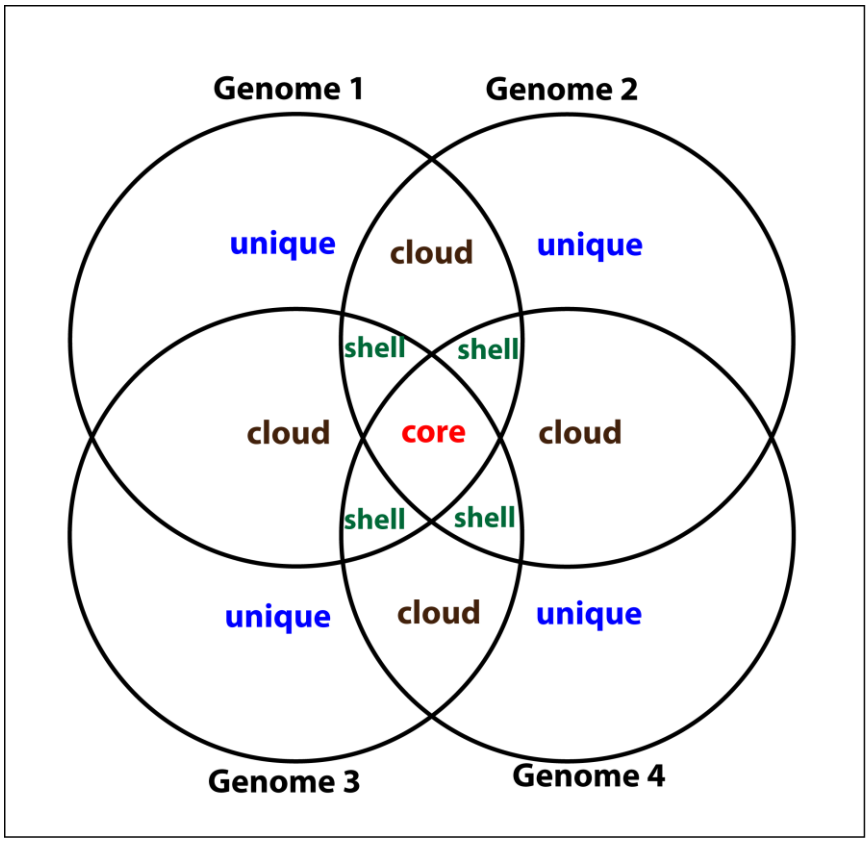

Figure 5. Venn diagram representation of the pan genome. Core genes represent genes present in all genomes whereas unique genes are specific to each genome. Shell genes represent genes present in two or more genomes and cloud genes are shared in one or two genomes.

Phylogenetic analysis

Phylogenetic tree-based methods rely on evolutionary relationships between multiple organisms. Various approaches have been invented to construct a phylogenetic tree. Traditionally, 16S rRNA sequences have been used to classify phylogeny, however there are growing concerns in the accuracy of the method (Fox et al. 1992; Baker et al. 2003). Next, multi-locus sequence analysis approach using housekeeping genes have been suggested (Eisen 1995). Some studies have also used core and pan-genome to construct a phylogenetic tree (Medini et al. 2005; Lugli et al. 2014). A growing number of single nucleotide variants (SNVs) or single nucleotide polymorphism (SNPs) based methods have been developed to identify the phylogenetic relatedness between target genomes and a reference (Petkau et al. 2017). The pipeline SNVPhyl maps the genomes to the reference, identifies variants and generates maximum likelihood phylogeny (Medini et al. 2005; Lugli et al. 2014; Petkau et al. 2017).

Functional annotation

Functional annotation is the process of finding the functional elements of the genomes. The convenient and faster way of assigning functions to the novel genes and proteins sequences is to predict them computationally. Comparing proteins from diverse species groups can improve the functional annotation of newly sequenced genomes (Delsuc et al. 2005). Some of the functional annotation databases include Gene Ontology (GO), Clusters of Orthologous Groups of proteins (COGs), evolutionary genealogy of genes: Non-supervised Orthologous groups (eggNOG) and Kyoto Encyclopedia for Genes and Genomes (KEGG). The GO resource is a widely used database to predict three aspects of gene function such as biological process, cellular component and molecular function (Ashburner et al. 2000). COG database contains the orthologous protein coding genes with 26 functional categories (Galperin et al. 2019). The eggNOG database is a database of orthologs groups, functional annotations, and gene evolutionary histories (Huerta-Cepas et al. 2016). KEGG comprises GENES, PATHWAY and KEGG Orthology (KO) databases to understand the high-level functions of biological systems from cellular-, genome- and molecular-level information (Kanehisa & Sato 2020). The KO database consists of functional orthologs, which are identified by KO identifiers (K numbers) elucidated from experimentally characterized genes and proteins (Kanehisa & Sato 2020). Blast KOALA and Ghost KOALA perform BLAST and GHOSTX respectively and assigns K numbers to the query data set (Kanehisa et al. 2016).

AIMS OF STUDY

Main aim:

The main aim of this thesis was to study the temperature-dependent QS and their role in regulating various phenotypic traits, and to further analyze the pan-genome of *A. wodanis.*

Sub aim:

1. To study the roles of LitR and AinS; and the effect of cell density and temperature difference in regulating various phenotypic traits (growth, motility, protease, hemolysin and siderophores) potentially linked to virulence and environmental adaptation.

2. To investigate the genome-wide expression profiling of QS mutants *ΔainS* and *ΔlitR* at temperatures 6°C and 12°C and cell densities (OD_{600} 2.0 and OD_{600} 6.0) and their role in regulating genes related to QS, T6SSs and CRISPR-Cas systems.

3. To explore the pan-genome of *A. wodanis* to better understand the phylogeny and diversity in virulence potential, survival and adaptation mechanisms.

SUMMARY OF PAPERS

Paper 1

Quorum sensing in *Aliivibrio wodanis* 06/09/139 and its role in controlling various phenotypic traits

Amudha Deepalakshmi Maharajan, Hilde Hansen, Miriam Khider, Nils Peder Willassen // PeerJ., August 24, 2021., 10.7717/peerj.11980

Quorum Sensing (QS) is a cell-to-cell communication system that bacteria utilize to adapt to the external environment by synthesizing and responding to signalling molecules called autoinducers. The psychrotrophic bacterium *Aliivibrio wodanis* 06/09/139, originally isolated from a winter ulcer of a reared Atlantic salmon, produces the autoinducer N-3-hydroxy-decanoyl-homoserine-lactone (3OHC10-HSL) and encodes the QS systems AinS/R and LuxS/PQ, and the master regulator LitR. However, the role of QS in this bacterium has not been investigated yet. In the present work we show that 3OHC10-HSL production is cell density and temperature-dependent in *A. wodanis* 06/09/139 with the highest production occurring at a low temperature (6°C). Gene inactivation demonstrates that AinS is responsible for 3OHC10-HSL production and positively regulated by LitR. Inactivation of *ainS* and *litR* further show that QS is involved in the regulation of growth, motility, hemolysis, protease activity and siderophore production. Of these QS regulated activities, only the protease activity was found to be independent of LitR. Lastly, supernatants harvested from the wild type and the *ΔainS* and *ΔlitR* mutants at high cell densities show that inactivation of QS leads to a decreased CPE in a cell culture assay, and strongest attenuation of the CPE was observed with supernatants harvested from the *ΔlitR* mutant. *A. wodanis* 06/09/139 use QS to regulate several activities that may prove important for host colonization or interactions. The temperature of 6°C that is in the temperature range at which winter ulcer occurs plays a role in AHL production and development of CPE on a Chinook Salmon Embryo (CHSE) cell line.

Paper 2

Quorum Sensing Controls the CRISPR and Type VI Secretion Systems in *Aliivibrio wodanis* 06/09/139

Amudha Deepalakshmi Maharajan, Erik Hjerde, Hilde Hansen, and Nils Peder Willassen // PeerJ., February 08, 2022., https://doi.org/10.3389/fvets.2022.799414

For bacteria to thrive in an environment with competitors, phages, and environmental cues, they use different strategies, including Type VI Secretion Systems (T6SSs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) to compete for space. Bacteria often use quorum sensing (QS), to coordinate their behavior as the cell density increases. Like other aliivibrios, *Aliivibrio wodanis* 06/09/139 harbors two QS systems, the main LuxS/LuxPQ system and an N-acyl homoserine lactone (AHL)-mediated AinS/AinR system and a master QS regulator, LitR. To explore the QS and survival strategies, we performed genome analysis and gene expression profiling on *A. wodanis* and two QS mutants (*ΔainS* and *ΔlitR*) at two cell densities (OD_{600} 2.0 and 6.0) and temperatures (6°C and 12°C). Genome analysis of *A. wodanis* revealed two CRISPR systems, one without a *cas* loci (CRISPR system 1) and a type I-F CRISPR system (CRISPR system 2). Our analysis also identified three main T6SS clusters (T6SS1, T6SS2, and T6SS3) and four auxiliary clusters, as well as about 80 potential Type VI secretion effectors (T6SEs). When comparing the wildtype transcriptome data at different cell densities and temperatures, 13–18% of the genes were differentially expressed. The CRISPR system 2 was cell density and temperature-independent, whereas the CRISPR system 1 was temperature-dependent and cell density-independent. The primary and auxiliary clusters of T6SSs were both cell density and temperature-dependent. In the *ΔainS* and *ΔlitR* mutants, several CRISPR and T6SS related genes were differentially expressed. Deletion of *litR* resulted in decreased expression of CRISPR system 1 and increased expression of CRISPR system 2. The T6SS1 and T6SS2 gene clusters were less expressed while the T6SS3 cluster was highly expressed in *ΔlitR.* Moreover, in *ΔlitR,* the *hcp1* gene was strongly activated at 6°C compared to 12°C. AinS positively affected the *csy* genes in the CRISPR system 2 but did not affect the CRISPR arrays. Although AinS did not significantly affect the expression of T6SSs, the hallmark genes of T6SS (*hcp* and *vgrG*) were AinS-dependent. The work demonstrates that T6SSs and CRISPR systems in *A. wodanis* are QS dependent and may play an essential role in survival in its natural environment.

Paper 3

Pan-genome analysis of *Aliivibrio wodanis* provides insight into the genetic diversity of the CRISPR-Cas system, T6SS2 and phages present.

Aliivibrio wodanis is repeatedly isolated from the infected fish during winter-ulcer outbreaks. Even though *A. wodanis* is not the causative agent of winter-ulcer; the role of this bacterium in the course of the disease is unclear. Information about the genetic diversity of *A. wodanis* is essential to understand its virulence, environmental adaptation, and survival strategies. In this study, 22 *A. wodanis* isolates were analyzed from the perspective of their pan, core, and cloud genomes. The pan-genome analysis identified 3149 core, 2583 shell, and 6271 cloud genes revealed an open *A. wodanis* pan-genome.

Furthermore, functional annotation showed that the most enriched protein families in the pan-genome were *metabolism*, *signaling and cellular processes*, and *genetic information processing.* The presence of type VI secretion systems (T6SS), Clustered Regularly Interspaced Palindromic Repeats-CRISPR associated protein (CRISPR-Cas) systems, phages, biosynthetic gene clusters (BGCs) and several VFs may enable *A. wodanis* during environmental adaptation and survival. All strains had CRISPR arrays, although only half of the strains encoded a complete *cas* operon of type IF and two isolates do not encode any *cas* genes suggesting a diverse CRISPR system. The T6SS1 and T6SS3 were conserved in all the isolates, whereas only fifteen out of twenty-two encoded T6SS2. Eleven of these fifteen isolates showed similarity to the T6SS of environmental *Aliivibrio* strains, while the other four showed similarity to pathogenic vibrios. Furthermore, the spacers and phages revealed great diversity suggesting acquisition at different time points. This study may provide better knowledge about the role of *A. wodanis* in developing the winter-ulcer disease.

RESULTS AND DISCUSSION

Bacteria exist as a community in nature and each bacterium performs some functions such as luminescence, motility, protease expression, antibiotic production, iron acquisition, biofilm formation, virulence factor expression, sporulation, and mating to thrive as a group through a mechanism called QS (Sheng et al. 2012; Jemielita et al. 2018; McRose et al. 2018; Gorelik et al. 2019). *A. wodanis* has been present during the winter ulcer outbreaks either alone or together with another bacterium *M. viscosa* (Lunder et al. 1995a; Benediktsdottir et al. 1998; Lunder et al. 2000). Though both *A. wodanis* and *M. viscosa* are known to be isolated together from the infected fish, the function of *A. wodanis* and the regulatory mechanisms behind its coexistence during winter ulcer outbreak remains unclear. In other aliivibrios, LitR the transcription regulator (LuxR homologs) and AinS the autoinducer synthase control various activities such as luminescence, motility, biofilm formation and colony morphology that may be important for virulence, survival, and host interaction (Higgins et al. 2007; Bjelland et al. 2012b; Chavez-Dozal et al. 2012; Khider et al. 2019; Bazhenov et al. 2021). Moreover, winter ulcer is a temperature-dependent disease where the fish recovers when the temperature rises above 8°C (Salte et al. 1994; Lunder et al. 1995a; Lunder et al. 2000). Hence, to investigate the roles of QS and temperature, in this thesis, QS genes *ainS* and *litR* were characterized with regards to the temperature in regulating various phenotypic traits (Paper I and II). Furthermore, in Paper III, the pan-genome of *A. wodanis* was explored by studying the phylogeny, strain-specific genes and genes related to CRISPR-Cas, phages, T6SSs, Biosynthetic gene clusters (BGCs) and VFs.

In this discussion, I will emphasize the following topics: (i) QS and temperature-dependent regulation of phenotypic traits connected to adaptation, survival mechanisms, virulence, and host-pathogen interaction in *A. wodanis* 06/09/139. (ii) Identification of T6SSs and CRISPR-Cas systems in *A. wodanis* 06/09/139. (iii) Expression profiling of *A. wodanis* and its QS mutants *ΔlitR* and *ΔainS* at two different temperatures (6°C and 12°C) and two different cell densities (OD_{600} 2.0 and OD_{600} 6.0). (iv) Pan-genome analysis and investigation of CRISPR-Cas, phages, T6SSs, VFs and BGCs in 22 *A. wodanis* isolates.

QS and temperature regulate various phenotypic traits in *A. wodanis*

QS regulates various phenotypic traits such as siderophores, motility, bioluminescence, biofilm formation and colony rugosity, T6SS, chitinases and virulence (Miller et al. 2002; Zhu et al. 2002; Defoirdt et al. 2010; Sheng et al. 2012; Thode et al. 2015; Elgaml & Miyoshi 2017; Khider et al. 2018; McRose et al. 2018). In previous studies, the reference *A. wodanis* 06/09/139 strain was shown to encode two QS systems AinS/AinR and LuxS/PQ and a QS regulator LitR and, most of the *A. wodanis* isolates tested produce only one AHL 3OHC10-HSL (Purohit et al. 2013; Hjerde et al. 2015). Temperature fluctuation is a major determinant of microbial diversity, and it structures the composition and function of the bacteria in different adaptive environments and hosts (Kokou et al. 2018). Moreover, in marine environments, water temperature below or above physiological optimum may lead to stress, immune suppression, infection, and mortality in the fish (Ermolenko et al. 1997; Warr 1997). Some of the other temperature-dependent fish diseases include Furunculosis, Ichthyophthiriasis, Lernaeosis and Columnaris (Ferguson 1977; Leibovitz 1980; Ermolenko et al. 1997). Several studies have also observed the association between temperature induced differences in various phenotypes and host responses (Bowden 2008; Hansen et al. 2015; Khider et al. 2019; Sepulveda & Moeller 2020). Therefore, to determine the roles of QS and temperature in *A. wodanis* 06/09/139, two mutant strains *ΔlitR* and *ΔainS* strains were constructed and examined for the regulation of various phenotypic traits at different cell densities OD_{600} 2.0 (LCD) and OD_{600} 6.0 (HCD) and temperatures at 6°C (low) and 12°C (high).

Our HPLC/MS-MS assay results showed that deletion of *ainS* resulted in loss of 3OHC10-HSL production suggesting AinS is the autoinducer synthase responsible for AHL production in *A. wodanis* (Paper I). Since QS is a cell density-dependent mechanism (Bassler 2002), the 3OHC10-HSL production in *A. wodanis* 06/09/139 was tested at different cell densities along the growth curve and the results showed that the AHL production increased with increase in cell density. This suggests that the QS dependent gene targets might be expressed at HCD when the AHL production is high. The AHL production was also found to be higher at 6°C than at 12°C, suggesting the low temperature at which winter ulcer occurs plays a significant role in its production and the expression of QS dependent target genes. These findings were further investigated through RNA-seq using transcriptomes profile (tp), high cell density against low cell density (tpHCD/LCD), elevated temperature against low temperature (tp12°C/6°C), mutants against wild type (tp*ΔlitR*/WT and tp*ΔlitR*/WT). The RNA-seq results showed that when wild type was compared against cell densities (tpHCD/LCD), the expression values of *ainS* and *litR* were higher at HCD than LCD at 6°C. This further supports that the *ainS* and *litR* expressions increase with increase in cell density. Likewise, increased expression of *ainS* was also observed in the mutant strain

ΔlitR(tpHCD/LCD), suggesting absence of LitR has no effect on the expression of *ainS*. Indeed, the expression value of *ainS* in *ΔlitR*(tpHCD/LCD) was slightly higher than that observed for *ainS* expression in WT(tpHCD/LCD). This together with the experimental data indicate that the *ainS* expression is not entirely dependent on LitR.

Temperature difference (tp12°C/6°C) revealed increased expression of only one QS related gene *qrr* sRNA at 12°C, LCD. This suggests that temperature alone does not have a big effect on genes belonging to the QS cascade in *A. wodanis*. The Qrr sRNAs in vibrios have been shown to regulate the LuxR homologs (Feng et al. 2015). For example, in *A. fischeri*, Qrr sRNAs negatively regulate the production of LitR (Miyashiro et al. 2010). Similarly, in this study, the increased expression of the *qrr* sRNA at 12°C may inhibit the translation of LitR.

We also identify here that the deletion of *litR* reduced 24% and 22% of AHL production compared to WT at 6°C and 12°C, respectively (Paper I). However, the impact was not as high as *A. salmonicida*, where the *litR* deletion led to 85-90% reduction in WT AHL production (Hansen et al. 2015). This indicates that LitR in *A. wodanis* weakly regulates the AHL production. Moreover, in the comparison of *ΔlitR/*WT at LCD and 12°C, there was a decreased expression of *ainS* (FC value = -1.93) in *ΔlitR* than in WT. This suggests that the *ainS* is still expressed in the *litR* mutant and therefore the *ainS* produced AHL production is not completely LitR-dependent. Several LitR-independent regulatory mechanisms such as *ainS*-autoregulation, cyclic AMP regulator protein or glucose-mediated mechanisms have been reported (Lupp & Ruby 2004; Lyell et al. 2013). Similarly, in *A. wodanis*, various other mechanisms together with LitR may regulate the *ainS* expression and the AHL production. In *ΔainS* mutant, *litR* expression was lower at all tested conditions which is like *A. fischeri* where AinS upregulates *litR* in a cell density dependent manner (Lupp et al. 2003). This indicates that AinS triggers the *litR* expression and thus the expression of target genes. Furthermore, in the tp*ΔainS*/WT, the expression of phosphorelay protein encoding gene *luxU* was significantly higher in the *ΔainS* at 6°C. LuxU is the essential point in the cascade where all the environmental signal converges, and it is responsible for integrating the signaling events from sensor kinases to the response regulator protein LuxO (Freeman & Bassler 1999). This suggests that the lower expression of *luxU* in *A. wodanis* could repress *qrr* sRNA and activate the *litR* expression at 6°C.

VFs are factors produced by bacteria and may cause pathogenesis because of interaction between the VFs and the response of the host (Diard & Hardt 2017). Synergistic effect between QS and VFs expression have been extensively studied in several human and fish pathogens (Fuqua et al. 2001; Kong et al. 2005; Rambow-Larsen et al. 2008; Natrah et al. 2011; Cai et al. 2015; Wen et

al. 2016; Vieira et al. 2020; Inat et al. 2021). In addition to pathogenesis, VFs are also important to combat stressful conditions and suppress host responses (Denning et al. 1998; Wang et al. 2018). Among the phenotypic traits that were tested in the two mutants in our first paper, the motility assay showed that the *ΔlitR* and *ΔainS* mutants were hypermotile when compared to the wild type. This is like *A. salmonicida* where the LitR and AinS mutants showed higher motility than the wild type (Bjelland et al. 2012b). Studies have demonstrated that hypermotile strains poorly colonize the host (Brennan et al. 2013), similarly in the hypermotile *ΔlitR* and *ΔainS* mutant strains, colonising the host may be defective. Moreover, motility is an important virulence factor in many bacteria, and it has been linked to other functions like virulence and biofilm formation in several vibrios and aliivibrios (Gardel & Mekalanos 1996; Lee et al. 2004; Khider et al. 2019) (Wolfe et al. 2004; Silva & Benitez 2016). Similarly, motility in *A. wodanis* may play a role in the virulence mechanism of winter ulcer pathogenesis. Furthermore, the RNA-seq results showed that the hypermotile *litR* and *ainS* mutant strains when compared to WT showed only a few differentially expressed flagellar genes and chemotaxis genes related to motility **(Figure 6)**.

QS and temperature regulate growth in some bacteria (Fidopiastis et al. 2002; Hansen et al. 2015). In this study (Paper I), the wild type and mutant strains grew normally at 6°C and 12°C. However, at 20°C, the WT and *ΔainS* strains stopped growing during the early log phase, then later grew back, although *ΔlitR* strain grew without stopping, and reached a better cell density at 20°C than the wild type and *ΔainS*. This indicates that LitR reduces growth at 20°C. Studies have shown that bacteria stop growing to have a fitness advantage during lack of nutrient source or stress (Heurlier et al. 2005; Yan et al. 2007). Heat shock proteins like RpoS stop the growth and later after fixing the heat damage done to the proteins they grow again (Bukau 1993; Guisbert et al. 2004). *A. wodanis* encodes RpoS, and it may have stopped the growth and let the bacteria grow again after adaptation. Similarly, the reduction in growth and low growth yield in wild type and *ΔainS* may provide a fitness advantage for *A. wodanis* during nutrient limitation or stress.

Many *Vibrio* and *Aliivibrio* species produce siderophores for iron acquisition (Miethke & Marahiel 2007; Naka & Crosa 2012; Ahmed & Holmstrom 2014; Balado et al. 2018) and chitinases as a source of nutrients (Tran et al. 2011). The results of this study (Paper I) showed that *A. wodanis* produces siderophores and chitinases where LitR positively affects the siderophore production. QS had no effect on the chitinase production. In some bacteria, siderophores and chitinases may also function as VFs and are involved in suppression of host innate immunity (Naka & Crosa 2011; Naka & Crosa 2012). Similarly, chitinases and siderophores in *A. wodanis* may act as a virulence factor. Moreover, like what has been observed experimentally, the RNA-seq results in

Paper II have found a significant difference in the expression of a few genes of the siderophores clusters in tp*ΔlitR*/WT at 6°C.

In an Atlantic salmon bath challenge experiment, *A. wodanis* alone can cause clinical symptoms such as scale loss, fin rot and other internal symptoms such as swollen spleen, petecchia in liver and peritoneal fat tissues (Karlsen et al. 2014b). Moreover, it was reported that *A. wodanis* supernatants cause cytotoxicity in various salmon cell lines (Karlsen et al. 2014b). Furthermore, in a co-cultivation experiment, *A. wodanis* impedes the growth and virulence of *M. viscosa* (Hjerde et al. 2015). Previous study has shown that *A. wodanis* produces bacteriocin and it is hypothesized to be involved in the activity of impeding the growth and virulence of *M. viscosa* (Hjerde et al. 2015). Other than bacteriocin, the virulence and survival factors in *A. wodanis* have not been identified yet. These studies led us to further study the importance of the virulence potential in *A. wodanis*. In fish pathogens *V. anguillarum* and *A. salmonicida* numerous VFs connected to cytotoxicity have been described (Kashulin et al. 2017; Balado et al. 2018; Khider et al. 2019). For example, hemolysin and proteases are responsible for causing hemolysis and cytotoxicity of *V. anguillarum* in fish (Crisafi et al. 2014). Our results showed that *A. wodanis* 06/09/139 was hemolytic on blood agar plates and proteolytic on skim milk agar plates (Paper I). Our results also showed that LitR positively regulated hemolysin and AinS positively influenced the protease production. This suggests that the hemolysin and proteases produced in *A. wodanis* may have caused the cytotoxicity in salmon cell lines in Paper I and the previous study (Karlsen et al. 2014b). To know the role of QS and temperature in cytotoxicity of *A. wodanis* in salmon cell lines, supernatants from WT, *ΔlitR* and *ΔainS*, grown at 6°C and 12°C were harvested and treated on the CHSE cell lines. The results showed that the supernatants from *ΔlitR* and *ΔainS* mutant showed less CPE than the supernatants harvested from wild type at 12°C. This indicates that LitR and AinS positively influenced the CPE at 12°C. On the other hand, at 6°C, CPE was affected only by LitR and not by AinS, implying that at 6°C, the CPE is not dependent on the AHL-based QS system. Moreover, the CPE was cell density dependent at 12°C whereas at 6°C, a higher CPE was observed at OD_{600} 6.0. Either the LuxS/PQ system or some unknown mechanisms are involved in the CPE caused at 6°C. This shows that combination of cell density, temperature and QS play a role in regulating the CPE.

In summary *A. wodanis* 06/09/139 encodes one AHL-mediated QS system by producing only one AHL when compared to other aliivibrios that produce more than a single AHL. This could suggest that this AHL-based QS system is important in establishing various functions in *A. wodanis* 06/09/139. However, the results of this study and RNA-seq showed that the AHL-based QS system

has significant impact only on AHL production and CPE in CHSE cell line. From our observation, we suggest that these phenotypic traits are not solely dependent on AHL-mediated QS. Further research is required to understand the roles of AHL-based QS system under different environmental conditions or along with *M. viscosa* and the host.

The expression profiling revealed DEGs related to T6SSs and CRISPR systems

Since cell density, temperature, LitR and AinS influenced the AHL production and CPE in the CHSE cell line in the Paper I, we wished to know the main role of these factors in the expression profiling. Current transcription profiling has enhanced our ability to understand cellular processes and discover the novel genes involved in various functions. In Paper II, we analyzed the genome-wide transcriptomics of *A. wodanis* at two different cell densities OD_{600} 2.0 and OD_{600} 6.0 and two different temperatures 6°C and 12°C. Furthermore, using the same conditions (cell densities OD_{600} 2.0 and OD_{600} 6.0 and temperatures 6°C and 12°C), we grew *ΔlitR* and *ΔainS* mutants and performed RNA-seq. We identified that cell density, temperature and QS regulate genes related to T6SSs, CRISPR-Cas systems and several other functions. However, T6SSs and CRISPR-Cas systems were the ones found in the DEGs of *ΔlitR* compared against the wild type (*ΔlitR*/WT) at all tested conditions. Hence, we proceeded to focus on T6SSs and CRISPR-Cas systems in Paper II.

Interestingly, the highest expression value in *ΔlitR*/WT is found in genes related to T6SS, indicating LitR regulates T6SSs. T6SS is present in about 25% of Gram-negative bacteria including pathogens *V. cholerae*, *Salmonella enterica*, *V. anguillarum* and *V. parahaemolyticus* (Weber et al. 2009; Blondel et al. 2013; Salomon et al. 2013; Joshi et al. 2017). In many bacteria, one to several T6SSs are present, often each T6SS performs different functions and regulations (Bingle et al. 2008; Schwarz et al. 2010). In this study, three T6SSs (T6SS1-T6SS3) and four auxiliary clusters (Aux-1 to Aux-4) (see Figure 3 in paper 2) and about 80 potential Type VI secretion system effectors (T6SEs) were identified in the genome of *A. wodanis* 06/09/139. The multiple T6SSs in *A. wodanis* may indicate that each T6SS possesses divergent functions. The T6SS1 in *A. wodanis* showed high AA similarity to the T6SS of *A. fischeri* MJ11 (Speare et al. 2018), and the Aux-1 located close to the T6SS1 encoded a heme utilization gene cluster nearby. In addition to its pathogenic role, T6SSs have also been reported to be involved in the acquisition

of iron, zinc and manganese and these acquisitions provide fitness to bacteria through exploitation competition for micronutrients (Lin et al. 2017). Hence, the T6SS1 together with Aux-1 in *A. wodanis* may be involved in ions uptake. On the other hand, T6SS2 in *A. wodanis* is like the T6SS of *V. cholerae* O1E1 and *V. fluvialis* (Huang et al. 2017; Joshi et al. 2017). In *V. fluvialis*, T6SS is associated with survival in highly competitive environments via antibacterial activity (Pan et al. 2018). Similarly, in *A. wodanis*, T6SS2 may play a role in survival in a highly competitive marine environment. The T6SS3 in *A. wodanis* was like *M. viscosa*, *V. anguillarum*, *A. salmonicida* and *Vibrio tapetis* (Weber et al. 2009; Bjornsdottir et al. 2012; Rodrigues et al. 2018). In addition to the structural T6SS genes, the T6SS3 in *A. wodanis* contains genes encoding VtsA-D. Like the VtsA-D proteins in *V. anguillarum* that are involved in stress response (Weber et al. 2009), the T6SS3 in *A. wodanis* may be involved in stress response and not in virulence. Several studies have focused on various host related functions of T6SS where the T6SS mediates colonization, host cell invasion and adhesion (Lertpiriyapong et al. 2012). Similarly, the T6SSs in *A. wodanis* may participate in interaction with the hosts.

The comparisons tpHCD/LCD revealed that cell density has a significant effect on the expressions of T6SS1 and T6SS2 with higher expression values found at HCD. In contrast, temperature changes (tp12°C/6°C) alone decreased the expression of few genes of T6SS2, Aux-2 and Aux-3 at 12°C compared to 6°C, where significant differential expression was observed at HCD. A few studies have described the effect of cell density and temperature in regulating T6SSs (Wang et al. 2013; Huang et al. 2017). Similarly, the T6SS1 and T6SS2 may play a key role during the mid or late stages of growth or infection and at low temperature (6°C).

In contrast to the negative effect that LitR has on T6SS1 and T6SS2 gene clusters, it increased the expression of T6SS3 genes. Reciprocal regulation of T6SSs by OpaR in *V. parahaemolyticus* has been reported where the T6SS1 and T6SS2 act as an anti-bacterial and anti-eukaryotic weapon respectively (Sana et al. 2012; Salomon et al. 2013). Similarly, LitR regulation of T6SSs in *A. wodanis* appears to be complex and may play a role in both anti-bacterial and anti-eukaryotic activity. Compared to the T6SS1 and T6SS3, the whole T6SS2 apparatus showed differential expression in the *ΔlitR*/WT. Therefore, it is important to note that LitR in *A. wodanis* could be essential for the complete functioning of T6SS2. Additionally, in Paper I, the temperature 6°C played a significant role in AHL production and in the development of CPE than 12°C. Hence, it is possible that the T6SS2 is more active at low temperatures and thus influenced the high CPE at 6°C (Paper I). In contrast, AinS do not have a significant impact on the expression of T6SSs except

positively influencing the *hcp1* and *vgrG1* expressions, which suggests that only these genes of T6SS are AinS-dependent.

Besides the structural function of VgrGs and Hcps in T6SSs, they can also function as effectors or a chaperone of effector (Brooks et al. 2013; Sha et al. 2013). In *V. cholerae*, *Aeromonas hydrophila* and *Helicobacter hepaticus*, the T6SS effectors Hcps and VgrGs are responsible for cytotoxicity and diseases in eukaryotic hosts (Suarez et al. 2010; Bartonickova et al. 2013). *A. wodanis* encoded four *vgrGs* in the Aux clusters (1-4) and four *hcps* in both the Aux (2-4) and main cluster (T6SS3). Hcp family proteins are known to be involved in bacterial competition and pathogenesis (Peng et al. 2016; Wang et al. 2018; Wang et al. 2020b). VgrGs and Hcps are also secreted into the extracellular environment and can be detected in the supernatants of a functional T6SSs (Bartonickova et al. 2013). Multiple VgrGs are used for interbacterial competition and are functionally redundant in mediating Hcp secretion (Santos et al. 2019). This may suggest that the multiple VgrGs and Hcps in *A. wodanis* may mediate Hcp secretion and function in delivery of T6SEs for pathogenesis. The T6SS1 and Aux-1 do not encode any *hcp* and the Hcp1 and Hcp4 share 60% homology between them whereas the Hcp2 and Hcp3 show 100% similarity. Except Hcp, the other T6SS proteins do not share high similarity between each other suggesting the multiple T6SSs are not a result of recent duplication. Our data (Paper II) revealed that the LitR has a significant impact on *hcp1* expression and the entire T6SS2 apparatus at 6°C than at 12°C. The supernatants harvested from *ΔlitR* mutant grown at 6°C showed less CPE effect in CHSE cell line than the wild type and AinS mutant (Paper I). Similar to other findings (Bartonickova et al. 2013), we propose that the decreased expression of *hcp1* in *ΔlitR* may have resulted in the low CPE in the CHSE cell line.

T6SS effectors are toxins that result in bacterial killing or pathogenesis (Pukatzki et al. 2006). Genes encoding putative adaptor and effector proteins immediately followed the *vgrG1* and *vgrG4*. Our analysis identified about 80 potential T6SEs in *A. wodanis*. *A. wodanis* genome revealed genes encoding cell wall degrading effectors proteins such as N-acetylmuramoyl-L-alanine amidase and hydrolases, membrane degrading effectors including phospholipases, porin-like protein, RHS protein and several nucleotide-degrading nucleases (Paper II). Also in the natural environment, the effectors in *A. wodanis* may work as toxins during interspecies niche competition and host interaction. Even though *M. viscosa* is the main pathogen in winter ulcer disease, this study shows that *A. wodanis* also encodes one of the virulence factors (T6SSs) that many pathogens encode. We speculate that the T6SSs, four Aux clusters and the effectors in *A. wodanis* may have directly or indirectly mediated the CPE in CHSE cell lines (Paper I) and the

pathological symptoms in Atlantic salmon (Karlsen et al. 2014b), as well as competitive behaviour towards *M. viscosa* (Hjerde et al. 2015). Nevertheless, further research is required to investigate the Type VI secreted compounds and functions of each T6SSs, Aux and effectors.

CRISPR is an adaptive defense system against phage invasion and other foreign genetic elements and contains short repeat sequences and spacer sequences (Barrangou & Marraffini 2014; Koonin et al. 2017; McGinn & Marraffini 2019). Spacer sequences are derived from phages or plasmids and stored as immune memory to encounter future invaders (Barrangou & Marraffini 2014). As the complete *cas* operon is differentially expressed in the LitR mutant, we wanted to search for the defense mechanism CRISPR-Cas system in *A. wodanis.* The genome analysis showed that *A. wodanis* encodes two CRISPR systems CRISPR systems 1 and 2, where one was in chromosome 1, and the other was in chromosome 2. CRISPR system 2 was identified as a type IF CRISPR system whereas CRISPR system 1 did not encode the operon. The CRISPR array of CRISPR system 2 contained 40 spacers and the CRISPR system 1 contained 25 spacers. The difference in the number of spacers suggests that CRISPR system 2 is more functional than the CRISPR system 1 or that the latter is a remnant of a previously active CRISPR system (paper II). When the spacers of the two CRISPR systems were compared, no identity was found between them, suggesting that the two CRISPR systems work independently of each other or that the CRISPR system 2 was introduced after CRISPR system 1. Comparing the type IF *A. wodanis* CRISPR cluster with *M. viscosa* showed greater than 90% AA similarity, suggesting that the CRISPR system has been horizontally transferred from *M. viscosa.* We speculate here that the similar CRISPR systems in *A. wodanis* and *M. viscosa* may have similar functions against invading foreign elements favoring their coexistence during winter ulcer disease.

The transcriptomics results showed significantly DEGs involved in CRISPR systems where the expressions were cell density, temperature, and QS dependent. The RNA-seq results showed that the *cas* genes such as *cas1* which is involved in spacer acquisition (Rollins et al. 2017; Yoganand et al. 2017), *cas3* that is responsible for helicase/nuclease activity (Sinkunas et al. 2011; Jackson et al. 2014), and *csy3* were more expressed at wild type (tpHCD/LCD) at 6°C whereas no significant differences were observed at 12°C. The differential expressions of *cas1* and *cas3* genes suggest that the mechanism of spacer acquisition and DNA degradation are cell density-dependent. Previous studies have shown that bacteria are at elevated risk during the late phase of infection (Hoyland-Kroghsbo et al. 2017; Hoyland-Kroghsbo et al. 2018), suggesting the *cas* operon in *A.*

wodanis is more highly expressed during high cell density or late stage of infection. However, cell density did not affect the expression of the CRISPR arrays.

Comparative analysis between 6°C and 12°C at LCD and HCD showed that temperature influenced the expression of the two CRISPR arrays, however it had no effect on any *cas* genes. The expression profiling results also indicated that loss of LitR significantly increased the expression of genes related to CRISPR system 2, indicating LitR is essential for the entire functioning (spacer acquisition, expression and interference) of the system. Finally, the comparison tp*ΔainS*/WT showed that AinS increased the expression of *csy* genes (*csy2-4*), suggesting that AinS is involved in the expression and interference steps of CRISPR-Cas mechanism.

Moreover, the reference genome was found to encode one intact phage in chromosome 1 and several incomplete phages in both the chromosomes and plasmids. The presence of phages and CRISPRs in the same bacteria suggest that the acquisition of one has happened before the other. Moreover, other studies have suggested that the genes of incomplete phages may result in new phenotypes and can provide fitness and virulence to the host (Boyd et al. 2001; Wagner & Waldor 2002). In paper II, we show that the incomplete phages in *A. wodanis* encode several conserved proteins such as Type I restriction endonuclease subunit M, DUF559 domain-containing protein, type II toxin-antitoxin system RelE/ParE family toxin, ClbS/DfsB family four-helix bundle protein and other conserved hypothetical proteins. This suggests that these phage proteins may provide an adaptive fitness advantage to *A. wodanis*. To understand how phages escaped the CRISPR system in the host, we searched for anti-CRISPR proteins in the *A. wodanis* genome using AcrFinder (Yi et al. 2020). The results showed that there were no anti-CRISPR proteins suggesting that the self-targeted protospacers that prevent the CRISPR-Cas response are not found.

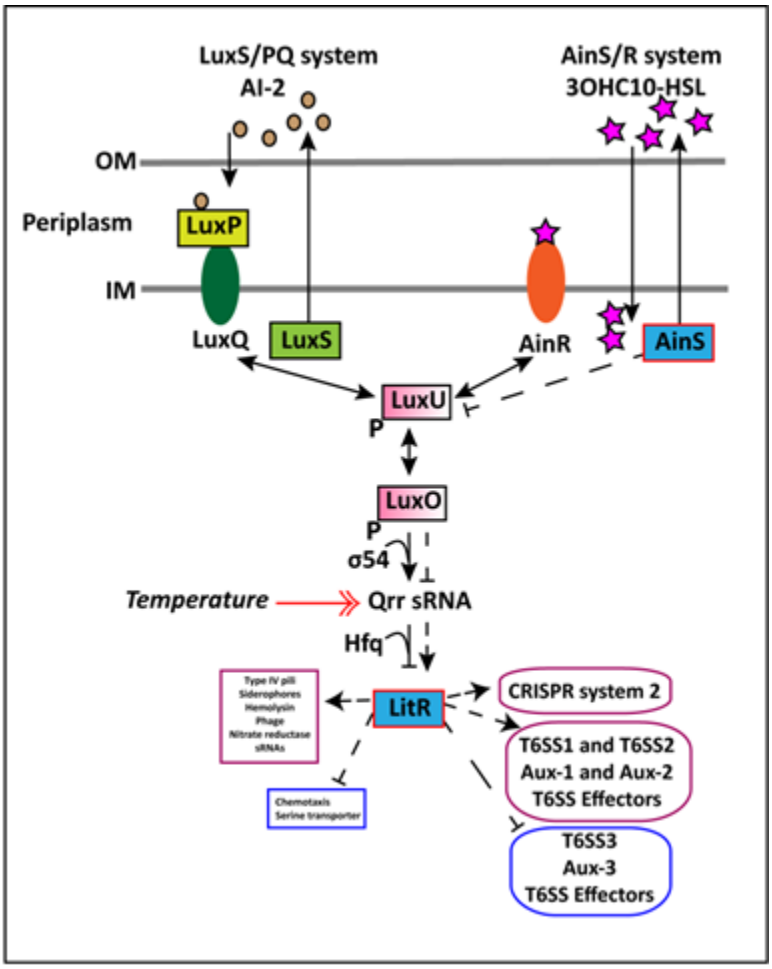

Figure 6: Proposed model for QS and its regulation of T6SSs and CRISPR systems in *A. wodanis* at 6°C (Paper II). Two QS systems AinS/AinR and LuxS/LuxPQ are believed to converge to activate the QS cascade. LitR increases the expression of CRISPR system 2, T6SS1, T6SS2, Aux-1, Aux-2 and T6SS effectors and decreases the expression of T6SS3, Aux-3 and T6SS effectors. LitR also increases the expression of type IV pili, siderophores, hemolysin, phages, nitrate reductase and sRNAs while decreasing the expression of chemotaxis and serine transporters. Temperature 6°C (red arrow) decreases the expression of *qrr* sRNA when compared to 12°C. AinS reduces the expression of *luxU*. The symbol "P" indicates the phosphorylated state, dotted lines with arrows indicate increased expression, and dotted lines with bars represent decreased expression.

A. wodanis pan-genome is open and reveals diversity in CRISPR-Cas, T6SS2 and phages

Few studies so far including the Paper I and II have explored the genome of reference strain *A. wodanis* 06/09/139. However, in a recent study with aliivibrios, a huge diversity has been observed among a few *A. wodanis* strains (Klemetsen et al. 2021), suggesting that the addition of genomes may reveal more diversity within the species. The reference *A. wodanis* 06/09/139 was isolated

together with *M. viscosa* from a head kidney of Atlantic salmon (Karlsen et al. 2014b). In addition to its co-existence with *M. viscosa*, *A. wodanis* exists as a free-living bacterium as well and has been isolated from other hosts such as Atlantic cod and Rainbow trout (Purohit et al. 2013). Hence, in paper III, we wanted to explore the pan-genome of twenty-two *A. wodanis* isolates to understand its phylogeny. Since in Paper II, the CRISPR-Cas system and T6SSs were significantly regulated by LitR and the T6SSs are speculated to be involved in the CPE observed in Paper I, we wanted to further investigate the pan-genome with regards to CRISPR-Cas system, T6SSs, phages and other functions like VFs and BGCs.

The genome analysis revealed that the size of all the *A. wodanis* genomes were approximately 4.5 MB and the variations in the genomes compared with reference were found to be linked to functional protein families: *secretion systems*, *prokaryotic defense mechanism*, *bacterial motility proteins*, *transporters*, *enzymes*, and *lipopolysaccharide biosynthesis proteins*. To reveal the evolutionary relationship between *A. wodanis* isolates, phylogenetic trees were constructed based on SNVs and the core genes. The results showed that the isolates were grouped into five different clades where the Groups 2, 4 and 5 comprised the most conserved isolates within each group. This observation suggests that the isolates within these groups evolved from the same ancestor and may have the same properties of genes related to niche adaptation, nutrient acquisition, interspecies and host-bacteria interaction in the fish farms. Even though all the isolates were from Norway and from the outbreak/ulcer region, the trees grouped into five different clades, where the diversity does not depend on the isolation place and time.

Bacteria have evolved a variety of mechanisms through HGT (Medini et al. 2005; Tettelin et al. 2005). The pan-genome analysis revealed that *A. wodanis* pan-genome is large and open suggesting a HGT in the genomes of *A. wodanis*. The analysis identified core (3149), shell (2583) and cloud (6271) genes indicating that the accessory genome content (shell, cloud and unique genes) varies about 73% (Paper III). The core genomes represent the set of homologous genes in the genomes, which perform housekeeping function or are involved in virulence in some bacteria (Wolfgang et al. 2003; Tettelin et al. 2005; Castillo et al. 2017). In Paper II, the core genome analysis showed that the core genome decreased gradually with addition of genomes suggesting a small core genome. The core genes are lower than that reported in *V. parahaemolyticus* and *P. aeruginosa* and higher than *V. anguillarum*, *Vibrio mimicus* (Hasan et al. 2010; Li et al. 2014; Ozer et al. 2014; Castillo et al. 2017). Despite finding three Groups with conserved isolates, the core genes in *A. wodanis* seem to be quite diverse. Unique genes that show no homology between

the same species and are often related to ecological adaptation (Rubin et al. 2000), were identified in all 22 isolates. The most enriched protein families in the pan and cloud genome were identified as *metabolism, signaling and cellular processes,* and *genetic information processing.* The isolate S7 encoded the highest number of unique genes (n=1007) which when functionally annotated revealed to encode several *Enzymes* (n=200) while the unique genes in other isolates encoded only a range of 4-30. Some isolates (Vw5, Vw12, Vw27 and Vw29 S7 S9, S8, S12, S11, Vw37, Vw35 and Vw8) encoded the genes related to T6SSs and CRISPR-Cas. The non-annotated unique gene clusters were manually annotated using BlastP and the results showed that the unique genes matched several bacteria including *Photobacterium* species, *Vibrio* genome sp. F6, *Vibrio* sp. 03-59-1 and *A. fischeri.* In *V. anguillarum*, selective pressure led to acquisition of genetic traits that could increase the virulence potential and fitness (Castillo et al. 2017). Similarly, *A. wodanis* may have acquired the unique genes under selective pressure, which may provide an advantage to the bacteria.

The genome analysis of the isolates showed that all isolated encoded CRISPR arrays suggesting that all isolates might have had a functional CRISPR-Cas system before. This study showed variations in the CRISPR-Cas systems, number of CRISPR arrays and spacers of the 20 isolates tested and two isolates (VwK7F1 and Vw7) do not encode any *cas* genes. Eight out of twenty-one (40%) isolates encoded the Type IF CRISPR system like the reference (Paper II) whereas the other isolates eleven out of twenty-one encoded different CRISPR-Cas systems in terms of number of *cas* genes and length of *cas* genes. This suggests that the isolates that encode incomplete *cas* operon or lack *cas* operon had a functional CRISPR system before. As expected, the isolates that showed incomplete CRISPR-Cas operon comprised less spacers than the isolates with a complete CRISPR-Cas operon confirming the findings that the incomplete CRISPR-Cas system is not functional as in the isolates with a complete *cas* operon.

Co-evolution and co-existence of CRISPR and phages have been reported in recent years (Watson et al. 2021), and these phages can escape CRISPR-Cas immunity through mutation in protospacer or PAM (Iranzo et al. 2013; Weissman et al. 2018). Moreover, phages may also encode anti-CRISPR proteins to inhibit CRISPR-Cas immunity (Li & Bondy-Denomy 2021). The PHASTER analysis showed that except for the Vw8 isolate, complete prophages were identified in all the isolates. However, as described above, two isolates (Vwk7F1 and Vw7) did not encode any *cas* genes suggesting that there is no correlation between the presence of CRISPR-Cas and incomplete prophages. The analysis also showed that about six out of twenty-two isolates Vwk7F1, S7,

5II_S9, S13, Vw11 and Vw7 did not encode any incomplete phages while the other isolates encoded incomplete prophages. These incomplete prophages encode genes that are believed to be involved in the adaptive functions in their host and be a putative source for phage-derived products such as bacteriocins and phage killer proteins (Bobay et al. 2014). Similarly, the genes in the incomplete prophages in *A. wodanis* could provide an advantage during the adaptation process.

The phylogenetic tree based on prophage sequences (intact and incomplete prophage) showed that the clustering of isolates was different from the grouping based on the core tree. This suggests that the phages are present in the accessory genomes, and they may have been acquired through HGT.

To efficiently adapt and thrive in specific environments, bacteria possess a wide variety of VFs and defense strategies that may contribute to pathogenicity and environmental survival. We identified three T6SSs and four Aux clusters in the genome of the reference strain (Paper II). Further genome analysis showed that all twenty-one isolates encoded T6SS1 and T6SS3 whereas only fifteen out of twenty-two isolates encoded T6SS2. Although the T6SS1 and T6SS3 were like the reference, the T6SS2 in many isolates were either different from the reference or completely absent. The same pattern was observed for the Aux cluster as well where the Aux-1, 3 and Aux-4 were like the reference whereas the Aux-2 was different from the reference in most of the isolates. However, the T6SS2 in 16 out of 21 (76%) isolates were less similar (AA similarity <50%) to the reference T6SS2 cluster whereas the 7 out of 21 (33%) isolates Vw7, Vw8, Vw37, Vw29, Vw130426, S10 and S6 did not encode the reference T6SS2 cluster. The proteins of the isolates that encoded the T6SS2 with low similarity to the reference T6SS2 were searched against the non-redundant protein database using BlastP. The results showed that the T6SS2 in the isolates were highly like *A. sifae, A. fischeri* and *A. logei*.

Together the results suggest that the T6SS1 and T6SS3 are more conserved and the T6SS2 is not conserved between the *A. wodanis* isolates. In our previous study, the reference T6SS2 has been identified to be highly like vibrios particularly *V. cholerae*, which is a pathogen (Maharajan et al. 2022). However, the 16 out of 21 isolates that showed low similarity to the reference T6SS2 showed high similarity to other *Aliivibrio* species such as *A. sifae, A. fischeri* and *A. logei*. *A. sifae, A. fischeri* and *A. logei* are environmental strains associated with symbiosis and not pathogens (Engebrecht et al. 1983; Fidopiastis et al. 1998; Sawabe et al. 2014). This may indicate that the T6SS2 in these 16 out of 21 isolates might behave more like environmental strains rather than a potential pathogen that interacts with the host. Further research is required to investigate the functional differences between the T6SS2 of reference and other isolates.

The isolates used in Paper III were mostly taken from outbreak regions and ulcers (Table 1 of Paper III). As described above, VFs are not necessarily required only for pathogenesis, they may play multiple roles in survival, adaptation, and host interactions. Thus, to identify the VFs in *A. wodanis*, the protein sequences were searched against the VFDB. VFs analysis results showed that all twenty-two *A. wodanis* isolates contained approximately 200 genes encoding various VFs. The VFs included *flagellum gene cluster*, *Type II secretion system*, *Ferric-anguibactin transport system*, *Chemotaxis gene cluster*, *Thermolabile hemolysin, RNA polymerase sigma factor RpoD* and *Twitching motility protein.* In *V. anguillarum,* the VFs found were similar from strains isolated from the same geographical location (Hansen et al. 2020). Similarly, the VFs in *A. wodanis* are conserved due to the lack of diversity in the isolation place.

Secondary metabolites synthesizing BGCs are especially important for bacteria to compete against other bacteria (Osbourn 2010; Srinivasan et al. 2021). Therefore, we aimed to identify the secondary metabolite producing clusters in *A. wodanis.* The genomes encoded around five potential BGCs with genes responsible for biosynthesis, regulation, and transport of secondary metabolites such as thioamititdes, ectoine, siderophores, arylploye ne and resorcinol. The siderophore cluster in the reference strain showed 60% gene similarity to *Vibrio crassostreae* strain 8T5 whereas the clusters producing ectoine, arylploye ne and resorcinol showed 50-95% gene similarity to the clusters of *A. sifae*. The siderophore clusters showed 100% gene similarity in seventeen out of twenty-one isolates while the ectoine clusters revealed gene similarity in fifteen out of twenty-one isolates when compared to the reference. The results showed that the siderophore and ectoine clusters are the most conserved BGCs between reference and many other isolates. The BGCs such as ectoine, arylpolyene and resorcinol are known to be involved in stress response and host-pathogen interaction (Cimermancic et al. 2014; Schoner et al. 2016; Czech et al. 2018). Even though the *A. wodanis* strains were from a diverse isolation period (1905-2013), these predcited BGCs are quite conserved between isolates and this suggests the importance of these BGCs in *A. wodanis*.

CONCLUDING REMARKS

There is a huge knowledge of why and how bacterial populations use QS to communicate and control diverse behaviours. This knowledge has provided a foundation to study QS dependent regulation in several bacterial species. *A. wodanis* is like *A. fischeri*, *A. salmonicida*, *A. logei* and *Aliivibrio* sp. *"*friggae*"*, however it lacks the *lux* operon and the *luxI*/*luxR* QS system. Reference *A. wodanis* has only one AHL based QS system AinS/AinR and a LitR regulator and produces one AHL. Thus, the AHL based QS system has been believed to function like other aliivibrios.

Previous studies have reported that *A. wodanis* could be a competitor as it inhibits the growth of *M. viscosa* in a co-cultivation experiment. In this study, *A. wodanis* encodes multiple VFs such as T6SSs, siderophores, motility, hemolysin and protease and defense mechanism CRISPR-Cas that may be involved in environmental adaptation, survival, host-pathogen interaction, and phage defense. Our results also demonstrate that LitR, the QS master regulator regulates motility, growth, hemolysin, siderophores and AinS autoinducer synthase positively influences the protease production in *A. wodanis.* More importantly, LitR and AinS play a vital role in controlling CPE in the CHSE salmon cell line at 6°C and 12°C, where the effect was significantly high at 6°C. This is further supported by the transcription profiling where LitR increases the expressions of T6SS2 and Aux-2 gene clusters more at HCD and 6°C than at LCD and 12°C. Additionally, LitR in *A. wodanis* increases the expression of the complete CRISPR-*cas* operon. However, AinS do not have a significant impact on the expressions of T6SSs and CRISPR-Cas systems suggesting that LitR regulates these systems through either the LuxS/PQ QS system or some other unknown functions.

Overall, the results obtained in the present work shows that LitR together with cell density and temperature regulate various functions potentially linked to anti-bacterial and anti-eukaryotic activity. Since QS regulates CPE, T6SSs and T6SEs in *A. wodanis*, blocking or inducing QS could be used in treatment of winter ulcer disease or inhibiting *M. viscosa* infections, respectively. This approach may overcome the limitations caused by antibiotics, since the blocking of QS only suppresses the virulence and does not favour the development of antibiotic resistant bacteria.

Pan-genome analysis of twenty-two isolates revealed the phylogenetic distribution and the genetic variations/similarity in CRISPR-Cas system, T6SSs, phages, VFs and BGCs between the isolates. These findings may aid in understanding the genetic mechanisms that contribute to the co-existence with *M. viscosa* and winter ulcer pathogenesis.

FURTHER PERSPECTIVES

Environmental pathogenic bacteria survive and replicate in a community while maintaining the mechanisms to infect the hosts. *A. wodanis* has been repeatedly isolated together with *M. viscosa* during the winter ulcer outbreaks. Despite the co-existence of *A. wodanis* with *M. viscosa*, it has not been identified as the main pathogen. While the exact function of *A. wodanis* in winter ulcer remains unclear, several potential VFs (hemolysin, protease, siderophore, T6SS, chitinase and motility) in *A. wodanis* have been identified in this study. However, the role of these VFs with regards to interplay between *A. wodanis* and *M. viscosa* has not been studied yet. The expression profiling studies revealed that QS and temperature regulate T6SSs and CRISPR-Cas systems. However, further laboratory experiments are required to check the secreted substances of the T6SSs apparatus and verify whether they target *M. viscosa* or fish cells. QS regulation of virulence has been studied by treating the CHSE cell lines with supernatants harvested from wild type and QS mutants grown 6°C and 12°C. However, cytotoxicity assays with live bacteria would provide a contact-dependent role of T6SSs in CPE than just the supernatants. Additionally, the virulence assay was performed only with salmon cell lines and not the whole fish. Future studies will be aimed at challenging the fish with the QS mutants to understand the QS regulation of virulence. Moreover, the pan-genome analysis was performed only with strains isolated from Norway and outbreak/ulcer regions, therefore including environmental strains from various locations would improve the genomic variations.

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Papers I-III

Paper 1

Quorum sensing in *Aliivibrio wodanis* 06/09/139 and its role in controlling various phenotypic traits

Amudha Deepalakshmi Maharajan, Hilde Hansen, Miriam Khider, Nils Peder Willassen // PeerJ., August 24, 2021., 10.7717/peerj.11980

Quorum sensing in *Aliivibrio wodanis* 06/09/139 and its role in controlling various phenotypic traits

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ABSTRACT

Background. Quorum Sensing (QS) is a cell-to-cell communication system that bacteria utilize to adapt to the external environment by synthesizing and responding to signalling molecules called autoinducers. The psychrotrophic bacterium *Aliivibrio wodanis* 06/09/139, originally isolated from a winter ulcer of a reared Atlantic salmon, produces the autoinducer N-3-hydroxy-decanoyl-homoserine-lactone (3OHC10-HSL) and encodes the QS systems AinS/R and LuxS/PQ, and the master regulator LitR. However, the role of QS in this bacterium has not been investigated yet.

Results. In the present work we show that 3OHC10-HSL production is cell density and temperature-dependent in *A. wodanis* 06/09/139 with the highest production occurring at a low temperature (6 °C). Gene inactivation demonstrates that AinS is responsible for 3OHC10-HSL production and positively regulated by LitR. Inactivation of *ainS* and *litR* further show that QS is involved in the regulation of growth, motility, hemolysis, protease activity and siderophore production. Of these QS regulated activities, only the protease activity was found to be independent of LitR. Lastly, supernatants harvested from the wild type and the Δ*ainS* and Δ*litR* mutants at high cell densities show that inactivation of QS leads to a decreased cytopathogenic effect (CPE) in a cell culture assay, and strongest attenuation of the CPE was observed with supernatants harvested from the Δ*litR* mutant.

Conclusion. *A. wodanis* 06/09/139 use QS to regulate a number of activities that may prove important for host colonization or interactions. The temperature of 6 °C that is in the temperature range at which winter ulcer occurs, plays a role in AHL production and development of CPE on a Chinook Salmon Embryo (CHSE) cell line.

Subjects Aquaculture, Fisheries and Fish Science, Microbiology, Molecular Biology, Veterinary Medicine

Keywords Quorum sensing, AHL, *Aliivibrio wodanis*, Winter ulcer, Atlantic salmon, Motility, Protease, Cytopathogenicity, Siderophores, CHSE

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INTRODUCTION

Quorum sensing (QS) is a cell–cell communication mechanism regulated by secretion and accumulation of small diffusible signalling molecules called autoinducers (AI) in a cell density-dependent manner (*Bassler, 1999*). In Gram-negative bacteria, the most common signalling molecules used for intra-species communication are N-acyl homoserine lactones (AHLs) referred to as AI-1. The AHLs consist of a common homoserine lactone (HSL) ring attached to an acyl side chain with four to 18 carbon atoms and a carbonyl substitution at third carbon (*Parsek et al., 1999*; *Kumari et al., 2006*). The type and number of AHLs produced differ between bacteria and also between bacteria of the same species (*Purohit et al., 2013*; *Girard et al., 2019*). In addition to the AHLs, bacteria may use autoinducer-2 (AI-2) for both intra- and interspecies QS signalling (*Forsyth & Cover, 2000*; *Schauder et al., 2001*; *Li et al., 2010*; *Marques et al., 2011*).

QS was first described in *Aliivibrio fischeri*, previously known as *Vibrio fischeri*, and later in other vibrios such as *Vibrio harveyi* (*Nealson & Hastings, 1979*; *Fuqua, Winans & Greenberg, 1994*; *Freeman, Lilley & Bassler, 2000*). *A. fischeri* possesses two AHL based QS systems, the AinS/AinR and LuxI/LuxR, in addition to a LuxS/LuxPQ system where AinS, LuxI and LuxS are the autoinducer synthases and AinR, LuxR and LuxPQ are the receptors. LuxI synthesizes N-3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL) and AinS synthesizes N-octanoyl- homoserine lactones (C8-HSL) while the LuxS synthesizes a furanosyl borate diester (AI-2) (*Lupp & Ruby, 2004*; *Miyashiro & Ruby, 2012*). In *A. fischeri*, the AinS/R and LuxS/LuxPQ QS systems work in parallel to transfer the signal responses to LuxO *via* LuxU. At a low cell density and low autoinducers concentration, the receptors AinR and LuxPQ phosphorylate LuxO. Phosphorylated LuxO activates the transcription of the sRNA *qrr* gene to repress the transcription factor LitR through RNA chaperone Hfq (*Lupp et al., 2003*). Alternatively, at high cell density, the autoinducers bind to their cognate receptors leading to dephosphorylation of LuxO and *litR* expression. In addition to the phosphorelay system, in *A. fischeri*, LitR directly activates *luxR* expression to regulate bioluminescence production from the *luxICDABEG* operon (*Engebrecht & Silverman, 1984*; *Lupp & Ruby, 2005*; *Bose, Rosenberg & Stabb, 2008*; *Verma & Miyashiro, 2013*). *A. fischeri* also uses QS to control other activities such as motility, colonization and biofilm formation (*Lupp et al., 2003*; *Lupp & Ruby, 2005*; *Studer, Mandel & Ruby, 2008*; *Ray & Visick, 2012*).

The marine bacterium *Aliivibrio wodanis* belongs to the genus *Aliivibrio* within the *Vibrionaceae* family (*Urbanczyk et al., 2007*; *Ast, Urbanczyk & Dunlap, 2009*). The bacterium is motile and psychrotrophic with the ability to grow at temperatures between 4 and 25 °C in the laboratory (*Lunder et al., 1995*; *Lunder et al., 2000*; *Soderberg et al., 2019*). *A. wodanis* is repeatedly isolated together with *Moritella viscosa* from Atlantic salmons (*Salmo salar*) suffering from winter ulcer disease (*Lunder et al., 1995*; *Benediktsdottir et al., 2000*; *Lunder et al., 2000*; *Whitman et al., 2001*). The disease has only been reported in reared salmons, and when the sea water temperature drops below 8 °C (*Lunder et al., 1995*; *Benediktsdottir, Helgason & Sigurjonsdottir, 1998*; *Whitman et al., 2001*). The role of *A. wodanis* in pathogenicity of winter ulcer disease is uncertain, and *M. viscosa* is considered

to be the main pathogen (*Lunder et al., 1995*; *Benediktsdottir, Helgason & Sigurjonsdottir, 1998*; *Bruno et al., 1998*).

Karlsen et al. (2012) and *Karlsen et al. (2014)* have attempted to study the interaction between *A. wodanis* and *M. viscosa* using different approaches. The studies suggest that *A. wodanis* may influence the progression of a *M. viscosa* infection. In particular, they show that predisposing Atlantic salmons to *A. wodanis* prior to infection with *M. viscosa* led to lower mortalities (*Karlsen et al., 2014*). As a follow-up *Hjerde et al. (2015)* studied the transcriptome of co-cultured *A. wodanis* and *M. viscosa*. In this study the authors concluded that the presence of *A. wodanis* alters the transcriptome of *M. viscosa* and impedes its growth. *A. wodanis* genome encodes bacteriocin, a proteinaceous toxin that inhibits the growth of closely or distantly related bacteria (*Hjerde et al., 2015*). It was speculated that the expression of bacteriocin in *A. wodanis* might have possibly impeded the growth of *M. viscosa* (*Hjerde et al., 2015*). Although the contribution of *A. wodanis* in the development of winter ulcer is unclear and may have an alleviating effect on a *M. viscosa* infection, laboratory experiments have shown that Atlantic salmons infected with *A. wodanis* alone is able to cause clinical symptoms such as scale loss, fin rot and internal pathological symptoms such as swollen spleen, peritoneal fat tissues and petecchia in liver (*Karlsen et al., 2014*). Furthermore, supernatants harvested from high cell density *A. wodanis* cultures induce severe and rapid cytopathogenic effect (CPE) in four differ cell lines of salmonid origins (*Karlsen et al., 2014*). Hence, the bacterium is able to produce and secrete some yet unknown agent(s) that is cytotoxic to eukaryotic fish cells.

We have previously shown that *A. wodanis* strain 06/09/139 produces N-3-hydroxy-decanoyl-homoserine-lactone (3OHC10-HSL) (*Purohit et al., 2013*), and encodes the QS systems LuxS/LuxPQ and AinS/AinR and the master regulator LitR (*Hjerde et al., 2015*). In the study presented here we investigate the QS system of *A. wodanis* by studying the functional roles of the autoinducer synthase AinS and the master regulator LitR. We performed the analyses at different temperatures since *A. wodanis* has been associated with the winter ulcer disease, and also due to the fact that temperature is an important factor for AHL production and QS regulation in the closely related *Aliivibrio salmonicida*, the bacterium known to cause cold-water vibriosis in Atlantic salmons (*Bjelland et al., 2012*; *Hansen et al., 2014*; *Hansen et al., 2015*). Our analysis show that the QS system in *A. wodanis* regulates various phenotypic traits such as motility, growth, hemolysis, protease, siderophore production, as well as cytotoxicity in a cell line. We speculate that QS regulation of various potential virulent factors in *A. wodanis* may play a vital role during winter ulcer development. To our knowledge, this is the first study on the QS system of *A. wodanis*.

MATERIALS & METHODS

Bacterial strains, plasmids and growth conditions

Bacterial cells and plasmids used in this study are listed in Table 1. The wild type *A. wodanis* 06/09/139 and the constructed *A. wodanis* mutants were grown from glycerol stocks (−80 °C) on Luria-Bertani Agar (Difco BD Diagnostics Sparks, MD, USA) plates containing 1.5% agar (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with a

Table 1 Bacterial strains, plasmids and primers used in this study.

Strains, plasmids and primers	Description	Source or reference
A. wodanis		
A. wodanis 06/09/139	Wild type from head kidney of Atlantic salmon from west coast of Norway	*Karlsen et al. (2014)*
Δ*litR*	*A. wodanis* 06/09/139 with a complete deletion of *litR* gene	This study
Δ*ainS*	*A. wodanis* 06/09/139 with a partial deletion of *ainS* gene	This study
litR^{+}	Δ*litR* complemented with the wild type *litR* gene	This study
E. coli		
JM109	Competent strain for transformation of pGEM vector with insert	*Yanisch-Perron, Vieira & (2005)*
SY327	Strain for replicating suicide vector, λ pir	*Miller & Mekalanos (1988)*
S.17-1	Donor strain used for conjugation, λ pir	*Simon et al. (1983)*
Plasmids		
pDM4	Suicide vector with *sacB*, cm^{R}, R6K origin (λpir)	*Milton et al. (1996)*
pGEM®-T Easy vector	Cloning vector with β-galactosidase, Amp^{r}, lacz,3'T overhangs, blue /white screening	Promega
pGEM Δ*litR*	pGEM®-T Easy vector with Δ*litR*	This study
pDM4 Δ*litR*	pDM4 with regions flanking the deleted *litR* gene	This study
pDM4 *litR*$^{+}$	pDM4 with flanking regions and full length *litR* gene	This study
pDM4 Δ*ainS*	pDM4 with regions flanking the deleted *ainS* gene	This study
Primers		
LitRA-F	ATATACTCGAGTTTACAACAAAAGCGCACCTG	This study
LitRB-R	CATATTTATTTATATCCTTGCCAACAA	This study
LitRC-F	GATATAAATAAATATGTAATATTCAGAACTCAGAAAGTAGATA	This study
LitRD-R	TATAATACTAGTGAGCTTCTTGGTGAAATTGG	This study
LitRG-F	GAGCCACGTAATAAACCAATCATC	This study
LitRH-R	CGTGTTATCGGTGGTGCTATT	This study
AinSA-F	AATAACTCGAGGGCTGATTATACAATAAGGTTGTG	This study
AinSB-R	CTAGATTGTTTAGATCAAATGTTGATA	This study
AinSC-F	GATCTAAACAATCTAGACGAGCCACCAAGATATCAA	This study
AinSD-R	TATATACTAGTCAACCTCCATCCGATCTTTA	This study
AinSG-F	TCACGACGAGAACCAAGACC	This study
AinSH-R	TTAGGTTGATAGCGAGAGCAAAG	This study
NQCAT	TAACGGCAAAAGCACCGCCGGACATCA	*Milton et al. (1996)*
NQREV	TGTACACCTTAACACTCGCCTATTGTT	*Milton et al. (1996)*

final concentration of 2.5% NaCl (wt/vol) (LA2.5) for 3 days at 12 °C. The Pre-culture of *A. wodanis* was grown overnight in 2 ml of Luria-Bertani broth (LB2.5) at 12 °C and 220 rpm.

The *Escherichia coli* (*E. coli*) strains JM109 and S.17-1lambdapir were grown on LA or LB supplemented with 1% (wt/vol) NaCl (LA1 and LB1, respectively) at 37 °C and 220 rpm overnight. The TA plasmid pGEM-T was propagated in *E. coli* JM109 (Promega). The suicide plasmid pDM4 (GenBank: KC795686.1) was propagated in S.17-1lambdapir cells. The *E.coli* JM109 and S.17-1lambdapir transformants were selected on LA1 with 100 µg/ml

ampicillin and 25 µg/ml chloramphenicol respectively. The potential transconjugants of *A. wodanis* were selected on LA2.5 supplemented with 2 µg/ml chloramphenicol at 12 °C for 5 days.

A sea water-based medium (SWT) was used in the biofilm and colony morphology assays consists of 5 g/L of Bacto peptone (BD), 3 g/L of yeast extract (Sigma-Aldrich, St. Louis, MO, USA) supplemented with different sea salt (Instant Ocean, Aquarium systems, Sarrebourg, France) concentrations (1.0, 2.8 and 4.0% per litre). For solid SWT plates, 1.5% agar (Sigma-Aldrich, St. Louis, MO, USA) was added. The hemolysin assay was carried on blood agar plates (BA) base no.2 (OXOID, Thermo Scientific) supplemented with 5% blood and 2.5% NaCl. Leibowitch-15 (L-15) medium (Thermo Fisher Scientific, USA) was used for cytotoxicity assay and supplemented with 200 mM L-glutamine, antibiotic-antimycotic solution 100 units/ml penicillin, 100 µg/ml Streptomycin, 250 ng/ml amphotericin B (P/S/A) (Sigma-Aldrich, St. Louis, MO, USA) and fetal bovine serum (FBS) (8%).

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 using NanoDrop™ 2000c spectrophotometer (Thermo Scientific, DE, USA). The PCR amplification was performed using Phusion® polymerase (Thermo Fisher Scientific, Waltham, MA, USA) or Taq polymerase (Sigma, St. Louis, MO, USA) in a Arktik™ thermal cycler (Thermo Fisher Scientific, USA). Restriction digestion using XhoI and SpeI restriction enzymes and DNA ligation using T4 DNA ligase were performed as recommended by the manufactures and were obtained from New England Biolabs (Ipswich, MA, USA). The PCR products and the digested DNA fragments were separated using agarose gel electrophoresis and extracted using Montage® gel extraction kit (Millipore, MA, USA). DNA sequencing was performed using Big Dye (Applied biosystems, CA, USA). The primers used in the PCR and sequencing reactions were synthesized by Sigma-Aldrich and are listed in Table 1.

Construction of Δ*litR* and Δ*ainS* mutants

The *litR* (AWOD_I_0419) and *ainS* (AWOD_I_1040) genes were in-frame deleted in *A. wodanis* using allelic exchange as described by others (*Milton et al., 1996*; *Bjelland et al., 2012*; *Hansen et al., 2015*; *Khider, Willassen & Hansen, 2018*). Briefly, *litR* and *ainS* genes were deleted by amplifying and fusing regions flanking these genes. The upstream (280 bp) and downstream (263 bp) flanking regions of *litR* gene were amplified by primer pairs LitRA/LitRB and LitRC/LitRD respectively. The upstream region contains the start codon (ATG), and the downstream region contains the last three codons (TAA) at the C-terminal end of *litR* gene. The upstream (253 bp) and downstream (271 bp) flanking regions of *ainS* gene were amplified using primer pairs AinSA/AinSB, and AinSC/AinSD, respectively. The upstream PCR product ends before the start codon (ATG), and the downstream PCR product contained the last 149 bp (50 codons) of the *ainS* gene. PCR amplification was performed with an initial denaturation at 98 °C for 30 s (s), followed by 30 cycles of 98 °C

for 10 s, 60 °C for 20 s, 72 °C for 30 s, finishing with a final extension at 72 °C for 5 min and storage at 4 °C thereafter. The upstream and downstream PCR products of either *litR* or *ainS* were fused by overlap extension PCR. This overlap PCR was performed by mixing the upstream and downstream PCR products with DNA Phusion polymerase, deoxynucleoside triphosphates (dNTPs) and buffer and cycling for seven times. Then the primer pairs LitRA/D or AinSA/D were added, and 25 more cycles were run (PCR amplification was performed similarly to the stated above). A'overhangs were added to the fused PCR products and ligated into pGEM-T Easy vector. The ligated constructs were transformed into *E. coli* JM109 cells. The inserts (PCR overlap products) were digested from the pGEM plasmid using *SpeI* and *XhoI* restriction enzymes as the primer pairs LitRA/D and AinSA/D contain restriction sites (*SpeI* and *XhoI*) to enable further ligation. The digested fused PCR products were further ligated into corresponding restriction sites of the suicide vector pDM4. The pDM4 plasmid with either *litR* or *ainS* fused PCR product was transformed to *E.coli* S.17-1lambdapir cells. The resulting plasmids were designated as pDM4Δ*litR* and pDM4Δ*ainS*.

The pDM4Δ*litR* and pDM4Δ*ainS* constructs were transferred into wild type *A. wodanis* by bacterial conjugation as described previously (*Milton et al., 1996*; *Bjelland et al., 2012*; *Khider, Willassen & Hansen, 2018*). Briefly, the donor cells *E.coli* S.17-1lambdapir harboring the pDM4Δ*litR* or pDM4Δ*ainS* were grown until mid-exponential phase to OD_{600} (optical density at 600 nm) of 1.0 and the recipient strain (*A. wodanis*) to an early-exponential phase OD_{600} of 2.0. One ml from each culture was centrifuged separately at room temperature and the pellets were separately washed twice with LB1 medium. The washed bacterial pellets were mixed and resuspended in 10 µl of LB1. The resuspended pellet was spotted onto LA1 plates and incubated at 19 °C for 6 h. The plates were further incubated at 12 °C for 48 h. The spotted cells were then resuspended in 2 ml LB2.5 and incubated overnight at 12 °C before plating (20, 40, 60, 80, and 100 µl) on LA2.5 plates with 2 µg/ml chloramphenicol. Potential transconjugants were selected after 3 to 5 days and confirmed using colony PCR. To complete the allelic exchange needed to generate the complete deletion mutants (Δ*ainS* and Δ*litR*) potential transconjugants (*A.wodanis*-pDM4-Δ*ainS* or *A.wodanis*-pDM4-Δ*litR*) were plated on LA2.5 plates with 5% sucrose to allow the second cross over. The cells that were able to grow were selected based on their sensitivity to chloramphenicol. The antibiotics sensitive cells were confirmed by colony PCR and further verified by sequencing.

Construction of Δ*litR* complementary strain

The complementary strain ($litR^+$) of the Δ*litR* mutant was constructed by amplifying the full-length wild type *litR* gene using primers LitRA and LitRD. Briefly, the length of both the parental *litR* gene and its flanking region used to generate $litR^+$ was 1,145 bp, which was amplified from wild type using primers LitRA and LitRD. The PCR product was then cloned into pDM4 using restriction digestion and ligation as described above. The resulting plasmid pDM4$litR^+$ was transformed into *E.coli* S.17-1lambdapir cells and further transferred to the *A. wodanis* Δ*litR* mutant by bacterial conjugation (described above). The selection and verification of the potential complementary strain were performed as

described above. The region that flanks the complemented region (*litR*$^{+}$) after allelic exchange was confirmed using primers LitRG and LitRH. The length of products amplified using LitRG and LitRH was 1,365 bp.

Preparation of bacterial supernatants for AHL measurements

The wild type *A. wodanis*, Δ*litR* and *litR*$^{+}$ were cultivated in parallel at 6 and 12 °C. The cultures were diluted to a start OD_{600} of 0.001 in a total volume of 60 ml LB2.5 in a 250 ml baffled flasks. The cultures were grown further at the selected temperatures and 220 rpm. Cultures of 1 ml (wild type, Δ*litR* and *litR*$^{+}$) were collected at seven different cell densities in total, six at the log phase (OD_{600} of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0) and one at the stationary phase (8.0). For Δ*ainS* AHL measurements, samples were only harvested at the early stationary phase (OD_{600} of 6.0). The cultures were centrifuged at 13,000× g for 2 min at 4 °C (Heraeus fresco 21; Thermo Scientific, Waltham, MA, USA). Seventy-five microliters of each supernatant were acidified with 4 µl of 1M HCl and stored in three technical replicates at −20 °C before measuring the AHLs. A commercial 3-OH-C10-HSL was used as a standard (Sigma-Aldrich, St. Louis, MO, USA). The sample preparations for High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) were done as described by others (*Purohit et al., 2013*; *Hansen et al., 2015*). 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 was pooled together into a 1 ml 96 well plate and dried in a rotary vacuum centrifuge at −90 °C for 2 h (SpeedVac Savant^{TM} 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 3-oxo-C12-HSL (Sigma-Aldrich, St. Louis, MO, USA).

HPLC-MS/MS analysis

The HPLC-MS/MS analysis was performed as described in *Purohit et al. (2013)* and *Hansen et al. (2015)*. Briefly HPLC-MS/MS was performed using an Ascentis Express C18 reversed-phase column (50 × 2.1 mm, 2.7 µm particle size; Sigma). A sample of 20 µl 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 Quadrapole (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 3OHC10-HSL and segment 2 scanned the internal standard 3O-C12-HSL with a retention time of 0–3.15 min and 3.15–6.00 min, 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/OD_{600}. The AHL measurements at different temperatures were performed twice.

Motility assay

Motility assay was performed in LA2.5 soft agar plates with 0.25% agar (*Bjelland et al., 2012*; *Khider, Willassen & Hansen, 2018*). Pre-cultures of *A. wodanis* wild type, Δ*ainS*, Δ*litR* and

litR^{+} were diluted 1:100 in LB2.5 and grown overnight at 12 °C to an OD_{600} corresponding to 1.0. Then 2 µl of each culture was spotted onto the LA2.5 soft agar plates and incubated at 6 °C and 12 °C. The diameter of motility zones were measured in millimeters every 24 h for 5 days.

Siderophore, hemolysis, chitinase and protease assays

Siderophore production was screened using Chromeazurol S (CAS) agar plates as described by others (*Lauzon et al., 2008*), with the exception that 2.5% of NaCl was used in this study. Culture supernatants harvested at OD_{600} 6.0 (100 µl) from strains grown at 6 and 12 °C were added to six mm wells casted in CAS agar. The CAS agar plates were incubated at 20 °C for 2 days. Hemolysin production was estimated by spotting 2 µl cultures of each strain on BA plates. The protease production was estimated by spotting 2 µl cultures of each strain on LA2.5 agar plates supplemented with 2% skim milk (Sigma-Aldrich, St. Louis, MO, USA). The chitinase assay was performed by spotting 2 µl cultures of each strain on LA2.5 supplemented with 2% colloidal chitin (Sigma-Aldrich, St. Louis, MO, USA) and the plates were stained with 0.5% congo red (Sigma-Aldrich, St. Louis, MO, USA) for 30 min before destaining with 1 M NaCl for 20 min for chitinase zones measurement. All the assays were performed with at least three biological replicates of strains *A. wodanis* wild type, Δ*ainS*, Δ*litR* and *litR*$^{+}$ and were incubated at 6 and 12 °C. The clear zones ratio values for hemolysis, protease and chitinase assays were calculated as clear zone diameter/colony diameter.

Biofilm and colony morphology assays

The biofilm and colony morphology assays were performed as described previously (*Hansen et al., 2014*) using SWT media and plates, respectively. Pre-cultures of *A. wodanis* wild type, Δ*ainS*, and Δ*litR* were diluted 1:100 in LB2.5 and grown overnight at 12 °C to an OD_{600} of 1.0. For the biofilm assay, the cultures were further diluted 1:10 in SWT media, and a total volume of 300 µl was added to Falcon 24 well plates (BD Biosciences) and incubated statically at 6 °C. The plates were monitored every 24 h. For the colony morphology assay, a 250 µl of each bacterial culture was harvested by centrifugation, and the pellet was resuspended in 250 µl SWT. Then, 2 µl of each culture was spotted onto SWT plates and incubated at 6 °C for up to 4 weeks. The biofilm formation and colony morphology was visualized using Ziess Primovert microscope at 10x and 4x magnification, respectively and were photographed with AxioCam ERc5s.

Cytotoxicity assay and crystal violet staining

Chinook salmon embryo (CHSE-214) cells were purchased from American Type Culture Collection (*Nicholson & Byrne, 1973*). Chinook salmon embryonic (CHSE) cells (passage 55) were grown in L-15. The CHSE cells were seeded 1×10^5 cells/ml in a flat-bottom tissue culture 24-well plates (Falcon; BD Biosciences) and incubated for 48 h at 20 °C. Supernatants of *A. wodanis*, Δ*ainS*, Δ*litR* and *litR*$^{+}$ grown at 6 °C and 12 °C were harvested at OD_{600} of 6.0, 7.0 and 8.0 and filter sterilized through 0.22 µm filter. The 100% confluent fish cells were washed with L-15 without supplements and treated with bacterial supernatants (1:10 to L-15 with antibiotics) before incubating at 12 °C. LB2.5 was used

as a negative control. The plates were monitored after 4 and 24 h. The treated CHSE cells were quantified using crystal violet staining. Briefly, the wells with treated CHSE cells were stained with 500 µL of 0.1% crystal violet for 20 min before washing with water. The plates were air-dried for 1 or 2 days, and the cells were dissolved in 96% ethanol before measuring the absorbance at 590 nm (100 µL) using a spectrophotometer (Spectromax, Molecular devices).

All assays were carried out in biological triplicates, unless otherwise indicated. The assays were also performed in two to three independent experiments to validate the results.

RESULTS

In order to study the roles of QS in *A. wodanis* 06/09/139, we deleted parts of the *ainS* gene (347 of 396 codons) and the *litR* gene (200 of 201 codons) using allelic exchange. A complementary Δ*litR* strain (*litR*$^+$) was constructed by re-inserting the full-length copy of the wild type *litR* gene into the Δ*litR* mutant to ensure that observed phenotypes are due to the targeted gene inactivation and not to other factors. Despite several trials, we were unable to rescue the Δ*ainS* mutant. The schematic presentation of the *litR* and *ainS* genes in the genome of *A. wodanis* is shown in Fig. S1.

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

In previous work, we mapped AHL profiles among members of the *Vibrionaceae* family. Only one single AHL, the 3OHC10-HSL, was identified in *A. wodanis* 06/09/139 (*Purohit et al., 2013*). In *A. salmonicida*, the autoinducer synthase AinS was responsible for 3OHC10-HSL synthesis (*Hansen et al., 2015*). Thus our first aim was to verify if 3OHC10-HSL is produced by AinS. To this end, supernatants harvested from the wild type and mutants (Δ*ainS*, Δ*litR* and *litR*$^+$) were analyzed by HPLC-MS/MS as previously described (*Purohit et al., 2013*; *Hansen et al., 2015*). A peak corresponding to 3OHC10-HSL was present only in supernatants harvested from the wild type, Δ*litR* mutant and the *litR*$^+$ (Fig. 1). This suggests that AinS is the autoinducer synthase responsible for 3OHC10-HSL production in *A. wodanis* 06/09/139. Unfortunately, we were not able to complement the *ainS* mutant which could have given absolute proof for AinS being the 3OHC10-HSL synthase in *A. wodanis*. Since *ainS* is the only AHL-linked gene annotated in *A. wodanis* we find other explanations unlikely.

LitR represses growth of *A. wodanis* at 20 °C

Mutations are known to affect the growth of bacteria. *A. wodanis* strains grow in a range of 4–25 °C and in a recent study in our lab, the optimal growth temperatures were found to be 12–18 °C (*Lunder et al., 1995*; *Lunder et al., 2000*; *Soderberg et al., 2019*). We, therefore tested the strains ability to grow at three different temperatures (6 °C, 12 °C and 20 °C) within the reported temperature range for *A. wodanis*. As shown in Fig. 2, the *litR* or *ainS* mutations did not alter the growth rate of *A. wodanis* at 6 °C and 12 °C, and all strains reached a maximum OD_{600} of ~8.0. The strains grew considerably faster at 12 °C than at 6 °C, and the duration of the log/exponential phase (OD_{600} 0.5 to 8.0) for the wild type

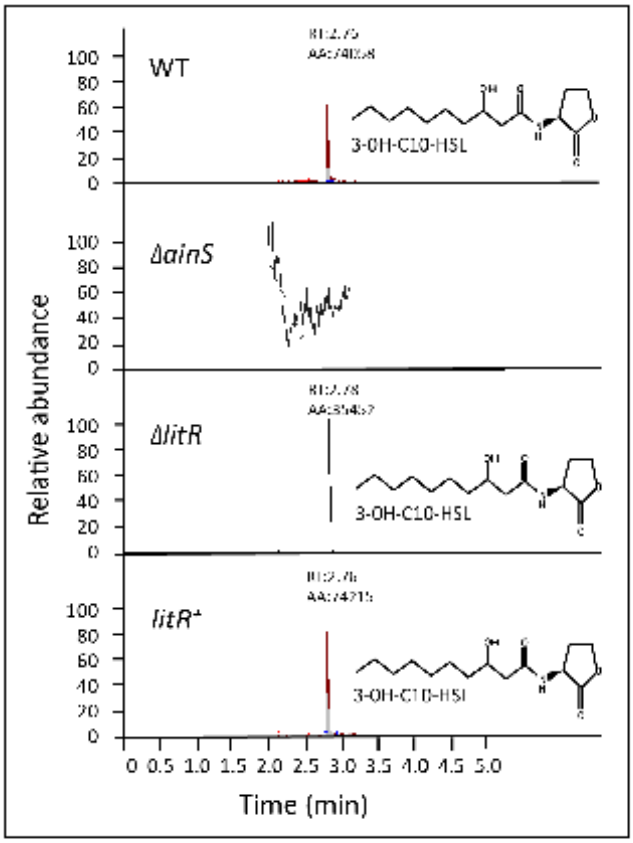

Figure 1 **3OHC10-HSL screening in wild type, Δ*ainS*, Δ*litR* and *litR*$^+$.** HPLC-MS/MS peaks showing the relative abundance of 3OHC10-HSL in supernatants harvested at OD_{600} of 6.0 after growth of the different bacterial strains at 12 °C. LB2.5 was used as a blank. RT: Retention Time, AA: Peak area count.
Full-size DOI: 10.7717/peerj.11980/fig-1

lasted for 22 h when grown at 12 °C compared to 45 h at 6 °C (Fig. 2). At 20 °C, which is a non-optimal temperature for growth of *A. wodanis* in the laboratory (*Soderberg et al., 2019*), the wild type and Δ*ainS* mutant showed a growth deficiency, and the growth halted after reaching an OD_{600} of 2.0–3.0 before it finally reached maximum OD_{600} of 5.0–6.0. On the other hand, the Δ*litR* mutant grew steadily and was able to reach an OD_{600} of 8.0. Neither of the strains grew in liquid media (LB2.5) at 25 °C, and after streaking single colonies of the different strains onto blood agar plates (BA2.5) only the Δ*litR* mutant was able to form small colonies when incubated at 25 °C (Fig. S2). Thus, LitR represses the ability of *A. wodanis* to grow at 20 °C and 25 °C.

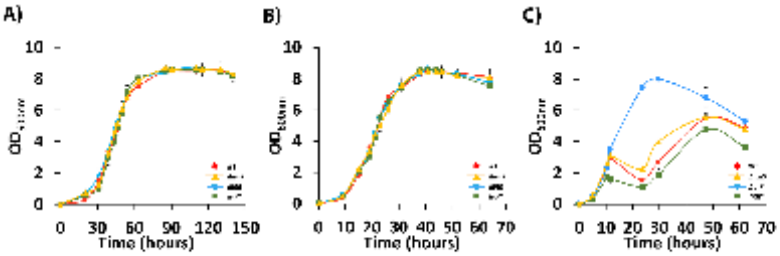

Figure 2 **Growth curves of wild type *A. wodanis* 06/09/139, and the isogenic mutants Δ*ainS*, Δ*litR* and *litR*⁺.** The strains were grown in LB2.5, 220 rpm at 6 °C (A), 12°C (B) and 20 °C (C). The error bars indicate the standard deviation of three biological replicates.

Full-size DOI: 10.7717/peerj.11980/fig-2

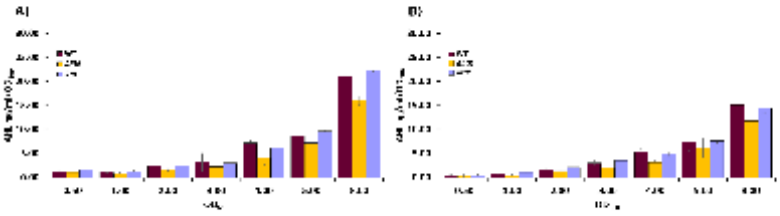

Figure 3 **AHL profiling of supernatants harvested from wild type *A. wodanis* 06/09/139, Δ*litR* and *litR*⁺.** (A) The 3OHC10-HSL concentrations (ng/ml/OD_{600}) were measured in acidified supernatants by HPLC-MS/MS after growth of the different strains at 6 °C and (B) at 12 °C. The error bars indicate the standard deviation of three biological replicates.

Full-size DOI: 10.7717/peerj.11980/fig-3

3OHC10-HSL production in *A. wodanis* is cell density and temperature-dependent, and weakly regulated by LitR

To explore the role of temperature on the production of 3OHC10-HSL in *A. wodanis*, we analyzed supernatants harvested from the wild type 06/09/139 at different cell densities after growth at 6 and 12 °C. The HPLC-MS/MS analyses showed that the 3OHC10-HSL production was detectable from the measurements started at OD_{600} of 0.5 and increased along the growth curve in a cell density-dependent manner. The bacterium produced higher concentrations of 3OHC10-HSL when it was grown at 6 °C compared to at 12 °C ($P < 0.05$, by Students *t* test). Highest 3OHC10-HSL concentrations were measured in the stationary phase (OD_{600} of 8.0) where the wild type reached concentrations of 21.06 ± 0.43 ng/ml/OD_{600} and 15.12 ± 0.94 ng/ml/OD_{600} after growth at 6 °C and 12 °C, respectively (Table S1, Fig. 3).

We also analyzed supernatants harvested from the Δ*litR* mutant to examine if LitR is a regulator of AHL production in *A. wodanis*, similar to what has been shown for other aliivibrios (*Lupp & Ruby, 2004*; *Hansen et al., 2015*). As shown in Fig. 3, the Δ*litR* mutant produced lower concentrations of 3OHC10-HSL than the wild type did in the stationary

phase, and the deletion of *litR* led to an 24% reduction when the maximum concentrations (measured at $OD_{600} = 8.0$) of the wild type and Δ*litR* were compared at 6 °C (WT = 21.06 ± 0.43 ng/ml/OD_{600} and Δ*litR* = 16.01 ± 0.96 ng/ml/OD_{600}; $P < 0.05$ by Students *t* test) and 22% reduction after growth at 12 °C (WT = 15.12 ± 0.94 ng/ml/OD_{600} and Δ*litR* = 11.78 ± 0.94 ng/ml/OD_{600}; $P < 0.05$ by Students *t* test). The complementary mutant *litR*$^+$ behaved as the wild type with regard to 3OHC10-HSL production.

Phenotypic traits regulated by QS in *A. wodanis* 06/09/139

QS is known to regulate several activities or phenotypic traits in vibrios and allivibrios (*Croxatto et al., 2002*; *Zhu et al., 2002*; *Lee et al., 2004*; *Tsou & Zhu, 2010*; *Bjelland et al., 2012*; *Khider et al., 2019*). Therefore, we analyzed the wild type *A. wodanis* 06/09/139 and QS mutants (Δ*ainS* and Δ*litR*) with regard to motility, protease and siderophore production, hemolysis, chitinase activity, biofilm formation and colony morphology. The experiments were performed at 6 °C and 12 °C to determine if the temperature has an influence on the phenotypic traits exhibited by the wild type, Δ*ainS* and Δ*litR* mutants.

Motility

The motility assay showed that the wild type *A. wodanis* 06/09/139 was motile at both 6 °C and 12 °C. The motility of wild type *A. wodanis* was 57% higher at 12 °C compared to at 6 °C (12 °C = 42.17 ± 3.19 mm and 6 °C = 18.00 ± 0.89 mm; $P < 0.05$ by Students *t* test) (Fig. 4A). The Δ*ainS* and Δ*litR* mutants showed significantly higher motility than the wild type at both temperatures. Compared to wild type, the Δ*litR* mutant showed 27% larger motility zones both at 6 °C (WT = 18.00 ± 0.89 mm and Δ*litR* = 24.58 ± 1.74 mm; $P < 0.05$ by Students *t* test) and at 12 °C (WT = 42.17 ± 3.19 mm and Δ*litR* = 57.67 ± 1.97 mm; $P < 0.05$ by Students *t* test). Similarly, the Δ*ainS* mutant showed 17% larger motility zones at 6 °C (WT = 18.00 ± 0.89 mm and Δ*ainS* = 21.67 ± 1.51 mm; $P < 0.05$ by Students *t* test) and 26% larger zones at 12 °C (WT = 42.17 ± 3.19 mm and Δ*ainS* = 57.17 ± 3.87 mm; $P < 0.05$ by Students *t* test) (Fig. 4A, Table S2).

Siderophore production

Siderophores are produced by the bacterium and secreted into the growth medium (*Sandy & Butler, 2009*). Hence, the activity was analyzed in supernatants harvested at OD_{600} of 6.0 from the wild type and mutants after growth at 6 and 12 °C. The CAS assay showed that supernatants harvested from wild type at 12 °C produced 19% larger zones than supernatants harvested at 6 °C (Fig. 4B, Table S2). Siderophore production was negatively affected by the *litR* mutation, and the zones formed by Δ*litR* mutant supernatants were 19% smaller at 6 °C (WT = 14.00 ± 1.00 mm and Δ*litR* = 11.33 ± 1.53 mm; $P < 0.05$ by Students *t* test) and 29% smaller at 12 °C (WT = 17.33 ± 1.15 mm and Δ*litR* = 12.33 ± 1.15 mm; $P < 0.05$ by Students *t* test) when compared to the zones produced by wild type supernatants. The size of siderophore zones formed by the supernatants from Δ*ainS* mutant was not significantly different from the wild type at neither 6 °C nor at 12 °C (Fig. 4B, Table S2).

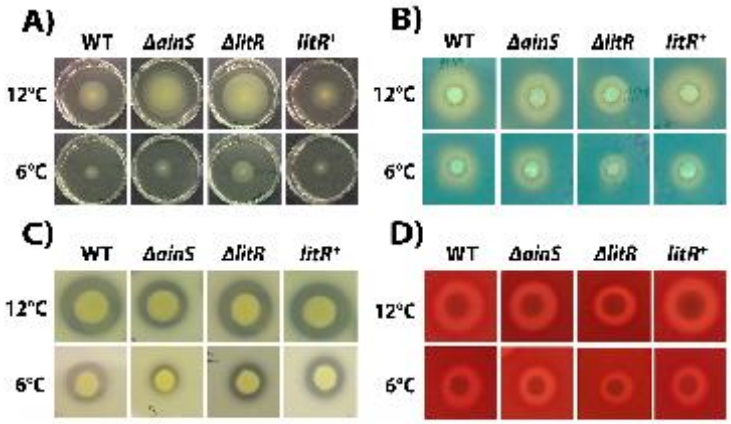

Figure 4 **Motility, siderophore- and protease production, and hemolytic activity in *A. wodanis*, Δ*ainS*, Δ*litR* and *litR*⁺ mutants at 6 °C and 12 °C.** (A) Soft agar plates showing the motility zones after 2 days. (B) Siderophores produced at OD_{600} of 6.0 visible as yellow halos on CAS agar. (C) Protease production visible as cleared zones on skim milk agar plates. (D) Hemolytic zones on blood agar.
Full-size DOI: 10.7717/peerj.11980/fig-4

Protease activity

The protease assay showed that the wild type *A. wodanis* 06/09/139 was able to cleave the skim milk embedded in the agar (Fig. 4C). When compared to wild type, the average proteolytic zone ratio of the Δ*ainS* mutant was 16% smaller at 6 °C (WT = 1.47 ± 0.20 and Δ*ainS* = 1.23 ± 0.08; $P < 0.05$ by Students *t* test) and 17% smaller at 12 °C (WT = 1.78 ± 0.15 and Δ*ainS* = 1.47 ± 0.07; $P < 0.05$ by Students *t* test). The proteolytic zones produced by the Δ*litR* mutant were not significantly different from the ones produced by the wild type at neither 6 °C nor 12 °C (Fig. 4C, Table S2).

Hemolytic activity

The hemolysis assay showed that the wild type *A. wodanis* 06/09/139 was hemolytic (Fig. 4D). The Δ*litR* mutant produced hemolytic zones ratio that were 10% smaller than the corresponding zones produced by the wild type at 6 °C (WT = 1.76 ± 0.07 and Δ*litR* = 1.58 ± 0.07; $P < 0.05$ Students *t* test) and 10% smaller at 12 °C (WT = 1.84 ± 0.03 and Δ*litR* = 1.66 ± 0.06; $P < 0.05$ by Students *t* test). Deletion of *ainS* had no significant effects on the hemolytic activity, as both Δ*ainS* and wild type produced similar hemolytic zones on the blood agar plates ($P > 0.05$ by Students *t* test) (Fig. 4D, Table S2).

Chitinase activity

The assay showed no significant differences ($P > 0.05$ by Students *t* test) in chitinase zones between the wild type and mutants (Δ*ainS* and Δ*litR*) (Fig. S3, Table S2).

Colony morphology and biofilm formation

The ability of the mutants to form biofilm and colony morphology was analyzed in the SWT media with different salt conditions. The strains (*A. wodanis*, ΔainS and ΔlitR) did not form biofilm (Fig. S4). Similarly, on the SWT plates, the colonies of Δ*ainS* and Δ*litR* looked smooth similar to the wild-type with no rugosity both microscopically and macroscopically (Fig. S5).

Cytopathogenic effect on Chinook salmon embryonic cells (CHSE)

To test whether QS affects the cytopathogenic effect (CPE) of *A. wodanis* 06/09/139, CHSE cells were treated with supernatants harvested at different cell densities (OD_{600} of 6.0, 7.0 and 8.0) from the wild type and mutants (Δ*ainS* and Δ*litR*) after growth at 6 and 12 °C. The CPE was observed microscopically (Fig. 5A), and cells that survived and remained attached to the substratum were thereafter quantified using a crystal violet staining method (Figs. 5B and 5C). Supernatants harvested from the wild type had a CPE on the CHSE cells similar to what has been described earlier (*Karlsen et al., 2014*). The temperature at which the bacterium was grown and the time of harvest (cell density) determined the severity of CPE. After growth at 6 °C, wild type supernatants harvested at OD_{600} of 6.0 showed highest CPE with complete lysis of the cells (Figs. 5A and 5B). Wild type supernatants harvested at OD_{600} of 6.0 after growth at 12 °C were less cytotoxic to the cells (Fig. 5A), but the CPE increased with increasing cell density, and after treatment with supernatants harvested at OD_{600} of 8.0 the cells suffered from severe CPE and few cells remained viable and attached to the substratum (Fig. 5C).

Compared to the negative control, some CPE was observed for cells treated with supernatants harvested from the Δ*litR* mutant, but most cells remained intact without losing the cell to cell contact (Fig. 5A). On the other hand, supernatants harvested at OD_{600} of 6.0 from the Δ*ainS* mutant grown at 6 °C induced severe CPE and few cells survived (Figs. 5A and 5B). However, after growth at 12 °C the supernatants harvested from the Δ*ainS* and Δ*litR* induced similar CPE and were less cytotoxic than the corresponding supernatants harvested from the wild type. Hence, in particular, QS and LitR play a role in regulation of CPE towards CHSE cells in *A. wodanis*, and this regulatory role is somewhat stronger at 6 °C compared to at 12 °C (Fig. 5, Table S3).

DISCUSSION

QS is known to regulate several phenotypes or traits in vibrios and aliivibrios such as motility, siderophore production, hemolysis, biofilm formation, protease production and virulence (*Croxatto et al., 2002*; *Zhu et al., 2002*; *Sandy & Butler, 2009*; *Bjelland et al., 2012*; *Yang & Defoirdt, 2015*; *Elgaml & Miyoshi, 2017*; *Balado et al., 2018*).

Before the study presented here, we had a limited knowledge regarding the QS systems in *A. wodanis*. However, from previous genome analysis and HPLC-MS/MS analyses of supernatants we knew that *A. wodanis* encoded two QS systems (LuxS/PQ and AinS/AinR) and produced one AHL (3OHC10-HSL) (*Purohit et al., 2013*; *Hjerde et al., 2015*). Thus, to explore the role of the QS in *A. wodanis*, the essential genes *ainS* and *litR*, were inactivated and their functional roles were investigated when the bacteria was grown at 6 °C and 12 °C.

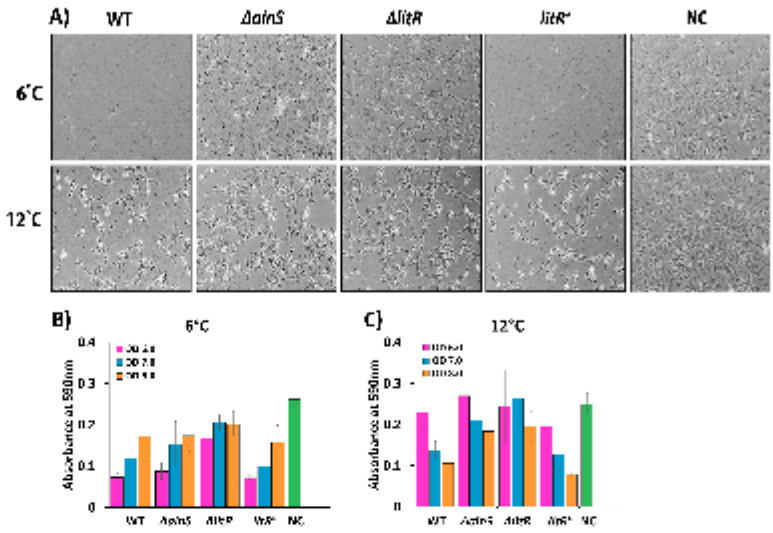

Figure 5 Cytopathogenic effect (CPE) in CHSE cells inoculated with supernatants of wild type *A. wodanis*, Δ*ainS*, Δ*litR* mutants and *litR*⁺ harvested at 6 and 12 °C. (A) CPE observed in CHSE cells treated with supernatants harvested at OD_{600} of 6.0 from strains grown at 6 °C (top) and 12 °C (bottom). The images were taken after 24 h incubation at 12 °C with a Nikon Eclipse TS100 Inverted Phase contrast Microscope at 10x magnification. The bar charts represent the absorbance measured after crystal violet staining the remaining attached CHSE cells after being exposed to supernatants harvested at OD_{600} of 6.0–8.0 from strains grown at 6 °C (B) and 12 °C (C). NC denotes negative control. The error bars indicate the standard deviation of three biological replicates.

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Our study shows that AinS is the autoinducer synthase responsible for cell density dependent 3OHC10-HSL production in *A. wodanis*. This is similar to AinS in *A. salmonicida*, which produces the same type of AHL (3OHC10-HSL) (*Hansen et al., 2015*). Previous studies of pathogenic vibrios and aliivibrios have pointed to a relationship between a temperature closest to disease temperature and AHL production (*Hansen et al., 2015*; *Bhedi et al., 2017*). Similarly, the temperature was found to regulate 3OHC10-HSL production in *A. wodanis*, where the concentration was higher at 6 °C than at 12 °C. Thus, the effect of temperature on 3OHC10-HSL production in *A. wodanis* may correlate to the winter ulcer threshold temperature, 8 °C.

LitR in *A. salmonicida* and *A. fischeri* are activators of AHL production while the LitR homologue VanT in *Vibrio anguillarum* does not affect AHL production (*Croxatto et al., 2002*; *Lupp & Ruby, 2004*; *Hansen et al., 2015*). Interestingly, LitR is only a weak activator of 3OHC10-HSL synthesis in *A. wodanis*, suggesting that other mechanisms may be involved in regulation of AHL production. Several LitR-independent regulations such as AinS autoregulation or cyclic adenosine monophosphate (cAMP) - cAMP receptor protein (CRP)-, response regulator (GacA)-, posttranscriptional regulator (RsmA)- and regulator of

general stress response (RpoS)- mediated regulation have been reported in *A. fischeri* (*Lupp & Ruby, 2004*; *Lyell et al., 2013*). The genome of *A. wodanis* encodes homologs of GacA (AWOD_I_1749), RpoS (AWOD_I_2147 and AWOD_II_1179), RsmA (AWOD_I_2393) and CRP (AWOD_I_0320) (*Hjerde et al., 2015*). However, further studies are needed to investigate if these regulators are involved in 3OHC10-HSL production in addition to LitR in *A.wodanis*.

The *A. wodanis* Δ*litR* mutant exhibited better growth than the wild type and Δ*ainS* mutant at the non-optimal temperatures 20 and 25 °C. At 20 °C the wild type and Δ*ainS* mutant stopped growing in the early log phase and then continued growing after few hours, whereas the Δ*litR* mutant grew well without this pause. This suggests that LitR is a negative regulator of growth and temporarily prevents growth of *A. wodanis* at 20 °C. Others have reported that QS upregulate growth at non-optimal temperatures such as in *A. fischeri* and *A. salmonicida* where deletion of *litR* led to slower growth than their respective wild types (*Fidopiastis et al., 2002*; *Hansen et al., 2015*). However, in some bacteria like *Pseudomonas aeruginosa*, the mutation of QS transcriptional regulators (LasR and RhlR) provided a growth advantage to the *lasR* and *rhlR* mutants over the wild type (*Heurlier et al., 2005*; *Yan et al., 2007*). Moreover, during alkaline stress, the *lasR* mutant in *P. aeruginosa* showed better cell viability than the wild type (*Heurlier et al., 2005*). Bacteria experience various fluctuations in the environment, and suboptimal temperature is a key stressor, which the bacteria have to react and respond to in order to survive. This is well-known from studies with *E.coli* where a temperature shift from 37 to 42 °C results in accumulation of heat shock proteins to maintain homeostasis and later, after the bacteria have adapted to the temperature, the heat shock proteins are down regulated to assist the growth again (*Bukau, 1993*; *Guisbert et al., 2004*). Thus, *A. wodanis* may respond to non-optimal temperatures by inducing heat shock proteins and start to grow again after adapting to the temperature shock. However, the mechanisms LitR may play in the response to stress and non-optimal temperatures needs to be further investigated.

A. wodanis is considered a secondary pathogen in winter ulcer disease, and little is known about virulence factors in this bacterium. In a community, bacteria produce various virulent and non-virulent factors that provide an opportunity for adaptation and survival, such as motility, biofilm formation, siderophore and protease production (*Hibbing et al., 2010*; *Cullen & McClean, 2015*; *Diard & Hardt, 2017*). In this study, we found that *A. wodanis* 06/09/139 was motile and produced siderophores, hemolysin, protease and chitinase. *A. wodanis* grows faster at 12 °C than at 6 °C, and the aforementioned phenotypes or activities were strongest at 12 °C. Deletion of *litR* and *ainS* in *A. wodanis* changed several phenotypes in this study. QS regulation of motility has been shown in numerous *Vibrionaceae* members, where the effect of QS on motility varies between bacteria. QS positively regulates motility in *V. harveyi* and *Vibrio cholerae*, whereas it negatively affects motility in *A. salmonicida, A. fischeri*, *Vibrio parahaemolyticus* and *Vibrio alginolyticus* (*Lupp & Ruby, 2004*; *Nielsen et al., 2006*; *Rui et al., 2008*; *Bjelland et al., 2012*; *Kernell Burke et al., 2015*; *Yang & Defoirdt, 2015*). Similarly, in our study, QS negatively regulates motility in *A. wodanis*. In a planktonic state, bacteria require higher motility to reach towards the host or surface, as they attach, the motility decreases to facilitate colonization

(*Lupp & Ruby, 2005*; *Liu et al., 2008*). Since LitR negatively regulates motility, it is likely that *A. wodanis* is more motile at low cell density and reduces its motility as it reaches higher cell density by activating the LitR-AinS pathway. In *A. fischeri* and *V. cholerae*, hypermotility in QS mutants have led to low colonization of the hosts (*Gardel & Mekalanos, 1996*; *Lupp et al., 2003*; *Lupp & Ruby, 2005*). We speculate that the hypermotile Δ*ainS* and Δ*litR* strains may behave like planktonic cells and result in low colonization in the host.

The motility is often linked to biofilm formation and colony rugosity in many *Vibrio* and *Aliivibrio spp.* (*Yildiz & Visick, 2009*; *Bjelland et al., 2012*; *Jemielita, Wingreen & Bassler, 2018*; *Khider et al., 2019*). In the present study, neither the wild type nor the hypermotile strains (Δ*ainS* and Δ*litR*) formed biofilm or colony rugosity under the tested conditions.

Proteases play an essential role in numerous bacterial biological processes and also act as virulence factors in many pathogens (*Rui et al., 2009*; *Syngkon et al., 2010*). QS master regulators such as VanT in *V. anguillarum*, HapR in *V. cholera*, SmcR in *Vibrio vulnificus*, OpaR in *V. parahaemolyticus* are known to be associated with regulation of proteases (*Croxatto et al., 2002*; *Wang et al., 2011*; *Elgaml & Miyoshi, 2017*; *Chang & Lee, 2018*). However, in *A. wodanis* LitR did not affect protease production. Interestingly, AinS in *A. wodanis* seems to positively affect protease production. This observation suggests that protease production is activated by AinS independently of LitR. LitR independent regulations of proteases have also been reported in other bacteria (*Chancey, Wood & Pierson, 1999*; *Elgaml & Miyoshi, 2017*). As AHL is still produced in the Δ*litR* mutant, the AHL could bind to some unknown LitR-independent regulators and express the wild type proteases. Thus, there is a possible linkage between other regulators and the AHL 3OHC10-HSL.

Deletion of *litR* in *A. wodanis* led to a reduction in siderophores production and hemolytic activity. The bacterium secretes siderophores to acquire iron from the environment and is a potential virulent factor (*Ratledge & Dover, 2000*; *Balado et al., 2018*). The genome of *A. wodanis* encodes two siderophores clusters (AWOD_I_1553-1563 and AWOD_II_0923-0927) and several putative hemolysin genes (AWOD_I_0727, AWOD_I_2361, AWOD_I_2612, AWOD_II_0256 and AWOD_II_1158) with high similarity to *A. salmonicida* and *A. fischeri* MJ11 (*Hjerde et al., 2015*). Hemolysin and siderophores are under QS regulation in other *Vibrionaceae* members (*Gao et al., 2018*; *McRose et al., 2018*). The finding that LitR is a positive regulator of siderophore and hemolysin production suggests that these phenotypes are more significant at high cell densities in *A. wodanis*. However, consistent with the LitR regulation of AHL production, LitR seems only to be a weak activator of siderophore and hemolysin production and may include other regulation mechanisms. In *A. fischeri*, a mutation in *ainS* showed no effect on siderophore production (*Lupp et al., 2003*). Similarly, neither siderophore nor hemolysin production was affected in Δ*ainS* mutant, suggesting that their productions are not dependent on AHL-mediated QS system. Additionally, the performed hemolysis and proteases assays in this study are semi-quantified test and conducted mainly due to their importance in virulence. However, further experiments and quantification methods are required to draw a better conclusion. While QS is known to negatively regulate chitinase in *V. harveyi*, the QS does not affect the chitinase production in *A. fischeri*

(*Defoirdt et al., 2010*; *Cao et al., 2012*). Like *A. fischeri*, deletion of *ainS* and *litR* in *A. wodanis* did not have an effect on chitinase production, suggesting the production is independent of the QS system.

In the study of Karlsen et al. supernatants (OD_{600} of 6.0–7.5) harvested from *A. wodanis* grown at 8 °C were cytotoxic to four different salmon cell lines including CHSE (*Karlsen et al., 2014*). Similarly, in the work presented here *A. wodanis* supernatants caused CPE on CHSE, but the severity varied with time of harvest and the temperature at which the bacterium was grown. When grown at 6 °C, a severe CPE was observed with supernatants harvested in the early in the stationary phase (OD_{600} of 6.0). However, when the cells were exposed to supernatants harvested at later stages in the stationary phase more cells survived and remained attached. This suggests that the factor(s) responsible for causing cell death is more strongly expressed early in the stationary phase at this temperature. The situation is opposite when the wild type was grown at 12 °C where a higher cell density seems to be vital for expression of the cytotoxic factor(s). Thus, if QS is involved in regulation of CPE the "quorum" needed to turn on this activity may be achieved at lower cell densities when the bacterium is grown at 6 °C compared to at 12 °C. Several pathogenic vibrios such as *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus* use QS to regulate cytotoxicity (*Cao et al., 2010*; *Gode-Potratz & McCarter, 2011*; *Shao et al., 2011*; *Gao et al., 2018*). Similarly, our results show that LitR and AinS are activators of cytopathogenicity. However, only the *litR* mutation led to reduced CPE when the CHSE cells were treated with supernatants harvested after growth at 6 °C suggesting that the cytotoxic effect is independent of AinS and AHL mediated QS at this temperature. In addition to the AinS/AinR system *A. wodanis* encodes the genes needed for the LuxS/LuxPQ system. Perhaps at 6 °C, the virulence or CPE is more dependent on this latter QS system or, so far, other unknown factors.

The temperature has been shown to regulate QS in some bacteria, where a difference in phenotypes between the wild type and QS mutants is clearly different at one temperature compared to another. This was seen for *A. salmonicida* where a *litR* mutation led to biofilm formation and rugose colonies when the bacteria were grown at a low temperature (*Hansen et al., 2014*). However, when the same bacteria were grown at higher temperatures the Δ*litR* mutant behaved like the wild type and was not able to produce biofilm and rugose colonies (*Hansen et al., 2014*). AinS in *A. salmonicida* produces the same AHL as *A. wodanis* in addition to seven LuxI produced AHLs, and the concentration of 3-OH-C10-HSL was much higher at low temperature (*Hansen et al., 2015*). *A. wodanis* is not able to produce rugose colonies or biofilm, and the difference in AHL production at different temperatures is modest. However, the different QS regulated phenotypes are expressed at 6 °C in *A. wodanis*, and at this temperature the CHSE cells showed highest CPE.

CONCLUSION

Based on the findings presented in this study, *A. wodanis* 06/09/139 produces some virulent factors that may be used for inter- or intraspecies co-operation and competition for niche adaptations during winter ulcer development. Many bacteria use AHL-mediated QS for

regulation of various phenotypic traits (*Papenfort & Bassler, 2016*). Like other *Vibrionaceae* members, the LuxS/PQ and AinS/AinR QS systems in *A. wodanis* probably convey into the same cascade to activate LitR and downstream genes. In this study, we found that AinS is responsible for autoinducer production. We have shown that temperature is an essential factor in regulating AHL production, growth and cytotoxicity. Although QS in *A. wodanis* may not be a crucial activator or repressor of virulence-associated phenotypic traits, the minor role in regulation can add knowledge to the winter ulcer disease development. The regulatory mechanisms other than QS that regulates the phenotypic traits in *A. wodanis* need to be further investigated.

Abbreviations

AA	Peak area count
AHL	N-acyl-homoserine-lactone
3OHC10-HSL	N-3-hydroxy-decanoyl-homoserine-lactone
AI	Autoinducers
CAS	Chrome azurol S
cAMP	cyclic adenosine monophosphate
CHSE	Chinook Salmon Embryo Cells
CPE	Cytopathogenic Effect
CRP	cAMP receptor protein
HPLC-MS/MS	High performance liquid chromatography mass spectrometry
LB	Luria-Bertani
Mm	millimetre
OD_{600}	Optical density measured at 600nm
PCR	Polymerase chain reaction
QS	Quorum Sensing
RT	Retention Time
WT	Wild type

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Amudha Deepalakshmi Maharajan conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Hilde Hansen conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Miriam Khider conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Nils Peder Willassen conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The sequences of mutations performed for genes litR AWOD_I_0419 and ainS AWOD_I_1040 are available in a Supplementary File.

The *A.wodanis* 06/09/139 genome are available at NCBI: GCA_000953695.1, PRJEB6963, LN554848, LN554849, LN554847, LN554850, LN554851, LN554846.

Data Availability

The following information was supplied regarding data availability:

The raw measurements are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11980#supplemental-information.

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Additional file 1

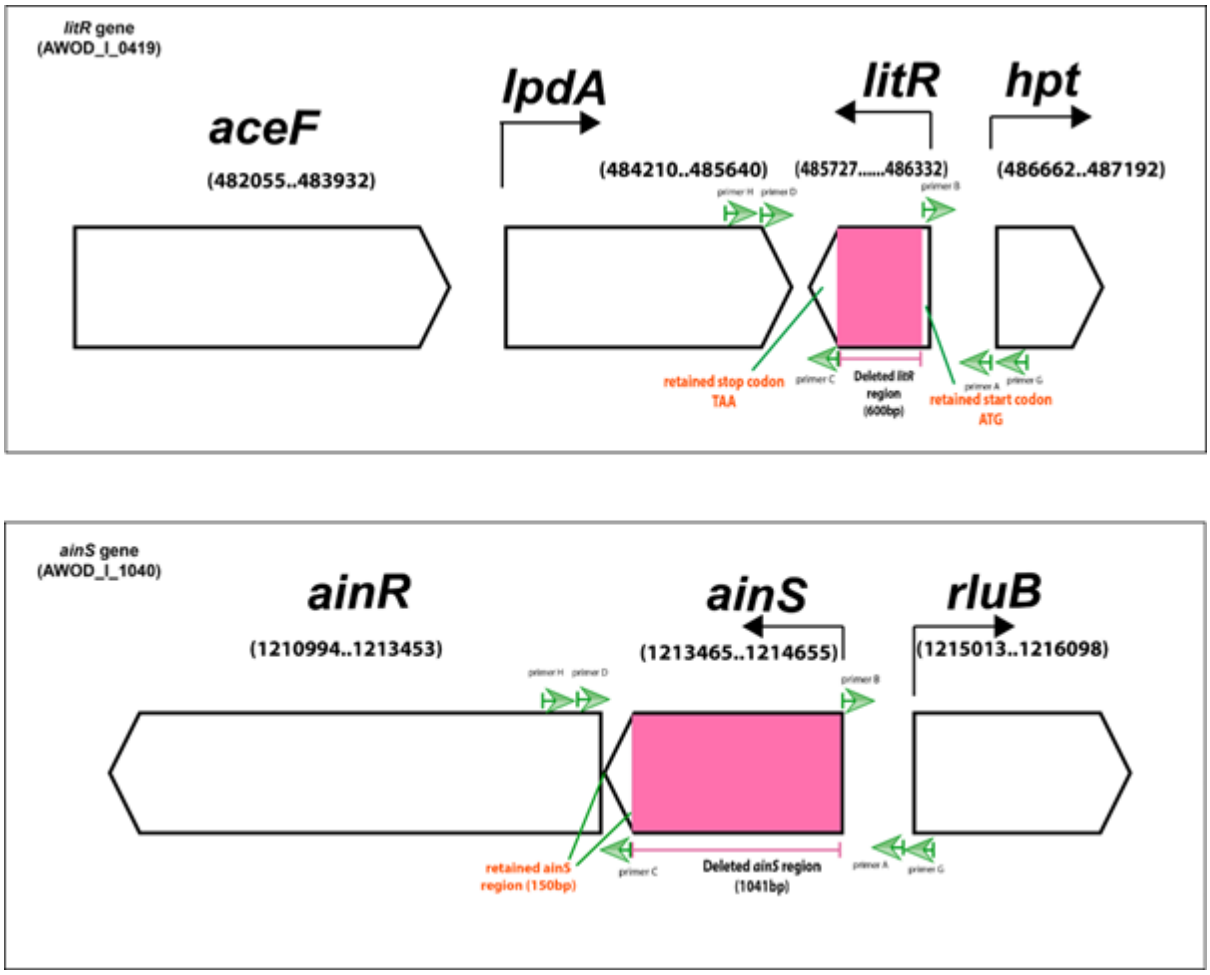

Figure S1. Schematic representation of *litR* and *ainS* gene context of *A. wodanis* 06/09/139. Pink blocks indicate the deleted region. Green arrows indicate the primer-binding sites. Black arrows indicate the transcription start sites. Green lines indicate the retained gene region. Gene position in the genome is presented in parentheses.

Additional file 2

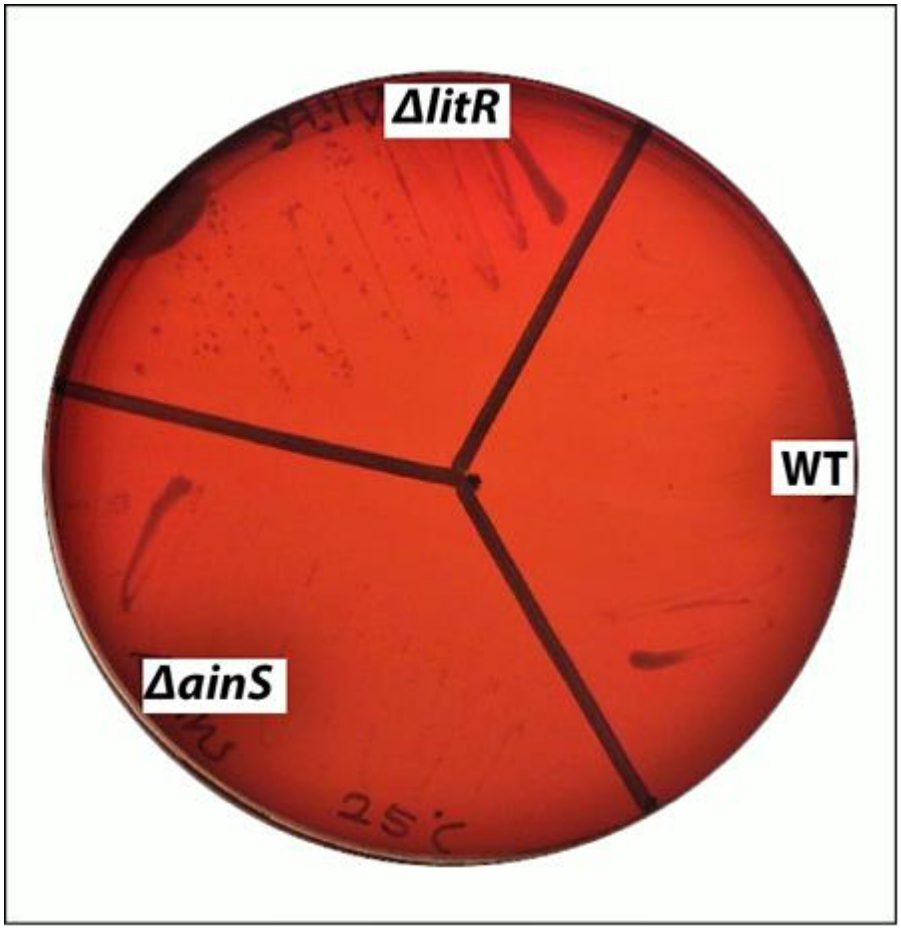

Figure S2. Growth of wild type *A. wodanis* 06/09/139, *ΔainS* and *ΔlitR*. The strains were grown on BA 2.5 plates, at 25 °C for 2 days.

Additional file 3

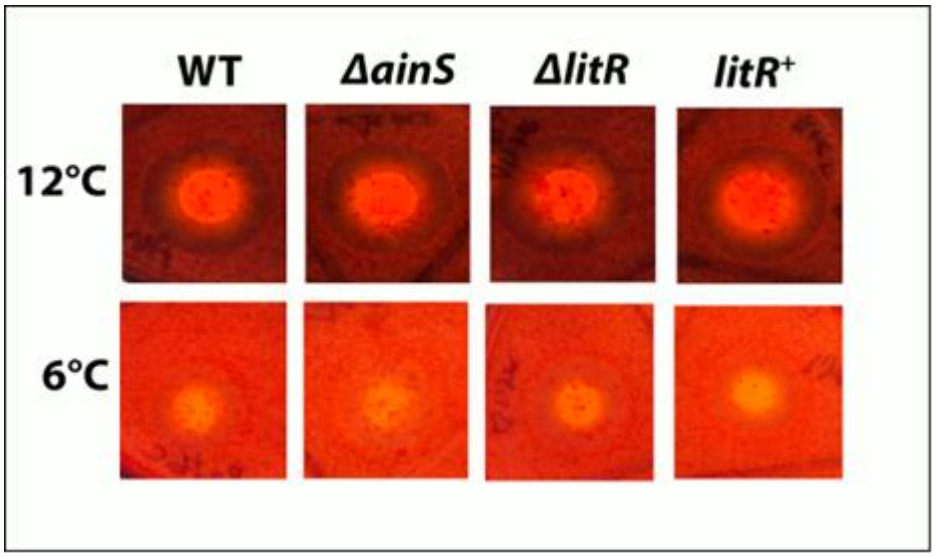

Figure S3. Chitinase production of wild type, *ΔainS*, *ΔlitR* and *litR*$^{+}$. The bacterial cultures were spotted on colloidal chitin plates and incubated at 6 and 12°C. The zones were measured after 4 days.

Additional file 4

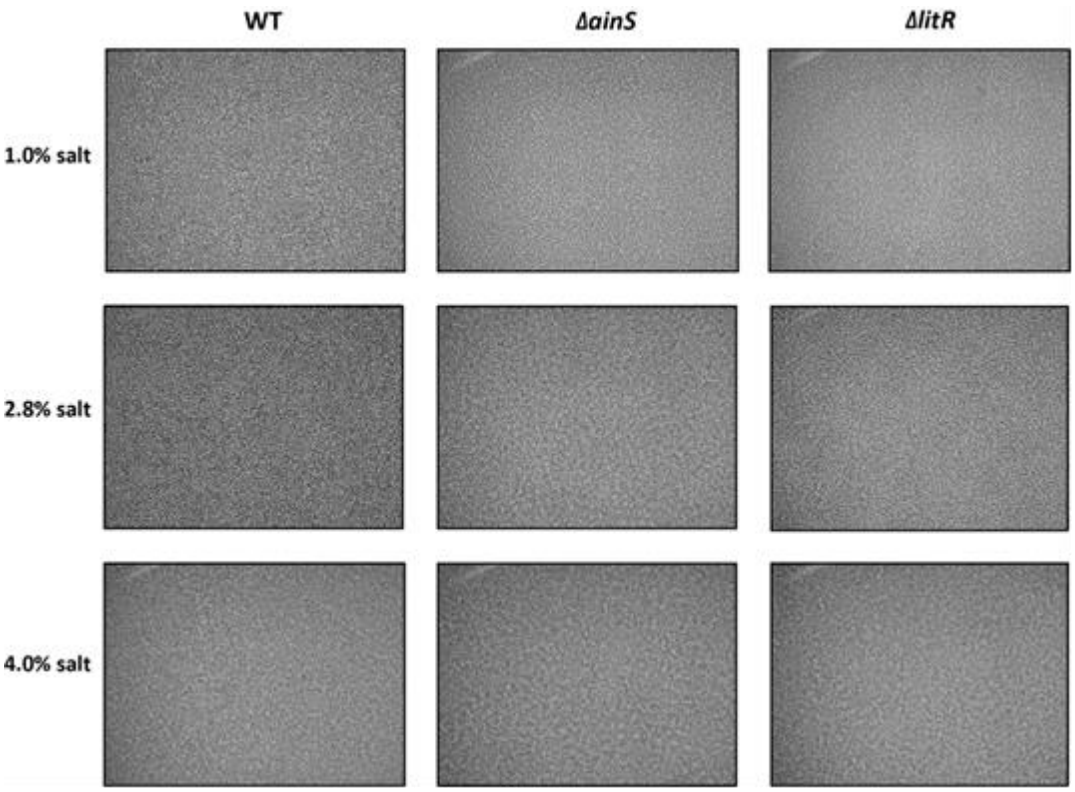

Figure S4. Biofilm assay of wild type, *ΔainS* and *ΔlitR* in SWT media at 6°C. Biofilm assay of wild type, *ΔainS* and *ΔlitR* in SWT media at 6°C. Cultures were visualized using Ziess Primo Vert microscope at 10x magnification and was photographed with AxioCam ERc5s after 2 days of incubation.

Additional file 5

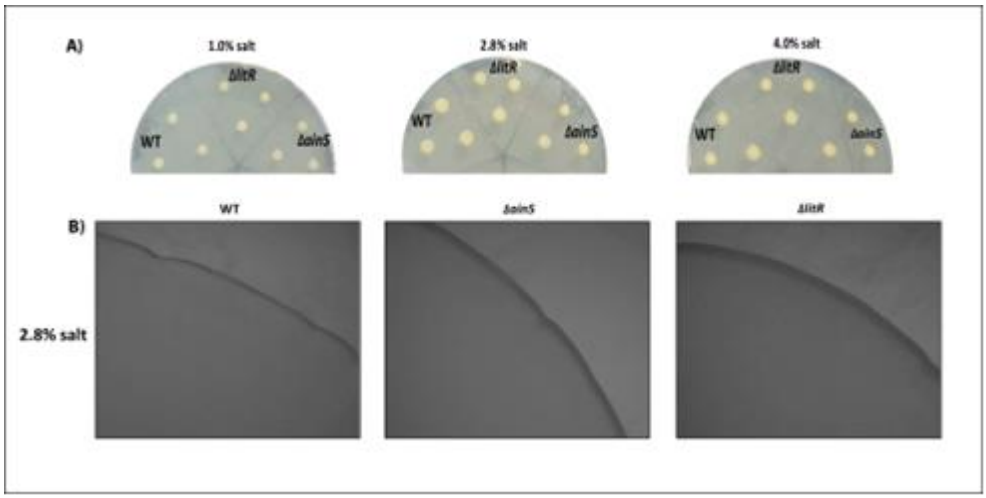

Figure S5. Colony morphology of wild type, *ΔainS* and *ΔlitR* on SWT plates at 6°C. (A) The colonies on 1.0%, 2.8% and 4.0% SWT plates were photographed after 3 days of incubation. (B) Colony morphology was visualized using Zeiss primo vert microscope at 4x magnification and was photographed with Axiocam ERc5s after two weeks of incubation.

Additional file 6

Table S1. 3OHC10-HSL concentrations produced by wild type *A. wodanis* 06/09/139, *ΔlitR* and *litR*$^+$ at different cell densities and temperatures.

	3OHC10-HSL production (ng/ml/OD_{600})					
	6°C			12°C		
OD_{600nm}	WT	*ΔlitR*	*litR*$^+$	WT	*ΔlitR*	*litR*$^+$
0.5	1.25± 0.14	1.05± 0.19	1.51± 0.11	0.51± 0.10	0.37± 0.20	0.54± 0.23
1.0	1.11± 0.14	0.92± 0.07	1.32± 0.16	0.74± 0.09	0.50± 0.16	1.04± 0.18
2.0	2.46± 0.53	1.61± 0.17	2.35± 0.24	1.67± 0.11	1.37± 0.29	2.18± 0.14
3.0	3.26± 1.88	2.25± 0.15	3.04± 0.16	3.07± 0.39	2.01± 0.29	3.51± 0.16
4.0	7.17± 0.49	4.21± 1.47	6.21± 0.46	5.43± 0.52	3.14± 0.29	4.83± 0.43
5.0	8.80± 0.24	7.17± 0.96	9.67± 0.05	7.50± 1.89	6.27± 2.06	7.67± 0.50
8.0	21.06± 0.43	16.01± 0.96	22.26± 0.17	15.12± 0.94	11.78± 0.94	14.41± 0.91

Additional file 7

Table S2. Phenotypic activities tested for *A. wodanis* 06/09/139, *ΔainS*, *ΔlitR* and *litR*$^+$ at 6°C and 12°C.

Phenotypes	WT		*ΔainS*		*ΔlitR*		*litR*$^+$	
	12°C	6°C	12°C	6°C	12°C	6°C	12°C	6°C
Motility Zone (mm)	42.17 ± 3.19	18.00 ± 0.89	57.17 ± 3.87†	21.67 ±1.51†	57.67 ± 1.97†	24.58 ± 1.74†	42.67 ± 4.59	19.33 ± 0.52
Siderophore Zone (mm)	17.33 ± 1.15	14.00 ± 1.00	15.50 ± 1.32	13.67 ± 1.15	12.33 ± 1.15†	11.33 ± 1.53†	18.00 ± 2.00	13.67 ± 1.15
Protease zone ratio	1.78± 0.15	1.47± 0.20	1.47± 0.07†	1.23± 0.08†	1.72± 0.10	1.42± 0.19	1.73± 0.06	1.42± 0.25
Hemolytic zone ratio	1.84± 0.03	1.76± 0.07	1.81± 0.06	1.83± 0.07	1.66± 0.06†	1.58± 0.07†	1.74± 0.06	1.75± 0.04
Chitinase zone ratio	2.28± 0.14	2.28± 0.12	2.23± 0.12	2.28± 0.12	2.28± 0.14	2.30± 0.10	2.28± 0.14	2.19± 0.20

† Symbol denotes significant difference ($P < 0.05$) between *ΔlitR*/*ΔainS* mutants and wild type *A. wodanis*

Additional file 8

Table S3. Absorbance measured after crystal violet staining of CHSE cells treated with supernatants harvested from strains grown at 6°C and 12°C.

	Absorbance measured at 590nm				
	WT	***ΔainS***	***ΔlitR***	***litR*⁺**	**Negative control**
12°C					
OD_{600nm} = 6.0	0.23 ± 0.04	0.27 ± 0.09	0.24 ± 0.04	0.19 ± 0.03	0.25 ± 0.02
OD_{600nm} = 7.0	0.13 ± 0.02	0.20 ± 0.03	0.26 ± 0.03	0.12 ± 0.03	
OD_{600nm} = 8.0	0.10 ± 0.01	0.18 ± 0.04	0.19 ± 0.03	0.07 ± 0.01	
6°C					
OD_{600nm} = 6.0	0.07 ± 0.01	0.09 ± 0.02	0.17 ± 0.01	0.07 ± 0.01	0.26 ± 0.02
OD_{600nm} = 7.0	0.12 ± 0.02	0.15 ± 0.01	0.20 ± 0.02	0.10 ± 0.02	
OD_{600nm} = 8.0	0.17 ± 0.05	0.17 ± 0.03	0.20 ± 0.03	0.16 ± 0.04	

Paper 2

Quorum Sensing Controls the CRISPR and Type VI Secretion Systems in *Aliivibrio wodanis* 06/09/139

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Quorum Sensing Controls the CRISPR and Type VI Secretion Systems in *Aliivibrio wodanis* 06/09/139

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For bacteria to thrive in an environment with competitors, phages and environmental cues, they use different strategies, including Type VI Secretion Systems (T6SSs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) to compete for space. Bacteria often use quorum sensing (QS), to coordinate their behavior as the cell density increases. Like other aliivibrios, *Aliivibrio wodanis* 06/09/139 harbors two QS systems, the main LuxS/LuxPQ system and an N-acyl homoserine lactone (AHL)-mediated AinS/AinR system and a master QS regulator, LitR. To explore the QS and survival strategies, we performed genome analysis and gene expression profiling on *A. wodanis* and two QS mutants (Δ*ainS* and Δ*litR*) at two cell densities (OD600 2.0 and 6.0) and temperatures (6 and 12°C). Genome analysis of *A. wodanis* revealed two CRISPR systems, one without a *cas* loci (CRISPR system 1) and a type I-F CRISPR system (CRISPR system 2). Our analysis also identified three main T6SS clusters (T6SS1, T6SS2, and T6SS3) and four auxiliary clusters, as well about 80 potential Type VI secretion effectors (T6SEs). When comparing the wildtype transcriptome data at different cell densities and temperatures, 13–18% of the genes were differentially expressed. The CRISPR system 2 was cell density and temperature-independent, whereas the CRISPR system 1 was temperature-dependent and cell density-independent. The primary and auxiliary clusters of T6SSs were both cell density and temperature-dependent. In the Δ*litR* and Δ*ainS* mutants, several CRISPR and T6SS related genes were differentially expressed. Deletion of *litR* resulted in decreased expression of CRISPR system 1 and increased expression of CRISPR system 2. The T6SS1 and T6SS2 gene clusters were less expressed while the T6SS3 cluster was highly expressed in Δ*litR*. Moreover, in Δ*litR*, the *hcp1* gene was strongly activated at 6°C compared to 12°C. AinS positively affected the *csy* genes in the CRISPR system 2 but did not affect the CRISPR arrays. Although AinS did not significantly affect the expression of T6SSs, the hallmark genes of T6SS (*hcp* and *vgrG*) were AinS-dependent. The work demonstrates that T6SSs and CRISPR systems in *A. wodanis* are QS dependent and may play an essential role in survival in its natural environment.

Keywords: CRISPR, T6SS, QS, *Aliivibrio wodanis* 06/09/139, LitR and AinS

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INTRODUCTION

Quorum sensing (QS) is a cell density-dependent cell-to-cell communication system in which bacteria produce and respond to signaling molecules called autoinducers (AIs), which subsequently activates the QS transcriptional regulator to control specific functions such as bioluminescence, motility, biofilm, secretion and virulence (1–5). Multiple QS systems have been described in several *Vibrio* species (6). Two QS systems, AinS/AinR and LuxS/LuxPQ that are believed to work through phosphorelay mechanism have been identified in the genome of *Aliivibrio wodanis* (7). The AinS/AinR QS system produces AI-1 known as N-acyl homoserine lactones (AHLs) and is present in many Gram-negative bacteria (2, 8). These AHL-mediated QS systems are used for intra-species communication (8, 9). The LuxS/LuxPQ QS system is present in a wide variety of Gram-negative and Gram-positive bacteria, and produces the AI-2 called furanosyl borate diester and is involved in inter-species communication (10, 11). These two QS systems are known to work in parallel in *Vibrio harveyi*, *Aliivibrio fischeri* and *Vibrio cholerae* (12–14). At low cell density, when the AI concentrations are low, the AI receptors act as kinases and relay phosphate to the RpoN (σ54)-dependent activator LuxO via phosphotransferase LuxU. This, in turn, activates the expression of *qrr* sRNAs, which together with RNA chaperone Hfq represses translation of the mRNA encoding the master regulator *LitR* (9, 14, 15). At high cell density, the signaling molecules reach a threshold concentration and bind to the receptors to dephosphorylate LuxO, which terminates the *qrr* sRNA transcription. In the absence of Qrr sRNA, *litR* is activated to regulate hundreds of genes (1, 14, 16).

In the environment, bacteria co-exist in communities with multiple competitors, including other bacterial species and phages, and have to respond to various cues such as changes in temperature, nutrient and iron availability, pH, osmolarity and salinity (17–22). Hence, bacteria have developed various strategies such as protein secretion, contact-dependent growth inhibition, bacteriocin production and antibiotic production to survive and thrive (23–27). Some strategies are not necessarily harmful to competitors, such as adhesion, exopolysaccharide production, siderophore production, motility, biofilm formation, heat shock response and quorum quenching (28–34). Other strategies developed, such as defense mechanisms against phages or mobile genetic elements (MGEs), including restriction-modification, receptor modification and clustered regularly interspaced short palindromic repeats-CRISPR associated (CRISPR-Cas) are for protection (35–37).

The CRISPR-Cas system is an adaptive immune system against invading nucleic acids from phages and other MGEs and is composed of *cas* genes, a leader sequence and a CRISPR array with repeats separated by several spacer sequences (35, 38). Two CRISPR classes have been identified with six main types and several subtypes, which are categorized based on the types of *cas* genes, direct repeats and gene arrangement where class 1 includes the type I, III and IV whereas type II, V and VI belong to class 2 (39–41). Several *Vibrio* species harbor the type I CRISPR system classified into subtypes such as type I-A, I-B, I-C, I-D, I-E and I-F (40, 42, 43). QS regulation of CRISPR system has been described in bacteria like *Pseudomonas aeruginosa*, *Serratia* sp. and *Chromobacterium violaceum* (44–47).

QS regulates type VI secretion systems (T6SSs) in several vibrios such as *V. cholerae*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio fluvialis* and *Vibrio alginolyticus* (48–52). T6SS is one of the largest contact-dependent secretion system bacteria use to transport T6SS effectors (T6SEs) into eukaryotic hosts, bacterial competitors or the environment (53–56). In some bacteria, T6SSs are also known to be involved in the uptake of metal ion (57, 58). The T6SS was first identified in *V. cholerae* as a virulence-associated secretion (*vas*) gene cluster and later in many other bacteria (59–61). The T6SEs are toxin molecules with anti-bacterial or anti-eukaryotic activity (62–64). Several anti-bacterial effector molecules such as amidases, glycoside hydrolases, lipases, phospholipases and nucleases and anti-eukaryotic effectors such as VasX, the Multifunctional-autoprocessing repeats-in-toxin and EvpP have been identified (65–69). T6SS gene clusters often encode immunity proteins close to the effector genes in order to neutralize their effector molecules to prevent self or sibling-killing (70). For instance, immunity proteins such as antitoxin TsaB in *V. cholerae* have been reported to protect self-killing against effectors VgrG-3 and Tse2, respectively (71). In addition, immunity protein-independent mechanisms like envelope stress response and two-component systems can facilitate self-protection (72).

A. wodanis is a Gram-negative, rod-shaped, non-luminescent and motile bacterium with multiple polar flagella (73). *A. wodanis* strains grow in the range of temperatures and salt concentrations between 4–25°C and 1–4% respectively (73). The genome of *A. wodanis* 06/09/139 contains two chromosomes and 4 plasmids (7). *A. wodanis* has been repeatedly isolated together with *Moritella viscosa (M. viscosa)* from Atlantic salmon (*Salmo salar*) during outbreaks of winter ulcer that mainly occurs at a temperature below 8°C (74, 75). Infected fish can survive when the temperature rises above 8°C (74, 76). Winter ulcer causes mortality and significant losses in farming industry and is characterized by large ulcers, hemorrhages and internal tissue necrosis in the infected fish (74, 77). Although *M. viscosa* is the primary agent for the disease, the role of *A. wodanis* and the mechanism behind the co-existence with *A. wodanis* in the winter ulcers are still unclear (74, 77). Experimental study reproducing field observation reveal that *A. wodanis* affects the progression of *M. viscosa* infection and is responsible for the chronic pathogenesis in fish (78). *A. wodanis* adheres to Atlantic salmon head kidney cells and in a bath challenge, *A. wodanis* separately produces clinical symptoms such as fin rot and other internal pathological symptoms in Atlantic salmon and it can co-infect Atlantic salmon together with *M. viscosa* (78). In a co-cultivation experiment, *A. wodanis* impedes the growth of *M.*

Abbreviations: AHL, N-acyl homoserine lactone; Aux, Auxiliary; Bp, Base pair; CHSE, Chinook salmon embryo; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DEGs, Differentially expressed genes; FC, Fold change; HCD, High cell density; LCD, Low cell density; MGE, Mobile genetic element; Min, minutes; OD_{600}, Optical density measured at 600 nm; QS, Quorum sensing; RHS, Rearrangement hotspot protein; RPKM, reads per kilobase of gene per million reads mapped; T6SE, Type VI Secretion Effector; T6SS, Type VI Secretion System; tp, Transcriptome profile.

viscosa and when both the bacteria were implanted together in fish abdomen, *A. wodanis* alters the gene expression of *M. viscosa* (7). It has been further hypothesized that *A. wodanis* perhaps uses bacteriocin to impede the growth and virulence of *M. viscosa* (7). In our previous studies we have reported that *A. wodanis* produces one AHL and encodes two QS systems (7, 79). In the cell culture studies, *A. wodanis* is known to be cytotoxic to different salmon cell lines when treated with supernatants harvested at cell densities higher than OD_{600} (optical density measured at 600 nm) of 6.0 (78, 80). Moreover, in a HPLC-MS/MS analysis, the AHL production in *A. wodanis* begins at the early log phase and increases with increase in cell density along the growth curve (80). Hence in this transcriptomics study, we chose two cell densities one at the early log phase (OD_{600} 2.0) and the other at the cell density close to the end log phase (OD_{600} 6.0) to study the role of cell densities in gene expression. Furthermore, in our recent study, we found that the temperature 6°C which is lower than the winter ulcer disease threshold temperature 8°C, has more impact on AHL production and cytotoxicity in CHSE cell line than 12°C (80). Therefore, in this study, we wanted to analyze the effects of 6°C and 12°C in gene expression. We have also shown that *A. wodanis* uses the QS to regulate growth, motility, siderophore- and protease production, hemolysis as well as cytotoxicity in the Chinook salmon embryo (CHSE) cell line (80). Considering the importance of understanding the QS and survival strategies in *A. wodanis*, we performed genome analysis and RNA sequencing (RNA-Seq) to reveal the global gene expression in the wild type and its QS mutant strains Δ*ainS* and Δ*litR*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *A. wodanis* 06/09/139 used was originally isolated from the head kidney of an Atlantic salmon on the west coast of Norway in 2006 (78). The construction of the Δ*ainS* and Δ*litR* in-frame mutants by allelic exchange has been described in a recent study (80). *A. wodanis* 06/09/139 and the mutants from glycerol stocks were grown at 12°C for 3 days on Luria-Bertani Agar (Difco BD Diagnostics Sparks, MD, USA) with a total concentration of 1.0% (wt/vol) 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). The pH of the media was adjusted to 7.5. Three biological replicates of pre-cultures of *A. wodanis* 06/09/139 and the mutants Δ*ainS* and Δ*litR* were grown from a single colony in 2 ml of Luria-Bertani Broth (LB2.5) overnight at 12°C, 220 rpm.

RNA Extraction and rRNA Depletion

Pre-culture biological triplicates of *A. wodanis* 06/09/139, Δ*ainS* and Δ*litR* were diluted to a start OD_{600} of 0.01 in LB2.5 in a final volume of 60 ml using a 250 ml baffled flasks. The cultures were grown further in parallel using two Infors HT Multitron incubators set at 6 and 12°C at 220 rpm. The cultures were diluted 1:10 for OD_{600} measurement and harvested at low cell density (LCD) OD_{600} of 2.0 and high cell density (HCD) OD_{600} of 6.0. A small culture volume (1 ml) was harvested and mixed with two volumes of RNAprotect Bacteria reagent (Qiagen, Hilden, Germany). The treated cultures were then incubated for 5 min at room temperature and vortexed for a few seconds before centrifuging at 13,000 rpm for 2 min in a cold Heraeus fresco 21 centrifuge (Thermo Scientific, Waltham, MA, USA). The pellets were flash-frozen with liquid nitrogen and stored at −80°C until RNA isolation. The total RNA from the cell pellets was isolated using the Masterpure™ complete DNA/RNA purification kit (Epicenter, Madison, WI, USA) according to the manufacturer's instructions. The RNA concentration was measured in NanoDrop™ 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). Ribosomal RNA (rRNA) was depleted from the total RNA using a Ribo-zero rRNA removal kit for bacteria (Illumina, San Diego, CA, USA). The RNA quality before and after rRNA depletion was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

RNA Sequencing and Data Analysis

The mRNA libraries were prepared using the TrueSeq stranded mRNA library kit (Illumina, San Diego, CA, USA) and sequenced on Nextseq 500 (Illumina, USA) using 150 cycles mid-output kit and run as a 75 bp paired-end reads at Norwegian Sequencing Center. The image analysis and base calling were performed using Illumina's RTA software version 2.4.6. Reads with low base call quality were removed using Illumina's default chastity criteria. The quality of the raw sequencing data was controlled using FastQC version 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The gene expression levels were determined using EDGE-pro v1.0.1 (81) and DESeq2 (82) with default parameters. EDGE-pro was used to align the reads to the reference genome (GCA_000953695.1) and convert the raw coverage into reads per kilobase of gene per million reads mapped (RPKM). DEseq2 was used to estimate the comparative differential gene expression values and provide the output as a log2fold change value with *p-value*. The log2fold change values were converted into fold change (FC) values, and the cutoff values of $\geq$ 2.0 or ≤ -2.0 with padj values of < 0.05 were counted as significantly differentially expressed genes (DEGs). The transcriptome profiles of each strain was compared as high cell density against low cell density (tpHCD/LCD), high growth temperature 12°C against low temperature 6°C (tp12°C/6°C) and mutants against wild type (tpΔ*litR*/WT and tpΔ*ainS*/WT). These abbreviations of comparisons are used throughout this paper. The RNA sequence data of wild type *A. wodanis*, Δ*ainS* and Δ*litR* have been deposited in the European Nucleotide Archive (www.ebi.ac.uk/ena) under study accession number PRJEB34433.

Functional Gene Family and Pathway Analysis

KEGG BRITE and KEGG pathway mapper were used to map the DEGs (FC values $\geq$ 2.0 or ≤ -2.0) found when wild type was compared against cell densities (tpHCD/LCD) and temperatures (tp12°C/6°C) and also when mutants were compared against wild type (tpΔ*litR*/WT and tpΔ*ainS*/WT) at different cell

densities and temperatures against the reference organism *A. wodanis* 06/09/139 (83).

Identification of CRISPR Systems, Protospacers and Prophage

The CRISPR-Cas operon in *A. wodanis* 06/09/139 genome was identified using CRISPRCasFinder (84), and a homology search was performed using KEGG Sequence Similarity Database (SSDB) (83). An intergenic distance of < 100 bp between genes was considered as a single operon in this study. The CRISPR arrays with direct repeats and spacers were identified from the genome of *A. wodanis* using the CRISPR finder tool (85), while the protospacers were identified using CRISPR target (86). The prophages in *A. wodanis* were identified using the phage search tool (PHASTER) (87, 88).

Identification of Type VI Secretion Systems and Type VI Secretion Effectors

The T6SS gene clusters in *A. wodanis* 06/09/139 were identified using SecReT6 (89) and a homology search against other T6SS gene clusters using KEGG SSDB database (83). The naming of T6SS genes of *A. wodanis* 06/09/139 in this study follows the naming of *V. cholerae* T6SS (90). Potential T6SEs in *A. wodanis* were predicted using the Bastion 6 tool (91).

RESULTS

We have previously shown that *A. wodanis* 06/09/139 produces one AHL (3OHC10-HSL) and encodes genes for QS (7, 79). Moreover, in a recent study, the QS system in *A. wodanis* 06/09/139 was found to affect various phenotypes that are possibly linked to the winter ulcer disease development (80). Genome analysis and transcriptome profiling was therefore performed to further study the QS and survival strategies in *A. wodanis* by comparing wild type and its Δ*litR* and Δ*ainS* mutants at low (OD_{600} of 2.0) and high cell density (OD_{600} of 6.0) and two different temperatures, 6 and 12°C.

Differential Expressed Genes

The transcriptome profiles of *A. wodanis* and mutant strains Δ*litR* and Δ*ainS* at different cell densities and temperatures are listed in **Supplementary Table 1**. The average of mapped reads to the reference genome of *A. wodanis* was above 90% in all samples suggesting that the transcriptome data were sufficient for further analysis.

The total number of DEGs at all tested conditions are shown in **Figure 1**, and the complete lists of all DEGs are given in **Supplementary Table 2**. When comparing the transcriptome profiles at HCD relative to LCD (tpHCD/LCD) of the wild type after growth at 6°C, 18% of the total genes (n = 4,282) of *A. wodanis* were differentially expressed. Increasing the growth temperature to 12°C lowered this number of DEGs to 15%. Among the tested tpHCD/LCD conditions of wildtype, Δ*litR* and Δ*ainS* (**Figure 1A**), the highest numbers of DEGs were observed for the Δ*litR* mutant at 6°C, where the number of DEGs account for about 32% of the total genes in of *A. wodanis*. On the other side, the lowest number of DEGs (8% of the total 4282 genes) was observed in the Δ*litR* mutant tpHCD/LCD at 12°C. The *ainS* mutant tpHCD/LCD at 6°C and 12°C showed 15 and 18% DEGs, respectively. Further, the DEGs in tpHCD/LCD (WT_12°C) and tpHCD/LCD (WT_6°C) were sorted using KEGG BRITE and KEGG pathway mapper using *A. wodanis* 06/09/139 as a reference organism. The functional families with at least 15 DEGs were *enzymes, transporters, secretion system, bacterial motility proteins* and *ribosome* as shown in **Supplementary Figure 1A**. In addition, in the wild type tpHCD/LCD comparison, at 6°C, a few DEGs (n = 5) with FC values ≥ 2.0 were also mapped into the functional families *prokaryotic defense system*, which includes the CRISPR related genes. The KEGG pathway mapping revealed that the DEGs (n ≥ 30) in the wild type tpHCD/LCD were involved in *metabolic pathways, microbial metabolism in diverse environments, biosynthesis of secondary metabolites, ABC transporters, biosynthesis of antibiotics, two-component system, carbon metabolism* and the *ribosome*. The numbers of DEGs involved in these pathways were similar in wild type tpHCD/LCD at both temperatures except in the *ribosome* pathway. At tpHCD/LCD (6°C), 42 DEGs mapped into *the ribosome* pathway, whereas only one DEG mapped at tpHCD/LCD (12°C) (**Supplementary Figure 2A**).

Comparison of the transcriptome profiles of the wild type grown at 12°C and 6°C (tp12°C/6°C) revealed DEGs, accounting for 13 and 17% of the total 4282 *A. wodanis* genes at HCD and LCD, respectively (**Figure 1A**). In the Δ*ainS* mutant (tp12°C/6°C), 10 and 14% of total genes were differentially expressed at HCD and LCD, respectively. Compared to the wild type and the Δ*ainS* mutant, 31% of the total genes in the Δ*litR* mutant were differentially expressed (tp12°C/6°C) at LCD, whereas at HCD, 4% of the total genes were differentially expressed. Further, the DEGs in wild type (tp12°C/6°C) with FC values ≥ 2.0 and ≤−2.0 were sorted into KEGG BRITE and KEGG pathway mapper. The functional families with DEGs of ≥ 15 were *enzymes, transporters, ribosome, ribosome biogenesis* and *non-coding RNAs* (**Supplementary Figure 1B**). Few DEGs (n < 10) in wild type (tp12°C/6°C) mapped into families such as *secretion system* and *prokaryotic defense system*. The KEGG pathway mapping revealed that the DEGs (n ≥ 30) in the WT(tp12°C/6°C) were involved in pathways such as *metabolic pathways, microbial metabolism in diverse environments, biosynthesis of secondary metabolites, ABC transporters, biosynthesis of antibiotics, two-component system, carbon metabolism, purine metabolism, Aminoacyl-tRNA biosynthesis* and *ribosome*. The numbers of DEGs involved in these pathways were higher at tp12°C/6°C (LCD) than at tp12°C/6°C (HCD). For example, 43 DEGs at LCD mapped to the *ribosome pathway* while only 6 DEGs mapped at HCD (**Supplementary Figure 2B**).

When comparing the profile of the Δ*litR* mutant to the wild type tpΔ*litR*/WT at HCD, 3 and 4% of the total genes were differentially expressed at 12 and 6°C, respectively. In the tpΔ*litR*/WT (HCD_6°C), 4% of the total genes were differentially expressed. In tpΔ*litR*/WT (LCD_12°C), 5% of total genes whereas in tpΔ*litR*/WT (LCD_ 6°C), 11% of the total genes were found to be differentially expressed. The number of DEGs was two times higher at 6°C and LCD compared to 12°C and

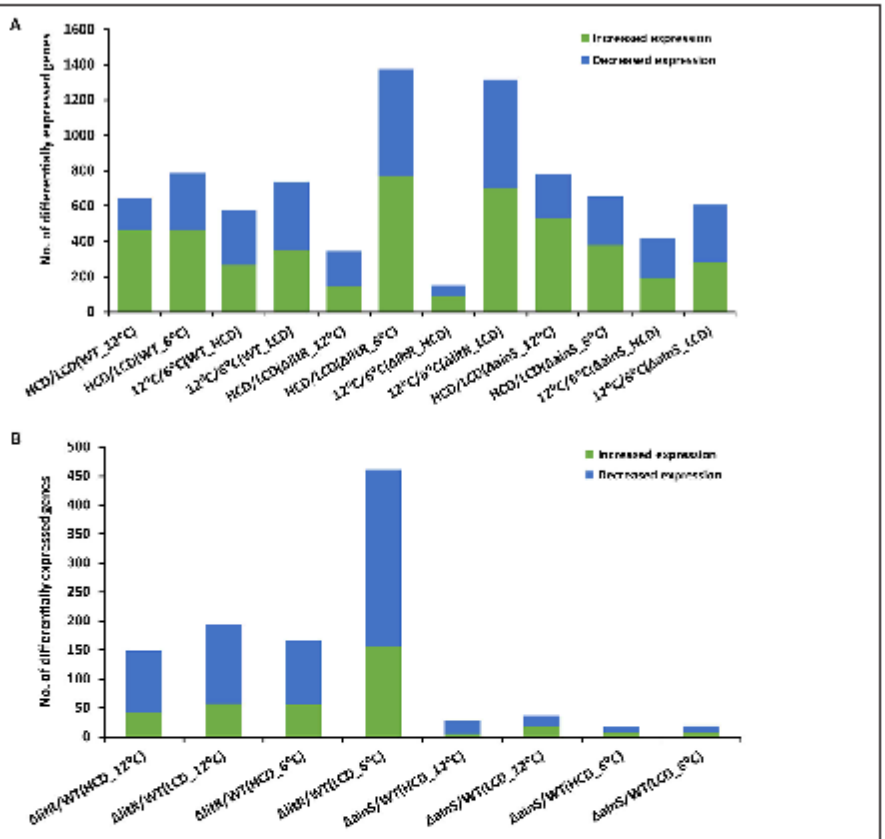

FIGURE 1 | The total number of differentially expressed genes in *A. wodanis* 06/09/139, Δ*ainS* and Δ*litR*. **(A)** The bar chart shows the number of increased (green) and decreased (blue) expression of genes when comparing high cell density (HCD) to low cell density (LCD) and growth temperature at 12 to 6°C. **(B)** The bar chart shows the number of increased and downregulated genes in comparisons Δ*litR*/WT and Δ*ainS*/WT.

HCD (**Figure 1A**). When comparing the expression profile of the Δ*ainS* mutant with the wild type (tpΔ*ainS*/WT) at HCD, 0.7 and 0.4% of the total genes were differentially expressed at 12 and 6°C, respectively. In tpΔ*ainS*/WT, during LCD and 12°C, 0.9% of the total genes and at LCD and 6°C, 0.4% of the total genes were found to be differentially expressed. The DEGs in Δ*litR* and Δ*ainS* mutants were sorted using KEGG BRITE (**Supplementary Figure 3A**) and mapped into KEGG reference pathway for *A. wodanis* 06/09/139 (**Supplementary Figure 4A**). The DEGs ($n \geq 5$) in the Δ*litR* and Δ*ainS* mutants compared to wild type with FC values of ≥ 2.0 and ≤ -2.0 were associated with functional families such as *enzymes*, *secretion system*, *bacterial motility proteins*, *transporters* and *prokaryotic defense system*. The KEGG pathway mapping revealed that the DEGs in the tpΔ*litR*/WT and tpΔ*ainS*/WT mutants affected several pathways, including *metabolic pathways*, *two-component system*, *biosynthesis of secondary metabolites*, *Quorum sensing* and *bacterial chemotaxis* (**Supplementary Figures 3B**, **4B**). Together our results suggest that, LitR is a crucial global regulator of genes at 6°C and at low cell density. As well, LitR has more impact on gene expression than AinS.

Key QS Genes of *A. wodanis* Are Cell Density and Temperature Dependent

Only a few genes known to be involved in the *A. wodanis* QS system were differentially expressed under the experimental conditions tested (**Supplementary Table 3**). When comparing the wild type transcriptome profile at HCD to LCD

(tpHCD/LCD), the AI synthase gene *ainS* (AWOD_I_1040) and master QS regulator gene *litR* (AWOD_I_0419) showed FC values of 2.01 and 2.24, at 6°C, respectively, while no significant differences were observed at 12°C.

When the wild type profile at high temperature was compared at low temperature (tp12°C/6°C), the *qrr* sRNA gene (AWOD_I_sRNA_054) was significantly higher with FC value of 2.92 at LCD, whereas no difference in its expression was observed at HCD. Temperature alone did not significantly impact other genes of the QS system at either HCD or LCD (**Supplementary Table 3**).

Comparing the profile of the Δ*litR* mutant relative to the wild type (tpΔ*litR*/WT), only the FC value of *ainS* at LCD and 12°C was significant (FC = −1.93, p_{adj} value < 0.05), although slightly lower than the cutoff FC value used in this study. The deletion of *litR* did not affect other QS related genes in *A. wodanis* under the conditions tested. On the other hand, when comparing the Δ*ainS* mutant profile to the wildtype (tpΔ*ainS*/WT), the *litR* expression was significantly lower at all conditions examined. The FC values of *litR* were notably lower at HCD compared to LCD. At HCD, the FC values for *litR* at 6°C and 12°C were −2.53 and −2.87, respectively. At LCD, the FC values at 6°C and 12°C were −1.93 and −2.04, respectively, indicating that the expression of *litR* in the Δ*ainS* mutant increases with increased density and independent of the temperature. Furthermore, the phosphorelay protein-encoding gene *luxU* (AWOD_I_0921) expression was significantly higher in the Δ*ainS* mutant at HCD (FC = 2.81) and LCD (FC = 2.17) at 6°C, whereas at 12°C no differences were observed. Hence, the *luxU* expression in the Δ*ainS* mutant was slightly higher at HCD compared to LCD and its expression was affected only at low temperature.

Differential Expression of CRISPR-Cas System in *A. wodanis*

We identified two CRISPR systems in the genome of *A. wodanis* using CRISPRCas finder (84), where CRISPR system 1, located on chromosome 1, did not contain any *cas* loci, whereas CRISPR system 2, located in chromosome 2, contains the *cas* loci (**Figure 2**). The CRISPR system 2 was classified as a Type I-F CRISPR system. A total number of 25 and 40 spacers with a length of 32-33 nucleotides were identified in CRISPR system 1 and 2, respectively (**Supplementary File S4**). When the spacers were searched against the phage databases using CRISPR Target, spacers matched to the protospacers with at least 5 single nucleotide polymorphisms. For example, spacer 11 of CRISPR system 1 matched to the *Vibrio* phage CTX plasmid pCTX-2, whereas spacer 19 of CRISPR system 2 matched to the *Staphylococcus* phage phiSP38-1 with a score of 20.

The data shows that in the wild type, the *csy3*, *cas1*, and *cas3* genes of CRISPR system 2 have significantly higher FC values (FC > 2.0) at HCD than at LCD after growth at 6°C. No significant differences in FC values of the *cas* genes were observed in wild type (tpHCD/LCD) at 12°C (**Supplementary Table 5**). Cell density had no impact on the expression of CRISPR arrays of CRISPR systems 1 and 2 at either temperature.

When the expression profile at 12°C was compared to 6°C, at LCD and HCD, the CRISPR arrays of system 1 displayed significantly lower FC values (FC value = ∼−2.0), suggesting temperature difference influences CRISPR system 1. However, temperature had no effect on the CRISPR system 2.

Further, the expression data showed that the expression of *cas* genes of CRISPR system 2 were strongly reduced in the Δ*litR* mutant at all conditions examined. The FC values of the *cas* genes in the Δ*litR* mutant increased with increasing cell density at both temperatures. Notably, in the Δ*litR* mutant at 6°C, the FC values of *csy3* and *csy1* were twice as high at HCD (FC = − approx. 10.0) compared to LCD (FC = − approx. 5.0). At LCD, the genes *csy1*, *csy2*, *csy3*, and *csy4* had lower FC values at 6°C compared to 12°C, whereas the *cas1* and *cas3* showed higher FC values (**Supplementary Table 5**). However, at HCD, the highest FC values for *cas* genes were observed at 6°C than at 12°C. The CRISPR array of system 2 in the Δ*litR* mutant showed significantly lower expression at LCD and 12°C (FC value = −1.96) and LCD and 6°C (FC value = −2.69). However, at HCD, the expression of the CRISPR array of system 2 was not significantly different. The data also showed that the CRISPR array of system 1 was highly expressed at HCD and 12°C, whereas the FC values were not significantly different at HCD and 6°C or at LCD at 6°C and 12°C. In the Δ*ainS* mutant compared to wild type, genes such as *csy2*, *csy3*, and *csy4* showed significantly lower expression at two experimental conditions, LCD, 12°C and HCD, 6°C. No significant differences were observed in expressions of the CRISPR array of system 1 and 2 in *the* Δ*ainS* mutant.

A. wodanis Harbors Three Main T6SSs and Four Auxiliary Clusters

A. wodanis was found to harbor three T6SSs, each encoding the conserved core and accessory T6SS genes. T6SS1 was located on chromosome 1, whereas the T6SS2 and T6SS3 were on chromosome 2. The T6SS1 gene clusters (AWOD_I_0981-0995) were composed of 3 operons with 15 consecutive genes, whereas the T6SS2 gene clusters consisted of 1 operon with 15 consecutive genes (AWOD_II_0111-0125) and T6SS3 gene clusters with 2 operons with 20 consecutive genes (AWOD_II_1008-1027) as shown in **Figure 3**. The main T6SS clusters comprised the core components *vasABDEFGHJKLQRS*, valine-glycine repeat protein G (*vgrG*) and Hemolysin Coregulated protein (*hcp*) and the accessory genes *vasCIUVX* (60, 92, 93). The genes *vasABEJL* and *vasDFK* may form the base-plate and membrane complex, respectively (94–96). The genes *vasRQ* and *vasC* may form the outer sheath and the FHA domain, respectively, while *vasG* is believed to act as a chaperone (97, 98). The gene *vasH* may serve as a sigma-54 dependent transcriptional regulator and *vasIS* as lysozyme-related proteins (99). The T6SS3 in *A. wodanis* does not encode the transcriptional regulator *vasH* (99). Four *vgrG* paralogous genes were identified in *A. wodanis*, and using them as markers, four auxiliary clusters were predicted, such as Aux-1 (AWOD_I_1435-1440, AWOD_I_2030), Aux-2 (AWOD_II_0126-0141), Aux-3 (AWOD_II_1028-1032) and Aux-4 (AWOD_II_1054-1060). The auxiliary clusters were

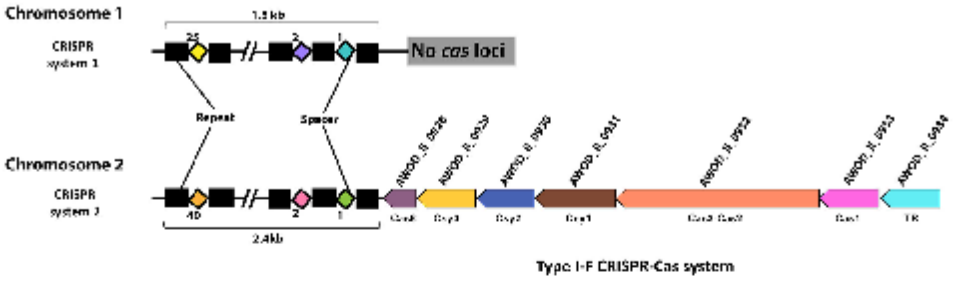

FIGURE 2 | CRISPR systems in *A. wodanis* 06/09/139. The figure shows the CRISPR arrays with repeats (black rectangle) and spacers (colored diamonds). In CRISPR system 2, the CRISPR array is located close to the *cas* operon (arrows) of the Type I-F CRISPR-Cas system. TR denotes transcriptional regulator.

located together with the core T6SS genes encoding Proline-alanine-alanine-arginine repeats (PAAR), VgrG and Hcp. The Aux-1 cluster does not encode Hcp, and Aux-4 does not encode a PAAR protein. Four genes encoding adaptor proteins of the DUF4123 family (unknown function) were identified adjacent to *vgrG1*, *vgrG2* and *vgrG4*, whereas no adaptor protein-encoding gene was present adjacent to *vgrG3*.

Differential Expression of T6SSs and Auxiliary Clusters of *A. wodanis*

In the wild type, at HCD, the main clusters T6SS1 (*vasR1Q1J1*) and T6SS2 (*vasH1I1J1K1L1*) were highly expressed at both 6 and 12°C, compared to LCD. No differential expression was observed for the T6SS3 (**Supplementary Table 6**). Furthermore, the complete auxiliary cluster Aux-1 showed higher expression at HCD at both temperatures. The Aux-2, Aux-3 and Aux-4 showed only significant differences in some genes such as AWOD_II_0126 - 0139, AWOD_II_1031 - 1032 and AWOD_II_1054 - 1059, respectively.

The temperature did not affect the expression of the T6SS1 and T6SS3 in the wild type. However, a few genes (*vasQ2, vasR2, vasS2, and vasD2*) of T6SS2 were differentially expressed at HCD, while at LCD, only *vasQ2* was differentially expressed. As for the auxiliary clusters, only the genes encoding membrane protein and DUF4123 (AWOD_I_1435 and AWOD_I_1439) of Aux-1, *hcp1* of Aux-2 and a putative uncharacterized protein (AWOD_II_1031) of Aux-3 were differentially expressed.

When the Δ*litR* mutant was compared to wild type (tpΔ*litR*/WT), the data revealed that the *vasR1Q1J1* operon of T6SS1 was differentially expressed at all tested conditions. The FC values of genes *vasR1Q1J1* were marginally higher at HCD compared to LCD. Particularly the FC values of *vasQ1* gene in Δ*litR* mutant were twice as high at HCD compared to LCD at both temperatures. The FC values of *vasR1Q1J1* operon in the Δ*litR* mutant were significantly higher at 6°C compared to 12°C at both cell densities (**Supplementary Table 6**). The genes *vasF1D1C1* showed higher FC values in Δ*litR* mutant at LCD than HCD. At 6°C, the complete T6SS2 cluster was differentially expressed in the Δ*litR* mutant at both HCD and LCD. At 12°C, differential expression was only observed in some of the genes in the T6SS2 cluster. Moreover, the FC values of T6SS2 genes were higher at low (6°C) compared to high (12°C) temperature. The T6SS3 gene cluster was only differentially expressed at LCD and 6°C with an average FC value of 2.0. In Δ*litR* mutant, some genes of Aux-1, Aux-2 and Aux-3 were differentially expressed, whereas the Aux-4 showed no differential expression (**Supplementary Table 6**). In Aux-1, the expression of *vgrG1* was lower in Δ*litR* mutant with an FC value of−4.90 at LCD and 6°C. The genes encoding Hcp1, VgrG2, PAAR motif-containing protein, a protein with ankyrin repeats and the rearrangement hotspot (RHS) protein in the Aux-2 were differentially expressed, where the FC values (FC = −22.75) of *hcp1* at HCD and 6°C was approximately thrice compared to LCD (FC = −8.32). Similarly, three times higher expression of *hcp1* was observed at 6°C compared to 12°C at both HCD and LCD. About 6 of the 16 total genes in the Aux-2 cluster showed significantly lower expression at LCD and 6°C. The Aux-3 was highly expressed in the Δ*litR* mutant, relative to wild type, at HCD and 12°C and at LCD and 6°C. When comparing the Δ*ainS* mutant with the wild type (tpΔ*ainS*/WT), only the expression of *vasR1* and *vasQ1* genes of the T6SS1 cluster were significantly reduced, whereas no differences in T6SS2 and T6SS3 gene clusters were observed. The *vgrG1* of Aux-1 showed lower expression (FC value = −2.07) at LCD and 6°C while the *hcp1* of Aux-2 showed significant lower expression (FC < −2.0) at all conditions except at LCD and 12°C. Additionally, the genes AWOD_II_1031 and *hcp3* of Aux-3 showed significant higher expression at HCD and 12°C. Other genes of auxiliary clusters in Δ*ainS* mutant did not show significant differential expression.

Potential T6SE Molecules Identified in *A. wodanis* 06/09/139

We predicted 80 potential effectors proteins using the T6SE prediction tool Bastion 6 (**Supplementary Table 7**) (91). Of the 80 potential effectors, 29 could be annotated, while 51 were identified as putative proteins. Of the potential effectors, 44 were located on chromosome 1, 33 effectors on chromosome 2 and 2 effectors were predicted to be located on plasmid p20. Among the

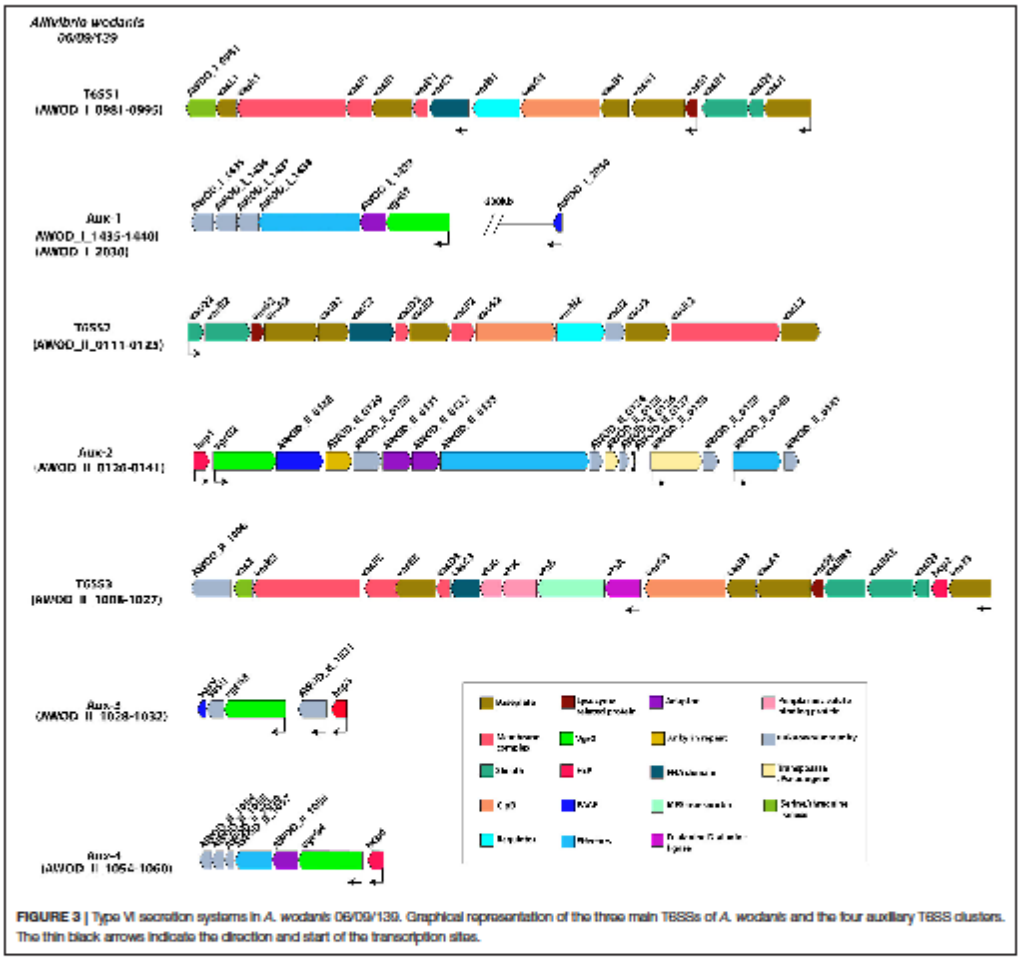

FIGURE 3 | Type VI secretion systems in *A. wodanis* 06/09/139. Graphical representation of the three main T6SSs of *A. wodanis* and the four auxiliary T6SS clusters. The thin black arrows indicate the direction and start of the transcription sites.

annotated T6SEs, we found potential cell wall degrading effectors such as N-acetylmuramoyl-L-alanine amidase and hydrolases, membrane degrading effectors including phospholipases, and several nucleotide degrading nucleases. Only a few effectors such as a porin-like protein H (AWOD_I_1000), a RHS protein (AWOD_II_0133) and type VI secretion system secreted protein Hcp (AWOD_II_1032) were located close to the main T6SS clusters, whereas the rest were located in different locations on both chromosomes.

Differential Expression of T6SEs in *A. wodanis*

In wild type, 24 effector genes were differentially expressed at HCD (tpHCD/LCD) at 6°C, while 19 effectors were differentially expressed at 12°C (**Supplementary Table 7**). These effector genes encoded several proteins including outer membrane proteins, membrane proteins, proteins with PAAR motif, lipoproteins with LysM domain, a sulfite reductase [NADPH] flavoprotein alpha-component, a secreted endonuclease I and a choline dehydrogenase. Interestingly, in the wild type at HCD and 6°C, an FC value of 173.29 was found for choline dehydrogenase (AWOD_II_1235), which was 20 times higher than at 12°C (FC = 8.99). When the wild type profiles at high and low temperatures were compared (tp12°C/6°C), 18 effectors and 22 effectors were differentially expressed at HCD and LCD, respectively. These effectors comprised putative exported protein, putative lipoprotein, putative beta-lactamase, amine oxidase and choline dehydrogenase.

In the Δ*litR* mutant, genes encoding effector molecules with significantly lower expression (FC < −2.0) were putative exported proteins (AWOD_I_0175, AWOD_I_1184, AWOD_II_0501, and AWOD_II_0656), outer membrane protein (AWOD_I_1120), putative lipoprotein (AWOD_I_1186, AWOD_II_0440, AWOD_II_0804, and AWOD_II_1206), putative polysaccharide deacetylase (AWOD_I_1338), membrane protein (AWOD_I_1567), amine oxidase (AWOD_II_0852) and phospholipase (AWOD_II_1212). The effector molecules genes differentially expressed in Δ*ainS* mutants included a putative histidine decarboxylase (AWOD_I_1509), and a secreted endonuclease (AWOD_II_0252). Additionally, genes encoding effectors such as a porin-like protein H (AWOD_I_1000), an endonuclease I precursor (AWOD_I_2248) and a 6-phospho-beta-glucosidase (AWOD_I_0029) were differentially expressed in both Δ*litR* and Δ*ainS* mutants. The differential expression of effector molecules in Δ*litR* mutant was seen more often during LCD and 6°C than at other experimental conditions like HCD and 12°C (**Supplementary Table 7**).

DISCUSSION

A. wodanis is frequently isolated together with *M. viscosa* during the winter ulcer outbreaks and believed to be involved in the progression of winter ulcer disease (7, 73, 74, 78). Although *M. viscosa* is the main agent causing the disease, the reason for its co-existence with *A. wodanis* is not yet clear. Bacteria use several strategies to compete for niche adaptions, which are known to be regulated by various mechanisms, including QS (49, 100–102). In our previous study we have shown that QS in *A. wodanis* regulates various phenotypic traits and cytotoxicity on CHSE cell line (80). In this study, we performed genome analysis and gene expression profiling of *A. wodanis* to explore the QS system and its role in regulating the survival strategies.

Total DEGs

When comparing the transcriptome profile of the *A. wodanis* wild type between cell densities (tpHCD/LCD) and temperatures (tp12°C/6°C), the strongest effect in terms of number of DEGs were at LCD (164 more genes than at HCD) and at 6°C (140 more genes than at 12°C). Furthermore, the functional mapping of DEGs revealed that families such as *secretion system*, *prokaryotic defense system* and *transporters* were highly expressed at HCD compared to LCD. As expected, the gene families related to protein synthesis such as *ribosomes*, *ribosome biogenesis* and *transfer RNA biogenesis* were less expressed at HCD compared to LCD (tpHCD/LCD). Similar cell density-dependent gene expression has been reported in *Aliivibrio salmonicida*, where about 1,000 genes were differentially expressed in response to increase in cell density (103). In the Δ*litR* mutant, 1.8 times more DEGs were observed at tpHCD/LCD at 6°C than for the wild type grown at the same condition, suggesting LitR is an important regulator in *A. wodanis* at low temperature. Furthermore, in *A. wodanis*, the lowly expressed genes in comparison tp12°C/6°C were related to protein synthesis such as *non-coding RNAs*, *ribosomes*, *transfer RNA biogenesis* and *ribosome biogenesis*. This suggests that genes related to protein synthesis are less expressed at 12°C compared to 6°C. Temperature is one of the major environmental stress factors that bacteria encounter in nature and many genes respond to temperature changes. In addition, the functional families such as the *secretion system* and *prokaryotic defense system* were also regulated by temperature in *A. wodanis*. For example, in *V. parahaemolyticus* 13% of DEGs were observed when the bacteria were grown at 15°C and 42°C compared to its optimal temperature 37°C, where genes related to energy metabolism were highly affected due to temperature change (104). In *A. wodanis*, more DEGs were identified in tpΔ*litR*/WT (LCD) and tpΔ*litR*/WT (6°C) compared to at HCD and 12°C, suggesting LitR is a global regulator when the temperature is low and the cells are in the early log phase. This is in contrast to *A. salmonicida*, where the numbers of DEGs in Δ*litR* mutant were higher at HCD compared to LCD at 12°C (103). The comparison between the transcriptome profiles of mutants and wildtype (tpΔ*litR*/WT and tpΔ*ainS*/WT) showed about 5 to 24 times more DEGs in tpΔ*litR*/WT compared to tpΔ*ainS*/WT at both cell densities and temperatures. This indicates that only a few genes seem to be regulated through the AinS-dependent QS system. This is similar to *A. salmonicida*, where only about 20 genes were differentially expressed in Δ*ainS* mutant when compared to wild type, whereas in *litR* mutant compared to wild type, 3 to 10 times more DEGs were found (103, 105). Moreover, in this study, the transcriptomics was performed at OD_{600} of 6.0, which is a late log phase in *A. wodanis* while it can reach an OD_{600} of ~8.0. Therefore, the AHL-mediated gene regulation in *A. wodanis* may differ at OD_{600} higher than 6.0. The functional mapping of DEGs from comparisons tpΔ*litR*/WT and tpΔ*ainS*/WT showed that the genes affected by LitR and AinS in *A. wodanis* mainly belongs to the families such as *secretion system* and *prokaryotic defense mechanism*, which contained the T6SS and CRISPR genes, respectively. Nevertheless, other families such as *enzymes*, *bacterial motility proteins* and *transporters* were also found to be affected by LitR and AinS in this study. LitR and AinS in *A. wodanis* also influenced various pathways such as metabolic pathways, *Quorum sensing*, and *two-component systems*, *suggesting* their role in signaling mechanisms and metabolic activities. QS regulation of metabolic functions such as glucose uptake, phosphoenolpyruvate-dependent sugar and nucleotide biosynthesis are known to enhance the co-operative behavior of bacteria to survive under limited nutrient conditions (101). Similarly, the LitR and AinS regulation of metabolic pathways in *A. wodanis* may play a role in co-operative behavior under limited nutrient conditions.

QS System in *A. wodanis*

In wild type, we found that an increase in cell density resulted in a higher expression of the AHL autoinducer synthase *ainS* and the master QS regulator *litR* when grown at 6°C and not at 12°C. This demonstrates that cell density only affects the expression of *litR* and *ainS* genes at low temperature. Similar increased expression of *ainS* was also observed in the Δ*litR* mutant strain when comparing HCD to LCD (tpHCD/LCD), indicating that the *ainS* expression is not entirely LitR-dependent. These results

confirm our previous work, where we showed that the 3OHC10-HSL production is cell density- and temperature-dependent (80). Similar to AinS in *A. salmonicida* (105), the AinS in *A. wodanis* negatively affected the *luxU* expression. This suggests that the decreased expression of *luxU* could repress *qrr* and activate *litR* in *A. wodanis*. Similarly, in this study, *luxU* was affected by AinS only at 6°C, which could suggest that low temperature affects the phosphorelay function of *luxU*. On the other hand, the *qrr* gene was more expressed at 12°C compared to 6°C. Qrr sRNAs in vibrios are known to regulate several target mRNAs, including the master QS regulator LuxR (106). In *A. fischeri*, Qrr sRNAs negatively regulates the production of LitR (16). Therefore, the increased expression of *qrr* in *A. wodanis* at 12°C may negatively affect the production of LitR and, subsequently, the QS dependent gene expression. Our results illustrate that *litR*, *ainS*, *luxU*, *qrr* sRNA in the *A. wodanis* QS system were differentially expressed in a cell density and temperature-dependent manner. The proposed QS model in *A. wodanis* and its regulation of target genes are presented in **Figure 4**.

In this study, LitR in *A. wodanis* did not affect the *ainS* expression, except at LCD and 12°C (FC = −1.93) in the Δ*litR* mutant. This confirms our previous work where we showed that the 3OHC10-HSL production is not completely controlled through LitR (80). However, LitR in *A. fischeri* and *A. salmonicida* has been shown to significantly upregulate AinS (11, 107). In *A. fischeri*, several LitR-independent regulatory mechanisms have been reported to regulate *ainS*, such as *ainS* auto-regulation or cyclic AMP receptor protein- and glucose-mediated mechanisms (11, 108). Similarly, in *A. wodanis*, other regulatory mechanisms may regulate the expression of *ainS*. The deletion of *ainS* in *A. wodanis* negatively affected the *litR* expression. In the Δ*ainS* mutants, the FC values of *litR* were slightly higher at HCD compared to LCD at both temperatures, which suggests that 3OHC10-HSL partly activates *litR* independently of the temperature. Similarly, the autoinducer synthase C8-HSL in *A. fischeri* positively affected *litR* in a cell density-dependent manner (14).

Regulation of CRISPRs and Spacers

A. wodanis harbors a type I-F CRISPR system and a CRISPR system without *cas* loci, the CRISPR system 1 and CRISPR system 2, respectively. CRISPR systems without *cas* loci are known as orphan arrays, where most of these are known to be non-functional (39). However, some of the orphan CRISPR arrays may function together with invading *cas* genes or the *cas* genes present in a different location within the same genome (109). In *A. wodanis*, the CRISPR system 1 consists of 25 spacers, whereas CRISPR system 2 consists of 40 spacers. The difference in the number of spacers in *A. wodanis* suggests that the CRISPR system 1 is less active than the CRISPR system 2 or is a remnant of a previously active CRISPR system. In addition, the spacers were not identical between CRISPR systems 1 and 2, indicating both are working independently of each other or that CRISPR system 2 has been introduced later than CRISPR system 1. The *cas* genes in *A. wodanis* shows ~90% amino acid similarity to the *cas* genes in *M. viscosa*, suggesting that the CRISPR system 2 has been horizontally transferred from *M. viscosa*. We speculate here that

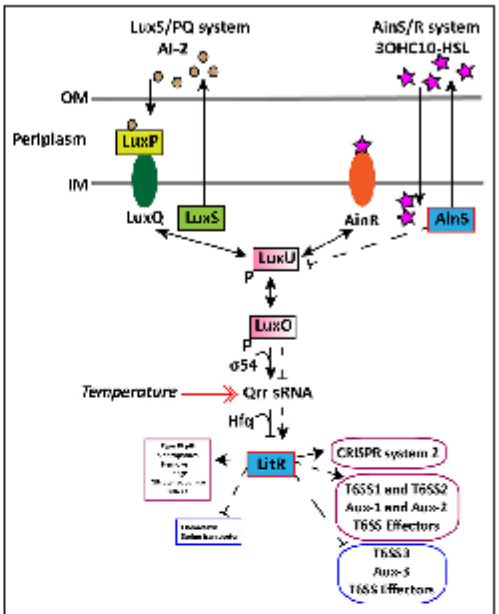

FIGURE 4 | Proposed model for regulation of CRISPR and T6SS in *A. wodanis* 06/09/139 at 6°C. The model includes the two QS systems LuxS/PQ and AinS/R in *A. wodanis*. At low cell density (LCD), when the AIs concentration is low, the receptors (AinR and LuxPQ) may act as kinases and relay phosphate to LuxO via LuxU, which activates the expression of *qrr* sRNA. The Qrr sRNA inhibits the expression of *litR*. When the AI concentration is high at high cell density (HCD), they bind to the receptors to dephosphorylate LuxO and inactivate *qrr* sRNA. Inactivation of *qrr* sRNA, in turn, activates *litR*, which regulates many genes. Arrowheads at both ends indicate phosphorylation relay, and the symbol "P" indicates the phosphorylated state. AinS seems to affect luxU negatively and the *litR* expression positively. LitR seems to positively affect the genes involved in T6SS1, T6SS2, Aux-1, Aux-2, T6SS effectors and CRISPR system 2 and, conversely, affect T6SS3, Aux-3 and T6SS effectors negatively. Thin line and dashed line with arrowhead indicate regulation at LCD and HCD, respectively, where arrowheads indicate increased expression (+) and bars indicate decreased expression (-). A double arrowhead red line indicates the increased expression of *qrr* sRNA at 12°C compared to 6°C. Genes with significantly higher and lower expression are presented in purple and blue boxes, respectively.

both bacteria can defend themselves against the same agents, which may favor their co-existence during the development of the winter ulcer disease. Similarity search of the spacers in *A. wodanis* against the phage databases revealed *Vibrio* phage CTX plasmid pCTX-2 and *Staphylococcus* phage phiSP38-1.

The expressions of *cas* genes (*cas1*, *cas3*, and *csy3*) in wild type seems to be cell density-dependent at 6°C, where the *cas* genes showed increased expression at HCD compared to LCD. However, cell density had no significant effect on the expression of CRISPR arrays of CRISPR systems 1 and 2 regardless of temperatures. Therefore, the CRISPR systems are

other. Besides the structural role of Hcp, it is involved in the inter-bacterial competition, bacterial invasion, adherence, and cytotoxicity against host cells, also known to have other functions in different bacteria (127, 128). Therefore, the multiple copies of *hcp* in *A. wodanis* may have different functions.

V. cholerae utilizes T6SS to compete against diverse eukaryotic and prokaryotic organisms (66, 129). In *V. fluvialis*, the T6SS2 is anti-bacterial and provides a better competitive fitness in the marine environment (123). The T6SS2 in *A. wodanis*, which shows higher homology to *V. fluvialis* and *V. cholerae*, may enhance *A. wodanis* through inter-bacterial competition and virulence.

The T6SS3, unlike T6SS1 and T6SS2 in addition to structural components, contains additional genes *vtsABCD* encoding transporter proteins and does not contain *vasH*. VtsA-D plays a role in stress responses, transport function and *hcp* expression in *V. anguillarum*, and VasH is essential to drive the expression of the T6SS operon by inducing *hcp* and *vgrG* expression (48, 130). The mutation in *vasH* repressed *hcp* expression and impaired its anti-bacterial activity in other vibrios (123, 131). In *V. anguillarum*, VtsA-D proteins are involved in stress responses, however, plays no role in virulence (48). Similarly, T6SS3 in *A. wodanis* is probably involved in stress responses. After *V. anguillarum*, the T6SS similar to T6SS3 in *A. wodanis* is in *M. viscosa* (mts1). The genes encoding Hcp (AWOD_II_1028 and AWOD_II_1032), which are located close to each other, shows 71% similarity to *M. viscosa hcp*, MVIS_3030 (7). This implies that the T6SS3 in *A. wodanis* might have a similar function as mts1 of *M. viscosa* during the winter ulcer disease development.

In this study, expression of the T6SS1 and T6SS2 genes and their auxiliary clusters in *A. wodanis* are dependent on cell density. Such cell density-dependent T6SS expression has been reported in *V. parahaemolyticus* (50). Some of the genes in the T6SS2 and auxiliary clusters (Aux-2 and Aux-3) were found to be altered by temperature. The expression was found to be higher at 6°C than at 12°C. In *V. fluvialis*, the T6SS2 is regulated by temperature (123). Temperature-dependent regulation of virulent factor genes may be an essential feature for many bacteria to survive in harsh environments (132).

LitR in *A. wodanis* seemed to positively affect two T6SSs (T6SS1 and T6SS2) and negatively affect the expression of T6SS3 gene cluster. This demonstrates that QS regulation of the T6SSs in *A. wodanis* is very complex. The regulation of T6SS3 by LitR in *A. wodanis* indicates T6SS3 may play different roles than T6SS1 and T6SS2. Such reciprocal regulation has also been shown in *V. parahaemolyticus*, where the QS regulator OpaR downregulates T6SS1 and upregulates T6SS2 where T6SS1 functions as anti-bacterial and T6SS2 as anti-eukaryotic (133).

LitR in *A. wodanis* seemed to positively affect only the genes encoding the outer sheath and base-plate proteins of T6SS1, suggesting these genes are QS dependent. Interestingly, LitR was involved in activating the expression of the whole apparatus of the T6SS2 system. Moreover, LitR also repressed the entire T6SS3 gene clusters but only at LCD at 6°C. Therefore, T6SS2 is ultimately QS dependent, whereas T6SS1 and T6SS3 are not completely QS dependent. Regulation of T6SS by LuxR homologs have been described in *V. cholerae* (HapR), *V. alginolyticus* (LuxR) and *V. anguillarum* (VanT) (48, 49, 51, 134). Furthermore, we observed that LitR in *A. wodanis* is a strong activator of *hcp1* expression at 6°C than at 12°C. Hence, this possibly implies that *hcp1* of T6SS2 is may be involved in the cytotoxicity in CHSE cell line (80). Temperature has been shown to influence *hcp* expression in other bacteria such as *Yersinia pestis* and *V. parahaemolyticus* (133, 135). Similarly, in *A. wodanis*, the high expression of *hcp* at 6°C indicates that the T6SS2 could be more active at low temperature (6°C).

We identified that the genes in Aux-2 encoding ankyrin repeat-containing proteins and RHS proteins were differentially expressed in tpΔ*litR*/WT at 6°C. Ankyrin repeat proteins are known to be involved in pathogenesis by imitating and impeding host function (136). RHS proteins are toxins that are exported to the cell surface through T6SS, and it mediates anti-bacterial activity (137–139). AinS had no significant effect on the main and auxiliary clusters of T6SSs like LitR. However, AinS positively influenced the expression of the *hcp1* and *vgrG1* genes and may indicate that only these T6SS genes are dependent on AHL-mediated QS. We predicted several T6SS effectors in *A. wodanis*, including lipoprotein, nucleases, membrane proteins, amidases and succinylglutamate desuccinylase. However, most of them are putative and hypothetical proteins, which require further research to confirm. From the transcriptomics data, we found that cell density regulated several predicted T6SEs in wild type. Choline dehydrogenase, an osmoprotectant enzyme that protects bacteria from adverse temperatures and other stresses, was more highly expressed at 6°C (140, 141). The temperature has affected expression of several T6SEs indicating a temperature-dependent production of effector proteins. Similar to the LitR regulation of T6SS main and auxiliary clusters, it also controlled several T6SEs. Few T6SEs were also found to be affected by AinS. This may indicate that several effectors are dependent on QS, where LitR influenced the expression higher at LCD and 6°C. Some of the known effectors included porin-like protein H (AWOD_I_1000), a molecular filter for hydrophilic compounds and bacteriocin (AWOD_p920_0063), which is a virulent factor in *A. wodanis* that modulates the growth and virulence of *M. viscosa* (7, 17). Genes encoding T6SS immunity proteins are usually located close to the genes encoding effector proteins (63). The potential immunity protein of Aux-1 (AWOD_I_1437) in *A. wodanis* shows 30% amino acid similarity to the immunity protein in *V. cholerae* strain O1E1 (90), while in Aux-2, the immunity protein (AWOD_II_0134) shows 29% amino acid similarity to *Mucilaginibacter gotjawali* (142). The immunity protein (AWOD_II_1056) in Aux-4 shows 77% amino acid similarity to a hypothetical protein in *A. fischeri* MJ11 (124).

CONCLUSION

In this present study we show that cell densities and temperatures influenced the expression of many genes in *A. wodanis*. Moreover, the QS related genes in *A. wodanis* are cell density- and temperature-dependent, where 6°C plays an essential role in activating the AHL-mediated QS system. *A. wodanis* harbors two CRISPR systems, three T6SSs and four auxiliary T6SSs in

its genome. We show that the CRISPR system 2 and T6SS3 of *A. wodanis* are similar to those in *M. viscosa*, the bacteria with which *A. wodanis* co-exists during winter ulcer disease. The low-temperature 6°C at which winter ulcer occurs exerts a significant effect on the expression pattern of *A. wodanis* than at high-temperature 12°C. We demonstrate here that LitR regulates CRISPR-Cas and T6SSs in a cell density- and temperature manner. Moreover, QS is found to regulate several potential T6SEs in *A. wodanis*. Thus, the QS regulation of T6SSs and CRISPR-Cas system of *A. wodanis* could be essential to understand the possible mechanisms used by *A. wodanis* during its co-existence with other bacteria like *M. viscosa* or the host.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AM, EH, HH, and NW conceived and designed the experiments. AM performed the experiments. AM, EH, and NW analyzed the transcriptomics data. AM and NW wrote the manuscript. All authors contributed to reviewing and proof reading the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.799414/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Additional file 1

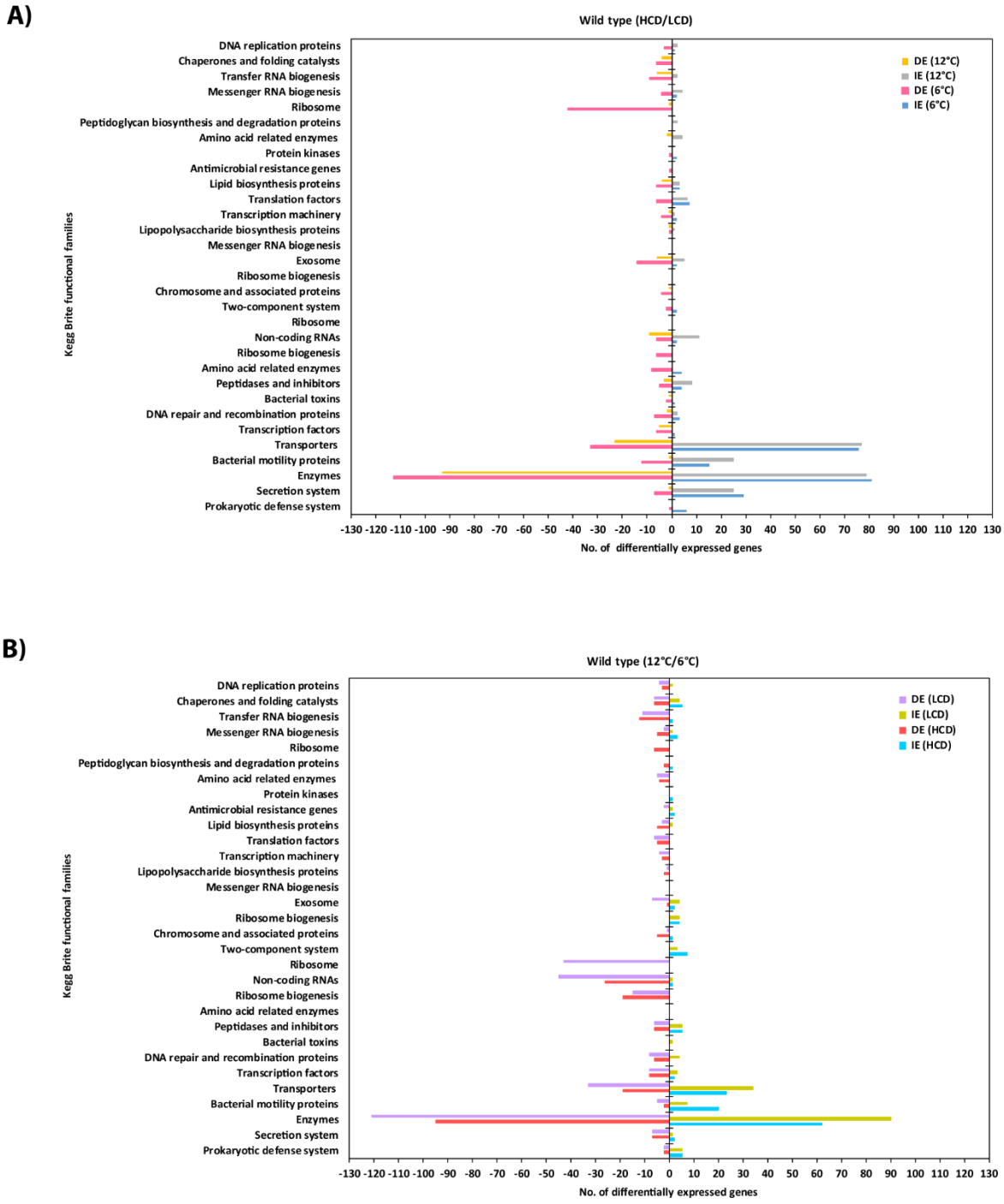


Figure S1. Functional gene family mapping of DEGs in wild type. (A) and **(B)** Bar chart showing the increased and decreased expression of genes in wild type compared between cell densities (HCD/LCD) and temperatures (12°C/6°C) sorted into different functional families respectively. IE and DE indicate increased and decreased expression respectively.

Additional file 2

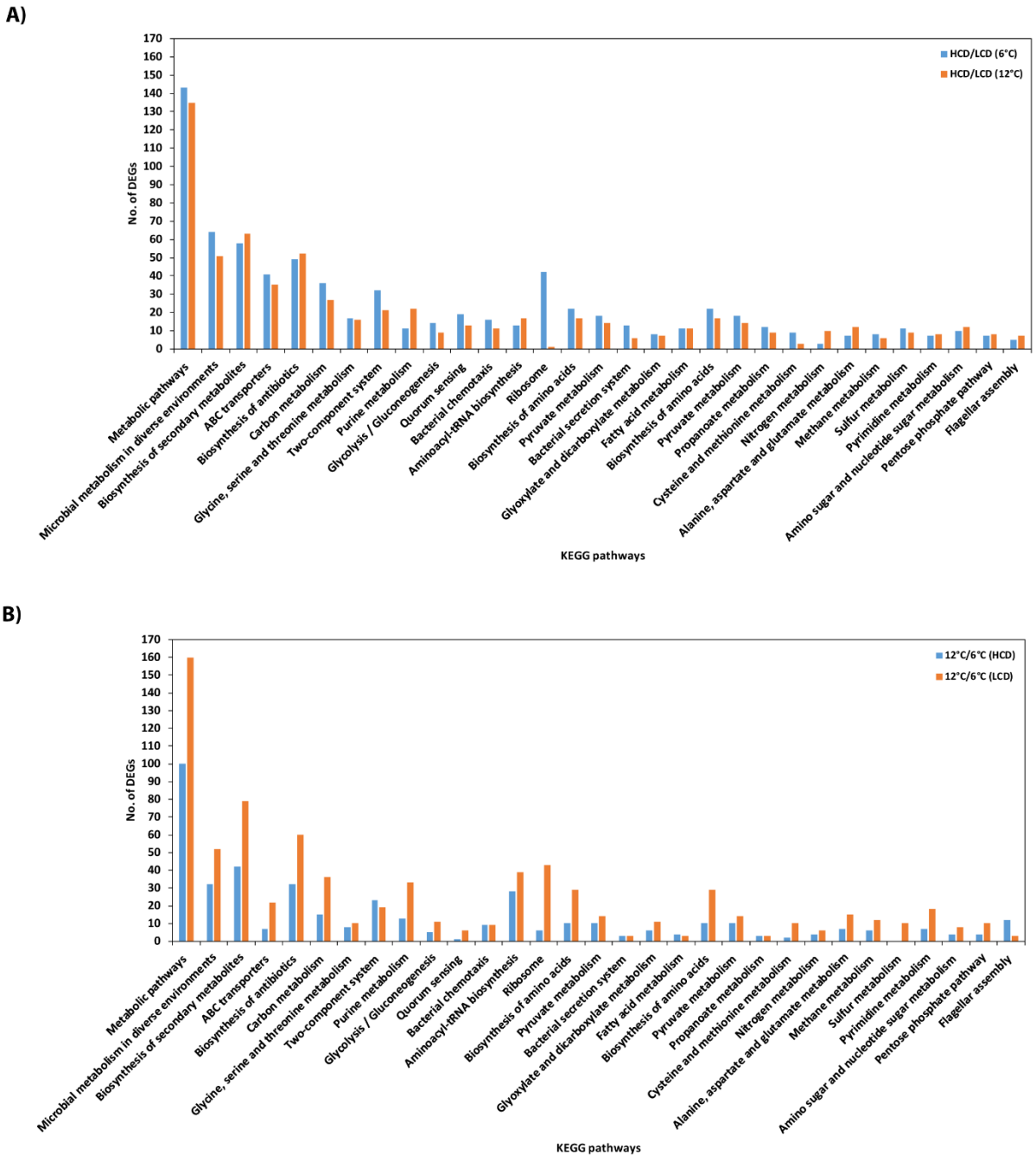
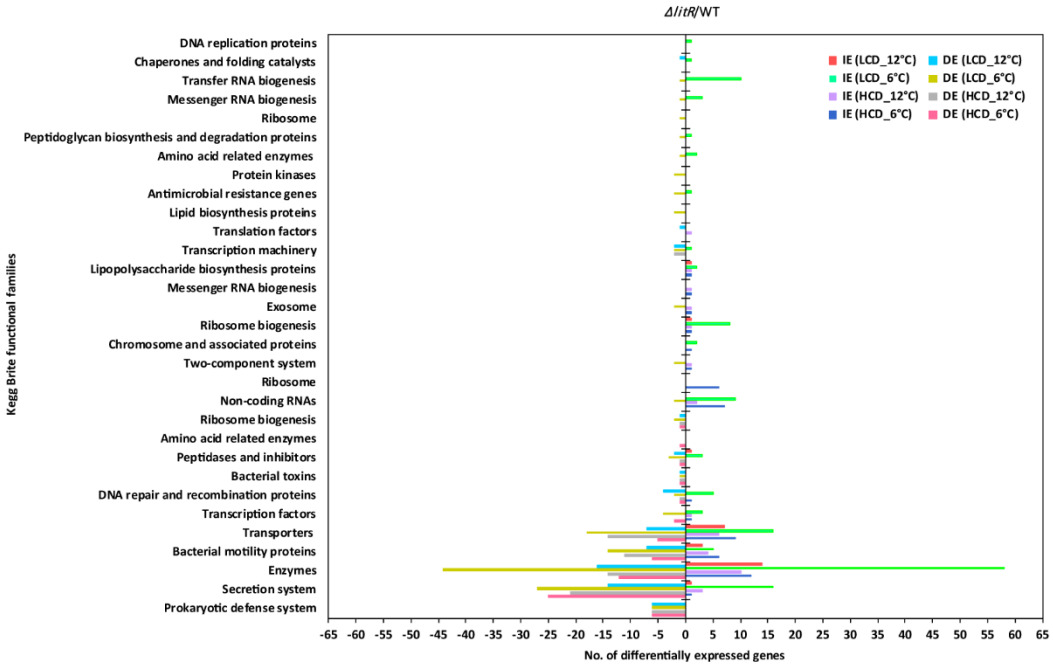


Figure S2. KEGG pathway mapping of DEGs in wild type. (A) and **(B)** Bar chart showing DEGs in comparisons WT (HCD/LCD) and WT (12°C/6°C) mapped into top 31 KEGG pathways of *A. wodanis* respectively.

Additional file 3

A)



B)

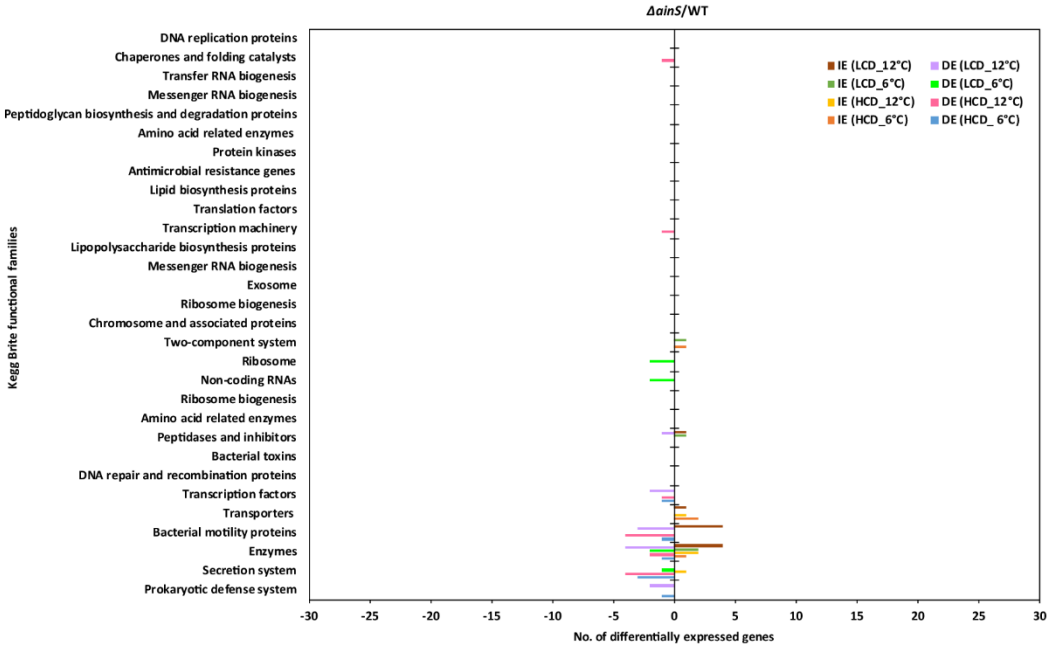


Figure S3. Functional gene family mapping of DEGs in *litR* and *ainS* mutants compared to wild type. (A) and **(B)** Bar chart showing DEGs from *ΔlitR*/WT and *ΔainS*/WT at two different cell densities and temperatures sorted into different functional families. IE and DE indicate increased and decreased expression respectively.

Additional file 4

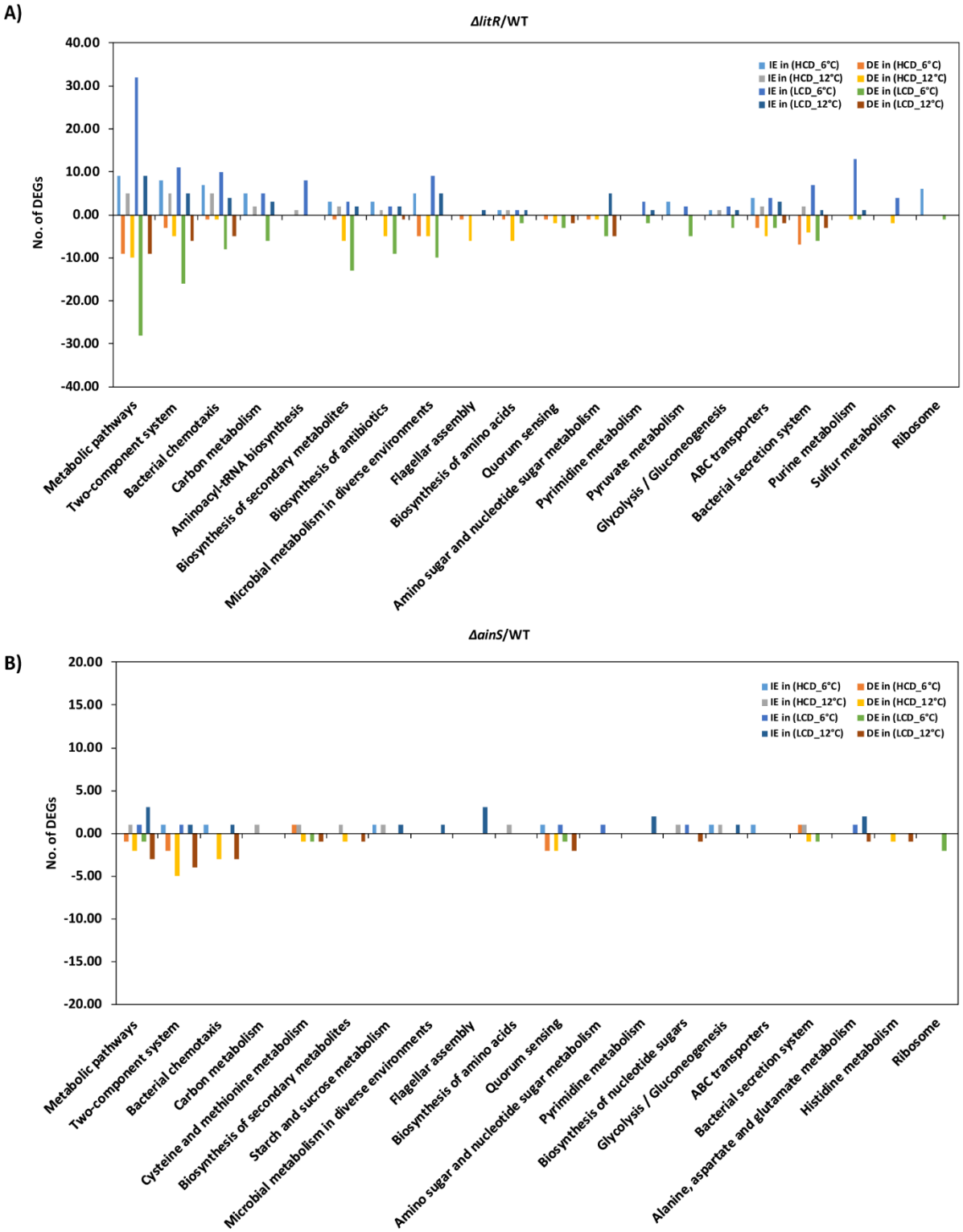


Figure S4. KEGG pathway analysis of DEGs in *litR* and *ainS* mutants compared to wild type. (A) and (B) Bar chart showing genes with increased and decreased expression in comparisons *ΔlitR*/WT and *ΔainS*/WT mapped into top 20 KEGG pathways of *A. wodanis* respectively. IE and DE indicate increased and decreased expression respectively.

Additional file 5

Spacers in CRISPR array 1 and 2

>spacer1
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>spacer2
TTGGTATTATTATTCGTGAATTTCCGCAAAAT
>spacer3
CGAGGGGACAGAGATCCCGCCTGAATGCAGAC
>spacer4
CTTGAGCAACTTGGATTTCTTTTGATTTGTCA
>spacer5
GCCGACAAAGCTATTAAATCCATGTTTATGAT
>spacer6
TGTTTGTTCATTCTATTTCACCATTTTAATTA
>spacer7
GAAAATCCAAGGGACAGCCTACGTATTTTTTA
>spacer8
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>spacer9
AATCAGCGTAATCGACTGGAAAAGTGTCTACT
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TTCTGTATGAGCAATTAAAATGTTATCGCAAG
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CRISPR array 2
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Additional file 6

Supplementary Table S1. Summary of RNA sequencing data					
		Total no. of reads	**Total no. of paired reads mapped to *A. wodanis***	**Total no. of singletons mapped to *A. wodanis***	**Percent mapped reads to *A. wodanis***
WT_LCD_12°C	**Replicate 1**	9318078	8683744	165736	94.97 %
	Replicate 2	8603498	8042848	131030	95.00 %
	Replicate 3	8039172	7628826	101488	96.15 %
WT_LCD_6°C	**Replicate 1**	6830012	6431918	94524	95.55 %
	Replicate 2	6764050	6275884	129210	94.69 %
	Replicate 3	16382490	13607574	291488	84.84 %
WT_HCD_12°C	**Replicate 1**	8405606	7924150	142350	95.96 %
	Replicate 2	7031754	6612690	102022	95.49 %
	Replicate 3	10520706	10038078	141765	96.76 %
WT_HCD_6°C	**Replicate 1**	9104484	8630120	133158	96.25 %
	Replicate 2	8794288	8404036	112444	96.84 %
	Replicate 3	7847740	7559668	76473	97.30 %
Δ*ainS*_LCD_12°C	**Replicate 1**	7143446	6738906	94882	95.66 %
	Replicate 2	8654686	8043884	145735	94.62 %
	Replicate 3	9865158	9091846	214675	94.33 %
Δ*ainS*_LCD_6°C	**Replicate 1**	7390374	6947748	101472	95.38%
	Replicate 2	8156088	7717366	105927	95.91%
	Replicate 3	8183570	7634498	134886	94.93%
Δ*ainS*_HCD_12°C	**Replicate 1**	8638822	8134650	167682	96.10 %
	Replicate 2	10776830	10216972	183895	96.51 %
	Replicate 3	6664550	6310982	97213	96.15 %
Δ*ainS*_HCD_6°C	**Replicate 1**	9212642	8683448	145152	95.83 %
	Replicate 2	8208020	7766162	104979	95.89 %
	Replicate 3	15229180	14602412	164224	96.96 %
Δ*litR*_LCD_12°C	**Replicate 1**	6867568	6487820	104444	95.99 %
	Replicate 2	8258068	7775178	109325	95.47 %
	Replicate 3	11600026	10966164	162825	95.93 %
Δ*litR*_LCD_6°C	**Replicate 1**	6469472	6151132	87641	96.43 %
	Replicate 2	6545258	6193608	93113	96.05 %
	Replicate 3	6707028	6308428	103580	95.00 %
Δ*litR*_HCD_12°C	**Replicate 1**	10079514	9496982	168175	95.88 %
	Replicate 2	7936052	7505174	145316	96.40 %
	Replicate 3	5773648	5419312	102581	95.63 %
Δ*litR*_HCD_6°C	**Replicate 1**	8032142	7611686	111371	96.15 %
	Replicate 2	8780408	8370148	109231	96.57 %
	Replicate 3	6454230	6182194	72719	96.91 %

Additional file 7

Supplementary Table S2. Fold change values in wild type *A. wodanis*, *ΔlitR* when comparing HCD to LCD, 12°C to 6°C, *litR* mutant compared to wild type and *ainS* mutant compared to wild type

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8861277/bin/Table_2.xlsx

Additional file 8

Supplementary Table S3. Fold change values in QS system related genes of *A. wodanis* 06/09/139

	litR		*ainS*		*ainR*		*luxU*		*luxO*		*σ54*		*qrr*		*hfq*		*luxS*		*luxP*		*luxQ*	
Comparisons	AWOD_I_0419		AWOD_I_1040		AWOD_I_1039		AWOD_I_0921		AWOD_I_0920		AWOD_I_2297		AWOD_I_sRNA_054		AWOD_I_0276		AWOD_I_0523		AWOD_I_0676		AWOD_I_0677	
Wild type	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**
HCD/LCD(12°C)	1.24	0.05	1.03	0.93	1.07	0.70	-1.31	0.18	-1.17	0.33	-1.31	0.07	-1.26	0.41	1.23	0.27	-1.04	0.80	-1.01	0.96	1.06	0.81
HCD/LCD(6°C)	**2.24**	**0.00**	**2.01**	**0.00**	1.16	0.28	-1.33	0.13	-1.46	0.01	-1.01	0.96	1.33	0.44	-1.04	0.75	-1.15	0.34	-1.28	0.05	1.45	0.02
12°C/6°C(HCD)	-1.51	0.00	-1.23	0.40	1.06	0.78	1.33	0.22	1.35	0.08	1.14	0.51	1.67	0.09	1.07	0.80	-1.54	0.01	1.28	0.09	-1.30	0.23
12°C /6°C(LCD)	1.23	0.12	1.61	0.03	1.18	0.25	1.35	0.09	1.12	0.44	1.51	0.00	**2.92**	**0.00**	-1.18	0.14	-1.68	0.00	1.04	0.80	1.09	0.60
Δ*litR*																						
HCD/LCD(12°C)	-1.77	0.07	1.64	0.06	1.06	0.80	-1.43	0.14	-1.23	0.24	-1.03	0.91	-1.34	0.38	1.08	0.80	-1.58	0.03	-1.09	0.68	1.21	0.32
HCD/LCD(6°C)	-1.51	0.07	**3.20**	**0.00**	1.43	0.02	-1.29	0.29	-1.02	0.93	1.45	0.00	1.18	0.65	1.23	0.05	-1.24	0.10	-1.04	0.80	1.14	0.41
12°C/6°C(HCD)	1.17	0.61	-1.67	0.02	-1.03	0.93	1.13	0.74	1.25	0.24	1.09	0.71	1.12	0.80	-1.15	0.52	-1.46	0.06	1.17	0.45	-1.04	0.89
12°C /6°C(LCD)	1.47	0.19	1.09	0.79	1.31	0.09	1.29	0.23	1.54	0.01	1.63	0.00	1.85	0.05	-1.01	0.96	-1.15	0.44	1.25	0.12	-1.12	0.50
Δ*ainS*																						
HCD/LCD(12°C)	-1.20	0.18	1.16	0.74	1.06	0.74	-1.48	0.01	-1.26	0.20	-1.68	0.00	1.01	0.99	1.23	0.30	-1.63	0.01	1.02	0.93	1.34	0.07
HCD/LCD(6°C)	1.61	0.04	1.59	0.34	1.17	0.54	1.11	0.77	-1.06	0.87	-1.00	0.99	-1.27	0.59	-1.01	0.99	-1.00	0.99	-1.37	0.19	1.20	0.48
12°C/6°C(HCD)	-1.51	0.01	-1.70	0.12	1.19	0.23	-1.98	0.00	-1.05	0.83	-1.20	0.20	1.31	0.40	1.03	0.90	-1.68	0.00	1.36	0.05	1.05	0.77
12°C /6°C(LCD)	1.27	0.30	-1.28	0.65	1.31	0.29	-1.22	0.50	1.12	0.74	1.37	0.27	1.04	0.94	-1.20	0.49	-1.04	0.90	-1.02	0.94	-1.06	0.86
Δ*litR*/WT																						
Δ*litR*/WT(HCD_12°C)	**-61.91**	NA	-1.22	0.76	-1.12	0.85	-1.11	0.89	1.06	0.93	1.13	0.81	-1.00	1.00	-1.04	0.98	-1.24	0.54	-1.09	0.88	1.02	0.99
Δ*litR*/WT(LCD_12°C)	**-24.11**	NA	**-1.93**	**0.01**	-1.14	0.50	-1.04	0.89	1.09	0.68	-1.13	0.36	1.04	0.92	1.06	0.84	1.17	0.43	-1.04	0.86	-1.14	0.46
Δ*litR*/WT(HCD_6°C)	**-110.98**	0.00	1.14	0.59	-1.01	0.95	1.06	0.87	1.17	0.41	1.20	0.18	1.41	NA	1.20	0.16	-1.28	0.12	1.01	0.97	-1.18	0.40
Δ*litR*/WT(LCD_6°C)	**-36.87**	0.00	-1.38	0.29	-1.26	0.19	1.00	1.00	-1.24	0.25	-1.22	0.23	1.57	0.26	-1.08	0.62	-1.23	0.17	-1.25	0.17	1.05	0.82
Δ*ainS*/WT																						
Δ*ainS*/WT(HCD_12°C)	**-2.87**	**0.00**	**-3.80**	**NA**	1.06	0.89	1.37	NA	1.14	0.68	-1.21	0.53	-1.08	NA	-1.02	0.96	-1.06	0.88	-1.01	0.98	1.14	0.75
Δ*ainS*/WT(LCD_12°C)	**-2.04**	**0.00**	**-4.41**	**NA**	1.06	0.85	1.53	0.01	1.21	0.41	1.03	0.93	-1.34	NA	-1.05	0.87	1.41	0.03	-1.04	0.87	-1.10	0.70
Δ*ainS*/WT(HCD_6°C)	**-2.53**	**0.00**	**-2.53**	**0.00**	-1.04	1.00	**2.81**	**0.00**	1.48	0.05	1.10	1.00	1.08	NA	1.01	1.00	1.02	1.00	-1.06	1.00	-1.14	0.94
Δ*ainS*/WT(LCD_6°C)	**-1.93**	**0.01**	**-2.34**	**NA**	-1.06	1.00	**2.17**	**0.00**	1.16	1.00	1.09	1.00	1.53	NA	-1.02	1.00	-1.10	1.00	1.00	1.00	1.02	1.00

Additional file 9

Supplementary Table S5.Fold change values of CRISPR system in *A. wodanis* 06/09/139

	AWOD_II_0928		AWOD_II_0929		AWOD_II_0930		AWOD_II_0931		AWOD_II_0932		AWOD_II_0933		AWOD_II_0934		Chromosome 1		Chromosome 2	
	csy4		*csy3*		*csy2*		*csy1*		*cas3*		*cas1*		TR					
Comparisons	CRISPR-associated protein		CRISPR-associated protein		CRISPR-associated protein		CRISPR-associated protein		CRISPR-associated helicase		CRISPR associated protein		putative transcriptional regulator		CRISPR array 1		CRISPR array 2	
Wild type(HCD/LCD)	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**	**FC**	**padj**
(HCD/LCD) 6°C	1.70	0.00	**2.05**	**0.00**	1.83	0.00	1.53	0.01	**2.42**	**0.00**	**2.27**	**0.00**	1.24	0.22	**-1.83**	0.00	1.04	0.82
(HCD/LCD) 12°C	1.30	0.23	1.30	0.10	1.21	0.25	-1.08	0.69	1.52	0.00	1.88	0.00	1.03	0.90	**-1.83**	0.00	**-1.26**	0.28
Wild type(12°C/6°C)																		
(12°C/6°C)HCD	1.33	0.24	-1.01	0.99	-1.01	0.96	-1.36	0.10	-1.07	0.73	1.08	0.75	-1.21	0.29	**-2.10**	**0.00**	**-1.8**	0.01
(12°C/6°C) LCD	1.80	0.00	1.61	0.00	1.54	0.00	1.25	0.15	1.52	0.00	1.33	0.04	1.02	0.92	**-2.05**	**0.00**	-1.32	0.04
Δ*litR*/WT																		
(Δ*litR*/WT)HCD_6°C	**-7.80**	**0.00**	**-11.15**	**0.00**	**-10.36**	**0.00**	**-10.35**	**0.00**	**-6.12**	**0.00**	**-10.32**	**0.00**	**-3.20**	**0.00**	1.61	0.00	-1.69	0.00
(Δ*litR*/WT)HCD_12°C	**-7.54**	**0.00**	**-8.66**	**0.00**	**-7.35**	**0.00**	**-6.50**	**0.00**	**-4.84**	**0.00**	**-6.65**	**0.00**	**-2.03**	**0.00**	**2.01**	**0.00**	-1.14	0.86
(Δ*litR*/WT)LCD_6°C	**-5.54**	**0.00**	**-4.94**	**0.00**	**-6.24**	**0.00**	**-5.87**	**0.00**	**-5.57**	**0.00**	**-8.12**	**0.00**	**-5.81**	**0.00**	-1.77	0.00	**-2.69**	**0.00**
(Δ*litR*/WT)LCD_12°C	**-7.21**	**0.00**	**-7.22**	**0.00**	**-6.55**	**0.00**	**-7.44**	**0.00**	**-4.86**	**0.00**	**-5.78**	**0.00**	**-2.70**	**0.00**	-1.24	0.49	**-1.96**	**0.00**
Δ*ainS*/WT																		
(Δ*ainS*/WT)HCD_6°C	-1.67	0.00	**-2.06**	**0.00**	**-1.92**	**0.00**	-1.87	0.00	-1.70	0.00	-1.52	0.00	-1.37	0.20	1.52	0.01	1.03	1.00
(Δ*ainS*/WT)HCD_12°C	-1.77	0.02	-1.78	0.02	-1.70	0.03	-1.80	0.00	-1.59	0.01	-1.72	0.00	-1.11	0.78	1.44	0.18	1.02	0.97
(Δ*ainS*/WT)LCD_6°C	-1.73	0.07	**-1.95**	**0.01**	**-1.94**	**0.01**	-1.90	0.02	-1.69	0.11	-1.52	0.31	-1.20	1.00	1.10	1.00	-1.05	1.00
(Δ*ainS*/WT)LCD_12°C	**-2.02**	**0.00**	**-2.03**	**0.00**	**-1.94**	**0.00**	-1.90	0.00	-1.83	0.00	-1.48	0.01	-1.12	0.69	1.04	0.93	-1.16	0.64

Additional file 10

Supplementary Table S6. Fold change values of T6SS gene clusters in wild type when comparing high cell density to low cell density

			Wildtype (WT)			
			HCD/LCD(6°C)		HCD/LCD(12°C)	
	AWOD_ID	Protein	FC	padj	FC	padj
T6SS1	AWOD_I_0981	serine/threonine protein kinase	1.31	0.10	1.52	0.02
	AWOD_I_0982	serine/threonine phosphoprotein phosphatase, VasL1	1.97	0.00	1.53	0.15
	AWOD_I_0983	putative type VI secretion protein VasK1	**2.26**	**0.00**	**2.36**	**0.00**
	AWOD_I_0984	putative type VI secretion protein VasF1	1.48	0.02	-1.23	0.26
	AWOD_I_0985	putative type VI secretion protein VasE1	1.52	0.01	1.17	0.50
	AWOD_I_0986	putative type VI secretion protein VasD1	1.43	0.20	1.38	0.30
	AWOD_I_0987	putative type VI secretion protein VasC1	1.51	0.08	1.82	0.03
	AWOD_I_0988	sigma-54 dependent transcriptional regulatory protein, VasH1	**3.12**	**0.00**	**2.45**	**0.00**
	AWOD_I_0989	chaperone ClpB, VasG1	**3.08**	**0.00**	**2.50**	**0.00**
	AWOD_I_0990	putative type VI secretion protein VasB1	**2.31**	**0.00**	1.83	0.11
	AWOD_I_0991	putative type VI secretion protein VasA1	-1.11	0.54	1.28	0.14
	AWOD_I_0992	putative type VI secretion protein VasS1	1.54	0.14	1.48	0.14
	AWOD_I_0993	putative type VI secretion protein VasR1	**2.22**	**0.00**	**2.30**	**0.00**
	AWOD_I_0994	putative type VI secretion protein VasQ1	**2.27**	**0.00**	**2.80**	**0.00**
	AWOD_I_0995	putative type VI secretion protein VasJ1	1.77	0.00	**2.08**	**0.00**
Aux-1	AWOD_I_1435	membrane protein	**2.92**	**0.00**	1.39	0.35
	AWOD_I_1436	membrane protein	**2.32**	**0.00**	**2.48**	**0.00**
	AWOD_I_1437	membrane protein	**2.81**	**0.00**	**2.49**	**0.00**
	AWOD_I_1438	membrane protein	**3.48**	**0.00**	**2.91**	**0.00**
	AWOD_I_1439	putative uncharacterized protein, DUF4123	**4.50**	**0.00**	**2.52**	**0.00**
	AWOD_I_1440	VgrG protein, VgrG1	1.42	0.05	1.93	0.00
	AWOD_I_2030	putative uncharacterized protein, PAAR motif	**2.04**	**0.00**	**2.44**	**0.00**
T6SS2	AWOD_II_0111	type VI secretion-related uncharacterized protein, VasQ2	-1.00	0.98	-1.62	0.01
	AWOD_II_0112	type VI secretion-related uncharacterized protein, VasR2	1.19	0.28	-1.39	0.03
	AWOD_II_0113	type VI secretion-related lysosyme protein, VasS2	1.40	0.05	-1.41	0.05
	AWOD_II_0114	type VI secretion-related uncharacterized protein VasA2	1.61	0.01	1.06	0.76
	AWOD_II_0115	type VI secretion-related uncharacterized protein VasB2	**2.01**	**0.00**	1.66	0.00
	AWOD_II_0116	type VI secretion-related FHA domain protein VasC2	1.98	0.00	1.43	0.02
	AWOD_II_0117	type VI secretion-related lipoprotein VasD2	**2.40**	**0.00**	-1.11	0.69
	AWOD_II_0118	type VI secretion-related protein VasE2	1.64	0.00	1.24	0.11
	AWOD_II_0119	type VI secretion-related protein VasF2	1.74	0.00	1.72	0.02
	AWOD_II_0120	type VI secretion ATPase, ClpB protein, VasG2	1.91	0.00	1.68	0.00
	AWOD_II_0121	Sigma-54 dependent transcriptional regulator VasH2	**2.17**	**0.00**	**2.00**	**0.00**
	AWOD_II_0122	type VI secretion-related protein VasI2	**2.09**	**0.00**	**2.17**	**0.00**
	AWOD_II_0123	type VI secretion-related protein (ImpA domain protein), VasJ2	**2.41**	**0.00**	**3.01**	**0.00**
	AWOD_II_0124	putative type VI secretion protein VasK2 (IcmF-related protein)	**2.05**	**0.00**	1.99	0.00
	AWOD_II_0125	type VI secretion-related protein VasL2	1.56	0.02	**2.04**	**0.00**
Aux-2	AWOD_II_0126	Hcp protein (haemolysin co-regulated protein), Hcp1	**2.63**	**0.00**	**4.80**	**0.00**
	AWOD_II_0127	VgrG protein, VgrG2	1.82	0.00	1.91	0.01
	AWOD_II_0128	type VI secretion-related protein, PAAR	**2.17**	**0.00**	**2.52**	**0.00**
	AWOD_II_0129	ankyrin repeat containing protein	1.67	0.01	1.55	0.08
	AWOD_II_0130	ankyrin repeat containing protein (pseudogene)	**2.29**	**0.00**	1.89	0.00
	AWOD_II_0131	putative uncharacterized protein, DUF4123	**2.69**	**0.00**	**2.34**	**0.01**
	AWOD_II_0132	putative uncharacterized protein, DUF4123	**2.86**	**0.00**	**2.00**	**0.01**
	AWOD_II_0133	RHS protein	-1.07	0.64	1.11	0.37
	AWOD_II_0134	putative uncharacterized protein	-1.19	0.35	-1.29	0.13
	AWOD_II_0135	pseudogene	-1.05	0.77	-1.24	0.12
	AWOD_II_0136	putative uncharacterized protein	1.18	0.39	-1.23	0.24
	AWOD_II_0137	transposase (fragment)	1.36	0.20	-1.06	0.84
	AWOD_II_0138	pseudogene	**-2.28**	**0.00**	-1.10	0.53
	AWOD_II_0139	putative uncharacterized protein	**-2.14**	**0.00**	-1.15	0.42
	AWOD_II_0140	RHS protein	-1.36	0.10	-1.09	0.65
	AWOD_II_0141	putative uncharacterized protein	-1.53	0.00	-1.31	0.06
T6SS3	AWOD_II_1008	putative uncharacterized protein	1.41	0.02	-1.03	0.85
	AWOD_II_1009	putative type VI secretion protein VasX	1.46	0.00	1.12	0.49
	AWOD_II_1010	putative type VI secretion protein, ImcF-related VtsI	1.30	0.03	1.15	0.28
	AWOD_II_1011	putative type VI secretion protein VasF3	1.24	0.21	1.08	0.66
	AWOD_II_1012	putative type VI secretion protein VasE3	1.06	0.79	-1.19	0.26
	AWOD_II_1013	putative type VI secretion protein VasD3	1.15	0.46	-1.12	0.58
	AWOD_II_1014	putative type VI secretion protein VasC3	1.35	0.03	-1.07	0.66
	AWOD_II_1015	putative type VI secretion protein, periplasmic solute-binding protein VtsD	1.47	0.01	1.08	0.70
	AWOD_II_1016	putative type VI secretion protein, periplasmic solute-binding protein VtsC	1.23	0.11	1.18	0.22
	AWOD_II_1017	putative type VI secretion protein, MFS transporter VtsB	-1.14	0.34	1.02	0.93
	AWOD_II_1018	putative type VI secretion protein, D-alanine-D-alanine ligase VtsA	1.02	0.91	1.02	0.92
	AWOD_II_1019	chaperone ClpB, VasG3	-1.16	0.29	-1.58	0.00
	AWOD_II_1020	putative type VI secretion protein VasB3	-1.12	0.40	-1.30	0.02
	AWOD_II_1021	putative type VI secretion protein VasA3	-1.18	0.32	-1.31	0.02
	AWOD_II_1022	putative type VI secretion protein VasS3	-1.05	0.82	-1.39	0.07
	AWOD_II_1023	putative type VI secretion protein VasRB3	1.01	0.97	-1.29	0.06
	AWOD_II_1024	putative type VI secretion protein VasRA3	-1.24	0.19	-1.26	0.11
	AWOD_II_1025	putative type VI secretion protein VasQ3	-1.07	0.69	-1.15	0.36
	AWOD_II_1026	hemolysin-coregulated protein Hcp2	1.09	0.57	-1.04	0.80
	AWOD_II_1027	putative type VI secretion protein VasJ3	1.20	0.22	-1.08	0.64
Aux-3	AWOD_II_1028	putative type VI secretion protein VasV, PAAR domain protein	-1.15	0.49	-1.31	0.26
	AWOD_II_1029	putative type VI secretion protein VasU	-1.11	0.59	-1.55	0.01
	AWOD_II_1030	putative type VI secretion protein VgrG3	-1.02	0.93	-1.77	0.00
	AWOD_II_1031	putative uncharacterized protein	-1.69	0.00	**-7.11**	**0.00**
	AWOD_II_1032	putative uncharacterized protein, Hcp3	-1.48	0.00	**-2.82**	**0.00**
Aux-4	AWOD_II_1054	membrane protein	1.48	0.16	**2.91**	**0.00**
	AWOD_II_1055	membrane protein	1.97	0.00	**3.27**	**0.00**
	AWOD_II_1056	membrane protein	1.45	0.30	1.99	0.09
	AWOD_II_1057	putative uncharacterized protein	**3.08**	**0.00**	**2.46**	**0.01**
	AWOD_II_1058	putative uncharacterized protein, DUF4123	**3.38**	**0.00**	**2.99**	**0.00**
	AWOD_II_1059	VgrG protein, VgrG4	**4.11**	**0.00**	**3.90**	**0.00**
	AWOD_II_1060	secreted protein Hcp4	1.62	0.15	1.45	0.38

Supplementary Table S6. Fold change values of T6SS gene clusters in wild type when comparing 12°C to 6°C

			Wild type (WT)			
			12°C/6°C(HCD)		12°C/6°C(LCD)	
	locus tag	Protein	FC	padj	FC	padj
T6SS1	AWOD_I_0981	serine/threonine protein kinase	1.38	0.09	1.23	0.21
	AWOD_I_0982	serine/threonine phosphoprotein phosphatase, VasL1	-1.03	0.94	1.27	0.40
	AWOD_I_0983	putative type VI secretion protein VasK1	1.22	0.54	1.15	0.50
	AWOD_I_0984	putative type VI secretion protein VasF1	-1.33	0.13	1.41	0.05
	AWOD_I_0985	putative type VI secretion protein VasE1	-1.01	0.98	1.33	0.11
	AWOD_I_0986	putative type VI secretion protein VasD1	1.22	0.54	1.29	0.41
	AWOD_I_0987	putative type VI secretion protein VasC1	1.41	0.21	1.21	0.46
	AWOD_I_0988	sigma-54 dependent transcriptional regulatory protein, VasH1	-1.08	0.85	1.20	0.49
	AWOD_I_0989	chaperone ClpB, VasG1	1.27	0.51	1.53	0.03
	AWOD_I_0990	putative type VI secretion protein VasB1	1.34	0.50	1.70	0.04
	AWOD_I_0991	putative type VI secretion protein VasA1	1.61	0.01	1.17	0.29
	AWOD_I_0992	putative type VI secretion protein VasS1	1.74	0.03	1.88	0.02
	AWOD_I_0993	putative type VI secretion protein VasR1	1.51	0.08	1.51	0.01
	AWOD_I_0994	putative type VI secretion protein VasQ1	1.01	0.97	-1.21	0.36
	AWOD_I_0995	putative type VI secretion protein VasJ1	1.04	0.86	-1.10	0.61
Aux-1	AWOD_I_1435	membrane protein	1.06	0.91	**2.33**	**0.01**
	AWOD_I_1436	membrane protein	1.44	0.27	1.38	0.31
	AWOD_I_1437	membrane protein	1.31	0.36	1.49	0.32
	AWOD_I_1438	membrane protein	1.43	0.18	1.74	0.00
	AWOD_I_1439	putative uncharacterized protein, DUF4123	1.16	0.69	**2.15**	**0.02**
	AWOD_I_1440	VgrG protein, VgrG1	1.24	0.37	-1.07	0.75
	AWOD_I_2030	putative uncharacterized protein, PAAR motif	1.40	0.25	1.20	0.58
T6SS2	AWOD_II_0111	type VI secretion-related uncharacterized protein, VasQ2	**-3.95**	**0.00**	**-2.45**	**0.00**
	AWOD_II_0112	type VI secretion-related uncharacterized protein, VasR2	**-2.48**	**0.00**	-1.48	0.00
	AWOD_II_0113	type VI secretion-related lysosyme protein, VasS2	**-2.29**	**0.00**	-1.16	0.38
	AWOD_II_0114	type VI secretion-related uncharacterized protein VasA2	-1.69	0.01	-1.09	0.64
	AWOD_II_0115	type VI secretion-related uncharacterized protein VasB2	-1.14	0.54	1.10	0.64
	AWOD_II_0116	type VI secretion-related FHA domain protein VasC2	-1.17	0.37	1.22	0.19
	AWOD_II_0117	type VI secretion-related lipoprotein VasD2	**-2.41**	**0.00**	1.12	0.62
	AWOD_II_0118	type VI secretion-related protein VasE2	-1.45	0.00	-1.06	0.70
	AWOD_II_0119	type VI secretion-related protein VasF2	-1.73	0.01	-1.69	0.01
	AWOD_II_0120	type VI secretion ATPase, ClpB protein, VasG2	-1.51	0.01	-1.29	0.11
	AWOD_II_0121	Sigma-54 dependent transcriptional regulator VasH2	-1.36	0.09	-1.22	0.24
	AWOD_II_0122	type VI secretion-related protein VasI2	-1.25	0.43	-1.26	0.35
	AWOD_II_0123	type VI secretion-related protein (ImpA domain protein), VasJ2	-1.15	0.56	-1.42	0.07
	AWOD_II_0124	putative type VI secretion protein VasK2 (IcmF-related protein)	-1.07	0.71	-1.01	0.95
	AWOD_II_0125	type VI secretion-related protein VasL2	-1.10	0.66	-1.39	0.09
Aux-2	AWOD_II_0126	Hcp protein (haemolysin co-regulated protein), Hcp1	-1.10	0.67	**-2.01**	**0.00**
	AWOD_II_0127	VgrG protein, VgrG2	-1.24	0.44	-1.30	0.12
	AWOD_II_0128	type VI secretion-related protein, PAAR	1.08	0.83	-1.09	0.67
	AWOD_II_0129	ankyrin repeat containing protein	-1.25	0.30	-1.14	0.63
	AWOD_II_0130	ankyrin repeat containing protein (pseudogene)	-1.14	0.62	1.07	0.76
	AWOD_II_0131	putative uncharacterized protein, DUF4123	1.08	0.85	1.21	0.44
	AWOD_II_0132	putative uncharacterized protein, DUF4123	1.03	0.94	1.47	0.16
	AWOD_II_0133	RHS protein	-1.08	0.59	-1.26	0.04
	AWOD_II_0134	putative uncharacterized protein	1.05	0.86	1.18	0.23
	AWOD_II_0135	pseudogene	1.20	0.22	1.46	0.00
	AWOD_II_0136	putative uncharacterized protein	1.13	0.54	1.69	0.00
	AWOD_II_0137	transposase (fragment)	1.23	0.43	1.82	0.01
	AWOD_II_0138	pseudogene	1.66	0.00	-1.22	0.12
	AWOD_II_0139	putative uncharacterized protein	1.82	0.00	1.00	0.99
	AWOD_II_0140	RHS protein	1.42	0.07	1.18	0.35
	AWOD_II_0141	putative uncharacterized protein	1.77	0.00	1.57	0.00
T6SS3	AWOD_II_1008	putative uncharacterized protein	-1.12	0.57	1.33	0.01
	AWOD_II_1009	putative type VI secretion protein VasX	-1.33	0.06	1.00	0.99
	AWOD_II_1010	putative type VI secretion protein, ImcF-related VtsI	1.06	0.67	1.24	0.08
	AWOD_II_1011	putative type VI secretion protein VasF3	1.06	0.80	1.25	0.13
	AWOD_II_1012	putative type VI secretion protein VasE3	1.17	0.40	1.51	0.01
	AWOD_II_1013	putative type VI secretion protein VasD3	1.09	0.69	1.45	0.03
	AWOD_II_1014	putative type VI secretion protein VasC3	1.08	0.66	1.60	0.00
	AWOD_II_1015	putative type VI secretion protein, periplasmic solute-binding protein VtsD	1.00	0.99	1.41	0.02
	AWOD_II_1016	putative type VI secretion protein, periplasmic solute-binding protein VtsC	1.18	0.30	1.26	0.08
	AWOD_II_1017	putative type VI secretion protein, MFS transporter VtsB	1.17	0.37	1.03	0.84
	AWOD_II_1018	putative type VI secretion protein, D-alanine-D-alanine ligase VtsA	-1.04	0.81	-1.02	0.92
	AWOD_II_1019	chaperone ClpB, VasG3	1.32	0.04	1.85	0.00
	AWOD_II_1020	putative type VI secretion protein VasB3	1.06	0.74	1.25	0.06
	AWOD_II_1021	putative type VI secretion protein VasA3	-1.01	0.98	1.13	0.31
	AWOD_II_1022	putative type VI secretion protein VasS3	-1.22	0.39	1.11	0.55
	AWOD_II_1023	putative type VI secretion protein VasRB3	-1.23	0.21	1.07	0.59
	AWOD_II_1024	putative type VI secretion protein VasRA3	1.27	0.15	1.33	0.04
	AWOD_II_1025	putative type VI secretion protein VasQ3	1.15	0.42	1.28	0.05
	AWOD_II_1026	hemolysin-coregulated protein Hcp2	1.04	0.87	1.22	0.08
	AWOD_II_1027	putative type VI secretion protein VasJ3	-1.26	0.21	1.05	0.72
Aux-3	AWOD_II_1028	putative type VI secretion protein VasV, PAAR domain protein	-1.20	0.48	-1.02	0.93
	AWOD_II_1029	putative type VI secretion protein VasU	-1.64	0.01	-1.15	0.42
	AWOD_II_1030	putative type VI secretion protein VgrG3	-1.47	0.00	1.22	0.12
	AWOD_II_1031	putative uncharacterized protein	**-2.90**	**0.00**	1.46	0.00
	AWOD_II_1032	putative uncharacterized protein, Hcp3	-1.69	0.00	1.16	0.17
Aux-4	AWOD_II_1054	membrane protein	1.14	0.71	-1.77	0.11
	AWOD_II_1055	membrane protein	1.26	0.56	-1.47	0.27
	AWOD_II_1056	membrane protein	1.05	0.91	-1.33	0.51
	AWOD_II_1057	putative uncharacterized protein	1.13	0.79	1.42	0.24
	AWOD_II_1058	putative uncharacterized protein, DUF4123	1.09	0.85	1.17	0.72
	AWOD_II_1059	VgrG protein, VgrG4	1.14	0.69	1.16	0.70
	AWOD_II_1060	secreted protein Hcp4	1.08	0.87	1.19	0.68

Supplementary Table S6. Fold change values of T6SS gene clusters in the *litR* mutant compared to wild type

			Δ*litR*/WT							
			HCD_6°C		HCD_12°C		LCD_6°C		LCD_12°C	
	AWOD_ID	Function	FC	padj	FC	padj	FC	padj	FC	padj
T6SS1	AWOD_I_0981	serine/threonine protein kinase	-1.22	0.31	-1.22	0.64	-1.62	0.02	-1.22	0.34
	AWOD_I_0982	serine/threonine phosphoprotein phosphatase, VasL1	-1.33	0.26	-1.35	0.50	-1.17	0.71	-1.39	0.40
	AWOD_I_0983	putative type VI secretion protein VasK1	-1.13	0.59	-1.32	0.65	-1.80	0.02	-1.08	0.86
	AWOD_I_0984	putative type VI secretion protein VasF1	-1.60	0.01	-1.64	0.04	-1.46	0.14	**-2.11**	**0.00**
	AWOD_I_0985	putative type VI secretion protein VasE1	-1.40	0.05	-1.39	0.39	-1.72	0.01	-1.69	0.01
	AWOD_I_0986	putative type VI secretion protein VasD1	-1.47	NA	-1.83	0.08	**-2.86**	**NA**	-1.72	0.12
	AWOD_I_0987	putative type VI secretion protein VasC1	-1.32	0.17	-1.69	0.11	**-2.02**	**0.02**	-1.26	0.50
	AWOD_I_0988	sigma-54 dependent transcriptional regulatory protein, VasH1	-1.64	0.01	-1.42	0.50	-1.96	0.04	-1.08	0.88
	AWOD_I_0989	chaperone ClpB, VasG1	-1.74	0.00	-1.63	0.30	-1.92	0.02	-1.50	0.25
	AWOD_I_0990	putative type VI secretion protein VasB1	-1.36	0.28	-1.54	0.44	-1.40	0.42	-1.20	0.63
	AWOD_I_0991	putative type VI secretion protein VasA1	-1.34	0.09	-1.67	0.01	**-2.04**	**0.00**	-1.43	0.02
	AWOD_I_0992	putative type VI secretion protein VasS1	-1.55	NA	**-2.28**	**0.01**	**-2.25**	**NA**	-1.76	0.05
	AWOD_I_0993	putative type VI secretion protein VasR1	**-4.43**	**0.00**	**-4.03**	**0.00**	**-3.20**	**0.00**	**-2.80**	**0.00**
	AWOD_I_0994	putative type VI secretion protein VasQ1	**-7.19**	**0.00**	**-5.13**	**0.00**	**-2.69**	**0.00**	**-2.63**	**0.00**
	AWOD_I_0995	putative type VI secretion protein VasJ1	**-3.15**	**0.00**	**-2.43**	**0.00**	**-2.59**	**0.00**	-1.91	0.00
Aux-1	AWOD_I_1435	membrane protein	-1.75	NA	-1.41	0.57	-1.35	NA	-1.60	NA
	AWOD_I_1436	membrane protein	-1.22	0.53	-1.56	0.26	-1.70	NA	-1.25	0.61
	AWOD_I_1437	membrane protein	-1.14	NA	-1.80	0.10	-1.28	NA	-1.06	NA
	AWOD_I_1438	membrane protein	-1.26	0.20	-1.52	0.31	-1.87	0.02	-1.06	0.89
	AWOD_I_1439	putative uncharacterized protein, DUF4123	-1.41	0.18	-1.37	0.62	-1.98	NA	-1.22	0.68
	AWOD_I_1440	VgrG protein, VgrG1	**-2.09**	**0.00**	**-2.01**	**0.02**	**-4.90**	**0.00**	-1.88	0.04
	AWOD_I_2030	putative uncharacterized protein, PAAR motif	1.31	0.28	-1.11	0.93	-1.24	0.64	1.33	0.53
T6SS2	AWOD_II_0111	type VI secretion-related uncharacterized protein, VasQ2	**-20.28**	**0.00**	**-5.33**	**0.00**	**-21.38**	**0.00**	**-7.00**	**0.00**
	AWOD_II_0112	type VI secretion-related uncharacterized protein, VasR2	**-17.59**	**0.00**	**-6.69**	**0.00**	**-19.31**	**0.00**	**-8.66**	**0.00**
	AWOD_II_0113	type VI secretion-related lysosyme protein, VasS2	**-13.50**	**0.00**	**-4.27**	**0.00**	**-11.40**	**0.00**	**-6.09**	**0.00**
	AWOD_II_0114	type VI secretion-related uncharacterized protein VasA2	**-5.83**	**0.00**	**-2.67**	**0.00**	**-7.35**	**0.00**	**-2.96**	**0.00**
	AWOD_II_0115	type VI secretion-related uncharacterized protein VasB2	**-3.41**	**0.00**	**-2.26**	**0.01**	**-5.44**	**0.00**	**-2.15**	**0.00**
	AWOD_II_0116	type VI secretion-related FHA domain protein VasC2	**-3.13**	**0.00**	**-2.40**	**0.00**	**-5.48**	**0.00**	**-3.26**	**0.00**
	AWOD_II_0117	type VI secretion-related lipoprotein VasD2	**-8.32**	**0.00**	**-3.46**	**0.00**	**-3.87**	**0.00**	**-4.52**	**0.00**
	AWOD_II_0118	type VI secretion-related protein VasE2	**-4.30**	**0.00**	**-2.31**	**0.00**	**-4.53**	**0.00**	**-2.72**	**0.00**
	AWOD_II_0119	type VI secretion-related protein VasF2	**-2.43**	**0.00**	-1.34	0.58	**-4.32**	**0.00**	-1.43	0.27
	AWOD_II_0120	type VI secretion ATPase, ClpB protein, VasG2	**-4.19**	**0.00**	**-2.44**	**0.00**	**-5.03**	**0.00**	**-2.37**	**0.00**
	AWOD_II_0121	Sigma-54 dependent transcriptional regulator VasH2	**-2.45**	**0.00**	-1.52	0.09	**-3.65**	**0.00**	-1.52	0.04
	AWOD_II_0122	type VI secretion-related protein VasI2	**-3.19**	**0.00**	**-2.02**	**0.01**	**-2.69**	**0.00**	-1.50	0.27
	AWOD_II_0123	type VI secretion-related protein (ImpA domain protein), VasJ2	**-2.41**	**0.00**	-1.71	0.16	**-4.22**	**0.00**	-1.22	0.62
	AWOD_II_0124	putative type VI secretion protein VasK2 (IcmF-related protein)	**-2.69**	**0.00**	**-2.15**	**0.00**	**-3.51**	**0.00**	-1.67	0.00
	AWOD_II_0125	type VI secretion-related protein VasL2	**-2.39**	**0.00**	**-2.27**	**0.00**	**-5.19**	**0.00**	-1.37	0.21
Aux-2	AWOD_II_0126	Hcp protein (haemolysin co-regulated protein), Hcp1	**-22.75**	**0.00**	**-8.22**	**0.00**	**-8.32**	**0.00**	-1.86	0.00
	AWOD_II_0127	VgrG protein, VgrG2	**-3.09**	**0.00**	-1.74	0.07	**-4.16**	**0.00**	-1.10	0.76
	AWOD_II_0128	type VI secretion-related protein, PAAR	-1.71	0.00	-1.48	0.42	**-2.62**	**0.00**	1.07	0.88
	AWOD_II_0129	ankyrin repeat containing protein	**-2.42**	**0.00**	-1.96	0.00	**-2.41**	**0.00**	-1.11	0.78
	AWOD_II_0130	ankyrin repeat containing protein (pseudogene)	-1.43	0.08	-1.32	0.57	**-3.83**	**0.00**	1.10	0.79
	AWOD_II_0131	putative uncharacterized protein, DUF4123	-1.17	0.57	-1.30	0.73	-1.67	0.12	1.15	0.73
	AWOD_II_0132	putative uncharacterized protein, DUF4123	-1.87	0.00	-1.30	0.65	-1.03	0.95	1.08	0.87
	AWOD_II_0133	RHS protein	-1.28	0.05	-1.09	0.86	-1.68	0.00	1.18	0.24
	AWOD_II_0134	putative uncharacterized protein	-1.26	0.26	-1.14	0.86	-1.32	0.17	1.19	0.29
	AWOD_II_0135	pseudogene	-1.23	0.18	-1.08	0.94	-1.36	0.10	-1.04	0.88
	AWOD_II_0136	putative uncharacterized protein	-1.16	0.47	-1.07	0.96	-1.18	0.54	1.07	0.78
	AWOD_II_0137	transposase (fragment)	-1.17	0.61	-1.22	0.79	-1.19	0.65	1.08	0.82
	AWOD_II_0138	pseudogene	-1.03	0.91	-1.21	0.62	-1.56	0.00	1.20	0.26
	AWOD_II_0139	putative uncharacterized protein	-1.00	1.00	-1.43	0.17	-1.43	0.07	1.07	0.78
	AWOD_II_0140	RHS protein	1.05	0.86	-1.14	0.82	**-2.04**	**0.00**	-1.04	0.89
	AWOD_II_0141	putative uncharacterized protein	1.26	0.20	-1.02	0.99	-1.77	0.00	-1.00	0.98
T6SS3	AWOD_II_1008	putative uncharacterized protein	1.35	0.06	1.14	0.86	**2.43**	**0.00**	1.33	0.13
	AWOD_II_1009	putative type VI secretion protein VasX	1.35	0.03	1.23	0.55	**2.23**	**0.00**	1.24	0.31
	AWOD_II_1010	putative type VI secretion protein, ImcF-related VtsI	1.24	0.08	1.23	0.57	**2.37**	**0.00**	1.30	0.14
	AWOD_II_1011	putative type VI secretion protein VasF3	1.25	0.16	1.12	0.87	**2.50**	**0.00**	1.37	0.06
	AWOD_II_1012	putative type VI secretion protein VasE3	1.25	0.21	1.02	0.99	**2.21**	**0.00**	1.09	0.73
	AWOD_II_1013	putative type VI secretion protein VasD3	1.36	0.05	1.17	0.82	**2.27**	**0.00**	1.15	0.52
	AWOD_II_1014	putative type VI secretion protein VasC3	1.11	0.60	1.09	0.90	**2.27**	**0.00**	1.26	0.13
	AWOD_II_1015	putative type VI secretion protein, periplasmic solute-binding protein VtsD	1.17	0.38	1.16	0.76	1.99	0.00	1.36	0.06
	AWOD_II_1016	putative type VI secretion protein, periplasmic solute-binding protein VtsC	1.29	0.07	-1.01	0.99	**2.09**	**0.00**	1.19	0.43
	AWOD_II_1017	putative type VI secretion protein, MFS transporter VtsB	1.41	0.00	1.10	0.86	1.81	0.00	1.02	0.93
	AWOD_II_1018	putative type VI secretion protein, D-alanine-D-alanine ligase VtsA	1.15	0.38	-1.02	0.99	1.94	0.00	1.09	0.71
	AWOD_II_1019	chaperone ClpB, VasG3	1.44	0.00	1.13	0.86	**2.11**	**0.00**	-1.06	0.77
	AWOD_II_1020	putative type VI secretion protein VasB3	1.39	0.01	1.16	0.71	1.92	0.00	-1.21	0.27
	AWOD_II_1021	putative type VI secretion protein VasA3	1.31	0.09	1.00	0.99	1.83	0.00	-1.46	0.00
	AWOD_II_1022	putative type VI secretion protein VasS3	1.20	0.40	1.03	0.99	1.93	0.00	-1.18	0.46
	AWOD_II_1023	putative type VI secretion protein VasRB3	1.15	0.42	1.02	0.99	1.91	0.00	-1.53	0.01
	AWOD_II_1024	putative type VI secretion protein VasRA3	1.33	0.08	1.14	0.86	**2.04**	**0.00**	1.17	0.39
	AWOD_II_1025	putative type VI secretion protein VasQ3	1.28	0.11	1.16	0.80	**2.06**	**0.00**	1.26	0.11
	AWOD_II_1026	hemolysin-coregulated protein Hcp2	1.44	0.01	1.31	0.45	1.96	0.00	1.54	0.01
	AWOD_II_1027	putative type VI secretion protein VasJ3	1.37	0.01	1.42	0.20	1.94	0.00	1.50	0.00
Aux-3	AWOD_II_1028	putative type VI secretion protein VasV, PAAR domain protein	**2.11**	**0.00**	**2.03**	**0.00**	**2.11**	**0.00**	1.28	0.28
	AWOD_II_1029	putative type VI secretion protein VasU	1.84	0.00	**2.17**	**0.00**	**2.47**	**0.00**	1.49	0.01
	AWOD_II_1030	putative type VI secretion protein VgrG3	1.93	0.00	**2.00**	**0.00**	**2.35**	**0.00**	1.29	0.04
	AWOD_II_1031	putative uncharacterized protein	1.91	0.00	**5.79**	**0.00**	**2.85**	**0.00**	1.65	0.00
	AWOD_II_1032	putative uncharacterized protein, Hcp3	1.75	0.00	**3.15**	**0.00**	**2.47**	**0.00**	**2.05**	**0.00**
Aux-4	AWOD_II_1054	membrane protein	1.30	0.39	1.07	0.97	-1.48	0.36	1.84	NA
	AWOD_II_1055	membrane protein	1.33	0.29	1.10	0.96	-1.37	0.42	1.75	0.14
	AWOD_II_1056	membrane protein	1.05	NA	1.15	0.91	-1.32	NA	-1.05	NA
	AWOD_II_1057	putative uncharacterized protein	1.13	0.69	-1.17	0.89	1.01	0.99	1.34	0.52
	AWOD_II_1058	putative uncharacterized protein, DUF4123	-1.22	0.50	1.07	0.98	-1.14	NA	1.28	0.63
	AWOD_II_1059	VgrG protein, VgrG4	1.12	0.67	-1.02	0.99	1.07	0.89	1.44	0.40
	AWOD_II_1060	secreted protein Hcp4	-1.68	NA	-1.62	0.32	-1.16	NA	1.05	NA

Supplementary Table S6. Fold change values of T6SS gene clusters in the *ainS* mutant compared to wild type

			Δ*ainS*/WT							
			HCD_6°C		HCD_12°C		LCD_6°C		LCD_12°C	
	locus tag	Protein	FC	padj	FC	padj	FC	padj	FC	padj
T6SS1	AWOD_I_0981	serine/threonine protein kinase	-1.05	1.00	-1.05	0.91	-1.39	0.82	-1.16	0.51
	AWOD_I_0982	serine/threonine phosphoprotein phosphatase, VasL1	-1.28	NA	-1.04	NA	1.06	NA	-1.27	NA
	AWOD_I_0983	putative type VI secretion protein VasK1	-1.11	1.00	-1.01	0.98	-1.09	1.00	-1.07	NA
	AWOD_I_0984	putative type VI secretion protein VasF1	-1.38	0.23	-1.08	NA	-1.05	1.00	-1.44	NA
	AWOD_I_0985	putative type VI secretion protein VasE1	-1.26	0.76	-1.19	0.70	-1.11	1.00	-1.37	0.13
	AWOD_I_0986	putative type VI secretion protein VasD1	-1.17	NA	-1.22	NA	-1.30	NA	-1.23	NA
	AWOD_I_0987	putative type VI secretion protein VasC1	-1.16	0.96	-1.13	0.81	-1.52	NA	-1.20	NA
	AWOD_I_0988	sigma-54 dependent transcriptional regulatory protein, VasH1	-1.34	0.57	1.02	0.98	-1.10	NA	-1.01	NA
	AWOD_I_0989	chaperone ClpB, VasG1	-1.29	0.73	-1.07	0.91	-1.51	NA	-1.09	NA
	AWOD_I_0990	putative type VI secretion protein VasB1	-1.26	NA	-1.07	NA	-1.30	NA	-1.01	NA
	AWOD_I_0991	putative type VI secretion protein VasA1	-1.09	1.00	-1.47	0.10	-1.12	1.00	-1.19	0.40
	AWOD_I_0992	putative type VI secretion protein VasS1	-1.11	NA	-1.43	NA	-1.02	NA	-1.32	NA
	AWOD_I_0993	putative type VI secretion protein VasR1	-1.75	0.00	**-2.17**	**0.00**	-1.58	0.27	-1.70	0.00
	AWOD_I_0994	putative type VI secretion protein VasQ1	**-2.12**	**0.00**	**-2.35**	**NA**	-1.85	NA	-1.46	NA
	AWOD_I_0995	putative type VI secretion protein VasJ1	-1.78	0.00	-1.69	0.01	-1.26	0.98	-1.42	NA
Aux-1	AWOD_I_1435	membrane protein	-1.05	NA	-1.11	NA	-1.09	NA	-1.38	NA
	AWOD_I_1436	membrane protein	-1.16	NA	1.02	NA	-1.07	NA	1.01	NA
	AWOD_I_1437	membrane protein	-1.10	NA	-1.39	NA	-1.01	NA	1.01	NA
	AWOD_I_1438	membrane protein	-1.18	0.94	-1.13	0.82	-1.17	1.00	-1.22	NA
	AWOD_I_1439	putative uncharacterized protein, DUF4123	-1.25	NA	-1.12	NA	-1.13	NA	-1.12	NA
	AWOD_I_1440	VgrG protein, VgrG1	-1.37	0.48	-1.29	0.48	**-2.07**	**0.02**	-1.87	NA
	AWOD_I_2030	putative uncharacterized protein, PAAR motif	-1.01	NA	1.12	NA	1.14	NA	1.09	NA
T6SS2	AWOD_II_0111	type VI secretion-related uncharacterized protein, VasQ2	-1.36	0.11	-1.41	NA	-1.69	0.10	-1.19	0.49
	AWOD_II_0112	type VI secretion-related uncharacterized protein, VasR2	-1.44	0.02	-1.61	0.03	-1.78	0.05	-1.76	0.00
	AWOD_II_0113	type VI secretion-related lysosyme protein, VasS2	-1.34	0.26	-1.68	NA	-1.63	0.17	-1.70	0.00
	AWOD_II_0114	type VI secretion-related uncharacterized protein VasA2	-1.22	0.79	-1.29	0.32	-1.68	0.16	-1.53	0.00
	AWOD_II_0115	type VI secretion-related uncharacterized protein VasB2	-1.25	0.57	-1.32	0.30	-1.48	0.66	-1.41	0.14
	AWOD_II_0116	type VI secretion-related FHA domain protein VasC2	-1.29	0.32	-1.20	0.56	-1.37	0.79	-1.61	0.02
	AWOD_II_0117	type VI secretion-related lipoprotein VasD2	-1.54	0.03	-1.23	NA	-1.27	NA	-1.84	NA
	AWOD_II_0118	type VI secretion-related protein VasE2	-1.47	0.00	-1.24	0.51	-1.41	0.66	-1.57	0.00
	AWOD_II_0119	type VI secretion-related protein VasF2	-1.18	0.91	1.33	0.46	-1.74	0.26	-1.37	NA
	AWOD_II_0120	type VI secretion ATPase, ClpB protein, VasG2	-1.42	0.02	-1.18	0.63	-1.56	0.49	-1.71	0.00
	AWOD_II_0121	Sigma-54 dependent transcriptional regulator VasH2	-1.36	0.11	1.03	0.95	-1.53	0.49	-1.19	0.51
	AWOD_II_0122	type VI secretion-related protein VasI2	-1.22	0.83	1.05	0.93	-1.39	NA	-1.49	NA
	AWOD_II_0123	type VI secretion-related protein (ImpA domain protein), VasJ2	-1.32	0.40	-1.01	0.99	-1.33	0.94	-1.09	NA
	AWOD_II_0124	putative type VI secretion protein VasK2 (IcmF-related protein)	-1.38	0.04	-1.17	0.57	-1.17	1.00	-1.23	0.34
	AWOD_II_0125	type VI secretion-related protein VasL2	-1.25	0.68	-1.07	0.86	-1.40	0.77	-1.28	NA
Aux-2	AWOD_II_0126	Hcp protein (haemolysin co-regulated protein), Hcp1	**-2.01**	**0.00**	**-2.83**	**0.00**	**-2.40**	**0.00**	-1.10	0.76
	AWOD_II_0127	VgrG protein, VgrG2	-1.55	0.03	-1.10	0.84	-1.63	0.40	-1.01	0.98
	AWOD_II_0128	type VI secretion-related protein, PAAR	-1.14	1.00	1.08	0.89	-1.47	0.58	1.16	NA
	AWOD_II_0129	ankyrin repeat containing protein	-1.34	0.31	-1.24	NA	-1.21	0.99	1.28	NA
	AWOD_II_0130	ankyrin repeat containing protein (pseudogene)	-1.19	0.93	1.16	0.77	-1.65	NA	1.10	NA
	AWOD_II_0131	putative uncharacterized protein, DUF4123	-1.07	1.00	1.21	0.72	-1.46	NA	1.09	NA
	AWOD_II_0132	putative uncharacterized protein, DUF4123	-1.14	1.00	1.01	NA	-1.23	NA	1.16	NA
	AWOD_II_0133	RHS protein	-1.19	0.75	1.07	0.84	-1.06	1.00	1.11	0.57
	AWOD_II_0134	putative uncharacterized protein	-1.17	0.93	-1.13	0.82	1.02	1.00	1.08	0.77
	AWOD_II_0135	pseudogene	-1.12	1.00	-1.08	0.87	1.14	1.00	-1.04	0.89
	AWOD_II_0136	putative uncharacterized protein	-1.04	1.00	-1.07	0.87	1.23	0.98	-1.02	0.97
	AWOD_II_0137	transposase (fragment)	-1.32	NA	-1.22	NA	1.11	NA	-1.08	NA
	AWOD_II_0138	pseudogene	1.10	1.00	-1.03	0.93	-1.07	1.00	-1.05	0.87
	AWOD_II_0139	putative uncharacterized protein	1.04	1.00	-1.11	0.83	-1.05	1.00	1.09	0.78
	AWOD_II_0140	RHS protein	-1.08	1.00	-1.05	0.92	-1.26	0.98	1.01	0.97
	AWOD_II_0141	putative uncharacterized protein	-1.03	1.00	-1.32	0.26	-1.28	0.98	-1.07	0.83
T6SS3	AWOD_II_1008	putative uncharacterized protein	1.12	1.00	1.10	0.84	1.52	0.18	-1.00	1.00
	AWOD_II_1009	putative type VI secretion protein VasX	1.01	1.00	1.12	0.82	1.32	0.83	1.02	0.97
	AWOD_II_1010	putative type VI secretion protein, ImcF-related VtsI	1.03	1.00	1.07	0.82	1.39	0.66	-1.15	0.57
	AWOD_II_1011	putative type VI secretion protein VasF3	1.07	1.00	1.04	0.94	1.45	0.58	-1.06	0.87
	AWOD_II_1012	putative type VI secretion protein VasE3	1.12	1.00	-1.06	0.88	1.37	0.78	-1.07	0.82
	AWOD_II_1013	putative type VI secretion protein VasD3	1.02	1.00	1.04	0.94	1.50	0.53	1.00	1.00
	AWOD_II_1014	putative type VI secretion protein VasC3	-1.05	1.00	-1.01	0.99	1.55	0.23	1.12	0.59
	AWOD_II_1015	putative type VI secretion protein, periplasmic solute-binding protein VtsD	-1.14	0.97	-1.04	0.93	1.46	0.57	1.08	0.80
	AWOD_II_1016	putative type VI secretion protein, periplasmic solute-binding protein VtsC	1.01	1.00	-1.04	0.91	1.36	0.75	1.03	0.91
	AWOD_II_1017	putative type VI secretion protein, MFS transporter VtsB	1.17	0.86	-1.02	0.94	1.24	0.98	-1.18	0.50
	AWOD_II_1018	putative type VI secretion protein, D-alanine-D-alanine ligase VtsA	-1.02	1.00	1.01	0.97	1.42	0.55	1.04	0.91
	AWOD_II_1019	chaperone ClpB, VasG3	1.03	1.00	-1.02	0.98	1.39	0.54	-1.17	0.48
	AWOD_II_1020	putative type VI secretion protein VasB3	-1.01	1.00	-1.13	0.70	1.24	0.97	-1.24	0.20
	AWOD_II_1021	putative type VI secretion protein VasA3	1.06	1.00	-1.19	0.45	1.29	0.83	-1.34	0.02
	AWOD_II_1022	putative type VI secretion protein VasS3	-1.01	1.00	-1.18	0.72	1.28	0.94	-1.14	0.57
	AWOD_II_1023	putative type VI secretion protein VasRB3	-1.00	1.00	-1.12	0.73	1.23	0.97	-1.32	0.05
	AWOD_II_1024	putative type VI secretion protein VasRA3	1.09	1.00	1.06	0.92	1.30	0.82	-1.01	0.97
	AWOD_II_1025	putative type VI secretion protein VasQ3	-1.05	1.00	1.04	0.94	1.28	0.89	-1.01	0.97
	AWOD_II_1026	hemolysin-coregulated protein Hcp2	1.02	1.00	1.17	0.72	1.22	0.98	1.01	0.98
	AWOD_II_1027	putative type VI secretion protein VasJ3	-1.17	0.81	1.26	0.57	1.15	1.00	-1.02	0.95
Aux-3	AWOD_II_1028	putative type VI secretion protein VasV, PAAR domain protein	1.43	0.10	1.56	NA	1.31	0.94	1.08	NA
	AWOD_II_1029	putative type VI secretion protein VasU	1.39	0.05	1.63	0.07	1.44	0.69	1.32	0.16
	AWOD_II_1030	putative type VI secretion protein VgrG3	1.41	0.02	1.56	0.02	1.44	0.57	1.18	0.35
	AWOD_II_1031	putative uncharacterized protein	1.73	0.00	**2.97**	**0.00**	1.61	0.22	1.26	0.32
	AWOD_II_1032	putative uncharacterized protein, Hcp3	1.77	0.00	**2.32**	**0.00**	1.49	0.36	1.42	NA
Aux-4	AWOD_II_1054	membrane protein	-1.04	NA	1.31	NA	-1.26	NA	1.30	NA
	AWOD_II_1055	membrane protein	1.03	1.00	1.31	NA	-1.30	NA	1.36	NA
	AWOD_II_1056	membrane protein	-1.10	NA	1.23	NA	-1.47	NA	1.06	NA
	AWOD_II_1057	putative uncharacterized protein	-1.10	1.00	1.05	NA	1.03	NA	1.22	NA
	AWOD_II_1058	putative uncharacterized protein, DUF4123	-1.07	NA	1.18	NA	-1.04	NA	1.10	NA
	AWOD_II_1059	VgrG protein, VgrG4	-1.09	1.00	1.21	0.71	1.15	NA	1.18	NA
	AWOD_II_1060	secreted protein Hcp4	-1.20	NA	-1.21	NA	-1.02	NA	-1.11	NA

Additional file 11

Supplementary Table S7. Type VI secretion system effectors predicted by Bastion 6 and their fold change values in wild type when compared between cell densities (HCD/LCD)

T6SE predicted by Bastion 6		6°C		12°C	
locus tag	Gene product	FC	padj	FC	padj
AWOD_I_0029	6-phospho-beta-glucosidase	-1.83	0.00	-1.52	0.01
AWOD_I_0055	TatD related DNase	1.15	0.32	1.00	0.98
AWOD_I_0090	putative lipoprotein	-1.04	0.80	-1.31	0.12
AWOD_I_0096	membrane protein	**-2.13**	**0.00**	-1.06	0.80
AWOD_I_0154	putative lipoprotein	-1.26	0.11	-1.27	0.04
AWOD_I_0175	putative exported protein	-1.27	0.18	-1.17	0.46
AWOD_I_0346	outer membrane protein	**2.15**	**0.00**	**2.25**	0.00
AWOD_I_0414	N-acetylmuramoyl-L-alanine amidase	-1.12	0.53	1.14	0.47
AWOD_I_0448	chitobiose phosphorylase (glycosyl transferase)	-1.02	0.91	**-5.55**	**0.00**
AWOD_I_0451	putative endochitinase	1.68	0.00	**-3.07**	**0.00**
AWOD_I_0522	putative lipoprotein	-1.63	0.00	-1.47	0.00
AWOD_I_0717	rare lipoprotein A	-1.13	0.53	-1.30	0.09
AWOD_I_0809	putative lipoprotein	1.12	0.49	-1.56	0.00
AWOD_I_1000	porin-like protein H	1.09	0.72	**5.50**	**0.00**
AWOD_I_1095	putative HNH endonuclease	-1.35	0.03	1.14	0.52
AWOD_I_1120	outer membrane protein	**2.46**	**0.00**	1.94	0.00
AWOD_I_1184	putative exported protein	-1.53	0.01	1.43	0.28
AWOD_I_1186	putative lipoprotein	-1.44	0.02	1.33	0.18
AWOD_I_1189	putative exported protein	1.16	0.41	-1.25	0.11
AWOD_I_1230	lipoprotein NlpC	-1.52	0.00	-1.06	0.78
AWOD_I_1294	putative nuclease	-1.45	0.05	1.04	0.88
AWOD_I_1338	putative polysaccharide deacetylase	1.87	0.00	1.61	0.03
AWOD_I_1428	succinylglutamate desuccinylase	**2.06**	**0.00**	1.77	0.00
AWOD_I_1438	membrane protein	**3.48**	**0.00**	**2.91**	**0.00**
AWOD_I_1509	putative histidine decarboxylase	**2.10**	**0.00**	-1.02	0.91
AWOD_I_1519	putative lipoprotein	-1.02	0.91	1.13	0.61
AWOD_I_1529	putative uncharacterized protein	**-2.63**	**0.00**	1.76	0.02
AWOD_I_1536	putative lipoprotein	**-3.66**	**0.00**	-1.30	0.19
AWOD_I_1540	putative lipoprotein	1.00	0.99	-1.01	0.98
AWOD_I_1567	membrane protein	1.28	0.15	-1.01	0.95
AWOD_I_1613	putative lipoprotein	1.70	0.01	1.97	0.00
AWOD_I_1647	phospholipase A1 precursor	-1.34	0.12	1.39	0.14
AWOD_I_1762	putative uncharacterized protein	**-3.44**	**0.00**	-1.58	0.01
AWOD_I_2007	putative porin	-1.76	0.01	-1.31	0.03
AWOD_I_2009	putative exported protein	-1.76	0.00	1.23	0.22
AWOD_I_2022	periplasmic nitrate reductase NapB	-1.24	0.26	**-4.75**	**0.00**
AWOD_I_2023	periplasmic nitrate reductase precursor NapA	-1.17	0.47	**-5.07**	**0.00**
AWOD_I_2030	putative uncharacterized protein, PAAR motif	**2.04**	0.00	**2.44**	0.00
AWOD_I_2061	putative hydrolase	-1.54	0.00	-1.14	0.51
AWOD_I_2148	lipoprotein, LysM domain	**2.63**	0.00	**2.24**	**0.00**
AWOD_I_2248	endonuclease I precursor	**2.00**	0.02	1.28	0.49
AWOD_I_2370	lfite reductase [NADPH] flavoprotein alpha-compone	**2.94**	0.00	**22.38**	0.00
AWOD_I_2451	putative lipoprotein	-1.31	0.02	1.07	0.67
AWOD_I_2491	putative lipoprotein	1.53	0.00	-1.02	0.94
AWOD_II_0128	type VI secretion-related protein	**2.17**	0.00	**2.52**	0.00
AWOD_II_0133	RHS protein	-1.07	0.64	1.11	0.37
AWOD_II_0252	secreted endonuclease	1.19	0.47	**-2.08**	**0.17**
AWOD_II_0254	secreted endonuclease I	**3.96**	**0.00**	**-2.17**	**0.10**
AWOD_II_0263	putative lipoprotein	**-7.81**	**0.00**	**-5.96**	**0.00**
AWOD_II_0339	putative lipoprotein	1.23	0.26	1.52	0.14
AWOD_II_0346	putative lipoprotein	-1.80	0.00	-1.68	0.01
AWOD_II_0356	aldose 1-epimerase	-1.19	0.18	-1.21	0.25
AWOD_II_0366	putative beta-lactamase	1.97	0.00	**2.01**	**0.00**
AWOD_II_0424	putative DNA-binding protein	1.93	0.00	-1.93	0.01
AWOD_II_0440	putative lipoprotein	1.03	0.87	-1.60	0.00
AWOD_II_0501	putative exported protein	**-2.36**	**0.00**	-1.14	0.68
AWOD_II_0639	putative uncharacterized protein	1.17	0.21	-1.28	0.08
AWOD_II_0656	putative exported protein	-1.08	0.69	-1.24	0.19
AWOD_II_0676	putative chloramphenicol acetyltransferase	-1.62	0.01	-1.15	0.46
AWOD_II_0680	putative chloramphenicol acetyltransferase	-1.49	0.02	-1.15	0.41
AWOD_II_0686	putative uncharacterized protein	1.62	0.00	-1.18	0.18
AWOD_II_0804	putative lipoprotein	-1.12	0.63	1.26	0.21
AWOD_II_0806	putative lipoprotein	1.77	0.08	1.80	0.08
AWOD_II_0830	putative lipoprotein	**-2.41**	**0.00**	-1.01	0.93
AWOD_II_0852	amine oxidase	1.66	0.02	1.91	0.00
AWOD_II_0964	N-succinylarginine dihydrolase	1.18	0.43	1.48	0.00
AWOD_II_1001	putative lipoprotein	-1.40	0.21	-1.16	0.51
AWOD_II_1005	putative uncharacterized protein	-1.13	0.35	-1.63	0.01
AWOD_II_1031	putative uncharacterized protein	-1.69	0.00	**-7.11**	0.00
AWOD_II_1032	type VI secretion system secreted protein Hcp	-1.48	0.00	**-2.82**	0.00
AWOD_II_1038	putative exported protein	-1.73	0.00	-1.71	0.00
AWOD_II_1045	putative uncharacterized protein	**3.05**	**0.02**	1.97	0.20
AWOD_II_1158	putative exported hemolysin	**5.02**	**0.00**	1.53	0.00
AWOD_II_1182	putative lipoprotein	1.41	0.17	-1.45	0.07
AWOD_II_1206	putative lipoprotein	**-5.83**	**0.00**	**-3.30**	**0.00**
AWOD_II_1212	putative uncharacterized phospholipase	**2.73**	**0.00**	1.91	0.01
AWOD_II_1235	choline dehydrogenase	**173.29**	**0.00**	**8.99**	0.00
AWOD_p920_006	putative bacteriocin	**2.73**	**0.00**	-1.46	0.10
AWOD_p920_0078	phospholipase A1 precursor	-1.60	0.00	1.58	0.01
AWOD_I_1183	Iron-regulated protein A	-0.43	0.04	-0.39	0.42

Supplementary Table S7. Type VI secretion system effectors predicted by Bastion 6 and their fold change values in wild type when compared between temperatures (12°C/6°C)

T6SE predicted by Bastion 6		12°C/6°C			
		HCD		LCD	
locus tag	Gene product	FC	padj	FC	padj
AWOD_I_0029	6-phospho-beta-glucosidase	1.39	0.07	1.19	0.27
AWOD_I_0055	TatD related DNase	1.17	0.33	1.38	0.02
AWOD_I_0090	putative lipoprotein	1.19	0.35	1.56	0.00
AWOD_I_0096	membrane protein	-1.07	0.78	**-2.10**	0.00
AWOD_I_0154	putative lipoprotein	1.14	0.45	1.19	0.13
AWOD_I_0175	putative exported protein	1.40	0.11	1.33	0.06
AWOD_I_0346	outer membrane protein	1.05	0.85	1.03	0.90
AWOD_I_0414	N-acetylmuramoyl-L-alanine amidase	-1.14	0.54	-1.42	0.01
AWOD_I_0448	chitobiose phosphorylase (glycosyl transferase)	1.43	0.01	**8.07**	0.00
AWOD_I_0451	putative endochitinase	1.48	0.05	**8.04**	0.00
AWOD_I_0522	putative lipoprotein	-1.22	0.23	-1.33	0.01
AWOD_I_0717	rare lipoprotein A	1.04	0.88	1.22	0.21
AWOD_I_0809	putative lipoprotein	1.02	0.93	1.83	0.00
AWOD_I_1000	porin-like protein H	**6.01**	**0.00**	1.27	0.18
AWOD_I_1095	putative HNH endonuclease	1.30	0.21	-1.15	0.35
AWOD_I_1120	outer membrane protein	1.55	0.05	**2.09**	0.00
AWOD_I_1183	Iron-regulated protein A	**-3.53**	**0.00**	**-3.83**	**0.00**
AWOD_I_1184	putative exported protein	**-2.77**	**0.00**	**-6.78**	**0.00**
AWOD_I_1186	putative lipoprotein	**-2.38**	**0.00**	**-4.50**	**0.00**
AWOD_I_1189	putative exported protein	-1.38	0.04	1.08	0.67
AWOD_I_1230	lipoprotein NlpC	**2.02**	0.00	1.46	0.00
AWOD_I_1294	putative nuclease	1.87	0.00	1.29	0.16
AWOD_I_1338	putative polysaccharide deacetylase	1.32	0.22	1.58	0.01
AWOD_I_1428	succinylglutamate desuccinylase	-1.22	0.39	-1.01	0.95
AWOD_I_1438	membrane protein	1.43	0.18	1.74	0.00
AWOD_I_1509	putative histidine decarboxylase	1.14	0.55	**2.54**	0.00
AWOD_I_1519	putative lipoprotein	1.19	0.47	1.06	0.75
AWOD_I_1529	putative uncharacterized protein	**3.45**	0.00	-1.28	0.34
AWOD_I_1536	putative lipoprotein	-1.74	0.00	**-4.80**	0.00
AWOD_I_1540	putative lipoprotein	-1.36	0.37	-1.32	0.29
AWOD_I_1567	membrane protein	1.21	0.35	1.61	0.00
AWOD_I_1613	putative lipoprotein	**2.26**	0.00	**2.07**	0.00
AWOD_I_1647	phospholipase A1 precursor	1.25	0.36	-1.46	0.04
AWOD_I_1762	putative uncharacterized protein	1.60	0.02	-1.30	0.01
AWOD_I_2007	putative porin	1.03	0.94	-1.30	0.03
AWOD_I_2009	putative exported protein	**2.51**	0.00	1.23	0.09
AWOD_I_2022	periplasmic nitrate reductase NapB	**-2.46**	0.00	1.59	0.00
AWOD_I_2023	periplasmic nitrate reductase precursor NapA	**-2.76**	0.00	1.60	0.01
AWOD_I_2030	putative uncharacterized protein, PAAR motif	1.40	0.25	1.20	0.58
AWOD_I_2061	putative hydrolase	-1.15	0.47	-1.51	0.00
AWOD_I_2148	lipoprotein, LysM domain	-1.07	0.84	1.13	0.28
AWOD_I_2248	endonuclease I precursor	1.02	0.96	1.63	0.11
AWOD_I_2370	ulfite reductase [NADPH] flavoprotein alpha-componer	1.50	0.04	**-5.06**	0.00
AWOD_I_2451	putative lipoprotein	1.40	0.03	1.02	0.92
AWOD_I_2491	putative lipoprotein	-1.81	0.01	-1.15	0.35
AWOD_II_0128	type VI secretion-related protein	1.08	0.83	-1.09	0.67
AWOD_II_0133	RHS protein	-1.08	0.59	-1.26	0.04
AWOD_II_0252	secreted endonuclease	1.70	0.33	**8.19**	0.00
AWOD_II_0254	secreted endonuclease I	1.05	0.93	**11.97**	0.00
AWOD_II_0263	putative lipoprotein	1.80	0.14	**2.58**	0.00
AWOD_II_0339	putative lipoprotein	1.05	0.90	-1.16	0.49
AWOD_II_0346	putative lipoprotein	-1.20	0.40	-1.26	0.23
AWOD_II_0356	aldose 1-epimerase	-1.46	0.02	-1.40	0.00
AWOD_II_0366	putative beta-lactamase	**4.03**	0.00	**4.11**	0.00
AWOD_II_0424	putative DNA-binding protein	-1.02	0.95	**3.82**	0.00
AWOD_II_0440	putative lipoprotein	-1.04	0.86	1.63	0.00
AWOD_II_0501	putative exported protein	1.07	0.87	-1.93	0.00
AWOD_II_0639	putative uncharacterized protein	1.34	0.02	**2.07**	0.00
AWOD_II_0656	putative exported protein	1.54	0.01	1.84	0.00
AWOD_II_0676	putative chloramphenicol acetyltransferase	1.71	0.00	1.25	0.18
AWOD_II_0680	putative chloramphenicol acetyltransferase	1.65	0.00	1.31	0.08
AWOD_II_0686	putative uncharacterized protein	-1.00	0.99	1.97	0.00
AWOD_II_0804	putative lipoprotein	1.73	0.01	1.27	0.21
AWOD_II_0806	putative lipoprotein	1.72	0.14	1.75	0.07
AWOD_II_0830	putative lipoprotein	**3.16**	0.00	1.39	0.00
AWOD_II_0852	amine oxidase	**2.31**	0.00	**2.14**	0.00
AWOD_II_0964	N-succinylarginine dihydrolase	1.12	0.62	-1.08	0.61
AWOD_II_1001	putative lipoprotein	**2.14**	0.00	1.85	0.00
AWOD_II_1005	putative uncharacterized protein	-1.39	0.03	1.07	0.72
AWOD_II_1031	putative uncharacterized protein	**-2.90**	0.00	1.46	0.00
AWOD_II_1032	type VI secretion system secreted protein Hcp	-1.69	0.00	1.16	0.17
AWOD_II_1038	putative exported protein	**-2.09**	0.00	**-2.09**	0.00
AWOD_II_1045	putative uncharacterized protein	1.07	0.91	1.52	0.48
AWOD_II_1158	putative exported hemolysin	-1.74	0.00	1.95	0.00
AWOD_II_1182	putative lipoprotein	1.16	0.62	**2.45**	0.00
AWOD_II_1206	putative lipoprotein	**-2.89**	0.00	**-5.08**	0.00
AWOD_II_1212	putative uncharacterized phospholipase	1.06	0.85	1.52	0.07
AWOD_II_1235	choline dehydrogenase	**-31.69**	**0.00**	-1.66	0.13
AWOD_p920_006	putative bacteriocin	-1.08	0.85	**3.91**	0.00
AWOD_p920_0078	phospholipase A1 precursor	1.34	0.12	-1.84	0.00

Supplementary Table S7. Type VI secretion system effectors predicted by Bastion 6 and their fold change values in *litR* and *ainS* mutants compared to wild type

		Fold change values of T6S effectors in Δ*litR*/WT								Fold change values of T6S effectors in Δ*ainS*/WT							
T6SE predicted by Bastion 6		HCD_6°C		HCD_12°C		LCD_6°C		LCD_12°C		HCD_6°C		HCD_12°C		LCD_6°C		LCD_12°C	
locus tag	Gene product	FC	padj	FC	padj	FC	padj	FC	padj	FC	padj	FC	padj	FC	padj	FC	padj
AWOD_I_0029	6-phospho-beta-glucosidase	**10.66**	**0.00**	**7.07**	**0.00**	**4.00**	**0.00**	**8.33**	**0.00**	**2.11**	**0.00**	**2.43**	**0.00**	1.51	0.69	**2.69**	**0.00**
AWOD_I_0055	TatD related DNase	1.06	0.79	-1.07	0.92	1.29	0.15	1.09	0.70	1.03	1.00	1.03	0.93	1.22	0.98	1.00	0.99
AWOD_I_0090	putative lipoprotein	1.09	0.66	-1.04	0.98	-1.10	0.67	-1.01	0.98	-1.11	1.00	1.11	0.78	1.04	1.00	-1.01	0.98
AWOD_I_0096	membrane protein	1.23	0.18	1.06	0.96	**-2.09**	**0.00**	1.14	0.48	1.05	1.00	1.07	0.88	-1.35	0.94	1.16	0.45
AWOD_I_0154	putative lipoprotein	-1.28	NA	-1.27	0.47	1.09	0.60	-1.34	0.02	1.02	1.00	-1.06	0.84	1.11	1.00	-1.05	0.87
AWOD_I_0175	putative exported protein	**-4.94**	**0.00**	**-2.96**	**0.00**	**-6.49**	**0.00**	**-2.30**	**0.00**	1.04	1.00	-1.25	NA	-1.20	0.98	-1.17	0.54
AWOD_I_0346	outer membrane protein	1.11	0.62	-1.19	0.80	-1.36	0.17	-1.48	0.06	-1.03	1.00	-1.36	0.24	-1.24	0.98	-1.19	0.49
AWOD_I_0414	N-acetylmuramoyl-L-alanine amidase	-1.02	0.95	1.03	0.99	-1.18	0.40	-1.06	0.86	-1.19	0.89	1.00	1.00	1.01	1.00	1.13	0.61
AWOD_I_0448	chitobiose phosphorylase (glycosyl transferase)	1.21	0.26	-1.64	0.01	-1.05	0.85	**-2.38**	**0.00**	1.07	1.00	-1.05	0.88	-1.04	1.00	-1.37	0.39
AWOD_I_0451	putative endochitinase	1.29	0.18	-1.03	0.99	-1.61	0.07	**-2.07**	**0.00**	1.00	1.00	1.09	0.86	1.12	1.00	-1.76	0.01
AWOD_I_0522	putative lipoprotein	-1.16	0.41	-1.02	0.98	-1.60	0.00	1.16	0.39	-1.08	1.00	-1.09	0.77	-1.23	0.98	1.14	0.55
AWOD_I_0717	rare lipoprotein A	1.22	0.37	1.26	0.40	1.31	0.18	-1.25	0.28	1.22	0.80	-1.02	0.96	-1.01	1.00	-1.20	0.45
AWOD_I_0809	putative lipoprotein	-1.09	0.68	-1.14	0.79	-1.13	0.60	-1.68	0.00	-1.10	1.00	-1.17	0.68	1.07	1.00	-1.18	0.38
AWOD_I_1000	porin-like protein H	-1.64	0.01	**-3.99**	**0.00**	-1.94	0.00	-1.46	0.03	-1.05	1.00	**-2.20**	**0.00**	-1.20	0.99	-1.04	0.91
AWOD_I_1095	putative HNH endonuclease	1.10	0.66	-1.10	0.90	1.32	0.09	1.07	0.84	1.19	0.78	-1.41	0.19	1.08	1.00	-1.26	0.28
AWOD_I_1120	outer membrane protein	1.09	0.74	**-2.07**	**0.00**	-1.01	0.95	-1.06	0.75	1.03	1.00	-1.47	0.07	1.12	1.00	-1.06	0.80
AWOD_I_1183	Iron-regulated protein A	**-4.59**	**0.00**	-1.22	0.86	-1.35	0.03	-1.57	0.01	-1.36	0.21	1.08	0.90	-1.22	0.98	-1.37	0.07
AWOD_I_1184	putative exported protein	**-3.30**	**0.00**	-1.54	0.36	-1.22	0.22	-1.16	0.73	-1.27	0.71	-1.15	0.80	-1.25	0.98	-1.38	NA
AWOD_I_1186	putative lipoprotein	**-2.72**	**0.00**	-1.43	0.32	-1.03	0.88	1.12	0.73	-1.16	0.93	-1.03	0.94	-1.17	1.00	1.04	NA
AWOD_I_1189	putative exported protein	1.21	0.26	1.20	0.74	**2.38**	**0.00**	1.03	0.91	1.08	1.00	-1.00	0.99	1.50	0.68	-1.06	0.85
AWOD_I_1230	lipoprotein NlpC	1.32	0.09	-1.25	0.54	-1.27	0.20	-1.14	0.49	1.10	1.00	-1.40	0.14	1.09	1.00	-1.17	0.45
AWOD_I_1294	putative nuclease	1.14	0.64	-1.09	0.92	-1.60	0.04	1.02	0.95	-1.00	1.00	1.02	0.97	-1.44	0.63	-1.04	0.94
AWOD_I_1338	putative polysaccharide deacetylase	**-2.10**	**0.00**	-1.55	0.15	**-2.05**	**0.01**	-1.06	0.85	-1.50	0.02	-1.17	0.72	-1.06	1.00	1.05	0.89
AWOD_I_1428	succinylglutamate desuccinylase	-1.08	0.78	-1.58	NA	**-2.10**	**0.01**	-1.08	0.67	1.11	1.00	-1.16	0.70	-1.08	1.00	1.09	0.76
AWOD_I_1438	membrane protein	-1.26	0.20	-1.52	0.31	-1.87	0.02	-1.06	0.89	-1.18	0.94	-1.13	0.82	-1.17	1.00	-1.22	NA
AWOD_I_1509	putative histidine decarboxylase	-1.14	0.53	-1.68	0.03	-1.69	0.01	-1.78	0.01	-1.52	0.01	**-2.44**	**0.00**	-1.24	0.99	**-2.34**	**0.00**
AWOD_I_1519	putative lipoprotein	1.18	0.44	1.01	0.99	1.17	0.49	1.01	0.97	1.01	1.00	1.24	0.63	1.03	1.00	1.07	0.85
AWOD_I_1529	putative uncharacterized protein	1.54	NA	**-2.74**	**0.00**	**-2.43**	**0.01**	-1.45	0.28	-1.11	NA	-1.97	NA	-1.06	NA	-1.43	NA
AWOD_I_1536	putative lipoprotein	-1.85	0.00	-1.18	0.77	-1.25	0.30	1.20	0.29	-1.07	1.00	1.21	0.63	-1.10	1.00	1.19	0.50
AWOD_I_1540	putative lipoprotein	-1.26	0.44	1.15	0.91	-1.27	0.48	1.03	0.95	-1.08	NA	1.30	NA	1.09	NA	-1.03	NA
AWOD_I_1567	membrane protein	**-2.53**	**0.00**	**-2.29**	**0.00**	-1.23	0.38	**-2.06**	**0.00**	-1.96	0.00	-1.72	0.04	-1.18	1.00	-1.43	0.07
AWOD_I_1613	putative lipoprotein	1.01	0.97	-1.67	0.04	-1.56	0.01	-1.39	0.00	-1.08	1.00	-1.80	0.00	-1.26	0.98	-1.22	0.25
AWOD_I_1647	phospholipase A1 precursor	-1.36	0.14	-1.69	0.06	1.17	0.51	1.29	0.27	1.01	1.00	-1.18	0.72	1.23	0.98	1.52	0.02
AWOD_I_1762	putative uncharacterized protein	-1.14	0.55	-1.26	0.66	**-2.07**	**NA**	-1.02	0.93	-1.04	1.00	-1.17	0.72	-1.43	0.83	1.10	0.73
AWOD_I_2007	putative porin	1.49	0.06	-1.42	0.05	**-2.32**	**0.00**	-1.00	0.98	1.35	0.52	-1.05	0.92	-1.49	0.66	-1.05	0.91
AWOD_I_2009	putative exported protein	1.06	0.83	**-2.14**	**0.00**	**-2.85**	**0.00**	-1.97	0.00	-1.12	1.00	-1.68	0.00	-1.56	0.50	-1.30	0.10
AWOD_I_2022	periplasmic nitrate reductase NapB	**-3.20**	**0.00**	-1.08	0.92	1.35	NA	-1.14	0.51	-1.07	1.00	-1.21	0.53	1.01	1.00	-1.09	0.79
AWOD_I_2023	periplasmic nitrate reductase precursor NapA	**-3.73**	**0.00**	-1.36	0.29	1.43	0.07	-1.27	0.15	-1.07	1.00	-1.47	0.01	1.15	1.00	-1.22	0.48
AWOD_I_2030	putative uncharacterized protein, PAAR motif	1.31	0.28	-1.11	0.93	-1.24	0.64	1.33	0.53	-1.01	NA	1.12	NA	1.14	NA	1.09	NA
AWOD_I_2061	putative hydrolase	1.78	0.00	1.64	0.01	1.12	0.64	**2.05**	**0.00**	1.27	0.33	1.19	0.57	1.14	1.00	1.50	0.03
AWOD_I_2148	lipoprotein, LysM domain	-1.61	0.01	-1.95	0.00	**-2.92**	**0.00**	-1.67	0.00	-1.06	1.00	-1.40	0.32	-1.21	NA	-1.19	0.35
AWOD_I_2248	endonuclease I precursor	**3.71**	**0.00**	**2.79**	**0.00**	**6.85**	**0.00**	**3.17**	**0.00**	1.37	NA	1.62	NA	**2.16**	**NA**	1.47	NA
AWOD_I_2370	fite reductase [NADPH] flavoprotein alpha-compon	-1.81	0.00	-1.94	0.02	**2.29**	**0.00**	1.83	0.00	-1.25	0.77	-1.02	0.98	1.00	1.00	-1.04	0.94
AWOD_I_2451	putative lipoprotein	-1.11	0.54	-1.20	0.63	-1.18	0.34	1.13	0.51	-1.05	1.00	-1.11	0.75	1.05	1.00	1.10	0.69
AWOD_I_2491	putative lipoprotein	1.03	0.91	1.38	0.45	1.19	0.40	1.12	0.62	1.11	0.96	1.42	0.33	1.04	1.00	1.06	0.84
AWOD_II_0128	type VI secretion-related protein	-1.71	0.00	-1.48	0.42	**-2.62**	**0.00**	1.07	0.88	-1.14	1.00	1.08	0.89	-1.47	0.58	1.16	NA
AWOD_II_0133	RHS protein	-1.28	0.05	-1.09	0.86	-1.68	0.00	1.18	0.24	-1.19	0.75	1.07	0.84	-1.06	1.00	1.11	0.57
AWOD_II_0252	secreted endonuclease	1.18	0.36	-1.31	0.75	1.06	NA	-1.50	0.11	-1.08	1.00	-1.26	0.61	1.16	1.00	**-2.31**	**0.00**
AWOD_II_0254	secreted endonuclease I	-1.24	0.31	-1.09	0.97	1.99	0.00	-1.07	0.91	-1.63	0.01	-1.21	0.72	1.13	1.00	-1.38	0.36
AWOD_II_0263	putative lipoprotein	**3.73**	**0.00**	1.60	0.35	**2.34**	**0.00**	**2.85**	**0.00**	**2.02**	**0.00**	1.08	0.89	1.78	0.16	1.32	0.12
AWOD_II_0339	putative lipoprotein	-1.01	0.98	-1.00	1.00	-1.18	0.57	1.05	0.92	-1.02	1.00	1.15	NA	1.31	0.94	1.21	NA
AWOD_II_0346	putative lipoprotein	**2.56**	**0.00**	1.99	0.00	1.31	0.32	**3.48**	**0.00**	**2.03**	**0.00**	1.81	0.00	1.51	0.72	1.71	0.01
AWOD_II_0356	aldose 1-epimerase	-1.23	0.10	-1.42	0.12	**-2.43**	**0.00**	-1.31	0.14	-1.13	0.87	-1.20	0.68	-1.16	NA	-1.11	0.71
AWOD_II_0366	putative beta-lactamase	1.02	0.93	**-2.36**	**0.00**	**-3.15**	**0.00**	-1.61	0.00	1.04	1.00	-1.84	0.00	-1.37	0.94	1.04	0.88
AWOD_II_0424	putative DNA-binding protein	1.09	0.69	-1.04	0.98	**-2.48**	**0.00**	-1.37	0.01	1.21	0.80	-1.30	0.56	1.03	1.00	-1.49	0.00
AWOD_II_0440	putative lipoprotein	**-3.59**	**0.00**	**-2.44**	**0.00**	**-2.67**	**0.00**	**-3.07**	**0.00**	-1.50	0.01	-1.53	0.06	-1.29	0.94	-1.50	0.00
AWOD_II_0501	putative exported protein	-1.88	0.01	-1.30	0.69	**-2.22**	**0.00**	-1.67	0.04	-1.28	NA	-1.18	NA	-1.18	0.99	1.16	NA
AWOD_II_0639	putative uncharacterized protein	-1.52	0.00	-1.79	0.00	-1.61	0.00	**-3.26**	**0.00**	-1.13	0.94	-1.53	0.04	-1.21	0.98	**-2.03**	**0.00**
AWOD_II_0656	putative exported protein	**-2.25**	**0.00**	**-2.05**	**0.00**	**-2.64**	**0.00**	**-3.26**	**0.00**	-1.21	0.84	-1.50	0.05	-1.61	0.18	-1.59	0.00
AWOD_II_0676	putative chloramphenicol acetyltransferase	-1.48	0.04	-1.62	0.10	**-2.67**	**0.00**	-1.69	0.00	-1.15	0.99	-1.22	0.64	-1.60	0.31	-1.13	0.69
AWOD_II_0680	putative chloramphenicol acetyltransferase	-1.59	0.01	-1.60	0.12	**-2.61**	**0.00**	-1.71	0.00	-1.17	0.94	-1.23	0.60	-1.63	0.26	-1.18	0.53
AWOD_II_0686	putative uncharacterized protein	**-2.45**	**0.00**	**-2.24**	**0.00**	**-2.52**	**0.00**	**-3.74**	**0.00**	-1.09	1.00	-1.26	0.55	-1.38	0.77	-1.90	0.00
AWOD_II_0804	putative lipoprotein	1.03	0.92	-1.22	0.63	**-2.15**	**0.01**	-1.29	0.37	-1.08	1.00	-1.14	0.73	-1.34	0.94	-1.29	NA
AWOD_II_0806	putative lipoprotein	1.51	0.12	-1.15	0.89	-1.00	1.00	1.08	0.88	1.09	NA	1.21	NA	1.05	NA	-1.08	NA
AWOD_II_0830	putative lipoprotein	-1.12	0.65	-1.90	0.07	-1.02	0.94	-1.45	0.01	1.02	1.00	-1.52	0.05	-1.08	1.00	1.06	0.87
AWOD_II_0852	amine oxidase	-1.08	0.77	**-2.22**	**0.00**	-1.46	0.07	**-2.23**	**0.00**	1.06	1.00	-1.64	0.07	-1.18	0.99	-1.42	0.03
AWOD_II_0964	N-succinylarginine dihydrolase	-1.08	0.74	-1.49	0.03	**-2.18**	**0.00**	-1.09	0.71	1.02	1.00	-1.10	0.84	-1.18	1.00	1.04	0.91
AWOD_II_1001	putative lipoprotein	1.10	NA	-1.09	0.93	-1.48	0.26	-1.58	0.06	-1.02	NA	-1.05	NA	-1.14	NA	-1.24	NA
AWOD_II_1005	putative uncharacterized protein	1.32	0.01	1.12	0.82	**-2.47**	**NA**	-1.55	0.02	1.22	0.29	-1.20	0.50	-1.15	1.00	-1.51	0.04
AWOD_II_1031	putative uncharacterized protein	1.91	0.00	**5.79**	**0.00**	**2.85**	**0.00**	1.65	0.00	1.73	0.00	**2.97**	**0.00**	1.61	0.22	1.26	0.32
AWOD_II_1032	type VI secretion system secreted protein Hcp	1.75	0.00	**3.15**	**0.00**	**2.47**	**0.00**	**2.05**	**0.00**	1.77	0.00	**2.32**	**0.00**	1.49	0.36	1.42	NA
AWOD_II_1038	putative exported protein	-1.15	0.43	1.17	NA	-1.02	0.92	-1.28	0.15	-1.05	1.00	-1.15	0.72	-1.02	1.00	-1.00	0.99
AWOD_II_1045	putative uncharacterized protein	1.14	NA	-1.10	0.96	-1.08	NA	1.07	NA	-1.01	NA	1.04	NA	1.04	NA	-1.20	NA
AWOD_II_1158	putative exported hemolysin	**-3.72**	**0.00**	**-3.36**	**0.00**	**-2.60**	**0.00**	**-2.64**	**0.00**	-1.22	0.35	-1.39	0.14	-1.18	1.00	-1.77	0.00
AWOD_II_1182	putative lipoprotein	1.18	0.59	1.13	0.87	-1.73	0.10	-1.06	0.88	-1.04	NA	1.17	NA	1.02	NA	1.01	NA
AWOD_II_1206	putative lipoprotein	**-2.81**	**0.00**	-1.68	0.02	-1.01	0.96	-1.67	0.00	-1.19	0.93	1.09	0.84	1.03	1.00	-1.01	0.99
AWOD_II_1212	putative uncharacterized phospholipase	1.31	0.12	1.07	0.96	1.27	0.41	1.36	0.30	1.07	1.00	1.27	0.56	1.03	NA	-1.02	NA
AWOD_II_1235	choline dehydrogenase	**-2.03**	**0.00**	-1.19	0.80	1.69	0.11	1.66	NA	-1.14	1.00	1.16	0.76	1.53	NA	1.01	NA
AWOD_p920_0063	putative bacteriocin	-1.18	0.72	-1.15	0.89	**-2.12**	**0.00**	1.06	0.92	-1.16	1.00	-1.17	0.74	-1.96	0.08	-1.23	0.61
AWOD_p920_0078	phospholipase A1 precursor	-1.16	0.50	-1.25	0.54	1.10	0.66	1.83	NA	1.17	0.89	1.02	0.95	1.18	0.99	**2.04**	**0.00**

Additional file 12

Supplementary Table S8. Prophages identified in chromosome 1 by PHASTER

#	CDS Position	BLAST Hit	E-Value	BlastP hit	Identity %	Query cover %
1	1655630..1655651	attL	0			
2	complement (1660097..1661338)	PHAGE_Vibrio_X29_NC_024369: integrase; PP_01493; phage(gi658311188)	1.52E-81			
3	complement(1661339..1662313)	PHAGE_Halomo_phiHAP_1_NC_010342: tail protein; PP_01494; phage(gi167832370)	8.59E-67			
4	complement(1662313..1662516)	PHAGE_Vibrio_VP882_NC_009016: phage tail X; PP_01495; phage(gi126010875)	9.99E-06			
5	complement(1662500..1662880)	PHAGE_Halomo_phiHAP_1_NC_010342: putative tail protein; PP_01496; phage(gi167832369)	9.50E-09			
6	complement(1662891..1665734)	PHAGE_Halomo_phiHAP_1_NC_010342: putative tail tape measure; PP_01497; phage(gi167832368)	1.31E-56			
7	AWOD_1_1463 complement(1665888..1666559)	hypothetical; PP_01498	0	hypothetical protein [Vibrio coralliilyticus]	69.19	77.00
8	complement(1666615..1667103)	PHAGE_Halomo_phiHAP_1_NC_010342: putative tail tube protein; PP_01499; phage(gi167832366)	1.55E-07			
9	complement(1667093..1668277)	PHAGE_Halomo_phiHAP_1_NC_010342: major tail sheath protein; PP_01500; phage(gi167832365)	3.58E-88			
10	complement(1668590..1669132)	PHAGE_Shewan_1/40_NC_025470: hypothetical protein; PP_01501; phage(gi722121121)	4.43E-36			
11	AWOD_1_1468 complement(1669129..1670196)	hypothetical; PP_01502	0	hypothetical protein [Vibrio rotiferianus]	32.28	99.00
12	AWOD_1_1469 complement(1670193..1671083)	hypothetical; PP_01503	0	phage tail protein [Vibrio sp. 10N.261.55.A7]	59.12	69.00
13	complement(1671083..1671787)	PHAGE_Halomo_phiHAP_1_NC_010342: putative tail protein; PP_01504; phage(gi167832357)	3.94E-11			
14	complement(1671771..1672751)	PHAGE_Halomo_phiHAP_1_NC_010342: baseplate assembly protein; PP_01505; phage(gi167832356)	2.04E-41			
15	complement(1672748..1673080)	PHAGE_Halomo_phiHAP_1_NC_010342: putative baseplate assembly protein; PP_01506; phage(gi167832355)	3.51E-10			
16	complement(1673077..1673367)	hypothetical; PP_01507	0	phage baseplate assembly protein V [Vibrio sp. 03-59-1]	76.60	97.00
17	complement(1673762..1674268)	hypothetical; PP_01508	0	hypothetical protein [Vibrio sp. 03-59-1]	85.63	99.00
18	complement(1674234..1674761)	PHAGE_Halomo_phiHAP_1_NC_010342: hypothetical protein; PP_01509; phage(gi167832352)	8.08E-33			
19	complement(1674739..1675071)	hypothetical; PP_01510	0	hypothetical protein [Vibrio sp. 03-59-1]	84.55	100.00
20	complement(1675073..1675405)	PHAGE_Pseudo_MD8_NC_031091: hypothetical protein; PP_01511; phage(gi100006)	4.81E-05			
21	complement(1675469..1677631)	PHAGE_Entero_c_1_NC_019706: head maturation protease; PP_01512; phage(gi428781740)	4.17E-61			
22	complement(1677624..1679030)	PHAGE_Phage_Gifsy_2_NC_010393: bacteriophage portal protein; PP_01513; phage(gi169257301)	2.05E-96			
23	complement(1679104..1679307)	hypothetical; PP_01514	0	hypothetical protein [Vibrio sp. 03-59-1]	80.60	100.00
24	complement(1679307..1681397)	PHAGE_Salmon_Fels_1_NC_010391: bacteriophage terminase, large subunit; PP_01515; phage(gi169257185)	9.34E-137			
25	complement(1681378..1681848)	PHAGE_Entero_mEp460_NC_019716: terminase small subunit; PP_01516; phage(gi428782317)	8.40E-06			
26	complement(1682065..1682274)	hypothetical; PP_01517	0	hypothetical protein [Aliivibrio logei]	98.39	89.00
27	complement(1682264..1682632)	hypothetical; PP_01518	0	hypothetical protein [Aliivibrio fischeri]	96.72	100.00
28	complement(1682634..1683161)	PHAGE_Sodali_phiSG1_NC_007902: hypothetical protein; PP_01519; phage(gi89885987)	7.71E-35			
29	complement(1683142..1683363)	PHAGE_Bacter_APSE_2_NC_011551: phage 21-like group II holin; PP_01520; phage(gi212499716)	1.19E-06			
30	complement(1683379..1684902)	PROPHAGE_Pseudo_KT2440: ISPpu13, transposase Orf2; PP_01521; phage(gi26989833)	7.39E-115			
31	complement(1684988..1685332)	PROPHAGE_Pseudo_KT2440: ISPpu15, transposase Orf1; PP_01522; phage(gi26990788)	4.53E-20			
32	1698945..1698966	attR	0			
Region 2, total 13 CDS						
#	**CDS Position**	**BLAST Hit**	**E-Value**			
1	2401466..2401479	attL	0			
2	complement(2415338..2415922)	PHAGE_Escher_500465_1_NC_049342: tail assembly protein; PP_02168; phage(gi100053)	2.24E-25			
3	2416097..2416981	PHAGE_Escher_500465_1_NC_049342: hypothetical protein; PP_02169; phage(gi100051)	7.23E-111			
4	2417108..2418775	PHAGE_Escher_500465_1_NC_049342: putative single-stranded DNA binding protein; PP_02170; phage(gi100050)	0			
5	2418989..2419345	PHAGE_Escher_500465_1_NC_049342: recombination protein; PP_02171; phage(gi100049)	1.88E-22			
6	complement(2419412..2419729)	PHAGE_Escher_500465_1_NC_049342: exonuclease; PP_02172; phage(gi100048)	5.18E-27			
7	complement(2419716..2420153)	PHAGE_Escher_500465_1_NC_049342: primase; PP_02173; phage(gi100047)	1.95E-68			
8	2420255..2420740	PHAGE_Escher_500465_1_NC_049342: HNH endonuclease; PP_02174; phage(gi100046)	1.74E-74			
9	2420812..2421178	tRNA	0			
10	2421431..2422600	PROPHAGE_Escher_MG1655: CP4-57 prophage; integrase; PP_02175; phage(gi16130540)	1.92E-147			
11	complement(2422656..2425508)	PHAGE_Bordet_vB_BbrM_PHB04_NC_047861: hypothetical protein; PP_02176; phage(gi100102)	8.76E-31			
12	complement(2425529..2425921)	PHAGE_Arthro_Mufasa8_NC_049478: hypothetical protein; PP_02177; phage(gi100003)	3.23E-05			
13	2435755..2435768	attR	0			

Supplementary Table S8. Prophages identified in Chromosome II						
Region 1,						
#	**CDS Position**	**BLAST Hit**	**E-Value**	**BlastP hit**	**Identity %**	**Query cover %**
1	complement(500799..502031)	PHAGE_Bacill_SP_15_NC_031245: prohead protease; PP_00421; phage(gi100150)	6.91E-15			
2	502180..502494	hypothetical; PP_00422	0	late competence development ComFB family protein [Aliivibrio finisterrensis]	91.03	75.00
3	complement(502652..504712)	PHAGE_Lactoc_Q54_NC_008364: putative ribonuclease; PP_00423; phage(gi115304286)	2.26E-30			
4	505018..506970	PHAGE_Acinet_vB_AbaM_ME3_NC_041884: deoxynucleoside kinase; PP_00424; phage(gi100082)	2.33E-13			
5	507244..507765	PHAGE_Bacill_SPbeta_NC_001884: hypothetical protein; PP_00425; phage(gi9630137)	2.48E-09			
6	508000..508956	PROPHAGE_Neisse_MC58: IS30 family transposase; PP_00426; phage(gi15677611)	1.59E-59			
7	complement(508946..510430)	PHAGE_Klebsi_ST16_OXA48phi5.4_NC_049450: methyltransferase type 11; PP_00427; phage(gi100001)	5.65E-13			
Region 2,						
#	**CDS Position**	**BLAST Hit**	**E-Value**			
1	576185..577306	PHAGE_Strept_phiAR10460_1_NC_031913: putative single-stranded DNA binding protein; PP_00483; phage(gi100050)	7.29E-06			
2	577349..577993	hypothetical; PP_00484	0	hypothetical protein [Vibrio vulnificus]	49.30	98.00
3	complement(578127..579314)	hypothetical; PP_00485	0	hypothetical protein [Vibrio parahaemolyticus]	51.06	82.00
4	complement(579333..579521)	PHAGE_Pseudo_PS_1_NC_029066: hypothetical protein; PP_00486; phage(gi985757670)	3.58E-18			
5	complement(579564..580076)	PHAGE_Endosy_APSE_1_NC_000935: hypothetical protein; PP_00487; phage(gi9633594)	5.55E-09			
6	complement(580076..580372)	hypothetical; PP_00488	0	hypothetical protein [Vibrio tasmaniensis]	45.92	100.00
7	complement(580372..580977)	PHAGE_Vibrio_Valm_yong1_NC_049477: hypothetical protein; PP_00489; phage(gi100005)	4.29E-54			
8	581060..581281	PHAGE_Vibrio_8_NC_022747: Cox; PP_00490; phage(gi557307479)	8.12E-10			
9	581403..581924	PHAGE_Vibrio_8_NC_022747: CII protein; PP_00491; phage(gi557307480)	4.44E-41			
10	581935..582255	PHAGE_Vibrio_8_NC_022747: hypothetical protein; PP_00492; phage(gi557307481)	4.41E-24			
11	582337..582804	PHAGE_Vibrio_Valm_yong1_NC_049477: hypothetical protein; PP_00493; phage(gi100011)	6.93E-05			
12	582801..582929	hypothetical; PP_00494	0	hypothetical protein [Aliivibrio fischeri]	90.48	100.00
13	582932..583162	PHAGE_Vibrio_8_NC_022747: hypothetical protein; PP_00495; phage(gi557307485)	1.37E-13			
14	583159..583434	hypothetical; PP_00496	0	MULTISPECIES: hypothetical protein [unclassified Aliivibrio]	97.80	100.00
15	583677..586175	PHAGE_Vibrio_8_NC_022747: putative replication protein; PP_00497; phage(gi557307488)	0			
16	586185..586385	hypothetical; PP_00498	0	hypothetical protein [Aliivibrio fischeri]	100.00	100.00
17	586470..586685	PHAGE_Vibrio_8_NC_022747: hypothetical protein; PP_00499; phage(gi557307491)	2.48E-06			
18	complement(586705..586956)	PHAGE_Vibrio_8_NC_022747: zinc-finger protein; PP_00500; phage(gi557307492)	5.89E-38			
19	587376..587594	hypothetical; PP_00501	0	hypothetical protein [Vibrio splendidus]	44.44	87.00
20	587603..587977	hypothetical; PP_00502	0	hypothetical protein [Vibrio splendidus]	58.82	97.00
21	588023..588247	hypothetical; PP_00503	0	hypothetical protein [Vibrio sp. V12_P9A6T4]	68.92	100.00
22	588679..589014	hypothetical; PP_00504	0	helix-turn-helix domain-containing protein [Aliivibrio finisterrensis]	95.96	89.00
23	589011..589355	PROPHAGE_Pseudo_KT2440: ISPpu15, transposase Orf1; PP_00505; phage(gi26900788)	4.53E-20			
Region 3,						
#	**CDS Position**	**BLAST Hit**	**E-Value**			
1	832764..833720	PROPHAGE_Neisse_MC58: IS30 family transposase; PP_00770; phage(gi15677611)	1.59E-59			
2	complement(833986..834279)	PHAGE_Aeromo_Aes012_NC_020879: putative XRE family plasmid maintenance system antidote protein; PP_00771;	3.41E-24			
3	complement(834279..834557)	PHAGE_Mannhe_vB_MhS_535AP2_NC_028853: plasmid maintenance system killer HigB; PP_00772; phage(gi971749637)	5.09E-35			
4	834930..835886	PHAGE_Escher_500465_2_NC_049343: putative transcriptional regulator; PP_00773; phage(gi100045)	2.21E-29			
5	complement(835909..836385)	PHAGE_Serrat_Moabite_NC_048792: hypothetical protein; PP_00774; phage(gi100166)	5.20E-07			
6	complement(836457..837473)	PHAGE_Stx2_c_Stx2a_F451_NC_049924: tail assembly protein; PP_00775; phage(gi100053)	4.69E-58			
7	complement(837470..837979)	hypothetical; PP_00776	0	IS66 family transposase [Aliivibrio finisterrensis]	100.00	query cover 52%
8	complement(838065..838409)	PHAGE_Stx2_c_Stx2a_F451_NC_049924: tail tip protein; PP_00777; phage(gi100054)	6.34E-15			

Supplementary Table S8. Prophages identified in plasmid AWOD150						
Region 1,						
#	**CDS Position**	**BastP Hit**	**E-Value**	**BlastP hit**	**Identity %**	**Query cover %**
1	2172..2184	attL	0			
2	2809..4662	PHAGE_Entero_Tyrion_NC_031077: hypothetical protein; PP_00004; phage(gi100023)	7.10E-106			
3	4970..6010	PHAGE_Entero_Tyrion_NC_031077: hypothetical protein; PP_00005; phage(gi100023)	4.70E-94			
4	6004..6837	PHAGE_Salmon_BTP1_NC_042346: hypothetical protein; PP_00006; phage(gi100015)	6.59E-10			
5	7293..7565	hypothetical; PP_00007	0	transglutaminase family protein [Methylobacterium mesophilicum]	45.45	36.00
6	7623..8279	PHAGE_Sodali_phiSG1_NC_007902: ParA-like protein; PP_00008; phage(gi89886027)	1.21E-91			
7	8283..8579	PHAGE_Sodali_phiSG1_NC_007902: hypothetical protein; PP_00009; phage(gi89886028)	9.34E-20			
8	8967..9725	PHAGE_Bacill_phiCM3_NC_023599: integrase; PP_00010; phage(gi589892763)	1.18E-05			
9	9829..9972	hypothetical; PP_00011	0	hypothetical protein [Vibrio parahaemolyticus]	57.89	80.00
10	9969..10256	hypothetical; PP_00012	0	hypothetical protein [Vibrio parahaemolyticus]	70.97	97.00
11	10597..10962	PHAGE_Burkho_Bcep176_NC_007497: gp18; PP_00013; phage(gi77864643)	1.00E-10			
12	11068..11625	hypothetical; PP_00014	0	hypothetical protein [Vibrio alginolyticus]	67.04	96.00
13	11841..12161	PHAGE_Escher_520873_NC_049344: hypothetical protein; PP_00015; phage(gi100017)	5.01E-11			
14	12177..12383	hypothetical; PP_00016	0	hypothetical protein AYY26_06110 [Photobacterium phosphoreum]	61.76	100.00
15	12367..12379	attR	0			
16	complement(12445..12591)	hypothetical; PP_00017	0	replication initiation protein [Aliivibrio finisterrensis]	85.11	97.00
17	12740..12997	hypothetical; PP_00018	0	hypothetical protein [Shewanella sp. KX20019]	35.82	76.00
18	13063..13653	hypothetical; PP_00019	0	hypothetical protein A6E13_15755 [Aliivibrio fischeri]	64.29	100.00
19	complement(13706..14266)	PHAGE_Bacill_phi4B1_NC_028886: GNAT family acetyltransferase; PP_00020;	9.65E-06			

Supplementary Table S8. Prophages identified in plasmid AWOD920						
Region 1, total 27						
#	**CDS Position**	**BLAST Hit**	**E-Value**	**BlastP hit**	**Identity %**	**Query cover %**
1	34773..34784	attL	0			
2	complement(42809..44332)	PHAGE_Strept_T12_NC_028700: site-specific recombinase; PP_00051; phage(gi966201482)	7.69E-12			
3	complement(44338..45453)	PHAGE_Pseudo_D3_NC_002484: integrase; PP_00052; phage(gi9635596)	1.32E-14			
4	45472..45483	attL	0			
5	complement(45926..46225)	hypothetical; PP_00053	0	type I restriction endonuclease subunit M [Aliivibrio fischeri]	92.93	100.00
6	complement(46301..46537)	hypothetical; PP_00054	0	hypothetical protein [Aliivibrio fischeri]	87.18	100.00
7	complement(46564..46950)	PHAGE_Vibrio_SHOU24_NC_023569: hypothetical protein; PP_00055; phage(gi589890636)	7.68E-17			
8	complement(46971..47246)	hypothetical; PP_00056	0	hypothetical protein [Aliivibrio fischeri]	93.41	100.00
9	complement(47846..49126)	PHAGE_Caulob_karma_NC_019410: putative ArdC-like antirestriction protein; PP_00057; phage(gi414089431)	1.43E-69			
10	complement(50232..50486)	hypothetical; PP_00058	0	hypothetical protein [Aliivibrio salmonicida]	100.00	100.00
11	50623..51306	hypothetical; PP_00059	0	hypothetical protein [Aliivibrio fischeri]	97.36	100.00
12	51477..51605	hypothetical; PP_00060	0	4-hydroxy-3-methylbut-2-enyl diphosphate reductase [Bacteroidetes bacterium SW_10_40_5]	43.33	71.00
13	complement(51662..52189)	hypothetical; PP_00061	0	ClbS/DfsB family four-helix bundle protein [Aliivibrio fischeri]	99.42	97.00
14	complement(52335..52694)	hypothetical; PP_00062	0	MULTISPECIES: hypothetical protein [unclassified Vibrio]	58.93	94.00
15	complement(52875..53825)	hypothetical; PP_00063	0	DUF559 domain-containing protein [Aliivibrio fischeri]	100.00	100.00
16	complement(53983..54447)	PHAGE_Lactoc_bIL311_NC_002670: Orf17; PP_00064; phage(gi13095675)	3.31E-07			
17	complement(54613..54915)	PHAGE_Gordon_Blueberry_NC_030943: DNA adenine methyltransferase; PP_00065; phage(gi100041)	1.63E-07			
18	complement(54920..55264)	hypothetical; PP_00066	0	MULTISPECIES: type II toxin-antitoxin system RelE/ParE family toxin [Vibrionaceae]	100.00	100.00
19	complement(55471..56244)	PHAGE_Salmon_SSU5_NC_018843: hypothetical protein; PP_00067; phage(gi410491447)	2.69E-07			
20	complement(56440..56577)	hypothetical; PP_00068	0	hypothetical protein [Aliivibrio fischeri]	47.00	100.00
21	56950..57223	PROPHAGE_Escher_MG1655: predicted transposase; PP_00069; phage(gi16131287)	9.65E-26			
22	57210..57566	PROPHAGE_Brucel_1330: transposase, putative; PP_00070; phage(gi23502708)	3.67E-30			
23	57833..57949	hypothetical; PP_00071	0	IS6-like element ISVsa4 family transposase [Aliivibrio salmonicida]	86.84	100.00
24	complement(57987..58106)	hypothetical; PP_00072	0	DUF262 domain-containing protein [Psychrosphaera saromensis]	63.64	84.00
25	complement(58110..60212)	PHAGE_Bacill_SPbeta_NC_001884: ABC transporter; PP_00073; phage(gi9630145)	1.89E-33			
26	66843..66854	attR	0			
27	70425..70436	attR	0			

Paper 3

Pan-genome analysis of *Aliivibrio wodanis* provides insight into the genetic diversity of the CRISPR-Cas system, T6SS2 and phages present.

Amudha Deepalakshmi Maharajan, Terje K and Nils Peder Willassen // Manuscript

Pan-genome analysis of *Aliivibrio wodanis* provides insight into the genetic diversity of the CRISPR-Cas system, T6SS2 and phages present.

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Keywords

Aliivibrio wodanis, pan-genome, phylogeny, CRISPR-Cas and T6SS.

Abstract

Aliivibrio wodanis is repeatedly isolated from the infected fish during winter-ulcer outbreaks. Even though *A. wodanis* is not the causative agent of winter-ulcer; the role of this bacterium in the course of the disease is unclear. Information about the genetic diversity of *A. wodanis* is essential to understand its virulence, environmental adaptation and survival strategies. In this study, 22 *A. wodanis* isolates were analyzed from the perspective of their pan, core and cloud genomes. The pan-genome analysis identified 3149 core, 2583 shell, and 6271 cloud genes revealing an open *A. wodanis* pan-genome.

Furthermore, functional annotation showed that the most enriched protein families in the pan-genome were *metabolism*, *signaling and cellular processes*, and *genetic information processing*. The presence of type VI secretion systems (T6SS), Clustered Regularly Interspaced Palindromic Repeats-CRISPR associated protein (CRISPR-Cas) systems, phages, biosynthetic gene clusters (BGCs) and several virulence factors (VFs) may enable *A. wodanis* during environmental adaptation and survival. All strains had CRISPR arrays, although only half of the strains encoded a complete *cas* operon of type IF and two isolates do not encode any *cas* genes suggesting a diverse CRISPR system. The T6SS1 and T6SS3 were conserved in all the isolates, whereas only fifteen out of twenty-two encoded T6SS2. Eleven of these fifteen isolates showed similarity to the T6SS of environmental *Aliivibrio* strains, while the other four showed similarity to pathogenic vibrios. Furthermore, the spacers and phages revealed great diversity suggesting acquisition at different time points. This study may provide better knowledge about the role of *A. wodanis* in developing the winter-ulcer disease.

Introduction

Aliivibrio wodanis (formerly *Vibrio wodanis*) is a Gram-negative, motile, rod-shaped, non-luminescent bacterium that belongs to the family *Vibrionaceae* (Urbanczyk et al. 2007). This bacterium was initially isolated from farmed Atlantic salmon (*Salmo salar)* in Norway and Iceland (Benediktsdottir et al. 2000). During the cold season, *A. wodanis* has been linked to the winter-ulcer disease that affects farmed Atlantic salmon (Lunder et al. 1995b; Lunder et al. 2000). Although *A. wodanis* is not recognized as the primary causative agent, it is often co-isolated together with *Moritella viscosa* from infected Atlantic salmon (Lunder et al. 1995a). *A. wodanis* 06/09/139 was the first *A. wodanis* strain completely sequenced. Several experimental studies have been performed with this strain together with *M. viscosa* to unravel the role of *A.* wodanis in developing the winter-ulcer disease (Karlsen et al. 2014a; Hjerde et al. 2015; Maharajan et al. 2021; Maharajan et al. 2022). In addition to Atlantic salmon, *A. wodanis* isolates have also been isolated from *Gadus morhua* (Atlantic cod), *Oncorhynchus mykiss* (Rainbow trout) and *Sepiola robusta* (Bobtail squid) (Purohit et al. 2013).

Two complete genomes of *A. wodanis* are currently available at the European Nucleotide Archive for the strains 06/09/139 and 03/09/160 (Hjerde et al. 2015; Soderberg et al. 2019). The genome sequence analysis of *A. wodanis* 06/09/139 reveals the presence of three Type VI secretion systems (T6SS), four auxiliary (Aux) T6SS clusters and a type IF Clustered Regularly Interspaced Palindromic Repeats-CRISPR associated protein (CRISPR-Cas) system (Maharajan et al. 2022). In addition, the *A. wodanis* genome encodes other virulence factors (VFs) such as siderophores, bacteriocins, proteases and hemolysins (Hjerde et al. 2015; Maharajan et al. 2021). Seventeen *A. wodanis* isolates have been screened for AHLs using HPLC-MS/MS and were found to produce one AHL 3OHC10-HSL (Purohit et al. 2013). Out of seventeen, three isolates SR6, SA12 and Vw11 were also found to produce other AHLs (Purohit et al. 2013). However, a phylogenetic study has suggested that SR6 and SA12 belong to a separate clade named *Aliivibrio* sp. “friggae” while Vw11 belongs to *A. wodanis* (Klemetsen et al. 2021). Vw11 produces an additional AHL C8-HSL (Purohit et al. 2013). Moreover, the AHL 3OHC10-HSL production in *A. wodanis* 06/09/139 is temperature and density-dependent (Maharajan et al. 2021). The *A. wodanis* 06/09/139 strain encodes two Quorum sensing (QS) systems (LuxS/PQ and AinS/AinR) and a master QS regulator LitR (LuxR homolog), which is a well-studied regulator of various phenotypes such as motility, colonization, biofilm, rugose colony and luminescence in *A. salmonicida*, *A. fischeri* and *A.*

logei (Fidopiastis et al. 2002; Bjelland et al. 2012a; Hansen et al. 2014; Bazhenov et al. 2021). In our previous study, LitR in *A. wodanis* 06/09/139 regulates T6SSs, CRISPR-Cas, motility, growth, AHL production, siderophore, hemolysin, protease production and cytotoxicity in CHSE cell line (Maharajan et al. 2021; Maharajan et al. 2022). Compared to the wild-type *A. wodanis* 06/09/139, the deletion of *litR* resulted in decreased expression of the whole T6SS2 system and CRISPR-*cas* operon in the *litR* mutant, indicating the importance of the QS system (Maharajan et al. 2022).

Genetic diversity, as a consequence of evolutionary and ecological processes, is important in environmental adaptation and survival. Due to the horizontal gene transfer (HGT) in the bacterial ecosystems, an important driving force in the evolution of bacteria, it is crucial to study the pan-genome of closely related strains to better understand the genomic diversity (Tettelin et al. 2005). In a recent study, the phylogenetic relationship between several aliivibrios species was demonstrated (Klemetsen et al. 2021).

The study aimed to gain more insights into the *A. wodanis* species- and strain-specific genes. For this purpose, twenty-two *A. wodanis* isolates, including the reference *A. wodanis* 06/09/139, isolated from either infected fish during outbreaks of winter-ulcer or experimental challenge in Norway were used (Soderberg et al. 2019; Klemetsen et al. 2021). This pan-genome study may provide new information about the genomic similarity, variation and phylogeny between *A. wodanis* strains. In addition, we used the data to investigate further the diversity of CRISPR-Cas systems, phages, T6SSs, Biosynthetic gene clusters (BGCs) and other virulence-associated factors.

Materials and Methods

Genome information

The genomes of the reference strains *A. wodanis* 06/09/139 and *A. wodanis* 03/09/160 used in this study were retrieved from NCBI (Hjerde et al. 2015; Soderberg et al. 2019). The other 20 *A. wodanis* isolates used in this study were sequenced, assembled and applied in a recent phylogenetic study on the genus *Aliivibrio* (Klemetsen et al. 2021). Before further analysis, assembled genomes were annotated using the RAST annotation server (Aziz et al. 2008). A

circular map of whole genome DNA sequences of twenty-two isolates against the reference *A. wodanis* 06/09/139 was generated using BLAST Ring Image Generator (BRIG) with default parameters **(Supplementary Figure S1)**. The source and place of isolation of the isolates used in this study are listed in **Table 1**. The DNA sequences have been deposited in the European Nucleotide Archive (ENA) with accession number PRJEB55367 and the following genome accession numbers ERS12744122 to ERS12744141.

Table 1. General features of the *A. wodanis* isolates analyzed in this study.

Isolate names	Size (Mb)	Source	Location in Norway (County, Municipality /Fjord, place)	Date	Reference
A. wodanis 06/09/139 (reference)	4.63	*Salmo salar*, Head kidney	Møre and Romsdal, Volda, Kvangardsnes	Mar. 2006	(Hjerde et al. 2015)
Vw1	4.57	*Salmo salar*, outbreak,	Møre and Romsdal, Frei, Salkfjellvik	Dec. 1989	(Klemetsen et al. 2021)
Vw5	4.48	*Salmo salar*, outbreak	Troms and Finnmark, Gratangsbotn, Straumen	Jan. 1990	(Klemetsen et al. 2021)
Vwk7F1	4.35	*Salmo salar*, Experiment with outbreak	Viken, Frogn, Solbergstrand	Apr. 2013	(Klemetsen et al. 2021)
A-H1309-5-2_S7(S7)	4.46	*Salmo salar*, skin ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
A-H1309-1-3_S8(S8)	4.44	*Salmo salar*, ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
Vw7	4.49	*Salmo salar*, outbreak	Trøndelag, Heim, Halsanaustan	Jan. 2002	(Klemetsen et al. 2021)
Vw8	4.51	*Salmo salar*, outbreak	Møre and Romsdal, Frei, Bogen	Apr. 2002	(Klemetsen et al. 2021)
Vw35	4.68	Atlantic cod, *Gadus morhua*	VESO Vikan*	Sep. 2006	(Klemetsen et al. 2021)
03/09/160(Vw37)	4.97	*Salmo Salar*	?	?	(Soderberg et al. 2019)
A-Hdes-19_S12(S12)	4.73	*Salmo salar*, skin ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
Vw27	4.59	*Salmo salar*, Outbreak	Agder, Flekkefjord, Buksevika	Apr. 2006	(Klemetsen et al. 2021)
Vw29	4.52	Rainbow trout, *Oncorhynchus mykiss*	Møre and Romsdal, Gjemnes, Knivskjeneset	28 Jun. 1905	(Klemetsen et al. 2021)
Vw130426	4.57	*Salmo salar*, outbreak	Finnmark, Hammerfest, Husfjorden	Apr. 2013	(Klemetsen et al. 2021)
A-H1309-4-3_S10(S10)	4.58	*Salmo salar*, ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
A-H1309-4-4_S6(S6)	4.59	*Salmo salar*, skin ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
Vw12	4.56	*Salmo salar*, Vaccination experiments	Sogn and Fjordane, Kinn, Svanøybukt	Apr. 1988	(Klemetsen et al. 2021)
A-H1309-5II_S9(5II_S9)	4.56	*Salmo salar*, ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
A-Hdes-10_S9(S9)	4.61	*Salmo salar*, skin ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
A-Hdes-20-1_S13(S13)	4.65	*Salmo salar*, skin ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)

A-Hdes-12_S11(S11)	4.63	*Salmo salar*, skin ulcer	Finnmark, Hammerfest, Husfjorden	Sep. 2013	(Klemetsen et al. 2021)
Vw11	4.48	*Salmo salar*, outbreak	Sogn and Fjordane, Kinn, Svanøybukt	Apr. 2001	(Klemetsen et al. 2021)

*Fish health company
? source not known

Whole genome, single nucleotide variant phylogenetic analysis

The SNVPhyl (Single Nucleotide variant PHYLogenomics) Galaxy workflow was used to identify (single nucleotide variants) SNVs and construct a phylogenetic tree (Petkau et al. 2017). The genome assembly of strain 06/09/139 was the designated reference. No sequence masking was included. Raw reads (FASTQ format) for each isolate were uploaded to the Galaxy workflow. The pipeline was run on default settings except for two parameters; *Data type=Nucleic acid* and *Size of search window=100.* This allows for the detection of additional variant sites.

Pan/core genome analysis

GET_HOMOLOGUES and GET_PHYLOMARKERS (v. 16092021) was applied to identify the orthologous gene clusters which were further used to infer a core genome phylogeny (Contreras-Moreira & Vinuesa 2013; Vinuesa et al. 2018). The RAST annotated GenBank files were used in the homologous gene clustering. After generating a blast-all dataset, the MCL algorithm was engaged with the parameters "*-M -t 0 -A -c"*. This ensured the algorithm reported all gene clusters, generated an average amino acid identity matrix, and performed resampling (10 permutations). Similarly, an average nucleotide identity matrix was generated by a second run with the command "*-M -A -t 0 -a 'CDS'"*. The former dataset became the basis for further pan/core genome analysis. A third run was initiated to infer the phylogeny. Here all three algorithms were applied; Bidirectional best hit (BDBH), cCusters of Orthologous Groups (COG) and Markov clustering of orthologs (OrthoMCL). Following the GET_PHYLOMARKERS pipeline, core clusters from the algorithms were compared in regards to amino acid and nucleotide sequences. The core phylogeny was computed with the "*-R 1 -t DNA*" command parameters. The protein sequences of the isolates were functionally annotated using BlastKOALA (KEGG Orthology and links Annotation) (Kanehisa et al. 2016).

CRISPR-Cas and spacers identification

The genomes of twenty-two *A. wodanis* isolates were analyzed for the presence of CRISPR-Cas system, arrays and spacers using CRISPRCasFinder and BlastKOALA (Kanehisa et al. 2016; Couvin et al. 2018). The spacer sequences were aligned using MAFFT, and the phylogenetic PhyML Newick tree was generated by the maximum likelihood method using PhyML with default parameters at NGPhylogny.fr (Lemoine et al. 2019).

Phage identification

The prophages present in the *A. wodanis* isolates were identified using PHAST (Zhou et al. 2011). The prophage's DNA sequences were retrieved and aligned using MAFFT. Phylogenetic PhyML Newick tree was constructed by the maximum likelihood method using PhyML with default parameters at NGPhylogny.fr (https://ngphylogeny.fr) (Lemoine et al. 2019).

Identification of T6SS and Aux clusters

A local protein database was made using the makeblastdb wrapper for Galaxy on a national server with twenty-two protein faa files including the reference faa file. The protein sequences of *A. wodanis* 06/09/139 T6SS and Aux clusters were used as a reference. BlastP was performed for the reference T6SS and Aux clusters against the local database with protein data sets with default parameters. The BlastP hit with > 50% identity and the presence of at least 13 T6SS core components were counted as a T6SS positive isolate.

Virulence factors identification

Virulence factor database (VFDB) with protein sequences of the full data set was retrieved from http://www.mgc.ac.cn/VFs/ (Liu et al. 2022). A local database with twenty-two *A. wodanis*

protein sequences was used to predict the virulence factors against the VFDB using the NCBI BLAST+ blastp wrapper with default parameters and a percentage identity filter of > 60%.

Biosynthetic gene cluster analysis

The biosynthetic gene clusters (BGCs) responsible for secondary metabolites production in *A. wodanis* genomes were identified using the web tool antiSMASH bacterial version 6.1.1 https://antismash.secondarymetabolites.org/ (Blin et al. 2021). The analysis was performed using the Genbank files of twenty-two isolates with "relaxed detection" and "all on" options.

Results

Genome analysis

A total of twenty-two *A. wodanis* isolates from various locations and isolation periods in Norway **(Table 1)** were chosen to explore the *A. wodanis* pan-genome. The genome sizes ranged from 4.35 to 4.97 Mb with a mean of 4.59 Mb **(Table 1),** and the number of proteins ranged from 3808 to 4455 with a mean of 4096. The reference genome has two chromosomes and four plasmids of sequence lengths 4.52 and 0.12 Mb, respectively (Hjerde et al. 2015). The comparative BRIG genome circular map showed differences between these strains mainly in seven regions, as shown in **Supplementary Figure S1**. Among other isolates, S7 (ring 6) and Vw35 (ring 10) showed larger gaps. The isolates VwK7F1 (ring 5), Vw7 (ring 8), Vw130426 (ring 15), S10 (ring 16) and S6 (ring 17) showed less sequence similarity, whereas the isolates Vw1 and Vw5 revealed more similarities with reference than others. Furthermore, when mapped against the reference, regions with gaps were annotated with BlastKOALA and found to be linked with functional protein families *secretion systems*, *prokaryotic defence mechanisms*, *bacterial motility proteins*, *transporters*, *enzymes* and *lipopolysaccharide biosynthesis proteins* **(Supplementary Figure S1)**.

Phylogenetic relationship between the *A. wodanis* strains

The genome-wide SNVPhyl analysis identified 4096 SNVs, which were used to construct a phylogenetic tree as shown in **Figure 1A** and **Supplementary Figure S3**. The phylogenetic tree revealed a distribution of strains into roughly five groups, where group 2, 4 and 5 encompassed conserved isolates within each group. All twenty-two strains shared more than 95% ANI and AAI between the *A. wodanis* strains **(Figure 1B and C)**, which is within the commonly accepted species cut-off (≥ 95% ANI) (Konstantinidis & Tiedje 2005).

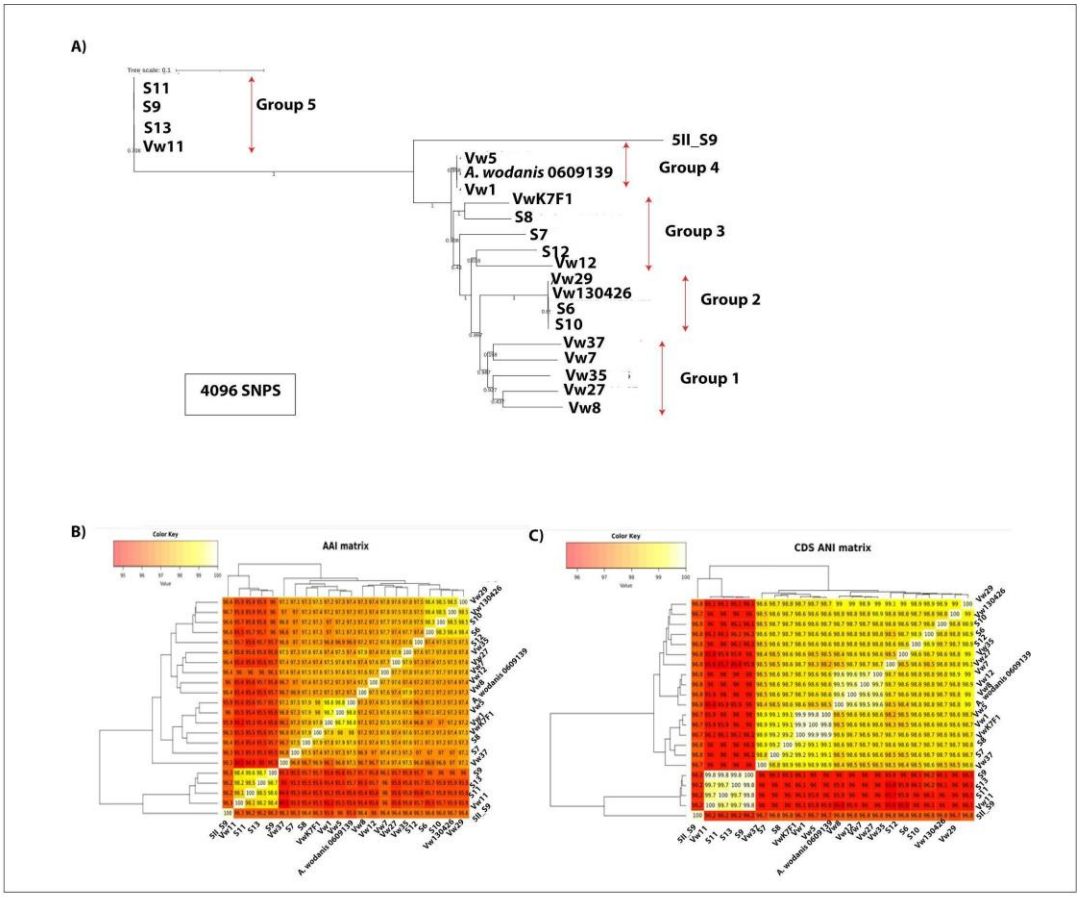

Figure 1. A) Phylogenetic tree of twenty-two *A. wodanis* strains based on 4096 SNVs. B) AAI matrix and C) ANI heatmap of the twenty-two included *A. wodanis* genomes, respectively.

A. wodanis pan and core genome

Pan-genome

A pan-genome analysis was performed on the twenty-two *A. wodanis* genome sequences using GET_HOMOLOGUES and the OMCL algorithm (Contreras-Moreira & Vinuesa 2013) **(Figure 2)**. Theoretical estimation of the pan-genome size and growth curve from the supplied

genomes did not flatten out based on the permutation analysis, as shown in **Figure 2A**. This graph also approximates 123 additional genes added in the pan-genome pool per genome, thus clearly indicating that saturation is not reached and *A. wodanis* has an open pan-genome. A total of 12003 gene clusters were determined by the OMCL algorithm, which categorized 3149 (26.23%) as extended core, 2583 as shell (21.51%) and 6271 as cloud and unique genes (52.24%) **(Figure 2B)**. Or, more strictly, 2862 (23.84%) gene clusters constitute the explicit core, 3653 the shared accessory (not core or unique) (30.43%), and 5488 unique genes (45.72%). Furthermore, functional characterization of all sequences showed that the most enriched functional families in all the isolates were *metabolism*, *signaling and cellular processes*, and *genetic information processing*. Specifically, more than 900 genes related to *enzymes* were assigned to metabolic functions in the pan-genome. The protein family *signaling and cellular processes* include families such as *transporters*, *secretion systems*, *bacterial toxins*, *prokaryotic defence mechanism*, *bacterial motility proteins*, *two-component system*, *exosome* and *antimicrobial resistance genes*. The *genetic information processing* includes families such as *transcription factors*, *ribosome*, *DNA repair and recombination proteins*, *transfer RNA biogenesis*, *ribosome biogenesis*, *membrane trafficking* and *chromosome and associated proteins* **(Supplementary Figure S2)**.

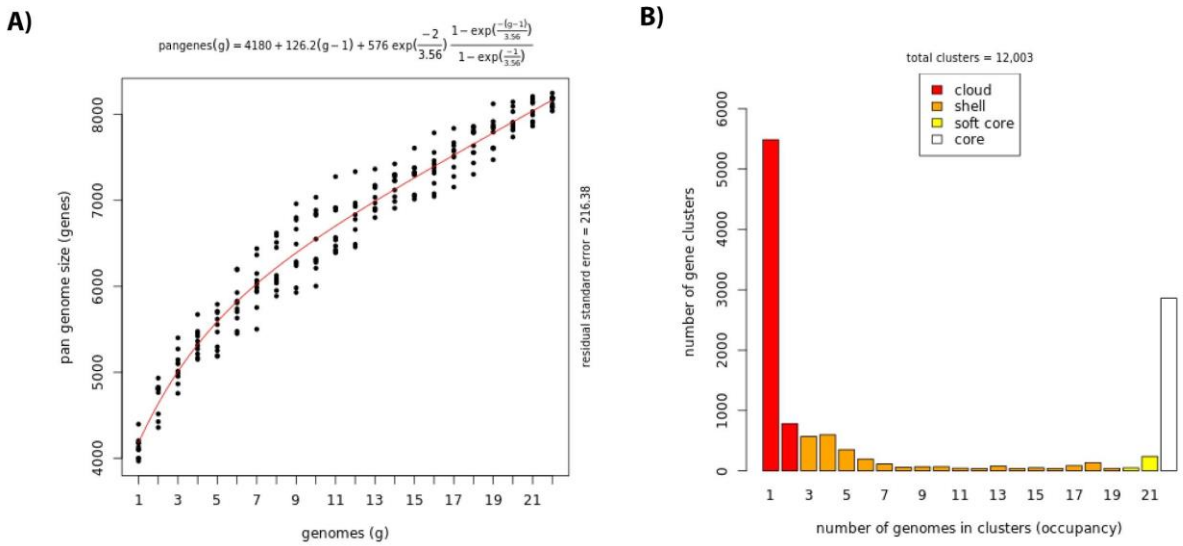

Figure 2. *A. wodanis* pan-genome. (A) *A. wodanis* pan-genome as a function of the included genomes. (B) Cluster distribution in *A. wodanis* genome.

Core genome

Core gene clusters, representing the homologous genes in all analyzed genomes (Tettelin et al. 2005), were derived using the OMCL algorithm in GET_HOMOLOGUES. Based on the twenty-two input genomes, the Willenbrock and Tettelin permutation analysis culminated on 2831 and 2943 theoretical core genes. These are declining, particularly the Tettelin algorithm, indicating a smaller core if additional genomes are added **(Figure 3A)**. The 2707 intersecting core-gene clusters from all three algorithms in GET_HOMOLOGUES were filtered down to 240 marker genes, concatenated and used to infer the core genome phylogeny. Similar to the SNV phylogeny, the core tree was resolved into five phylogenetic groups **(Figure 3B),** where group 2, 4 and 5 were highly conserved **(Supplementary Figure S3)**. However, similar to the SNV-based tree, strain 5II_S9 did not relate to any other strain group.

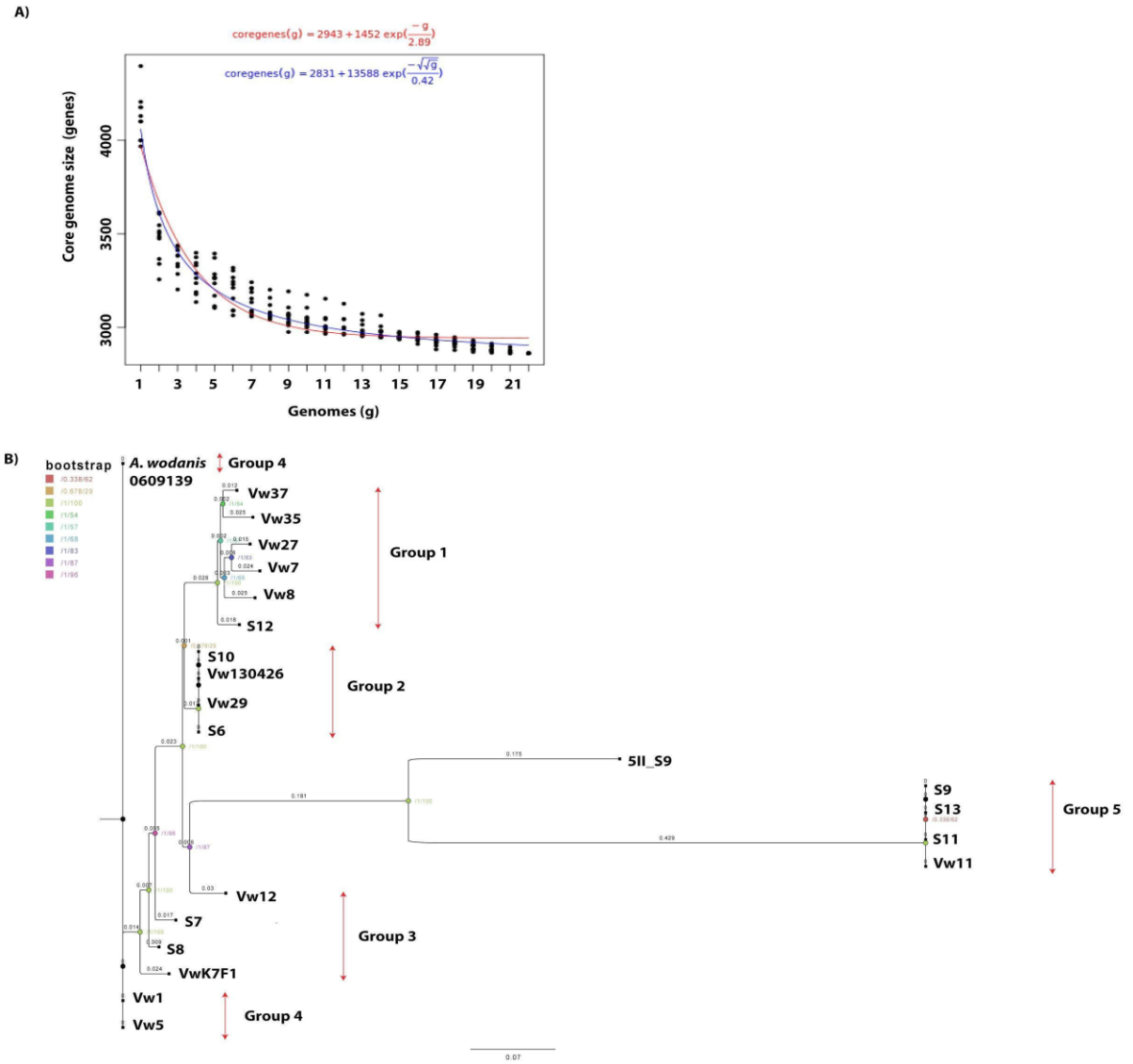

Figure 3. *A. wodanis* core genome A) as a permutation analysis of the 22 included genomes. (B) Phylogenetic tree of 240 selected marker genes of the *A. wodanis* core clusters. The tree is drawn to scale with branch lengths[/scale bar] reflecting nucleotide substitutions per site. Vw1 and Vw5 were grouped together with the reference *A. wodanis* 06/09/139 (Group 4). Group 3 included Vwk7f1, S8, and S7. Group 2 consisted of four isolates S6, S10, Vw130426 and Vw29, while Group 1 included six isolates Vw37, Vw35, Vw7, Vw27, S12 and Vw8. Four isolates S9, S13, S11, and Vw11 fell into Group 5.

A. wodanis cloud and unique genes

The accessory genome may include genes that provide fitness to the bacteria and may have been acquired through HGT (McNally et al. 2016). Hence, the identification and characterization of the accessory genome are important. The reference strain *A. wodanis* 06/09/139 showed 66 unique genes. The lowest number of unique genes (n < 50) were found in three isolates Vw1 (n = 34), S6 (n = 43) and S10 (n = 47) as shown in **Figure 4A**. One isolate, S7 from Finnmark, Hammerfest, Husfjorden, showed the highest number of unique genes (n = 1007) out of all isolates. Most of the isolates (16/22) consisted of a number of unique genes ranging from 100 to 500. Similar to the whole genome annotation, *metabolism, signaling and cellular processes,* and *genetic information processing* were the most enriched protein families in the cloud genes. The functional annotation showed that isolate S7 encoded about 200 *enzymes* while the cloud genes in other isolates encoded only a range of 4-30 **(Figure 4B)**. The *enzymes* included *oxidoreductases*, *nitrate reductases*, *hydrolases*, *transferases*, *excinucleases*, *DNA polymerases* and *lysases*. The isolates S9, S8, S12, S11, Vw37, Vw35 and Vw8 encoded the genes related to CRISPR-Cas in the cloud genes where the isolate S12 encoded a type III CRISPR system, and S8 encoded a subtype ID factor **(Figure 4B)**. The isolate Vwk7F1 encoded four, and other isolates S7, S9, S12, Vw5, Vw12, Vw27, and Vw29 encoded two to three T6SS proteins in the cloud genes. The other protein families encoded by the cloud genes include *exosomal proteins, bacterial motility proteins, transporters, two-component system, transcription factors* **(Figure 4B)**. However, only 16% of the cloud genome, gene clusters present in < 2 genomes, have been functionally annotated. Therefore, the unique gene clusters lacking annotation were manually analyzed using BlastP to understand where they were acquired from. The results showed that the unique genes in isolate S7 were mostly acquired from Class: *Gammaproteobacteria*, *Bacilli* and *Flavobacteriia*; Family: *Vibrionaceae, Pseudoalteromonadaceae, Alteromonadaceae, Deinococcaceae, Chromatiaceae,*

Lactabacillaceae, *flavobacteriaceae* and *Oceanospirillaceae*; *and* Genus: *Vibrio*, *Aliivibrio*, *Photobacterium* and *Enterovibrio*. Among other family members, unique genes were mostly acquired from *Vibrionaceae* which included several *Photobacterium* species, *Vibrio* genome sp. F6, *Vibrio* sp. 03-59-1 and *A. fischeri* as listed in **Supplementary Table S1**. In addition to many proteins, the unique genes in S7 were found to encode several hypothetical proteins, phage-related proteins and transposases as listed in **Supplementary Table S1**.

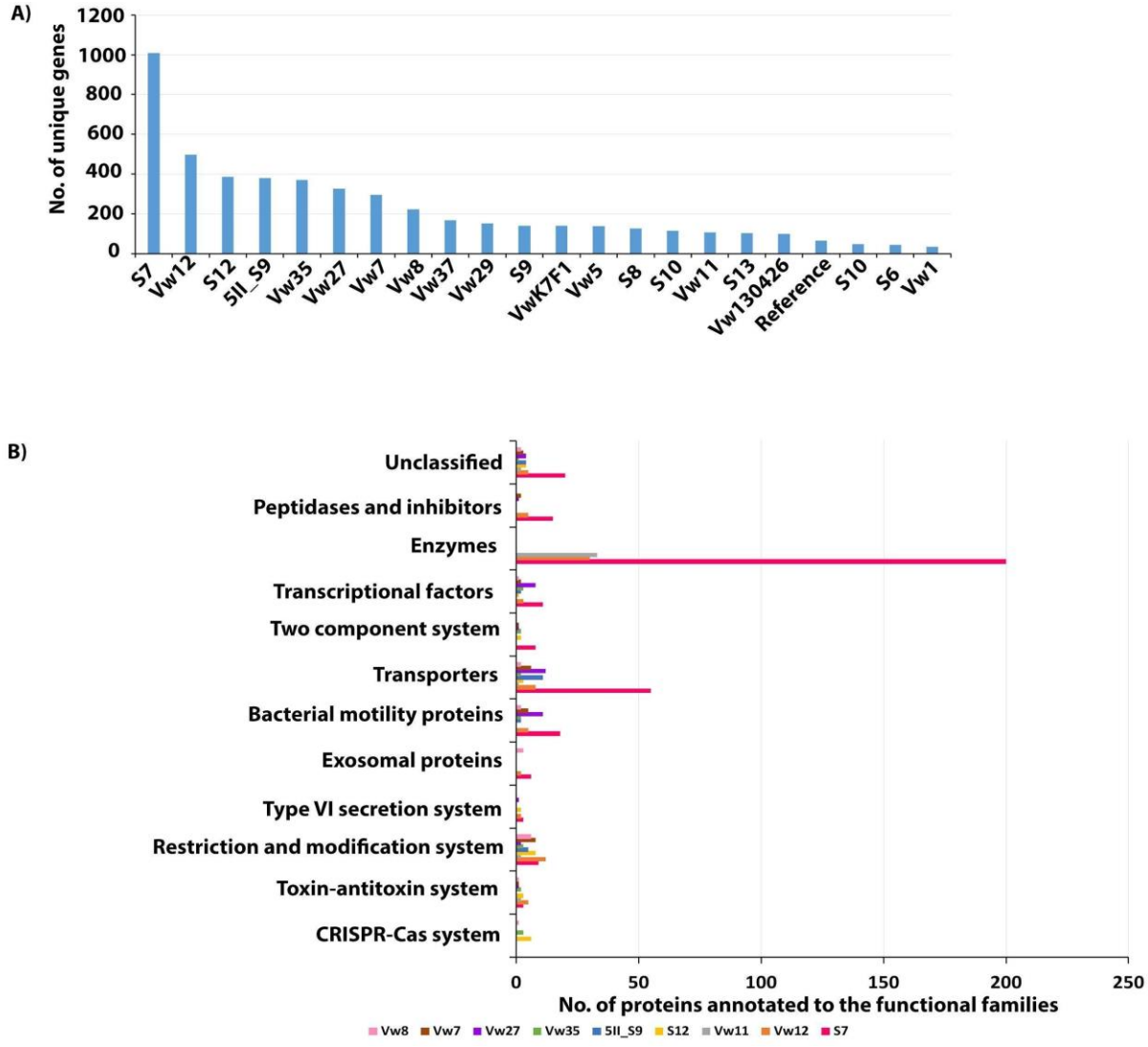

Figure 4. A) Unique genes present in twenty-two *A. wodanis* isolates. B) Functional annotation of cloud genomes of isolates Vw8, Vw7, Vw27, Vw35, 5II_S9, S12, Vw11, Vw12 and S7 are presented here.

CRISPR-Cas system differs between *A. wodanis* isolates

Many bacteria have an adaptive immune system called CRISPR-Cas (Makarova et al. 2020). Our previous work identified a type IF CRISPR-Cas system and two CRISPR arrays in the reference strain (Maharajan et al. 2022). In this study, we analyzed the CRISPR-Cas systems in *A. wodanis* isolates using CRISPRCasFinder and BlastKOALA (Kanehisa et al. 2016; Couvin et al. 2018). The results showed that out of twenty-two isolates tested, two isolates VwK7F1 and Vw7, did not encode any *cas* genes **(Figure 5)**. However, all isolates were found to contain the CRISPR arrays. Like the reference strain, eight out of twenty-one isolates (Vw1, Vw5, Vw27, 5II_S9, S12, S13, Vw11 and Vw12) revealed a type IF CRISPR-Cas system with the same gene order and gene length **(Figure 5)**. The isolate S12 is the only isolate that encoded an additional type III CRISPR system. The other eight isolates (Vw35, Vw130426, S6, S10, Vw29, Vw37, S7 and S8) did not contain a complete IF *cas* operon; however, the isolates encoded *cas6f* gene, a marker for the type IF CRISPR.

The isolates Vw35 contained *csy1234* genes and the isolates Vw37, and S7 encoded *csy234; however*, the isolates did not encode the *cas1* and *cas3-cas2* genes. The isolates Vw130426, S6, Vw29 and S10 contained the *cas6*, *cas3* and *cas7fv* genes. This suggests that these isolates encode a type IF variant CRISPR system. The isolates S9 and S11 encoded all the genes of type the IF CRISPR system; however, the gene order and orientation differed from the reference strain **(Figure 5)**. Vw8 is the only isolate that encodes the *cas5fv* and *cas7fv* genes next to the *cas1*, *cas3* and *cas6* genes, suggesting that Vw8 contains a type IF variant CRISPR system.

The isolates Vw1, Vw5, S8, Vw12, Vw27 and 5II_S9 showed high amino acid (AA) similarity (80-100%) to the *cas* operon of the reference strain, whereas the isolates Vw8, S12, S9, S13, S11 and Vw11 only a partial number of genes (30-40%) showed high similarity to the reference *cas* operon. The AA sequences of isolates that showed low similarity to the reference *cas* genes were retrieved, and BlastP was performed against the NCBI non-redundant protein DB. The similarity search revealed that the isolates S9, S13, S11 and Vw11 showed high AA similarity to *Aliivibrio finisterrensis* and *Colwellia* sp. RSH04. The protein Csy1 of S12 showed high AA similarity to *Poseidonibacter antarcticus*, whereas the other Cas proteins showed similarity to *A. finisterrensis* and Colwellia sp. RSH04. The isolate Vw35 showed approximately 50% query coverage and identity to *Vibrio spartinae* (Csy2), *A. fischeri* (Csy3) and *Nitrosococcus oceani* (Cas6) (99% query coverage, <50% identity). The S7 isolate showed AA similarity to *Vibrio splendidus*, while the Vw8 isolate showed AA similarity to *A. fischeri.*

When comparing the Cas proteins of Group 2 isolates S10, Vw130426, Vw29 and S6, the BlastP results showed that the Cas3, Cas6 and Cas7fv proteins were 100% identical between each other. The Cas3, Cas6 and Cas7fv proteins showed high AA similarity (>90%) to *Photobacterium* species when searched against the non-redundant protein DB. The Cas7fv of isolates Vw8 also showed hits to *Photobacterium* sp., whereas the Cas5fv showed 48% AA similarity to *Methylotuvimicrobium buryatense.* The Cas3 protein sizes of Vw8 and S8 were not similar; therefore they were searched against the non-redundant protein DB, and the results showed similarity to Cas3 of Type I and DEAD/DEAH box helicase or DinG of Type IV, respectively. The Cas3 and DEAD/DEAH box helicase belong to the helicase superfamily where both play an important role in the innate immunity mechanism (Makarova et al. 2020). DEAD/DEAH box helicases are generally involved in DNA recombination and repair mechanisms (Fairman-Williams et al. 2010). *M. viscosa* encodes a ferrous siderophore system upstream of the CRISPR-Cas system (Karlsen et al. 2017). Similar to *M. viscosa* the upstream of reference, the isolates Vw1, Vw27 and Vw12 CRISPR-Cas contain a ferrous siderophore system.

Mapping the CRISPR-Cas systems to the core tree revealed that Group 2 and 4 showed similar CRISPR-Cas systems. The other groups showed differences in CRISPR-Cas systems between the isolates **(Figure 5)**. Together the results showed that 9 out of 22 (40%) isolates showed a similar IF CRISPR system. The other isolates (60%) show a diverse CRISPR system in terms of the number of *cas* genes, gene length, gene order and orientation between the isolates.

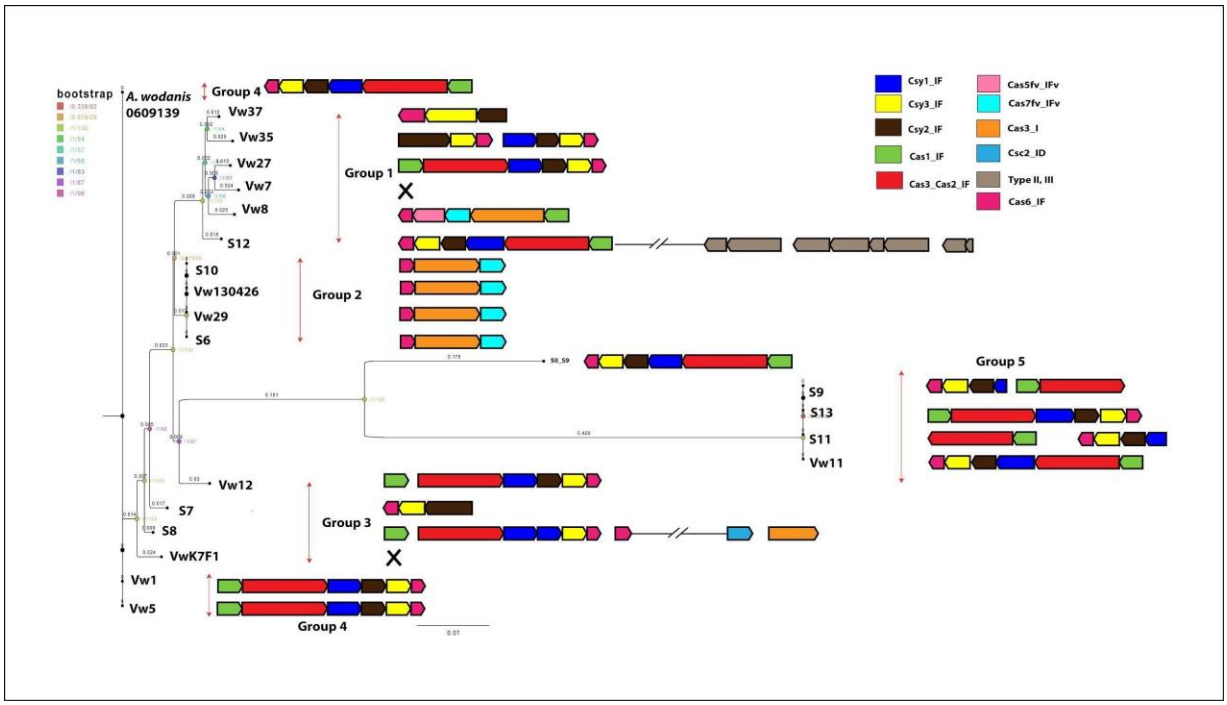

Figure 5. CRISPR-Cas systems in *A. wodanis* isolates mapped to the core *A. wodanis* tree. The cross mark indicates the absence of CRISPR-*cas* operon.

Spacers in *A. wodanis* isolates

Spacers are pieces of foreign sequences integrated into the CRISPR arrays (Mojica et al. 2005). Every time a phage or plasmid is encountered, a new spacer is added to the CRISPR array through adaptation (Mojica et al. 2005). Therefore, spacers are considered immune memories in bacteria. The spacers in the *A. wodanis* isolates were identified using the CRISPRCasFinder **(Table 2)**. The results showed that the spacer length varied from 20-70nt and the direct repeats ranged from 23-31nt **(Table 2)**. The spacer sequences were aligned using MAFFT, and a PhyML Newick tree was constructed using PhyML. A comparison of the spacer sequences revealed that similar to the core tree, the spacers of Group 4 (Vw1, Vw5 and reference) were grouped into their corresponding groups **(Supplementary Figure S4)**.

Table 2. Spacers and direct repeats in the isolates

Isolate names	No. of CRISPR arrays (No. of spacers in each array)	Direct Repeats length range	Spacers length range

A. wodanis 06/09/139	2 (25, 40)	28	32-33
Vw1	2 (39, 24)	28	32-33
Vw5	2 (32, 23,1)	28	32-33
Vw7	2 (2,1)	23-28	32-70
Vw8	5 (1,5,21,1,1)	28-29	31-32
Vw35	3 (6,7,2)	28-29	31-32
Vw37/0309160	2 (5,3)	28-38	35-41
S12	4 (12,29,10,4)	28	20-33
Vw27	2 (6,33)	26-28	31-32
Vw29	4 (2,2,10,1)	28-31	29-32
Vw130426	3 (2,2,6)	28-31	32-33
S10	3 (2,8,1)	24-28	32-36
S6	4 (2,8,1,1)	28	32-33
Vwk7f1	1 (13)	28	32-33
S8	4 (3,17,7,21)	23	46-52
S7	1 (2)	24-28	32-33
Vw12	2 (25,15)	27-30	30-33
5II_S9	3 (23, 35)	28	32-33
S9	3 (6,35,1)	28	20-33
S13	2 (35,6,1,1)	28	20-33
S11	2 (6,30,1)	28	20-33
Vw11	2 (6,35,1)	28	20-33

Phages in *A. wodanis* isolates

Except for the Vw8 isolate, at least 1-3 complete prophages were identified in all the isolates using the PHAST tool. The analysis showed that about six isolates Vwk7f1, S7, 5II_S9, S13, Vw11 and Vw7 encoded only complete prophages. To reveal the evolution of phages, we inferred the genetic diversity of phages using PhyML. The DNA sequences of the complete and incomplete prophages were aligned using MAFFT, and a PhyML Newick tree was constructed using the maximum likelihood method. The prophages tree showed variations in the grouping of isolates, which is different from the grouping based on the core- and SNVs-based

phylogenetic trees **(Figure 6)**. For example, Group 4 isolates Vw1 and Vw5 grouped closer to the reference in the SNVs-based tree, grouped away from the reference in the phage-based tree.

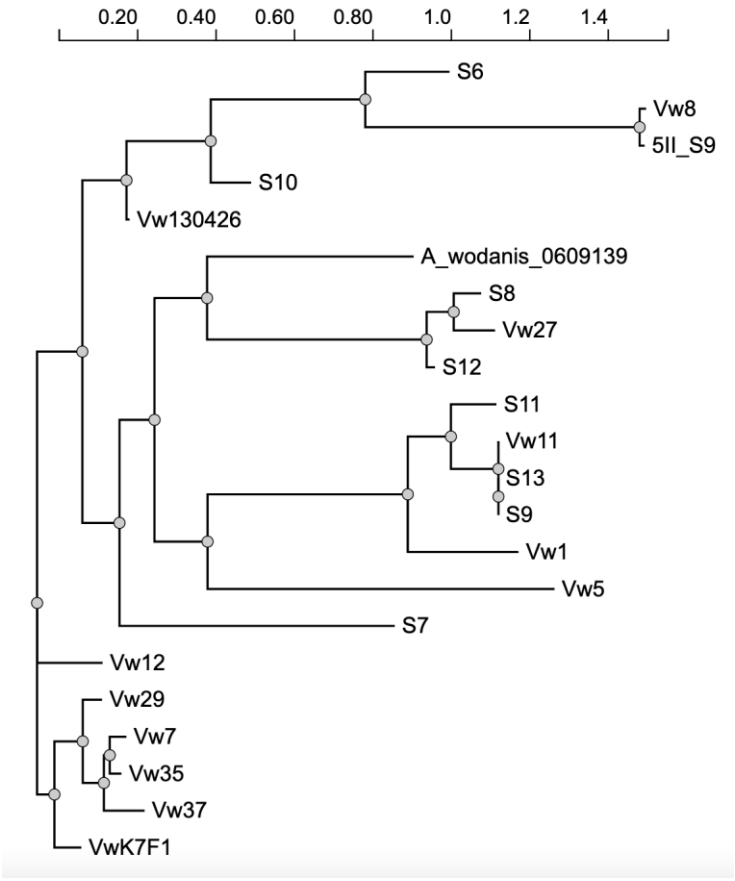

Figure 6. Phylogenetic relationship of complete and incomplete prophages identified in *A. wodanis* isolates.

Identification of T6SS and Aux clusters in *A. wodanis* isolates

Our previous work identified three T6SSs and four Aux clusters in the reference strain genome (Maharajan et al. 2022). Similar to the reference strain, the other 21 *A. wodanis* isolates comprised both main and Aux T6SS clusters. Group 4 (Vw1, Vw5 and reference) and Group 3 (VwK7F1) in the core tree showed similar T6SS2. However, 11 isolates showed low similarity (<50%) to the reference T6SS2, while seven isolates Vw7, Vw8, Vw37, Vw29, Vw130426,

S10 and S6, did not encode the reference T6SS2 cluster (**Table 3**). The T6SS2 proteins with low similarity to the T6SS2 reference were searched against the NCBI non-redundant protein database using BlastP. The results showed that the T6SS2 in the isolates were highly similar to aliivibrios *A. sifae*, *A. fischeri* and *A. logei*. The reference T6SS1-T6SS3 cluster proteins were BlastP searched against the protein sequences of 21 isolates. The results showed that three isolates Vw1, Vw5 and Vwk7f1 comprised three main clusters highly similar (>90%) to the T6SS1-T6SS3 clusters of the reference strain. In the other 17 isolates, the T6SS1 cluster was found to be 59-98% similar to the reference T6SS1. Except for S7, which showed low similarity (<50%), 16 of the isolates contained T6SS3 highly similar (>99%) to the T6SS3 of the reference strain (**Table 3**). The isolates 19 out of 21 except S7 and S8 showed high AA similarity to the reference Aux- 1, 3 and 4. The isolates S7 and S8 encoded only Aux-1 and 4 (**Table 3**). Furthermore, the Aux-2 in six of twenty-two isolates showed high AA similarity to the reference Aux-2. However, other 15/21 isolates showed either low similarity or the absence of Aux-2 genes. To identify the total number of VgrGs in the genomes, the four reference VgrGs, identified from the reference Aux1-4, were searched against the protein sequences of the 22 isolates using BlastP. The results showed that the number of VgrGs differed between isolates (**Table 3**).

Table 3. Similarity search of 21 isolates against reference T6SSs and Aux clusters

Isolate names	T6SS1	T6SS2	T6SS3	Aux-1	Aux-2	Aux-3	Aux-4	No. of VgrGs	BlastP hit*
A. wodanis 06/09/139	+	+	+	+	+	+	+	7	No similarity to aliivibrios; similar to vibrios
Vw1	+	+	+	+	+	+	+	7	No similarity to aliivibrios; similar to vibrios
Vw5	+	+	+	+	+	+	+	7	No similarity to aliivibrios; similar to vibrios
Vwk7f1	+	+	+	+	+	+	+	6	No similarity to aliivibrios; similar to vibrios
S7	+	<50%	<50%	+	-	-	+	2	*A. sifae*
S8	+	<50%	+	+	-	-	+	5	*A. sifae, A. fischeri and A. logei*
Vw7	+	-	+	+	-	+	+	5	-
Vw8	+	-	+	+	-	+	+	6	-
Vw35	+	<50%	+	+	-	+	+	6	*A. sifae, A. fischeri and A. logei*
Vw37/0309160	+	-	+	+	-	+	+	6	-
S12	+	<50%	+	+	-	+	+	5	*A. sifae* and *A. fischeri*
Vw27	+	<50%	+	+	-	+	+	5	*A. sifae, A. fischeri and A. logei*
Vw29	+	-	+	+	-	+	+	6	-
Vw130426	+	-	+	+	-	+	+	5	-
S10	+	-	+	+	-	+	+	5	-
S6	+	-	+	+	-	+	+	5	-
Vw12	+	<50%	+	+	-	+	+	6	*A. sifae* and *A. fischeri*
5II_S9	+	<50%	+	+	-	+	+	5	*A. sifae* and *A. fischeri*
S9	+	<50%	+	+	+	+	+	7	*A. sifae, A. logei* and *A. fischeri*
S13	+	<50%	+	+	+	+	+	6	*A. sifae, A. logei* and *A. fischeri*
S11	+	<50%	+	+	+	+	+	6	*A. sifae, A. logei* and *A. fischeri*
Vw11	+	<50%	+	+	-	+	+	3	*A. sifae, A. fischeri*

*T6SS2 genes that showed <50% AA similarity to reference were searched against protein database using BlastP

Biosynthetic gene cluster analysis (BGC)

BGCs encode the genes involved in the biosynthesis, regulation and transport of secondary metabolites (Osbourn 2010). The anti-smash analysis identified five potential BGCs in the genomes that produce secondary metabolites such as thioamititdes, ectoine, siderophores, arylploye ne and resorcinol. The siderophore cluster in the reference strain showed 60% gene

similarity to *Vibrio crassostreae* strain 8T5 while the clusters producing ectoine, arylploye ne and resorcinol showed 50-95% gene similarity to the clusters of *A. sifae*. The siderophore and ectoine genes clusters in the other *A. wodanis* isolates showed 62-100% gene similarity to the reference genes. Only one isolate, S7, encoded the thioamitides producing cluster, which showed 40% gene similarity to the strain *V. crassostreae* strain 25 P9 **(Supplementary Table S2)**. This may indicate an HGT of the thioamitides cluster in the genome of S7. Except for Vw5, Vwk7f1, Vw27 and Vw29, the arylpolyene and resorcinol clusters showed high similarity to the reference clusters. The BGC analysis results showed that the siderophore and ectoine clusters are the most conserved BGC.

Virulence factors identified in *A. wodanis* isolates

A. wodanis has been associated with the winter-ulcer disease outbreak (Lunder et al. 1995a; Lunder et al. 2000). Moreover, in our previous study, *A. wodanis* supernatants caused cytotoxicity in salmon cell lines (Karlsen et al. 2014a; Maharajan et al. 2021). Therefore, we wanted to predict the VFs in *A. wodanis.* Moreover, in our previous study, *A. wodanis* supernatants caused cytotoxicity in salmon cell lines (Karlsen et al. 2014a; Maharajan et al. 2021). Therefore, we wanted to predict the VFs in *A. wodanis*. The VFs in the *A. wodanis* were identified by performing BlastP against the virulence database (VFDB) set B, which contains the entire dataset of both experimentally verified and predicted VFs (Liu et al. 2022). The results showed, on average, that *A. wodanis* isolates encode about 200 VFs, as presented in **Supplementary Table S3**. The VFs identified are present in all the isolates. They include *flagellum gene cluster*, *type II secretion system*, *ferric-anguibactin transport system*, *chemotaxis gene cluster*, *thermolabile hemolysin*, *RNA polymerase sigma factor RpoD* and *twitching mobility protein.* This suggests that the VFs are well-conserved in all the isolates. Similar to *V. anguillarum* where the VFs were similar to strains isolated from the same geographical location (Hansen et al. 2020). In addition to the pathogenic role of VFs, they are also present in commensals and symbiotic bacteria (Niu et al. 2013). This suggests that the VFs may perform multiple host-associated roles.

Discussion

In this study, we investigated the pan-genome of 22 *A. wodanis* to reveal the phylogeny, strain-specific genes and properties of genes related to CRISPR-Cas, phages, T6SSs, VFs and BGCs. In a recent study, *A. wodanis* genomes showed a wider diversity among a few strains (Klemetsen et al. 2021).

Phylogenetic analysis

The phylogeny in this study showed considerable variations, suggesting the diversity could be due to the genome sizes. The phylogenetic analysis based on SNVs and core genes resulted in five clades where the isolates of Group 2, 4 and 5 showed high similarity to their corresponding groups. This suggests that these isolates evolved similarly from a common ancestor. The similarity could also indicate that these isolates may colonize the same niche in the fish farms. Even though all the isolates were from Norway and outbreaks of the winter-ulcer disease, this study does not find a correlation between the inferred phylogenies and the spatiotemporal data.

Pan, core and unique genes

Pan-genome analysis of *A. wodanis* showed an open pan-genome, suggesting the genomes have experienced HGT in the past. An open pan-genome in bacteria has been linked with susceptibility to HGT related to metabolism, virulence and antibiotic resistance (Hurtado et al. 2018). It has been reported that species colonizing different environments can exchange genetic material in multiple ways and have an open pan-genome (Costa et al. 2020). This has been observed in other species like *E. coli*, *Corallococci*, *M. bovis*, and *Helicobacter* (Livingstone et al. 2018; Costa et al. 2020; Reis & Cunha 2021). *A. wodanis* is known to exist as free-living bacteria and co-exist with *M. viscosa*, and the ability to cause clinical symptoms in Atlantic salmon has been documented (Benediktsdottir et al. 2000; Lunder et al. 2000; Karlsen et al. 2014a). In addition, the growth temperature of *A. wodanis* ranges from 4-20°C (Soderberg et al. 2019; Maharajan et al. 2021). This suggests that the accessory genomes play an important role in the adaptation process of *A. wodanis*. The pan-genome cluster numbers for *A. wodanis* are analogous to those pan-genome proportions reported for species of *Vibrio harveyi*, *Vibrio*

owensii, *Vibrio campbellii*, *Vibrio rotiferianus*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (Nathamuni et al. 2019; Maistrenko et al. 2020). In other vibrios, the variable genome content accounted for about 80% of the total pan-genome (Nathamuni et al. 2019; Maistrenko et al. 2020). Similar to other vibrios (Nathamuni et al. 2019), a huge variation of about 73% was observed in the shell and cloud genomes of *A. wodanis*, which is likely due to the high capacity to acquire new genes. Moreover, a larger part of the pan-genome contains genes related to the functional families *metabolism*, *signaling and cellular processes* and *genetic information processing*. As described in prior studies (Wang et al. 2020a), it is likely that the genes present in *A. wodanis* pan-genome are mainly associated with environment interaction and energy metabolism which were possibly obtained through environmental changes.

Furthermore, the core genomes decreased with the addition of genomes suggesting a small core genome. Put in context with current knowledge of core genes in the bacteria kingdom (from low ~500 to high ~6000), the size of *A. wodanis* is above the average (3100) and alongside other species related to aquatic, marine, soil and plant-associated environments (Makarova et al. 2020). The core genes are higher than that reported for *V. anguillarum*, *V. mimicus* and lower than *V. parahaemolyticus* and *P. aeruginosa* (Hasan et al. 2010; Li et al. 2014; Ozer et al. 2014; Castillo et al. 2017). Core genes represent the highly conserved genes between isolates, which majorly perform housekeeping functions or are involved in virulence in some bacteria (Wolfgang et al. 2003; Castillo et al. 2017). In our analysis, although isolates within three clades were conserved, the core genes in *A. wodanis* were found to be quite diverse.

This study identified 4961 unique genes between the 22 included *A. wodanis* genomes. Such plasticity indicates a capacity for ecological adaptation (Rubin et al. 2000) and is related to HGT events between other bacteria species (Gogarten & Townsend 2005; Lawrence 2005). As described for *V. anguillarum* (Castillo et al. 2017), selective pressure on *A. wodanis* may have caused the acquisition of unique genes to increase virulence potential and fitness. The functional analysis frequently coupled strain-specific genes to *enzymes*, *transporters* and the *CRISPR-Cas system*, showing potential for a wider strain-specific range of capabilities. Isolate S7, for instance, has six times the amount of enzyme-encoding unique genes relative to the other isolates in this study. These enzymes mostly corresponded to metabolic pathways such as *metabolism of cofactors and vitamins*, *carbohydrate metabolism*, *aminoacid metabolism* and *energy metabolism.* A key to bacterial survival and adaptation in diverse environments depends

on its wide metabolic functions (McNally et al. 2016; Goyal 2018). For example, *P. aeruginosa* has a larger genome encoding functionally diverse and metabolic versatile products to survive in multiple environments (Ozer et al. 2014). We speculate that the unique genes in isolate S7 may have additional metabolic advantages in nutrient uptake compared to other isolates to survive in diverse environments.

Major accessory genome components in many bacteria are conjugative elements, phages and transposons (Kung et al. 2010). However, these components are not present noticeably in the unique gene clusters of S7. The prevalence of unique genes could not be correlated with genome size, nor time and location of sampling in Norway.

CRISPR-Cas system

In this study, we identified a type IF CRISPR system in nine isolates and an incomplete Cas operon in ten other isolates. However, all isolates were found to contain the CRISPR arrays, suggesting that the CRISPR systems in VwK7F1 and Vw7 isolates probably have been lost later. In addition to the role of CRISPR-Cas in defence, some of them are involved in the regulation of envelope stress response and virulence (Louwen et al. 2014; Faure et al. 2019). Similarly, the diverse CRISPR-Cas systems may have different functions in *A. wodanis*. The variation in the CRISPR-Cas systems in *A. wodanis* may influence their ability to uptake or defend foreign genetic material in the natural environment. The CRISPR systems showed high amino acid similarity to *Colwellia* spp., suggesting *A. wodanis* acquired it from these bacteria. Members of the genus *Colwellia* have been isolated from marine organisms and other marine environments (Choi et al. 2010; Lin et al. 2019). It has been reported that bacteria belonging to the same species can reveal diverse spacer sequences (Lopatina et al. 2019). Similarly, in this study, the spacer sequences of 15 out of 22 isolates showed differences in grouping from core tree grouping; there were still few isolates grouped similar to the core tree. Moreover, the number of spacers in each CRISPR array varied between isolates. The isolates that showed incomplete CRISPR-Cas operon comprised fewer spacers than the isolates with a complete CRISPR-Cas operon. This result indicates that the incomplete CRISPR-Cas operon is not functional anymore in these isolates. However, they may play a role in other functions like DNA repair (Zhang & Ye 2017). The spacer phylogram showed differences in grouping from

the core and SNVs-based tree except for group 4. This suggests that the spacer sequences of *A. wodanis* isolates that did not match the core tree are diverse and were acquired independently. The phage analysis demonstrated that the grouping of *A. wodanis* showed no similarity to the core tree, which suggests that the phages have been acquired independently and may not be transmitted vertically.

T6SSs

All twenty-two isolates encoded the T6SS1 and T6SS3; however, a larger variation was seen in the T6SS2. The T6SS2 showed similarities to other aliivibrios such as *A. sifae, A. fischeri* and *A. logei*. These aliivibrios are mainly environmental strains associated with symbiosis and not pathogens (Engebrecht et al. 1983; Fidopiastis et al. 1998; Sawabe et al. 2014). This may indicate that the T6SS2 in the 16 isolates might behave more like environmental strains rather than a potential pathogen interacting with the host. The T6SS analysis suggests that the T6SS1 and T6SS3, unlike T6SS2, are highly conserved between the *A. wodanis* isolates. Further research is required to investigate the functional differences between the T6SS2 of reference and other isolates. Like the CRISPR-Cas systems, where all four isolates belonging to Group 2 in the core tree encoded an incomplete *cas* operon, these isolates lacked the T6SS2. Moreover, the isolate Vw37, which encoded an incomplete *cas* operon, also lacked T6SS2. The isolates that lack T6SS2 also encoded an incomplete *cas* operon or lack *cas* genes except isolate Vw8. The reference T6SS2 is highly similar to vibrios and particularly *V. cholerae* (Maharajan et al. 2022). The VgrG and HcP are the hallmark proteins of T6SSs (Mougous et al. 2006). VgrG is a bacteriophage-like cell-puncturing device, and Hcp acts as a tube component that delivers the effectors (Pukatzki et al. 2007b). Besides the structural role of VgrG and HcP in the functioning of T6SSs, they act as effectors or as chaperones to effectors (Santos et al. 2019). This suggests that the multiple VgrG may function as effectors.

Despite the genetic diversity in CRISPR-Cas and T6SS2, the BGCs and VFs were highly conserved in all strains. This suggests that the BGCs and VFs are essential for the isolates. *A. wodanis* encodes gene clusters that produce secondary metabolites thioamititdes, ectoine, siderophores, arylploye ne and resorcinol. Ectoine provides protection against salt and temperature stress (Czech et al. 2018). Arylpolyene is often found in host-associated bacteria

like commensals and pathogens (Cimermancic et al. 2014) and protects the bacterium from reactive oxygen species (Schoner et al. 2016). Similarly, these secondary metabolites may protect *A. wodanis* from stress and reactive oxygen species. In addition to the pathogenic role of VFs, they are also present in commensals and symbiotic bacteria (Niu et al. 2013). This suggests that the VFs may perform multiple host-associated roles. In *V. anguillarum* similar VFs were identified from strains isolated from the same geographical location (Hansen et al. 2020). Similarly, the reason for the similarity in the VFs could be that the isolates used were only from Norway and not from different countries.

Conclusion

The *A. wodanis* isolates analyzed in the present study represent the collection of whole genome sequenced strains isolated in Norway. The analysis reveals an open pan-genome and the evolutionary relationship between the isolates. The genome analysis found variations in the CRISPR system, phages and T6SS2 systems, while quite conserved regarding other T6SSs, biosynthetic gene clusters and virulence factors. The study adds knowledge to the virulence, survival and defence potential of *A. wodanis.*

Competing interests

The author declare that they have no competing interests.

Ethics statement

The research presented in this paper do not involve any human or animal subjects and we see no ethical issues.

Authors' contributions

AM, TK and NP conceived the study and drafted the manuscript. AM and TK performed the bioinformatic analyses. AM wrote the manuscript. AM, TK and NP authors reviewed and provided critical feedback to the final manuscript.

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Supplementary information

Additional file 1

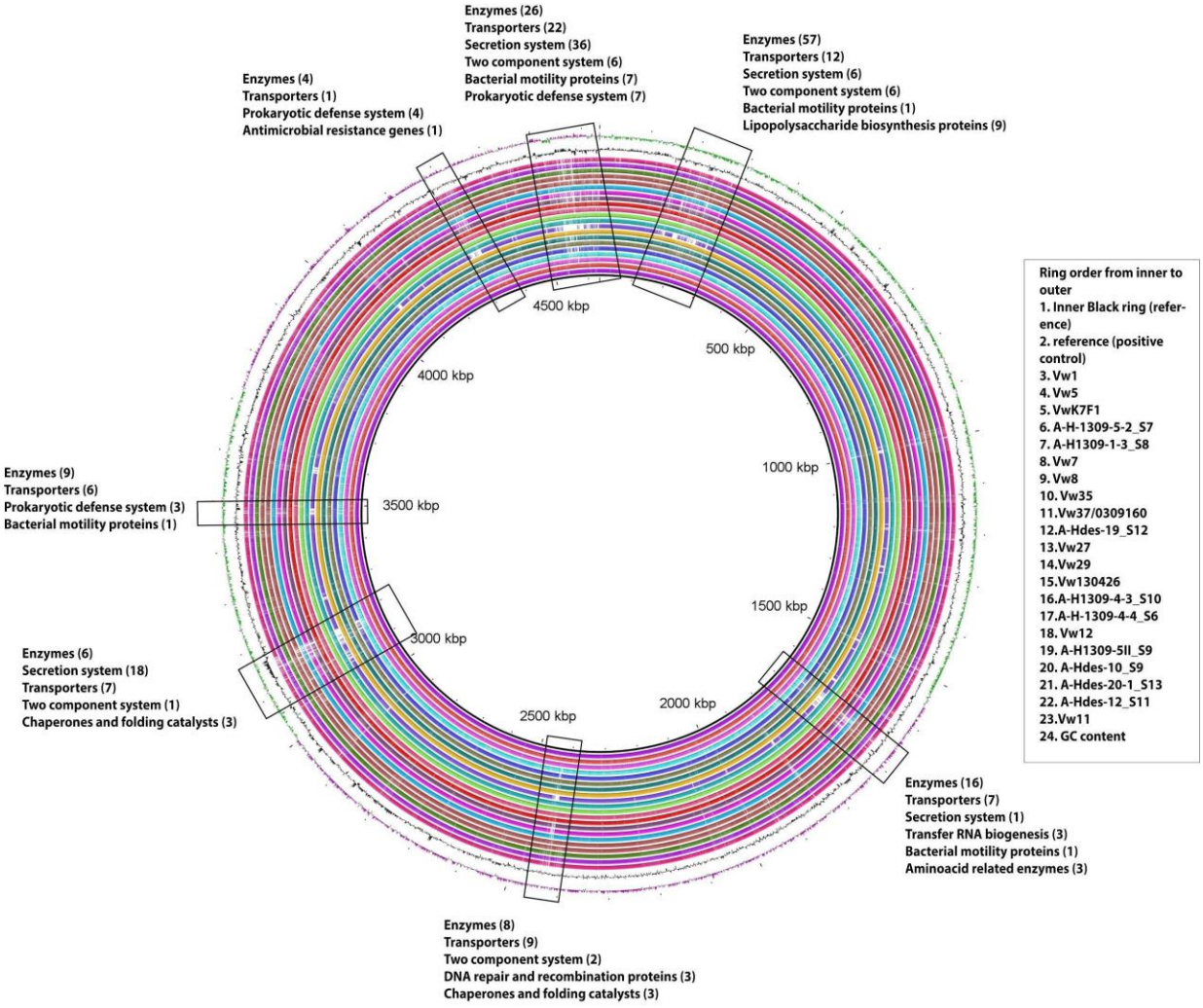
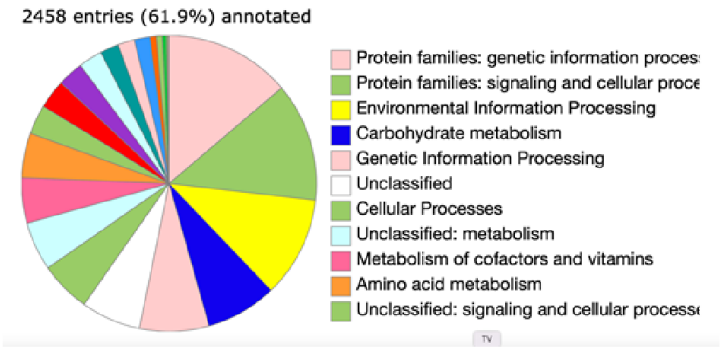


Figure S1. BRIG circular representation of genomes. The order of the rings is presented in the box (right). The regions showing gaps against the reference ring are highlighted in black rectangles.

Additional file 2

A)



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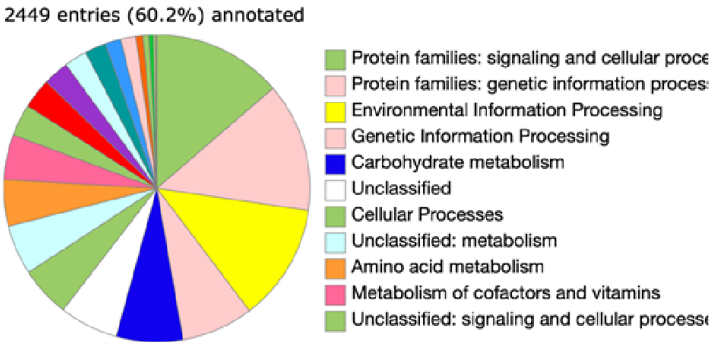

Figure S2. The BlastKOALA annotation of genomes S7 and Vw1. The functional annotation of genomes of the isolates (A) S7 and (B) Vw1 with the highest and lowest number of unique genes respectively are presented here.

Additional file 3

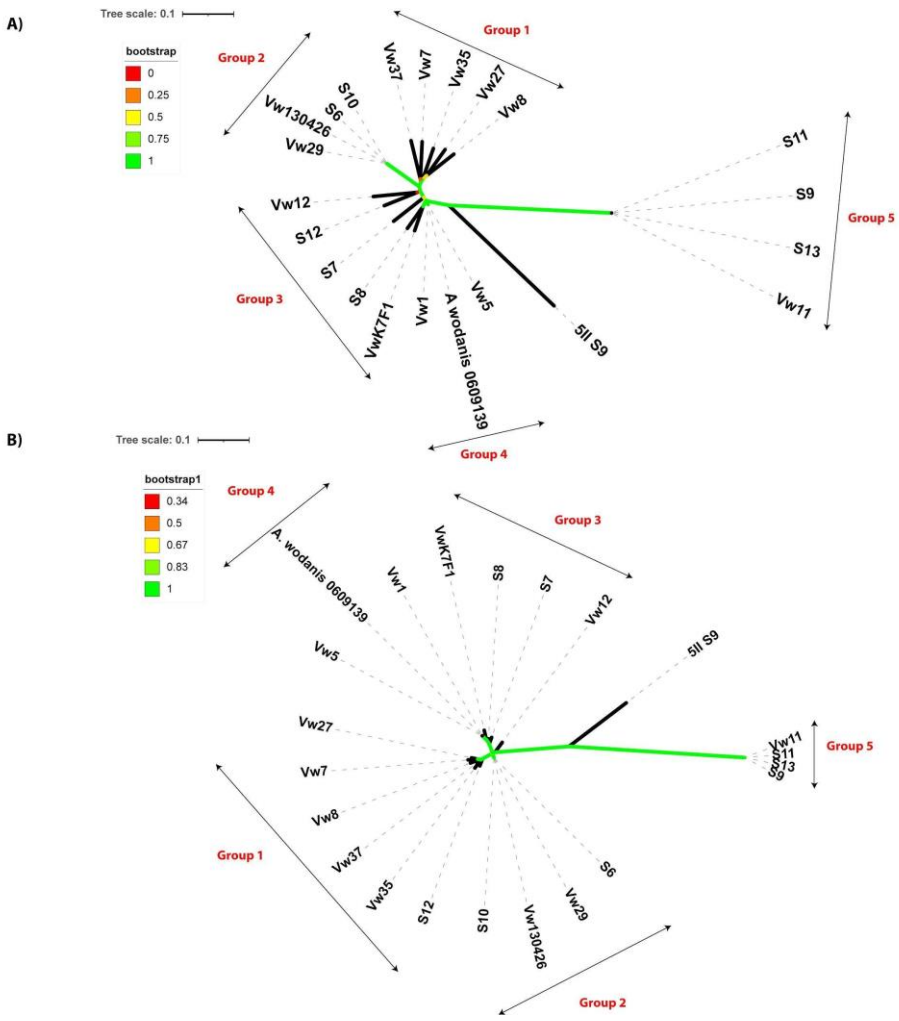

Figure S3: Phylogenetic trees based on SNVs and core genes. A) SNV and B) core tree.

Additional file 4

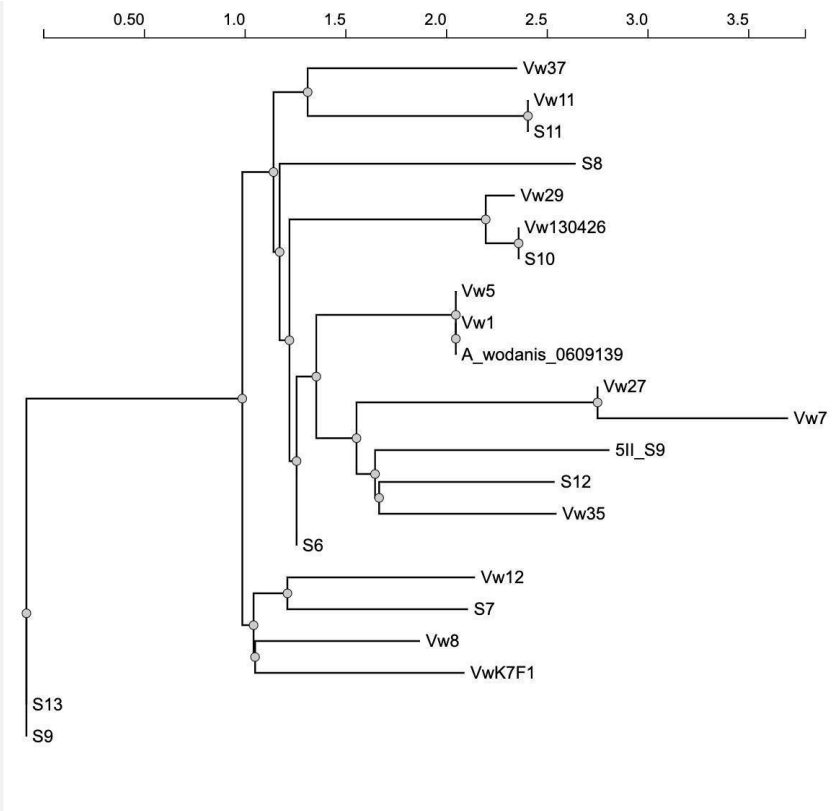

Figure S4. Phylogenetic analysis of spacers. Phylogram represents the phylogenetic relationships between isolates.

Additional file 5

Supplementary Table S1: BlastP analysis of unique genes present in S7 isolate					
Clusters no.	**Unique gene ID**	**BlastP hit**	**Product**	**Organisms**	**Identity %**
		Protein accession ID			
1	80852.49.peg.626	WP_075767609.1	formate dehydrogenase subunit alpha	*Photobacterium proteolyticum*	72.093
	80852.49.peg.627	WP_164918488.1	Ig-like domain-containing protein	*Photobacterium chitinilyticum*	82.968
	80852.49.peg.628	WP_197026917.1	tail fiber protein	*Methylomonas* sp. *11b*	40.708
	80852.49.peg.629	WP_130066352.1	ISAs1 family transposase, partial	*Aliivibrio finisterrensis*	91.919
	80852.49.peg.630	WP_102540820.1	FAD-dependent oxidoreductase	*Vibrio splendidus*	59.459
	80852.49.peg.632	WP_075765923.1	hypothetical protein	*Photobacterium proteolyticum*	90.82
	80852.49.peg.633	WP_075765925.1	YcaO-like family protein	*Photobacterium proteolyticum*	88.943
	80852.49.peg.634	WP_160648939.1	TfuA-like protein [	*Photobacterium alginatilyticum*	85.779
	80852.49.peg.635	WP_075765929.1	hypothetical protein	*Photobacterium proteolyticum*	82.301
	80852.49.peg.636	WP_160648937.1	FHA domain-containing protein	*Photobacterium alginatilyticum*	88.685
	80852.49.peg.637	NQY65349.1	MAG: phosphatidylinositol-specific phospholipase C	*Alteromonadaceae bacterium*	63.455
	80852.49.peg.638	WP_189005044.1	MACPF domain-containing protein	*Deinococcus roseus*	25.773
	80852.49.peg.639	NQZ06452.1	MAG: hypothetical protein HRT35_04750	*Algicola* sp.	36.066
2	80852.49.peg.2046	WP_065610204.1	AAA family ATPase	*Aliivibrio logei*	99.427
	80852.49.peg.2048	NTU53671.1	MAG: tetratricopeptide repeat protein	*Chlorobiaceae bacterium*	28.182
	80852.49.peg.2050	WP_102619189.1	DGQHR domain-containing protein	*Vibrio parahaemolyticus*	81.957
	80852.49.peg.2051	PMT73668.1	hypothetical protein C1S97_26475	*Vibrio parahaemolyticus*	65.436
	80852.49.peg.2058	ACB55709.1	hypothetical protein VF_A1183	*Aliivibrio fischeri ES114*	92.308
	80852.49.peg.2059	WP_024699529.1	ATP-binding protein	*Vibrio parahaemolyticus*	72.381
	80852.49.peg.2060	WP_077536767.1	recombinase family protein	*Pseudoalteromonas aliena*	63.158
	80852.49.peg.2061	WP_065600668.1	carboxypeptidase M32	*Aliivibrio sp. 1S128*	96.226
	80852.49.peg.2064	WP_130086538.1	endonuclease/exonuclease/phosphatase family proto	*Aliivibrio finisterrensis*	75.581
	80852.49.peg.2066	WP_122035732.1	nitrite reductase large subunit NirB	*Aliivibrio sp. EL58*	100
3					
	80852.49.peg.2140	WP_122032489.1	HNH endonuclease	*Aliivibrio* sp. *EL58*	93.75
	80852.49.peg.2142	WP_151653314.1	SulP family inorganic anion transporter	*Aliivibrio finisterrensis*	100
	80852.49.peg.2144	WP_155662497.1	CHAT domain-containing protein	*Aliivibrio fischeri*	94.247
	80852.49.peg.2145	WP_155662496.1;	hypothetical protein	*Aliivibrio fischeri*	94.18
	80852.49.peg.2146	WP_155659585.1;	hypothetical protein	*Aliivibrio fischeri*	99.038
	80852.49.peg.2147	WP_155659584.1;	hypothetical protein	*Aliivibrio fischeri*	100
	80852.49.peg.2148	WP_065599993.1	TatD family hydrolase	*Aliivibrio* sp. *1S128*	99.468
	80852.49.peg.2149	CED71478.1;	membrane associated GGDEF protein	*Aliivibrio wodanis*	100
	80852.49.peg.2151	WP_086713075.1	MotA/TolQ/ExbB proton channel family protein	*Vibrio gigantis*	80
	80852.49.peg.2153	WP_017051056.1	FAD-dependent oxidoreductase	*Vibrio genomosp. F6*	94.309
	80852.49.peg.2155	WP_009848399.1	5'-methylthioadenosine/S-adenosylhomocysteine nu	*Vibrio sp. MED222*	92.437
	80852.49.peg.2156	WP_017052894.1;	conjugative transfer relaxase/helicase TraI	*Vibrio genomosp. F6*	99.593
	80852.49.peg.2157	OCH16795.1	hypothetical protein A6E05_02275	*Aliivibrio* sp. *1S165*	95.833
4					
	80852.49.peg.2444	WP_061002801.1	DUF2057 family protein	*Aliivibrio sifiae*	81.818
	80852.49.peg.2445	WP_179022795.1	methyl-accepting chemotaxis protein	*Shewanella* sp. *Scap07*	66.667
	80852.49.peg.2446	WP_017053127.1	aromatic amino acid transporter	*Vibrio genomosp. F6*	94.161
	80852.49.peg.2447	WP_171755551.1	glycosyl hydrolase family 18 protein	*Vibrio sp. 03-59-1*	96.795
	80852.49.peg.2448	WP_065604742.1	ATP-binding protein	*Aliivibrio fischeri]*	98.03

	80852.49.peg.2451	WP_122033358.1	bifunctional GNAT family N-acetyltransferase/hotd	*Aliivibrio* sp. *EL58*	96.129
	80852.49.peg.2452	WP_065612260.1	hypothetical protein	*Aliivibrio logei*	99.605
	80852.49.peg.2454	WP_017053091.1	asparaginase	*Vibrio genomosp. F6*	100
	80852.49.peg.2455	WP_136994327.1	DUF1315 family protein	*Vibrio genomosp. F6*	97.872
	80852.49.peg.2456	WP_136994329.1	DUF2989 domain-containing protein	*Vibrio genomosp. F6*	100
	80852.49.peg.2457	WP_196582638.1	nucleotidyltransferase domain-containing protein	*Aliivibrio fischeri*	100
	80852.49.peg.2458	WP_107295952.1	hypothetical protein	*Photobacterium phosphoreum*	99.355
	80852.49.peg.2459	WP_107295952.1	hypothetical protein	*Photobacterium phosphoreum*	100
	80852.49.peg.2460	WP_054776311.1	hypothetical protein	*Aliivibrio fischeri]*	94.34
	80852.49.peg.2461	WP_122032150.1	helix-turn-helix transcriptional regulator	*Aliivibrio* sp. *EL58*	97.849
5					
	80852.49.peg.2559	OEE80729.1	type II secretion system protein GspC	*Vibrio genomosp. F6 str. FF-238*	93.939
	80852.49.peg.2560	WP_017052070.1	ribosome-associated heat shock protein Hsp15	*Vibrio genomosp. F6*	96.875
	80852.49.peg.2561	WP_113796180.1	Hsp33 family molecular chaperone HslO	*Vibrio sp. 03-59-1*	100
	80852.49.peg.2562	WP_017054093.1;	SCP2 domain-containing protein	*Vibrio genomosp. F6*	94.444
	80852.49.peg.2567	PMO50483.1	hypothetical protein BCT09_00330	*Vibrio splendidus*	79.012
	80852.49.peg.2568	WP_182110210.1	sulfurtransferase TusA	*Vibrio marinisediminis*	86.42
	80852.49.peg.2569	RBW64915.1	hypothetical protein DS893_12455	*Vibrionales bacterium C3R12*	96.721
	80852.49.peg.2570	WP_136995897.1	LysR family transcriptional regulator	*Vibrio genomosp. F6*	98.969
	80852.49.peg.2573	WP_171756982.1	nitrogen regulation protein NR(II)	*Vibrio* sp. *03-59-1*	98.63
	80852.49.peg.2575	WP_161156571.1	IS110 family transposase	*Vibrio* sp. *CAIM 722*	79.811
	80852.49.peg.2576	WP_103197387.1	IS110 family transposase	*Vibrio vulnificus*	98.462
	80852.49.peg.2580	WP_171755510.1	hypothetical protein	*Vibrio* sp. *03-59-1*	88.571
	80852.49.peg.2581	WP_004731882.1	hypothetical protein	*Vibrio splendidus*	55.705
	80852.49.peg.2582	WP_017023494.1	acetate--CoA ligase, partial	*Aliivibrio logei*	98.14
6					
	80852.49.peg.2801	WP_136995085.1	MoxR family ATPase	*Vibrio genomosp. F6*	97.833
	80852.49.peg.2802	WP_155672662.1	pleiotropic regulatory protein RsmS	*Aliivibrio fischeri*	85.714
	80852.49.peg.2803	WP_105063232.1	type VI secretion system protein TssA	*Aliivibrio sifiae*	60.556
	80852.49.peg.2806	WP_122036754.1	metalloprotease PmbA	*Aliivibrio* sp. *EL58*	98.98
	80852.49.peg.2807	WP_130044441.1	metalloprotease PmbA	*Aliivibrio finisterrensis*	100
	80852.49.peg.2808	WP_054775719.1	hypothetical protein	*Aliivibrio fischeri*	99.384
	80852.49.peg.2809	WP_188863627.1	hypothetical protein	*Aliivibrio fischeri*	94.57
	80852.49.peg.2810	VVV06357.1	hypothetical protein AW0309160_03847	*Aliivibrio wodanis*	38.06
	80852.49.peg.2811	WP_045378558.1	DUF1311 domain-containing protein	*Vibrio campbellii*	62.222
	80852.49.peg.2814	WP_017052592.1	MULTISPECIES: methylenetetrahydrofolate reduc	*Vibrio*	98.551
	80852.49.peg.2854	WP_153448704.1	MULTISPECIES: cold-shock protein	*Vibrio algicola*	100
	80852.49.peg.2855	WP_113799429.1	TRIC cation channel family protein	*Vibrio sp. 03-59-1*	99.394
	80852.49.peg.2855	WP_017051061.1	TRIC cation channel family protein	*Vibrio genomosp. F6*	99.394
	80852.49.peg.2856	WP_017052968.1	hypothetical protein	*Vibrio genomosp. F6*	95.522
	80852.49.peg.2857	EED28077.1	conserved hypothetical protein	*Vibrio* sp. *16*	72.727
	80852.49.peg.2860	RBW64918.1	acetyl-CoA C-acyltransferase FadA	*Vibrionales bacterium C3R12*	100
	80852.49.peg.2861	RBW64919.1	fatty acid oxidation complex subunit alpha FadB	*Vibrionales bacterium C3R12*	94.848
	80852.49.peg.2862	WP_155662495.1	hypothetical protein	*Aliivibrio fischeri*	97.826
	80852.49.peg.2863	WP_155659583.1	radical SAM protein	*Aliivibrio fischeri*	98.086
	80852.49.peg.2864	WP_155662493.1	dTMP kinase	*Aliivibrio fischeri*	97.872
	80852.49.peg.2865	WP_136995741.1	RNA-binding S4 domain-containing protein	*Vibrio genomosp. F6*	100

1826

1827

	80852.49.peg.2866	WP_170907738.1	sigma-54 dependent transcriptional regulator	*Vibrio sp. B4-6*	64.455
	80852.49.peg.2870	WP_017054306.1	DNA topoisomerase (ATP-hydrolyzing) subunit B	*Vibrio genomosp. F6*	98.261
	80852.49.peg.2871	WP_017054306.1	DNA topoisomerase (ATP-hydrolyzing) subunit B	*Vibrio genomosp. F6*	98.507
7					
	80852.49.peg.2971	WP_074191940.1	hypothetical protein	*Vibrio antiquarius*	95.833
	80852.49.peg.2972	RBW66270.1	YjbF family lipoprotein	*Vibrionales bacterium C3R12]*	88.75
	80852.49.peg.2973	WP_113798438.1	hypothetical protein	*Vibrio* sp. *03-59-1*	88.168
	80852.49.peg.2978	WP_029203404.1	acyltransferase	*Vibrio genomosp. F6*	92.462
	80852.49.peg.2979	WP_017051032.1	flavodoxin FldB	*Vibrio genomosp. F6*	93.333
	80852.49.peg.2980	WP_171756189.1	site-specific tyrosine recombinase XerD	*Vibrio* sp. *03-59-1*	98.529
	80852.49.peg.2981	WP_182105559.1	DNA circularization N-terminal domain-containing	*Vibrio marinisediminis*	59.274
	80852.49.peg.2982	WP_065545258.1	DNA circularization N-terminal domain-containing	*Vibrio scophthalmi*	57.692
	80852.49.peg.2983	HGY39092.1	MAG TPA: iron-containing alcohol dehydrogenase	*Candidatus Caldatribacterium sacch*	44.531
	80852.49.peg.2986	WP_038176298.1	hypothetical protein, partial	*Vibrio coralliilyticus*	91.479
	80852.49.peg.2987	WP_136995519.1	DUF3413 domain-containing protein	*Vibrio genomosp. F6*	90.61
	80852.49.peg.2988	WP_102500144.1	hypothetical protein	*Vibrio splendidus*	51.152
	80852.49.peg.2990	EGQ8512300.1	hypothetical protein GQ852_18335	*Vibrio parahaemolyticus*	29.412
	80852.49.peg.2991	WP_139173024.1	hypothetical protein	*Marinobacter* sp. *AC-23*	41.463
	80852.49.peg.2993	TFH88893.1	hypothetical protein ELS82_25380	*Vibrio ouci*	59.091
	80852.49.peg.2998	WP_065593552.1	MULTISPECIES: hypothetical protein	*unclassified Aliivibrio*	96.25
	80852.49.peg.3004	WP_080539600.1	hypothetical protein	*Vibrio* sp. *Y29_XK_CS5*	56.383
	80852.49.peg.3005	WP_136993865.1	propionyl-CoA synthetase	*Vibrio genomosp. F6*	96.903
	80852.49.peg.3006	WP_171756523.1	acetate--CoA ligase	*Vibrio sp. 03-59-1*	98.551
	80852.49.peg.3008	WP_096739050.1	glycosyltransferase	*Pseudoalteromonas atlantica*	61.449
	80852.49.peg.3009	WP_136995543.1	EAL domain-containing protein	*Vibrio genomosp. F6*	93.314
	80852.49.peg.3010	WP_136994747.1	enoyl-CoA hydratase	*Vibrio genomosp. F6*	99.057
	80852.49.peg.3011	WP_136994749.1	hypothetical protein	*Vibrio genomosp. F6*	99.301
	80852.49.peg.3012	WP_171756950.1	alkaline phosphatase family protein	*Vibrio sp. 03-59-1*	97.03
	80852.49.peg.3013	WP_032551148.1	MULTISPECIES: pantetheine-phosphate adenylylt	*Vibrio*	90.506
	80852.49.peg.3014	WP_017052182.1	glycosyltransferase family 4 protein	*Vibrio genomosp. F6*	93.103
	80852.49.peg.3016	WP_154169266.1	MULTISPECIES: metalloprotease TldD	*Vibrio*	89.068
	80852.49.peg.3017	VVV03257.1	hypothetical protein AW0309160_00625	*Aliivibrio wodanis*	100
8					
	80852.49.peg.3470	WP_172853923.1	serine protease	*Vibrio coralliilyticus*	47.619
	80852.49.peg.3471	GAK17264.1	ATP synthase epsilon chain	*Vibrio sp. JCM 19053*	96.552
	80852.49.peg.3472	ETX58079.1	ATP synthase alpha/beta chain, C terminal domain	*Vibrio parahaemolyticus EKP-028*	95.89
	80852.49.peg.3473	WP_050987529.1	M4 family metallopeptidase	*Vibrio vulnificus*	71.56
	80852.49.peg.3474	WP_017054182.1	hypothetical protein	*Vibrio genomosp. F6*	100
	80852.49.peg.3475	WP_017054181.1	S-ribosylhomocysteine lyase	*Vibrio genomosp. F6*	97.674
	80852.49.peg.3476	WP_171757372.1	TraB/GumN family protein	*Vibrio sp. 03-59-1*	92.672
	80852.49.peg.3477	WP_113796528.1	DNA repair protein RecN	*Vibrio sp. 03-59-1*	96.19
	80852.49.peg.3478	WP_171755885.1	DUF2590 family protein	*Vibrio sp. 03-59-1*	93.878
	80852.49.peg.3479	WP_171755886.1	baseplate J/gp47 family protein	*Vibrio sp. 03-59-1*	98.765
	80852.49.peg.3480	WP_088879211.1	phage tail protein	*Vibrio rotiferianus*	87.5
	80852.49.peg.3481	WP_171306844.1	phage baseplate assembly protein	*Vibrio rotiferianus*	91.62
	80852.49.peg.3482	WP_171306845.1	hypothetical protein	*Vibrio rotiferianus*	82.812

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	80852.49.peg.3483	WP_045420003.1	phage GP46 family protein	*Vibrio jasicida*	86.719
	80852.49.peg.3484	WP_182032938.1	baseplate J/gp47 family protein	*Vibrio diabolicus*	98.529
	80852.49.peg.3485	WP_089138326.1	DDE-type integrase/transposase/recombinase	*Vibrio rumoiensis*	95.935
	80852.49.peg.3486	WP_108196240.1	tryptophanase	*Vibrio splendidus*	97.458
	80852.49.peg.3487	WP_137032290.1	tryptophanase	*Vibrio kanaloae*	97.619
9					
	80852.49.peg.3599	WP_009846889.1	hypothetical protein	*Vibrio sp. MED222*	95.769
	80852.49.peg.3600	WP_009846890.1	DUF4391 domain-containing protein	*Vibrio sp. MED222*	94.035
	80852.49.peg.3601	WP_102532440.1	helicase-related protein	*Vibrio sp. 10N.286.48.B7*	98.201
	80852.49.peg.3602	WP_017035588.1	MULTISPECIES: hypothetical protein	*Gammaproteobacteria*	97.436
	80852.49.peg.3603	WP_039461214.1	hypothetical protein	*Vibrio navarrensis*	55.738
	80852.49.peg.3610	WP_182699703.1	ParA family protein	*Aliivibrio sp. SR45-2*	99.145
	80852.49.peg.3611	WP_171757494.1	2-iminoacetate synthase ThiH	*Vibrio sp. 03-59-1*	91.892
	80852.49.peg.3612	RBW65697.1	aminopeptidase P family protein	*Vibrionales bacterium C3R12*	98.544
	80852.49.peg.3613	WP_019613480.1	chemotaxis protein CheC	*Psychromonas ossibalaenae*	46.995
	80852.49.peg.3614	WP_193022055.1	response regulator	*Thalassotalea sp. LPB0316*	50.769
	80852.49.peg.3615	WP_017052106.1	HDOD domain-containing protein	*Vibrio genomosp. F6*	95.69
	80852.49.peg.3617	WP_171756261.1	alpha/beta fold hydrolase	*Vibrio sp. 03-59-1*	91.795
	80852.49.peg.3618	WP_136994184.1	alpha/beta hydrolase	*Vibrio genomosp. F6*	96.078
	80852.49.peg.3619	RBW63479.1	HlyD family secretion protein	*Vibrionales bacterium C3R12*	95.266
	80852.49.peg.3620	TKF22034.1	DHA2 family efflux MFS transporter permease sub	*Vibrio genomosp. F6*	98.261
	80852.49.peg.3621	WP_136993951.1	agmatine deiminase	*Vibrio genomosp. F6*	97.26
	80852.49.peg.3622	WP_017053347.1	APC family permease	*Vibrio genomosp. F6*	97.321
	80852.49.peg.3623	WP_017054675.1	GNAT family N-acetyltransferase	*Vibrio genomosp. F6*	97.222
	80852.49.peg.3624	WP_171756291.1	MFS transporter	*Vibrio sp. 03-59-1*	97.015
	80852.49.peg.3625	WP_010316906.1	DUF1145 domain-containing protein	*Vibrio anguillarum*	91.111
	80852.49.peg.3626	WP_006957309.1	MULTISPECIES: YhgN family NAAT transporter	*Vibrio*	87.629
	80852.49.peg.3628	WP_017051139.1	2-isopropylmalate synthase	*Vibrio genomosp. F6*	99.415
10	80852.49.peg.3714	AKN38625.1	hypothetical protein	*Vibrio splendidus*	78.378
	80852.49.peg.3715	WP_130954725.1	DUF1508 domain-containing protein	*Hafnia paralvei*	90.196
	80852.49.peg.3717	WP_171755930.1	response regulator	*Vibrio sp. 03-59-1*	98.529
	80852.49.peg.3718	WP_029203434.1	glutathione-regulated potassium-efflux system prot	*Vibrio genomosp. F6*	95.556
	80852.49.peg.3719	WP_017053164.1	YheV family putative metal-binding protein	*Vibrio genomosp. F6*	100
	80852.49.peg.3720	WP_171138354.1	peptidylprolyl isomerase	*Vibrio plantisponsor*	90.789
	80852.49.peg.3721	WP_136995517.1	exodeoxyribonuclease V subunit beta	*Vibrio genomosp. F6*	98.171
	80852.49.peg.3722	RBW64065.1	MSHA biogenesis protein MshJ	*Vibrionales bacterium C3R12*	96.237
	80852.49.peg.3723	WP_171756253.1	MSHA biogenesis protein MshI	*Vibrio sp. 03-59-1*	89.73
	80852.49.peg.3724	WP_136993714.1	phosphomethylpyrimidine synthase ThiC	*Vibrio genomosp. F6*	80.142
	80852.49.peg.3725	WP_136995127.1	YdcF family protein	*Vibrio genomosp. F6*	80.753
	80852.49.peg.3726	WP_113799245.1	2-amino-4-hydroxy-6-hydroxymethyldihydropteridi	*Vibrio sp. 03-59-1*	96.894
	80852.49.peg.3727	WP_017036674.1	3-methyl-2-oxobutanoate hydroxymethyltransferase	*Vibrio genomosp. F10*	100
	80852.49.peg.3728	WP_017051153.1	threonine/serine exporter family protein	*Vibrio genomosp. F6*	100
	80852.49.peg.3729	WP_171756262.1	type 3 dihydrofolate reductase	*Vibrio sp. 03-59-1*	91.111
	80852.49.peg.3730	WP_042503177.1	hypothetical protein	*Vibrio maritimus*	86.957
	80852.49.peg.3731	NOH85878.1	multidrug effflux MFS transporter	*Vibrio sp. 03-59-1*	94.301
	80852.49.peg.3732	WP_053409555.1	DUF1853 family protein	*Vibrio hepatarius*	66.026

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	80852.49.peg.3733	OXE30909.1	phosphoglucomutase, alpha-D-glucose phosphate-s	*Vibrio parahaemolyticus*	97.333
11					
	80852.49.peg.3880	WP_017053166.1	isoaspartyl peptidase/L-asparaginase	*Vibrio genomosp. F6*	91.667
	80852.49.peg.3881	WP_053411063.1	SlyX family protein	*Vibrio hepatarius*	86.667
	80852.49.peg.3882	WP_017051182.1	type II secretion system F family protein	*Vibrio genomosp. F6*	95.96
	80852.49.peg.3883	WP_113799602.1	type II/IV secretion system protein	*Vibrio sp. 03-59-1*	96.319
	80852.49.peg.3884	WP_171306855.1	terminase family protein	*Vibrio rotiferianus*	89.034
	80852.49.peg.3885	WP_072668905.1	MULTISPECIES: carbon storage regulator CsrA	*Vibrio*	98.462
	80852.49.peg.3886	WP_017054191.1	aspartate kinase	*Vibrio genomosp. F6*	85.714
	80852.49.peg.3887	WP_171757462.1	ABC transporter substrate-binding protein	*Vibrio sp. 03-59-1*	99.405
	80852.49.peg.3888	WP_176298421.1	outer membrane-stress sensor serine endopeptidase	*Vibrio sp. 05-20-BW147*	85.542
	80852.49.peg.3889	WP_017052805.1	MFS transporter	*Vibrio genomosp. F6*	95.276
	80852.49.peg.3890	WP_113799249.1	ABC transporter ATP-binding protein	*Vibrio sp. 03-59-1*	99.091
	80852.49.peg.3891	WP_113795782.1	signal peptidase I	*Vibrio sp. 03-59-1*	95.633
	80852.49.peg.3892	EHA1084964.1	hypothetical protein FG308_09790	*Vibrio parahaemolyticus*	77.612
	80852.49.peg.3893	WP_136994689.1	glucosaminidase domain-containing protein	*Vibrio genomosp. F6*	80.625
	80852.49.peg.3895	WP_017052367.1	rhodanese-like domain-containing protein	*Vibrio genomosp. F6*	90.598
	80852.49.peg.3896	WP_171755518.1	fimbria/pilus periplasmic chaperone	*Vibrio sp. 03-59-1*	80.24
	80852.49.peg.3897	NOH82302.1	fimbrial biogenesis outer membrane usher protein	*Vibrio sp. 03-59-1*	63.415
	80852.49.peg.3899	WP_017054636.1	holo-ACP synthase	*Vibrio genomosp. F6*	96.825
	80852.49.peg.3900	WP_171757170.1	two-component sensor histidine kinase BarA	*Vibrio sp. 03-59-1*	88
	80852.49.peg.3901	HAS6345173.1	TPA: hypothetical protein I7243_17860	*Vibrio vulnificus*	86.254
	80852.49.peg.3902	EGR1275458.1	PAS domain S-box protein	*Vibrio parahaemolyticus*	95.652
	80852.49.peg.3904	WP_171756799.1	UDP-N-acetylglucosamine--undecaprenyl-phosphat	*Vibrio sp. 03-59-1*	97.143
	80852.49.peg.3905	WP_102430374.1	restriction endonuclease subunit S	*Vibrio breoganii*	86.473
	80852.49.peg.3906	WP_139742787.1	anti-phage deoxyguanosine triphosphatase	*Aeromonas veronii*	64.238
	80852.49.peg.3907	WP_102536226.1	hypothetical protein	*Vibrio lentus*	76.712
	80852.49.peg.3908	WP_158141838.1	ogr/Delta-like zinc finger family protein	*Vibrio furnissii*	88.889
	80852.49.peg.3909	WP_192891084.1	hypothetical protein	*Vibrio bathopelagicus*	78.082
	80852.49.peg.3910	WP_088879214.1	baseplate J/gp47 family protein	*Vibrio rotiferianus*	81.579
	80852.49.peg.3911	WP_088879215.1	hypothetical protein	*Vibrio rotiferianus*	85
12	80852.49.peg.4153	WP_108188206.1	dihydroxy-acid dehydratase	*Vibrio splendidus*	97.163
	80852.49.peg.4154	WP_017054336.1	dihydroxy-acid dehydratase, partial	*Vibrio genomosp. F6*	97.08
	80852.49.peg.4156	RBW66268.1	YjbH domain-containing protein	*Vibrionales bacterium C3R12*	97.409
	80852.49.peg.4157	WP_113798843.1	rhomboid family intramembrane serine protease Glp	*Vibrio sp. 03-59-1*	95.694
	80852.49.peg.4158	WP_171756495.1	ATP-dependent RNA helicase HrpA	*Vibrio sp. 03-59-1*	98.246
	80852.49.peg.4159	WP_171756048.1	type II secretion system GspH family protein	*Vibrio sp. 03-59-1*	64.516
	80852.49.peg.4160	RBW66599.1	RluA family pseudouridine synthase	*Vibrionales bacterium C3R12*	78.03
	80852.49.peg.4161	ARP40289.1	Nitrite reductase	*Vibrio alginolyticus*	97.79
	80852.49.peg.4162	HAS6345173.1	TPA: hypothetical protein I7243_17860	*Vibrio vulnificus*	83.071
	80852.49.peg.4163	WP_072962833.1	AMP-binding protein	*Vibrio gazogenes*	98.643
	80852.49.peg.4231	VVV04920.1	Pyridoxal kinase PdxY	*Aliivibrio wodanis*	99.465
	80852.49.peg.4232	ETX58582.1	aminotransferase class I and II family protein, parti	*Vibrio parahaemolyticus EKP-028*	91.935
	80852.49.peg.4233	WP_102459390.1	glycine--tRNA ligase subunit beta, partial	*Vibrio lentus*	96.774
	80852.49.peg.4234	RBW66316.1	aminoacetone oxidase family FAD-binding enzyme	*Vibrionales bacterium C3R12*	97.665
	80852.49.peg.4235	WP_017054647.1	L-aspartate oxidase	*Vibrio genomosp. F6*	95.238
	80852.49.peg.4236	WP_141707850.1	hypothetical protein	*Vibrio genomosp. F6*	67.308

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	80852.49.peg.4237	WP_170961700.1	MULTISPECIES: PTS trehalose transporter subun	*Vibrio*	98.016
	80852.49.peg.4238	WP_171756884.1	alpha,alpha-phosphotrehalase	*Vibrio sp. 03-59-1*	98.99
	80852.49.peg.4239	WP_171756969.1	purine/pyrimidine permease	*Vibrio sp. 03-59-1*	97.72
	80852.49.peg.4240	WP_061065895.1	ATP-binding protein	*Vibrio harveyi*	64.062
	80852.49.peg.4242	WP_089138325.1	AAA family ATPase	*Vibrio rumoiensis*	95.885
	80852.49.peg.4243	RBW64341.1	UDP-glucose 4-epimerase GalE	*Vibrionales bacterium C3R12*	95.939
	80852.49.peg.4244	RBW65504.1	GGDEF domain-containing protein	*Vibrionales bacterium C3R12*	89.64
	80852.49.peg.4245	WP_047045656.1	prephenate dehydratase	*Vibrio mexicanus*	90.283
	80852.49.peg.4262	WP_065622408.1	CIA30 family protein	*Aliivibrio fischeri*	97.872
	80852.49.peg.4263	WP_155675674.1	CIA30 family protein	*Aliivibrio fischeri*	98.901
	80852.49.peg.4264	WP_063669148.1	DUF3081 domain-containing protein	*Aliivibrio fischeri*	100
	80852.49.peg.4268	WP_063648815.1	UvrD-helicase domain-containing protein	*Aliivibrio fischeri*	99.594
	80852.49.peg.4269	WP_155672238.1	hypothetical protein	*Aliivibrio fischeri*	98.919
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	80852.49.peg.4406	WP_096739050.1	glycosyltransferase	*Pseudoalteromonas atlantica*	67.407
	80852.49.peg.4407	WP_096739051.1	hypothetical protein	*Pseudoalteromonas atlantica*	66.038
	80852.49.peg.4408	SEG63503.1	primary replicative DNA helicase	*Vibrio hangzhouensis*	87
	80852.49.peg.4409	WP_017052867.1	EAL domain-containing protein	*Vibrio genomosp. F6*	80.952
	80852.49.peg.4411	WP_113795922.1	sodium-dependent transporter	*Vibrio* sp. *03-59-1*	100
	80852.49.peg.4413	WP_171757184.1	pyrroline-5-carboxylate reductase	*Vibrio* sp. *03-59-1*	100
	80852.49.peg.4414	WP_113795760.1	YggS family pyridoxal phosphate-dependent enzym	*Vibrio* sp. *03-59-1*	100
	80852.49.peg.4416	WP_017051905.1	S41 family peptidase	*Vibrio genomosp. F6*	95.785
	80852.49.peg.4417	WP_076586103.1	RNA polymerase sigma factor RpoS	*Vibrio ostreicida*	96.216
	80852.49.peg.4421	WP_017054345.1	LysR family transcriptional regulator	*Vibrio genomosp. F6*	93.855
	80852.49.peg.4422	WP_171755631.1	maltose ABC transporter substrate-binding protein	*Vibrio* sp. *03-59-1*	89.908
	80852.49.peg.4424	WP_171757484.1	putative DNA-binding domain-containing protein	*Vibrio* sp. *03-59-1*	90
	80852.49.peg.4425	WP_171757483.1	DUF692 family protein	*Vibrio* sp. *03-59-1*	95.906
	80852.49.peg.4426	WP_171756198.1	glutamate synthase small subunit	*Vibrio* sp. *03-59-1*	96.667
	80852.49.peg.4427	WP_029203239.1	autotransporter assembly complex protein TamA	*Vibrio genomosp. F6*	93.857
	80852.49.peg.4428	WP_113798130.1	DUF3135 domain-containing protein	*Vibrio genomosp. F6*	90.385
	80852.49.peg.4429	WP_017052607.1	transcriptional regulator	*Vibrio genomosp. F6*	90.244
	80852.49.peg.4430	WP_017052350.1	FHA domain-containing protein	*Vibrio genomosp. F6*	95.909
	80852.49.peg.4431	WP_136995769.1	heme biosynthesis protein HemY	*Vibrio genomosp. F6*	88.073
	80852.49.peg.4433	WP_017052866.1	5-amino-6-(5-phospho-D-ribitylamino)uracil phosp	*Vibrio genomosp. F6*	89.231
	80852.49.peg.4434	WP_017052865.1	tyrosine recombinase XerC	*Vibrio genomosp. F6*	99.074

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Additional file 6

Supplementary Table S2. Biosynthetic gene clusters in *A. wodanis* isolates

	Secondary metabolites				
Isolate names	**Thioamitides**	**Siderophore**	**Arylpolyene**	**Arylpolyene,resorcinol**	**Ectoine**
A. wodanis 06/09/139		60% gene similarity to *Vibrio crassostreae* strain 8T5	85% gene similarity to *Aliivibrio sifiae* strain	93% gene similarity to *Aliivibrio sifiae* strain	50% gene similarity to *Aliivibrio sifiae* strain
Vw1		100% gene similarity to reference strain	100% gene similarity to reference strain	97% gene similarity to reference strain	100% gene similarity to reference strain
Vw5		100% gene similarity to reference strain	72% gene similarity to reference strain	25% gene similarity to reference strain	75% gene similarity to reference strain
Vwk7F1		100% gene similarity to reference strain	75% gene similarity to reference strain	44% gene similarity to reference strain	75% gene similarity to reference strain
S7	40% genes similarity to *Vibrio crassostreae* strain 25 P	100% gene similarity to reference strain	31% gene similarity to reference strain	97% gene similarity to reference strain	100% gene similarity to reference strain
S8		100% gene similarity to reference strain	93% gene similarity to reference strain	83% gene similarity to reference strain	100% gene similarity to reference strain
Vw7		71% gene similarity to reference strain	72% gene similarity to reference strain	79% gene similarity to reference strain	62% gene similarity to reference strain
Vw8		100% gene similarity to reference strain	93% gene similarity to reference strain	100% gene similarity to reference strain	100% gene similarity to reference strain
Vw35		100% gene similarity to reference strain	93% gene similarity to reference strain	97% gene similarity to reference strain	100% gene similarity to reference strain
Vw37		100% gene similarity to reference strain	95% gene similarity to reference strain	97% gene similarity to reference strain	100% gene similarity to reference strain
S12		100% gene similarity to reference strain	95% gene similarity to reference strain	90% gene similarity to reference strain	100% gene similarity to reference strain
Vw27		85% gene similarity to reference strain	56% gene similarity to reference strain	39% gene similarity to reference strain	75% gene similarity to reference strain
Vw29		85% gene similarity to reference strain	75% gene similarity to reference strain	27% gene similarity to reference strain	75% gene similarity to reference strain
Vw130426		100% gene similarity to reference strain	72% gene similarity to reference strain	81% gene similarity to reference strain	75% gene similarity to reference strain
S10		100% gene similarity to reference strain	93% gene similarity to reference strain	97% gene similarity to reference strain	100% gene similarity to reference strain
S6		100% gene similarity to reference strain	93% gene similarity to reference strain	100% gene similarity to reference strain	100% gene similarity to reference strain
Vw12		100% gene similarity to reference strain	95% gene similarity to reference strain	97% gene similarity to reference strain	100% gene similarity to reference strain
5II_S9		85% gene similarity to reference strain	47% gene similarity to reference strain	69% gene similarity to reference strain	100% gene similarity to reference strain
S9		100% gene similarity to reference strain	90% gene similarity to reference strain	72% gene similarity to reference strain	100% gene similarity to reference strain
S13		100% gene similarity to reference strain	90% gene similarity to reference strain	72% gene similarity to reference strain	100% gene similarity to reference strain
S11		100% gene similarity to reference strain	90% gene similarity to reference strain	74% gene similarity to *Aliivibrio sifiae* strain and 7	100% gene similarity to reference strain
Vw11		100% gene similarity to reference strain	90% gene similarity to reference strain	74% gene similarity to *Aliivibrio sifiae* strain and 7	100% gene similarity to reference strain

Additional file 7

Supplementary Table S3: Virulence factors identified in reference *A. wodanis*

Protein ID	VFDB DB Hit	Identity %	E value	Product
AWOD_06/09/139				
CNLNGIJN_00057	VFG013197(gi:170717931)	72.781	1.58E-165	CNLNGIJN_00057 Delta-aminolevulinic acid dehydratase
CNLNGIJN_00082	VFG013205(gi:170718942)	63.797	0	CNLNGIJN_00082 Oxygen-independent coproporphyrinogen III oxidase
CNLNGIJN_00082	VFG013630(gi:113460617)	63.576	0	CNLNGIJN_00082 Oxygen-independent coproporphyrinogen III oxidase
CNLNGIJN_00108	VFG043365(gb|NP_206882)	71.212	3.98E-20	CNLNGIJN_00108 Methyl-accepting chemotaxis protein McpP
CNLNGIJN_00132	VFG013319(gi:33152306)	64.94	1.14E-106	CNLNGIJN_00132 SPBc2 prophage-derived glycosyltransferase SunS
CNLNGIJN_00137	VFG037905(gi:384141324)	78.827	0	CNLNGIJN_00137 UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase
CNLNGIJN_00137	VFG037921(gi:169632084)	78.571	0	CNLNGIJN_00137 UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase
CNLNGIJN_00138	VFG037920(gi:384141325)	65.879	3.08E-166	CNLNGIJN_00138 UDP-N-acetylbacillosamine transaminase
CNLNGIJN_00151	VFG013409(gi:148828275)	74.441	3.04E-152	CNLNGIJN_00151 ADP-L-glycero-D-manno-heptose-6-epimerase
CNLNGIJN_00160	VFG007646(gi:59710802)	67.774	3.11E-129	CNLNGIJN_00160 N-acetyl-alpha-D-glucosaminyl-diphospho-ditrans,octacis-undecaprenol 4-epimerase
CNLNGIJN_00161	VFG007649(gi:27364225)	88.095	3.31E-72	CNLNGIJN_00161 Putative undecaprenyl-phosphate N-acetylgalactosaminyl 1-phosphate transferase
CNLNGIJN_00162	VFG007655(gi:59710804)	72.072	5.38E-105	CNLNGIJN_00162 Redox-sensing transcriptional repressor Rex
CNLNGIJN_00163	VFG007655(gi:59710804)	83.292	0	CNLNGIJN_00163 UDP-N-acetyl-alpha-D-glucosamine C6 dehydratase
CNLNGIJN_00164	VFG007643(gi:59710763)	66.847	0	CNLNGIJN_00164 hypothetical protein
CNLNGIJN_00179	VFG038578(gi:507519909)	63.333	0.000434	CNLNGIJN_00179 Type II secretion system protein G
CNLNGIJN_00182	VFG006167(gb|NP_273931)	60	0.000521	CNLNGIJN_00182 hypothetical protein
CNLNGIJN_00182	VFG006166(gi:15794057)	60	0.000517	CNLNGIJN_00182 hypothetical protein
CNLNGIJN_00182	VFG006168(gi:121634665)	60	0.000488	CNLNGIJN_00182 hypothetical protein
CNLNGIJN_00182	VFG006169(gi:59800895)	60	0.000502	CNLNGIJN_00182 hypothetical protein
CNLNGIJN_00197	VFG005898(gi:125718225)	63.86	2.28E-116	CNLNGIJN_00197 Glucose-1-phosphate thymidylyltransferase 1
CNLNGIJN_00197	VFG047048	63.287	1.24E-111	CNLNGIJN_00197 Glucose-1-phosphate thymidylyltransferase 1
CNLNGIJN_00198	VFG007663(gi:37678487)	66.667	1.78E-75	CNLNGIJN_00198 dTDP-4-dehydrorhamnose 3,5-epimerase
CNLNGIJN_00198	VFG048804	60.234	2.65E-63	CNLNGIJN_00198 dTDP-4-dehydrorhamnose 3,5-epimerase
CNLNGIJN_00206	VFG047090	70.339	5.96E-159	CNLNGIJN_00206 dTDP-4-amino-4,6-dideoxy-D-glucose transaminase
CNLNGIJN_00232	VFG048789	75	0	CNLNGIJN_00232 UDP-glucose 6-dehydrogenase
CNLNGIJN_00288	VFG007621(gi:59712924)	92.381	1.92E-135	CNLNGIJN_00288 hypothetical protein
CNLNGIJN_00310	VFG042876(gi:27364773)	60	8.87E-72	CNLNGIJN_00310 hypothetical protein
CNLNGIJN_00312	VFG042878(gi:326423830)	65.093	0	CNLNGIJN_00312 Type IV pilus biogenesis and competence protein PilQ
CNLNGIJN_00317	VFG046603	65.766	2.11E-89	CNLNGIJN_00317 Ribulose-phosphate 3-epimerase
CNLNGIJN_00325	VFG042734(gi:15595849)	67.822	1.11E-88	CNLNGIJN_00325 cAMP-activated global transcriptional regulator CRP
CNLNGIJN_00334	VFG043366(gb|NP_206899)	69.444	7.69E-23	CNLNGIJN_00334 Methyl-accepting chemotaxis protein McpP

CNLNGIJN_00343	VFG016042(gi:152985810)	85,714	0,001	CNLNGIJN_00343 Long-chain-fatty-acid--CoA ligase FadD15
CNLNGIJN_00354	VFG009719(gi:145224520)	60,669	1,58E-88	CNLNGIJN_00354 RNA polymerase sigma factor RpoD
CNLNGIJN_00354	VFG022861(gi:339632715)	60,669	1,19E-88	CNLNGIJN_00354 RNA polymerase sigma factor RpoD
CNLNGIJN_00373	VFG000331(gb|NP_439675)	63,983	0	CNLNGIJN_00373 Bifunctional protein HldE
CNLNGIJN_00409	VFG013413(gi:68249693)	74,013	4,69E-153	CNLNGIJN_00409 UDP-3-O-acyl-N-acetylglucosamine deacetylase
CNLNGIJN_00416	VFG006919(gi:59712795)	87,919	2,59E-178	CNLNGIJN_00416 Type 4 prepilin-like proteins leader peptide-processing enzyme
CNLNGIJN_00417	VFG006913(gi:59712794)	80,34	0	CNLNGIJN_00417 Putative type II secretion system protein F
CNLNGIJN_00418	VFG006907(gi:59712793)	84,014	0	CNLNGIJN_00418 Putative type II secretion system protein E
CNLNGIJN_00419	VFG042920(gi:146328645)	70,588	1,23E-10	CNLNGIJN_00419 Fimbrial protein
CNLNGIJN_00458	VFG013190(gi:170717479)	66,272	1,12E-146	CNLNGIJN_00458 Iron uptake protein A1
CNLNGIJN_00474	VFG013203(gi:170719024)	71,028	0	CNLNGIJN_00474 Glutamate-1-semialdehyde 2,1-aminomutase
CNLNGIJN_00474	VFG013618(gi:113461372)	70,794	0	CNLNGIJN_00474 Glutamate-1-semialdehyde 2,1-aminomutase
CNLNGIJN_00489	VFG043487(gb|NP_760575)	62,356	1,47E-116	CNLNGIJN_00489 Porin-like protein L
CNLNGIJN_00495	VFG013515(gi:148826007)	73,756	0	CNLNGIJN_00495 Phosphoglucosamine mutase
CNLNGIJN_00495	VFG013514(gi:68250006)	73,529	0	CNLNGIJN_00495 Phosphoglucosamine mutase
CNLNGIJN_00538	VFG045727(gi:289165404)	81,818	4,21E-27	CNLNGIJN_00538 Translational regulator CsrA
CNLNGIJN_00554	VFG018246(gi:59711152)	92,353	1,86E-110	CNLNGIJN_00554 S-ribosylhomocysteine lyase
CNLNGIJN_00566	VFG026747(gi:386004335)	60	0,0000849	CNLNGIJN_00566 Energy-dependent translational throttle protein EttA
CNLNGIJN_00578	VFG049189	76,671	0	CNLNGIJN_00578 Chaperone protein ClpB
CNLNGIJN_00682	VFG045346(gb|NP_933683)	78,81	3,46E-123	CNLNGIJN_00682 D-methionine-binding lipoprotein MetQ
CNLNGIJN_00705	VFG043378(gb|NP_207394)	62,121	1,29E-18	CNLNGIJN_00705 Methyl-accepting chemotaxis protein McpG
CNLNGIJN_00721	VFG007603(gi:59711321)	96,457	3,01E-165	CNLNGIJN_00721 Chemotaxis protein PomA
CNLNGIJN_00722	VFG007609(gi:59711322)	92,532	0	CNLNGIJN_00722 Motility protein B
CNLNGIJN_00789	VFG013465(gb|NP_439706)	78,014	9,8E-150	CNLNGIJN_00789 2-dehydro-3-deoxyphosphooctonate aldolase
CNLNGIJN_00811	VFG000077(gb|NP_465991)	62,304	3,36E-81	CNLNGIJN_00811 ATP-dependent Clp protease proteolytic subunit
CNLNGIJN_00826	VFG000478(gi:16764063)	82,069	3,78E-78	CNLNGIJN_00826 Ferric uptake regulation protein
CNLNGIJN_00878	VFG043367(gb|NP_206903)	61,111	3,58E-23	CNLNGIJN_00878 Methyl-accepting chemotaxis protein CtpH
CNLNGIJN_00878	VFG043365(gb|NP_206882)	60,465	2,36E-22	CNLNGIJN_00878 Methyl-accepting chemotaxis protein CtpH
CNLNGIJN_00952	VFG001867(gb|YP_096960)	64,583	4,27E-82	CNLNGIJN_00952 Superoxide dismutase [Fe]
CNLNGIJN_00957	VFG007615(gi:59711533)	91,438	0	CNLNGIJN_00957 putative lipoprotein YiaD
CNLNGIJN_00957	VFG007612(gi:28898885)	66,323	1,64E-129	CNLNGIJN_00957 putative lipoprotein YiaD
CNLNGIJN_00957	VFG007611(gi:147674163)	66,096	3,18E-126	CNLNGIJN_00957 putative lipoprotein YiaD
CNLNGIJN_01042	VFG041016(gi:15596855)	75,407	0	CNLNGIJN_01042 hypothetical protein

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CNLNGIJN_01043	VFG041015(gi:15596854)	62,577	2,13E-63	CNLNGIJN_01043 hypothetical protein
CNLNGIJN_01049	VFG043487(gb|NP_760575)	64,103	0,0000103	CNLNGIJN_01049 Porin-like protein H
CNLNGIJN_01174	VFG043365(gb|NP_206882)	71,642	1,88E-21	CNLNGIJN_01174 Methyl-accepting chemotaxis protein McpP
CNLNGIJN_01206	VFG009578(gi:118470205)	62,069	0,000138	CNLNGIJN_01206 putative ABC transporter ATP-binding protein YknY
CNLNGIJN_01206	VFG022783(gi:340626364)	62,069	0,00033	CNLNGIJN_01206 putative ABC transporter ATP-binding protein YknY
CNLNGIJN_01206	VFG026710(gi:433641500)	62,069	0,00033	CNLNGIJN_01206 putative ABC transporter ATP-binding protein YknY
CNLNGIJN_01362	VFG006994(gi:28899470)	65,385	0,000262	CNLNGIJN_01362 hypothetical protein
CNLNGIJN_01362	VFG038498(gi:145300527)	63,158	0,00000103	CNLNGIJN_01362 hypothetical protein
CNLNGIJN_01362	VFG038499(gi:330831157)	62,857	0,000000455	CNLNGIJN_01362 hypothetical protein
CNLNGIJN_01490	VFG007271(gi:59711841)	91,53	0	CNLNGIJN_01490 Hemin receptor
CNLNGIJN_01498	VFG044246(gi:145300219)	71,856	1,15E-77	CNLNGIJN_01498 putative protein
CNLNGIJN_01498	VFG044437(gi:42491184)	68,605	4,12E-77	CNLNGIJN_01498 putative protein
CNLNGIJN_01499	VFG044438(gi:42491185)	67,516	1,94E-68	CNLNGIJN_01499 hypothetical protein
CNLNGIJN_01505	VFG044440(gi:42491190)	72,611	3,81E-128	CNLNGIJN_01505 Hemin transport system permease protein HmuU
CNLNGIJN_01505	VFG044249(gi:145300222)	60,121	1,12E-101	CNLNGIJN_01505 Hemin transport system permease protein HmuU
CNLNGIJN_01614	VFG044173(gi:28901507)	83,071	4,65E-148	CNLNGIJN_01614 Fe(3+) dicitrate transport ATP-binding protein FecE
CNLNGIJN_01615	VFG044174(gi:28901508)	83,591	6,55E-168	CNLNGIJN_01615 Fe(3+) dicitrate transport system permease protein FecD
CNLNGIJN_01616	VFG044175(gi:28901509)	79,456	7,17E-158	CNLNGIJN_01616 Fe(3+) dicitrate transport system permease protein FecC
CNLNGIJN_01617	VFG044176(gi:28901510)	71,987	1,5E-140	CNLNGIJN_01617 Fe(3+) dicitrate-binding periplasmic protein
CNLNGIJN_01618	VFG044177(gi:28901511)	82,353	0	CNLNGIJN_01618 Fe(3+) dicitrate transport protein FecA
CNLNGIJN_01619	VFG044178(gi:28901512)	85,337	0	CNLNGIJN_01619 Catecholate siderophore receptor Fiu
CNLNGIJN_01620	VFG044179(gi:28901513)	73,697	0	CNLNGIJN_01620 D-alanine--D-alanine ligase
CNLNGIJN_01621	VFG044180(gi:28901514)	73,65	0	CNLNGIJN_01621 hypothetical protein
CNLNGIJN_01622	VFG044181(gi:28901515)	73,566	0	CNLNGIJN_01622 Multidrug resistance protein MdtG
CNLNGIJN_01623	VFG044182(gi:28901516)	76,823	0	CNLNGIJN_01623 N(2)-citryl-N(6)-acetyl-N(6)-hydroxylysine synthase
CNLNGIJN_01624	VFG044183(gi:28901517)	83	0	CNLNGIJN_01624 L-glutamyl-[BtrI acyl-carrier protein] decarboxylase
CNLNGIJN_01664	VFG043365(gb|NP_206882)	72,222	1,39E-16	CNLNGIJN_01664 Methyl-accepting chemotaxis protein CtpH
CNLNGIJN_01664	VFG043367(gb|NP_206903)	70,175	2,09E-17	CNLNGIJN_01664 Methyl-accepting chemotaxis protein CtpH
CNLNGIJN_01790	VFG024067(gi:379760831)	65,385	0,001	CNLNGIJN_01790 Phosphate import ATP-binding protein PstB 3
CNLNGIJN_01790	VFG026711(gi:433630464)	65,385	0,000834	CNLNGIJN_01790 Phosphate import ATP-binding protein PstB 3
CNLNGIJN_01828	VFG015785(gi:170722540)	63,507	2,57E-84	CNLNGIJN_01828 Response regulator UvrY
CNLNGIJN_01839	VFG006986(gi:15640436)	65,517	9,73E-18	CNLNGIJN_01839 Type II secretion system protein G
CNLNGIJN_01952	VFG011430(gb|NP_540392)	63,158	7,08E-25	CNLNGIJN_01952 Acyl carrier protein

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CNLNGIJN_01953	VFG038840(gi:507521851)	78,279	5,18E-125	CNLNGIJN_01953 3-oxoacyl-[acyl-carrier-protein] reductase FabG
CNLNGIJN_02039	VFG007549(gi:59712433)	98,171	1,18E-106	CNLNGIJN_02039 Chemotaxis protein CheW
CNLNGIJN_02041	VFG038725(gi:330828823)	62,598	8,06E-104	CNLNGIJN_02041 Sporulation initiation inhibitor protein Soj
CNLNGIJN_02042	VFG007555(gi:59712437)	88,947	0	CNLNGIJN_02042 Chemotaxis response regulator protein-glutamate methylesterase of group 1 operon
CNLNGIJN_02043	VFG007561(gi:59712438)	84,78	0	CNLNGIJN_02043 Chemotaxis protein CheA
CNLNGIJN_02044	VFG007567(gi:59712439)	91,176	2,73E-139	CNLNGIJN_02044 Protein phosphatase CheZ
CNLNGIJN_02045	VFG007573(gi:59712440)	100	6,35E-80	CNLNGIJN_02045 Chemotaxis protein CheY
CNLNGIJN_02046	VFG007579(gi:59712441)	95,062	7,8E-159	CNLNGIJN_02046 RNA polymerase sigma factor FliA
CNLNGIJN_02047	VFG007585(gi:59712442)	95,27	0	CNLNGIJN_02047 Flagellum site-determining protein YlxH
CNLNGIJN_02048	VFG007591(gi:59712443)	88,844	0	CNLNGIJN_02048 Flagellar biosynthesis protein FlhF
CNLNGIJN_02049	VFG007597(gi:59712444)	96,557	0	CNLNGIJN_02049 Flagellar biosynthesis protein FlhA
CNLNGIJN_02049	VFG014600(gi:77457787)	62,774	0	CNLNGIJN_02049 Flagellar biosynthesis protein FlhA
CNLNGIJN_02051	VFG007399(gi:59712446)	93,617	0	CNLNGIJN_02051 Flagellar biosynthetic protein FlhB
CNLNGIJN_02052	VFG007405(gi:59712447)	95,385	2,38E-167	CNLNGIJN_02052 hypothetical protein
CNLNGIJN_02053	VFG007411(gi:59712448)	96,629	8,55E-55	CNLNGIJN_02053 hypothetical protein
CNLNGIJN_02054	VFG007417(gi:59712449)	92,388	3,03E-179	CNLNGIJN_02054 Flagellar biosynthetic protein FliP
CNLNGIJN_02055	VFG007423(gi:59712450)	85,938	1,64E-71	CNLNGIJN_02055 Flagellar protein FliO
CNLNGIJN_02055	VFG007420(gi:28899014)	60	2,68E-38	CNLNGIJN_02055 Flagellar protein FliO
CNLNGIJN_02056	VFG007429(gi:59712451)	94,574	1,37E-77	CNLNGIJN_02056 Flagellar motor switch protein FliN
CNLNGIJN_02057	VFG007435(gi:59712452)	96,802	0	CNLNGIJN_02057 Flagellar motor switch protein FliM
CNLNGIJN_02058	VFG007441(gi:59712453)	88,199	5,4E-89	CNLNGIJN_02058 hypothetical protein
CNLNGIJN_02060	VFG007453(gi:59712455)	89,51	6,1E-84	CNLNGIJN_02060 hypothetical protein
CNLNGIJN_02061	VFG007459(gi:59712456)	97,267	0	CNLNGIJN_02061 Flagellum-specific ATP synthase
CNLNGIJN_02062	VFG007465(gi:59712457)	86,742	4,8E-154	CNLNGIJN_02062 Flagellar assembly protein FliH
CNLNGIJN_02063	VFG007471(gi:59712458)	96,571	0	CNLNGIJN_02063 Flagellar motor switch protein FliG
CNLNGIJN_02064	VFG007477(gi:59712459)	93,761	0	CNLNGIJN_02064 Flagellar M-ring protein
CNLNGIJN_02065	VFG007483(gi:59712460)	91,262	1,39E-59	CNLNGIJN_02065 Flagellar hook-basal body complex protein FliE
CNLNGIJN_02065	VFG007481(gi:27365279)	73,786	6,7E-47	CNLNGIJN_02065 Flagellar hook-basal body complex protein FliE
CNLNGIJN_02066	VFG007489(gi:59712461)	92,034	0	CNLNGIJN_02066 Regulatory protein AtoC
CNLNGIJN_02067	VFG007495(gi:59712462)	94,186	0	CNLNGIJN_02067 Signal transduction histidine-protein kinase AtoS
CNLNGIJN_02068	VFG007501(gi:59712463)	87,071	0	CNLNGIJN_02068 DNA-binding transcriptional regulator NtrC
CNLNGIJN_02069	VFG007507(gi:59712465)	96,324	5,84E-88	CNLNGIJN_02069 Flagellar secretion chaperone FliS
CNLNGIJN_02070	VFG007513(gi:59712466)	76,042	8,12E-44	CNLNGIJN_02070 hypothetical protein

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CNLNGIJN_02071	VFG007519(gi:59712467)	89,024	0	CNLNGIJN_02071 B-type flagellar hook-associated protein 2
CNLNGIJN_02072	VFG007525(gi:59712468)	77,869	7,24E-57	CNLNGIJN_02072 hypothetical protein
CNLNGIJN_02073	VFG007531(gi:59712469)	90,476	0	CNLNGIJN_02073 Flagellin C
CNLNGIJN_02074	VFG007537(gi:59712470)	91,467	0	CNLNGIJN_02074 Flagellin D
CNLNGIJN_02075	VFG007543(gi:59712471)	86,17	0	CNLNGIJN_02075 Flagellin B
CNLNGIJN_02076	VFG007291(gi:59712472)	92,573	0	CNLNGIJN_02076 Flagellin C
CNLNGIJN_02077	VFG007297(gi:59712473)	89,446	0	CNLNGIJN_02077 Flagellin B
CNLNGIJN_02078	VFG007303(gi:59712474)	90,68	0	CNLNGIJN_02078 Flagellar hook-associated protein 3
CNLNGIJN_02079	VFG007309(gi:59712475)	95,527	0	CNLNGIJN_02079 Flagellar hook-associated protein 1
CNLNGIJN_02080	VFG007315(gi:59712476)	87,725	0	CNLNGIJN_02080 Peptidoglycan hydrolase FlgJ
CNLNGIJN_02081	VFG007321(gi:59712477)	95,89	0	CNLNGIJN_02081 Flagellar P-ring protein
CNLNGIJN_02082	VFG007327(gi:59712478)	94,922	1,75E-167	CNLNGIJN_02082 Flagellar L-ring protein
CNLNGIJN_02083	VFG007333(gi:59712479)	97,328	9,78E-175	CNLNGIJN_02083 Flagellar basal-body rod protein FlgG
CNLNGIJN_02084	VFG007339(gi:59712480)	95,181	1,32E-160	CNLNGIJN_02084 Flagellar basal-body rod protein FlgF
CNLNGIJN_02085	VFG007345(gi:59712481)	93,981	0	CNLNGIJN_02085 Flagellar hook protein FlgE
CNLNGIJN_02086	VFG007351(gi:59712482)	97,458	7,78E-155	CNLNGIJN_02086 Basal-body rod modification protein FlgD
CNLNGIJN_02087	VFG007357(gi:59712483)	99,275	4,24E-92	CNLNGIJN_02087 Flagellar basal-body rod protein FlgC
CNLNGIJN_02088	VFG007363(gi:59712484)	95,42	3,69E-84	CNLNGIJN_02088 Flagellar basal body rod protein FlgB
CNLNGIJN_02089	VFG007369(gi:59712485)	97,455	0	CNLNGIJN_02089 Chemotaxis protein methyltransferase Cher2
CNLNGIJN_02089	VFG007364(gi:15642200)	85,091	3,55E-164	CNLNGIJN_02089 Chemotaxis protein methyltransferase Cher2
CNLNGIJN_02090	VFG007375(gi:59712486)	94,771	0	CNLNGIJN_02090 Chemotaxis protein CheV
CNLNGIJN_02091	VFG007381(gi:59712487)	77,823	1,68E-122	CNLNGIJN_02091 hypothetical protein
CNLNGIJN_02092	VFG007387(gi:59712488)	84,466	1,37E-48	CNLNGIJN_02092 hypothetical protein
CNLNGIJN_02093	VFG007393(gi:59712489)	94,366	7,02E-88	CNLNGIJN_02093 hypothetical protein
CNLNGIJN_02094	VFG043121(gi:15642205)	64,138	9,13E-54	CNLNGIJN_02094 hypothetical protein
CNLNGIJN_02095	VFG043122(gi:15642206)	73,134	3,75E-101	CNLNGIJN_02095 hypothetical protein
CNLNGIJN_02124	VFG038405(gi:117619593)	65,333	5,79E-25	CNLNGIJN_02124 putative deoxyribonuclease RhsA
CNLNGIJN_02165	VFG013374(gb|NP_439075)	62,09	2,99E-128	CNLNGIJN_02165 UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferase
CNLNGIJN_02191	VFG009575(gi:41408512)	62,162	3,63E-08	CNLNGIJN_02191 Phosphate import ATP-binding protein PstB
CNLNGIJN_02247	VFG043648(gi:15598818)	74,275	1,11E-130	CNLNGIJN_02247 RNA polymerase sigma factor RpoS
CNLNGIJN_02255	VFG005579(gi:116516768)	61,111	3,15E-153	CNLNGIJN_02255 Enolase
CNLNGIJN_02258	VFG007537(gi:59712470)	70,822	1,04E-159	CNLNGIJN_02258 Flagellin C
CNLNGIJN_02275	VFG000121(gb|NP_249453)	66,842	1,89E-81	CNLNGIJN_02275 ECF RNA polymerase sigma-E factor

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CNLNGIJN_02351	VFG042880(gi:27364898)	76,087	0	CNLNGIJN_02351 Twitching mobility protein
CNLNGIJN_02352	VFG042879(gi:27364897)	84,638	0	CNLNGIJN_02352 Twitching mobility protein
CNLNGIJN_02359	VFG013626(gi:68249065)	64,675	1,29E-163	CNLNGIJN_02359 Oxygen-independent coproporphyrinogen-III oxidase-like protein YqeR
CNLNGIJN_02359	VFG013628(gi:148827660)	64,675	1,56E-163	CNLNGIJN_02359 Oxygen-independent coproporphyrinogen-III oxidase-like protein YqeR
CNLNGIJN_02425	VFG038496(gb|YP_858148)	66,667	0,000249	CNLNGIJN_02425 hypothetical protein
CNLNGIJN_02425	VFG038497(gi:507523386)	66,667	0,000251	CNLNGIJN_02425 hypothetical protein
CNLNGIJN_02426	VFG007003(gi:59710975)	62,694	1,69E-68	CNLNGIJN_02426 hypothetical protein
CNLNGIJN_02427	VFG006997(gi:59710974)	60,204	7,51E-56	CNLNGIJN_02427 hypothetical protein
CNLNGIJN_02428	VFG006991(gi:59710973)	80,519	7,93E-75	CNLNGIJN_02428 hypothetical protein
CNLNGIJN_02429	VFG006985(gi:59710972)	87,166	4,4E-100	CNLNGIJN_02429 Fimbrial protein
CNLNGIJN_02431	VFG006973(gi:59710970)	90,686	0	CNLNGIJN_02431 Type II secretion system protein F
CNLNGIJN_02432	VFG006967(gi:59710969)	91,188	0	CNLNGIJN_02432 Type II secretion system protein E
CNLNGIJN_02433	VFG006967(gi:59710969)	93,939	9,4E-13	CNLNGIJN_02433 hypothetical protein
CNLNGIJN_02435	VFG006955(gi:59710967)	81,206	7,97E-162	CNLNGIJN_02435 hypothetical protein
CNLNGIJN_02436	VFG006949(gi:59710966)	86,57	0	CNLNGIJN_02436 Type II secretion system protein D
CNLNGIJN_02436	VFG006947(gi:27364821)	67,225	0	CNLNGIJN_02436 Type II secretion system protein D
CNLNGIJN_02436	VFG006948(gi:37681131)	66,425	0	CNLNGIJN_02436 Type II secretion system protein D
CNLNGIJN_02436	VFG006946(gi:28899478)	65,461	0	CNLNGIJN_02436 Type II secretion system protein D
CNLNGIJN_02436	VFG006944(gi:15640429)	64,947	0	CNLNGIJN_02436 Type II secretion system protein D
CNLNGIJN_02437	VFG006943(gi:59710965)	62,281	6,01E-40	CNLNGIJN_02437 hypothetical protein
CNLNGIJN_02438	VFG006937(gi:59710964)	75,943	1,26E-107	CNLNGIJN_02438 hypothetical protein
CNLNGIJN_02439	VFG006931(gi:59710963)	71,784	0	CNLNGIJN_02439 hypothetical protein
CNLNGIJN_02440	VFG006925(gi:59710962)	83,891	0	CNLNGIJN_02440 RNase E specificity factor CsrD
CNLNGIJN_02494	VFG013531(gi:68249838)	73,406	0	CNLNGIJN_02494 Glucose-6-phosphate isomerase
CNLNGIJN_02527	VFG001206(gb|NP_273675)	68,182	0,0000365	CNLNGIJN_02527 Thiamine import ATP-binding protein ThiQ
CNLNGIJN_02527	VFG006259(gi:59800668)	68,182	0,0000375	CNLNGIJN_02527 Thiamine import ATP-binding protein ThiQ
CNLNGIJN_02528	VFG044147(gi:16766086)	70	0,0000018	CNLNGIJN_02528 Thiamine import ATP-binding protein ThiQ
CNLNGIJN_02528	VFG048551	62,5	0,00000175	CNLNGIJN_02528 Thiamine import ATP-binding protein ThiQ
CNLNGIJN_02563	VFG046459	68,957	4,46E-166	CNLNGIJN_02563 Elongation factor Tu 2
CNLNGIJN_02591	VFG001855(gb|YP_094724)	74,237	0	CNLNGIJN_02591 60 kDa chaperonin
CNLNGIJN_02595	VFG013287(gi:68248956)	62,202	7,91E-129	CNLNGIJN_02595 UDP-glucose 4-epimerase
CNLNGIJN_02595	VFG013286(gb|NP_438515)	61,905	4,17E-128	CNLNGIJN_02595 UDP-glucose 4-epimerase
CNLNGIJN_02595	VFG013288(gi:148825365)	61,607	6,42E-128	CNLNGIJN_02595 UDP-glucose 4-epimerase

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CNLNGIJN_02618	VFG013200(gi:170718716)	78,028	0	CNLNGIJN_02618 Uroporphyrinogen decarboxylase
CNLNGIJN_02618	VFG013612(gi:113461676)	77,465	0	CNLNGIJN_02618 Uroporphyrinogen decarboxylase
CNLNGIJN_02629	VFG046459	78,626	0	CNLNGIJN_02629 Elongation factor Tu 2
CNLNGIJN_02676	VFG007047(gi:59713071)	83,794	4,25E-148	CNLNGIJN_02676 Type II secretion system protein N
CNLNGIJN_02677	VFG007053(gi:59713072)	84,049	6,68E-94	CNLNGIJN_02677 Type II secretion system protein M
CNLNGIJN_02678	VFG007059(gi:59713073)	74,341	0	CNLNGIJN_02678 Type II secretion system protein L
CNLNGIJN_02679	VFG007065(gi:59713074)	82,609	0	CNLNGIJN_02679 Putative type II secretion system protein K
CNLNGIJN_02680	VFG007071(gi:59713075)	74,554	1,22E-99	CNLNGIJN_02680 Type II secretion system protein J
CNLNGIJN_02681	VFG007077(gi:59713076)	91,597	1,95E-70	CNLNGIJN_02681 Type II secretion system protein I
CNLNGIJN_02682	VFG007083(gi:59713077)	80,203	7,61E-95	CNLNGIJN_02682 Type II secretion system protein H
CNLNGIJN_02683	VFG007089(gi:59713078)	98	2,16E-99	CNLNGIJN_02683 Type II secretion system protein G
CNLNGIJN_02684	VFG007095(gi:59713079)	96,543	0	CNLNGIJN_02684 Type II secretion system protein F
CNLNGIJN_02685	VFG007101(gi:59713080)	92,585	0	CNLNGIJN_02685 Type II secretion system protein E
CNLNGIJN_02686	VFG007107(gi:59713081)	90,639	0	CNLNGIJN_02686 Type II secretion system protein D
CNLNGIJN_02687	VFG007113(gi:59713082)	80,936	5,67E-164	CNLNGIJN_02687 Type II secretion system protein C
CNLNGIJN_02752	VFG038904(gb]YP_857967)	62,736	2,95E-77	CNLNGIJN_02752 hypothetical protein
CNLNGIJN_02752	VFG038905(gi:507523204)	62,736	2,18E-77	CNLNGIJN_02752 hypothetical protein
CNLNGIJN_02752	VFG038906(gi:145297880)	61,792	1,76E-76	CNLNGIJN_02752 hypothetical protein
CNLNGIJN_02752	VFG038907(gi:330828499)	61,321	1,63E-75	CNLNGIJN_02752 hypothetical protein
CNLNGIJN_02802	VFG037029(gi:59802086)	74,477	0	CNLNGIJN_02802 Catalase
CNLNGIJN_02810	VFG006992(gi:15640437)	65,517	0,0000147	CNLNGIJN_02810 hypothetical protein
CNLNGIJN_02810	VFG042840(gi:18311266)	61,29	0,0000166	CNLNGIJN_02810 hypothetical protein
CNLNGIJN_02890	VFG017856(gb]YP_001007658)	62,887	1,34E-77	CNLNGIJN_02890 Recombination-promoting nuclease RpnA
CNLNGIJN_02890	VFG017855(gi:153948767)	62,887	6,9E-79	CNLNGIJN_02890 Recombination-promoting nuclease RpnA
CNLNGIJN_02897	VFG002092(gb]NP_232508)	83,626	1,06E-91	CNLNGIJN_02897 hypothetical protein
CNLNGIJN_02898	VFG002093(gb]NP_232509)	92,057	0	CNLNGIJN_02898 hypothetical protein
CNLNGIJN_02899	VFG002094(gb]NP_232510)	80	1,08E-78	CNLNGIJN_02899 hypothetical protein
CNLNGIJN_02900	VFG002078(gb]NP_232511)	88,285	0	CNLNGIJN_02900 hypothetical protein
CNLNGIJN_02901	VFG007117(gi:147671686)	82,54	1,04E-177	CNLNGIJN_02901 hypothetical protein
CNLNGIJN_02901	VFG002079(gb]NP_232512)	79,586	0	CNLNGIJN_02901 hypothetical protein
CNLNGIJN_02902	VFG002080(gb]NP_232513)	70,505	0	CNLNGIJN_02902 hypothetical protein
CNLNGIJN_02902	VFG007119(gi:147671976)	70,303	0	CNLNGIJN_02902 hypothetical protein
CNLNGIJN_02903	VFG002081(gb]NP_232514)	77,987	1,01E-80	CNLNGIJN_02903 hypothetical protein

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CNLNGIJN_02904	VFG007123(gi:147671947)	91,481	1,35E-166	CNLNGIJN_02904 hypothetical protein
CNLNGIJN_02904	VFG002082(gb|NP_232515)	91,441	0	CNLNGIJN_02904 hypothetical protein
CNLNGIJN_02905	VFG002083(gb|NP_232516)	82,879	2,97E-149	CNLNGIJN_02905 hypothetical protein
CNLNGIJN_02906	VFG002084(gb|NP_232517)	86,521	0	CNLNGIJN_02906 Protein ClpV1
CNLNGIJN_02906	VFG038395(gb|YP_856375)	60,203	0	CNLNGIJN_02906 Protein ClpV1
CNLNGIJN_02907	VFG002085(gb|NP_232518)	74,624	0	CNLNGIJN_02907 Transcriptional regulatory protein ZraR
CNLNGIJN_02908	VFG002086(gb|NP_232519)	62,963	7,36E-73	CNLNGIJN_02908 hypothetical protein
CNLNGIJN_02908	VFG007131(gi:147671784)	62,963	1,46E-72	CNLNGIJN_02908 hypothetical protein
CNLNGIJN_02909	VFG002087(gb|NP_232520)	78,085	0	CNLNGIJN_02909 hypothetical protein
CNLNGIJN_02910	VFG002088(gb|NP_232521)	85,521	0	CNLNGIJN_02910 hypothetical protein
CNLNGIJN_02910	VFG007135(gi:147671876)	85,303	0	CNLNGIJN_02910 hypothetical protein
CNLNGIJN_02911	VFG002089(gb|NP_232522)	64,407	0	CNLNGIJN_02911 hypothetical protein
CNLNGIJN_02912	VFG007138(gb|NP_232418)	94,186	5,32E-114	CNLNGIJN_02912 Major exported protein
CNLNGIJN_02974	VFG043366(gb|NP_206899)	73,333	1,01E-18	CNLNGIJN_02974 Methyl-accepting chemotaxis protein McpH
CNLNGIJN_03011	VFG043365(gb|NP_206882)	66,667	7,56E-20	CNLNGIJN_03011 Methyl-accepting chemotaxis protein McpQ
CNLNGIJN_03026	VFG043365(gb|NP_206882)	68,493	4,26E-22	CNLNGIJN_03026 Methyl-accepting chemotaxis protein McpS
CNLNGIJN_03034	VFG012509(gb|NP_753167)	68,421	0,000486	CNLNGIJN_03034 Trehalose import ATP-binding protein SugC
CNLNGIJN_03034	VFG044336(gb|YP_002920268)	68,421	0,000434	CNLNGIJN_03034 Trehalose import ATP-binding protein SugC
CNLNGIJN_03079	VFG006167(gb|NP_273931)	62,069	0,0000212	CNLNGIJN_03079 Type II secretion system protein G
CNLNGIJN_03137	VFG007277(gi:59713515)	76,231	0	CNLNGIJN_03137 Vitamin B12 transporter BtuB
CNLNGIJN_03149	VFG013287(gi:68248956)	61,31	8,29E-128	CNLNGIJN_03149 UDP-glucose 4-epimerase
CNLNGIJN_03203	VFG043366(gb|NP_206899)	82,609	5,57E-16	CNLNGIJN_03203 Methyl-accepting chemotaxis protein PctC
CNLNGIJN_03300	VFG043365(gb|NP_206882)	70,27	1,2E-21	CNLNGIJN_03300 Methyl-accepting chemotaxis protein McpH
CNLNGIJN_03300	VFG043366(gb|NP_206899)	66,667	1,19E-21	CNLNGIJN_03300 Methyl-accepting chemotaxis protein McpH
CNLNGIJN_03589	VFG038845(gi:507522470)	67,46	2,23E-109	CNLNGIJN_03589 3-deoxy-manno-octulosonate cytidylyltransferase
CNLNGIJN_03592	VFG013249(gi:68248611)	62,931	0	CNLNGIJN_03592 Lipid A export ATP-binding/permease protein MsbA
CNLNGIJN_03695	VFG043634(gi:27366547)	65,301	4,61E-177	CNLNGIJN_03695 Putative conjugal transfer protein
CNLNGIJN_03695	VFG013229(gi:33152383)	60,894	1,31E-138	CNLNGIJN_03695 Putative conjugal transfer protein
CNLNGIJN_03747	VFG007255(gi:15600999)	71,6	6,41E-121	CNLNGIJN_03747 Iron(3+)-hydroxamate import ATP-binding protein FhuC
CNLNGIJN_03748	VFG007250(gi:15600998)	66,772	3,79E-128	CNLNGIJN_03748 Ferric-anguibactin transport system permease protein FatC
CNLNGIJN_03748	VFG007252(gi:28900514)	66,667	1,11E-131	CNLNGIJN_03748 Ferric-anguibactin transport system permease protein FatC
CNLNGIJN_03748	VFG007253(gi:27366568)	62,626	4,85E-119	CNLNGIJN_03748 Ferric-anguibactin transport system permease protein FatC
CNLNGIJN_03749	VFG007248(gi:27366567)	74,113	1,21E-135	CNLNGIJN_03749 Ferric-anguibactin transport system permease protein FatD

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CNLNGIJN_03750	VFG007240(gi:15600996)	60	3,88E-113	CNLNGIJN_03750 putative ABC transporter solute-binding protein YclQ
CNLNGIJN_03839	VFG010785(gi:52841484)	77,778	0,00000912	CNLNGIJN_03839 hypothetical protein
CNLNGIJN_03839	VFG010786(gi:54296150)	72,222	0,0000826	CNLNGIJN_03839 hypothetical protein
CNLNGIJN_03854	VFG041187(gi:269139783)	72,165	2,04E-35	CNLNGIJN_03854 hypothetical protein
CNLNGIJN_03886	VFG041013(gi:15596709)	78,235	7,21E-90	CNLNGIJN_03886 Major exported protein
CNLNGIJN_03987	VFG007036(gi:27367835)	68,856	0	CNLNGIJN_03987 Thermolabile hemolysin
CNLNGIJN_04003	VFG043365(gb|NP_206882)	68,254	1,04E-18	CNLNGIJN_04003 Methyl-accepting chemotaxis protein CtpH
CNLNGIJN_04124	VFG043378(gb|NP_207394)	69,565	1,29E-20	CNLNGIJN_04124 Methyl-accepting chemotaxis protein McpA
CNLNGIJN_04154	VFG038131(gi:213155479)	60	8,34E-29	CNLNGIJN_04154 O-acetyltransferase OatA
CNLNGIJN_04245	VFG017856(gb|YP_001007658)	62,667	2,68E-24	CNLNGIJN_04245 Recombination-promoting nuclease RpnA
CNLNGIJN_04245	VFG017855(gi:153948767)	62,667	1,42E-24	CNLNGIJN_04245 Recombination-promoting nuclease RpnA

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