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Strain-specific quorum-sensing responses determine virulence properties in *Vibrio anguillarum*

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Abstract
Bacterial populations communicate using quorum-sensing (QS) molecules and switch on QS regulation to engage in coordinated behaviour such as biofilm formation or virulence. The marine fish pathogen *Vibrio anguillarum* harbours several QS systems, and our understanding of its QS regulation is still fragmentary. Here, we identify the VanT-QS regulon and explore the diversity and trajectory of traits under QS regulation in *Vibrio anguillarum* through comparative transcriptomics of two wildtype strains and their corresponding mutants artificially locked in QS-on (ΔvanO) or QS-off (ΔvanT) states. Intriguingly, the two wildtype populations showed different QS responses to cell density changes and operated primarily in the QS-on and QS-off spectrum, respectively. Examining 27 *V. anguillarum* strains revealed that ~11% were QS-negative, and GFP-reporter measurements of nine QS-positive strains revealed a highly strain-specific nature of the QS responses. We showed that QS controls a plethora of genes involved in processes such as central metabolism, biofilm formation, competence, T6SS, and virulence properties in *V. anguillarum*, with large strain-specific differences. Moreover, we demonstrated that the QS state is an important driver of virulence towards fish larvae in one of two *V. anguillarum* strains. We speculate that infections by mixed-strain communities spanning diverse QS strategies optimize the infection efficiency of the pathogen.

INTRODUCTION

Many bacteria can communicate by a process termed quorum-sensing (QS) driven by the production, release and detection of chemical signals termed autoinducers (Papenfort & Bassler, 2016). As bacteria divide, produce and release autoinducers, the accumulation of autoinducers becomes a proxy of the cell density in the environment, and, at high autoinducer concentrations, bacteria activate QS pathways to regulate gene expression governing several metabolic processes and cooperative behaviours in response to cell density. When QS regulation is activated (i.e. the QS-on state), bacteria engage in a coordinated manner in multi-cellular behaviours such as bioluminescence (Bassler et al., 1994; Engebret et al., 1983), biofilm formation (Miller & Bassler, 2001), swarming (Eberl et al., 1998; Givskov et al., 1998), and secretion of extracellular enzymes including virulence factors (Antunes et al., 2010; de Kievit & Iglewski, 2000).

Several studies have highlighted strain-specific differences in autoinducer release (Buchholtz et al., 2006; García-Aljaro et al., 2012; Purohit et al., 2013), variability in the accumulation of QS master regulator proteins (Joelsson et al., 2006), and deviations in QS responses (Geisinger et al., 2012). However, whether these differences represent differential regulation of QS-on/off states remain unknown. While the activation of the QS
response can be vital for adapting phenotypes according to the surroundings, the impact of the QS state on the bacterial functional properties is ultimately determined by the QS regulon. Most studies of QS regulons have been performed in single model strains; however, it has been emphasized that QS regulons are subject to strain specificity (Chugani et al., 2012; Simpson et al., 2021). Collectively, previous studies thus suggest that a vast diversity of autoinducer profiles, QS responses and regulons have evolved even within individual bacterial species, making it crucial to venture beyond the study of single model strains in order to reveal the true complexity of QS interactions and their influence on phenotypic heterogeneity in bacterial populations.

Here, we have studied V. anguillarum strains isolated from diseased fish (Table S1) to map the QS regulon in this important pathogen and to study the strain-specific diversity of the QS system centred on the master regulator VanT. V. anguillarum is a marine fish pathogen, which infects >50 fish species and causes vibriosis outbreaks in both wild fish populations and in aquaculture farms leading to large economic losses (Frans et al., 2011). Coordination of virulence is a hallmark of QS in pathogenic bacteria (Antunes et al., 2010). However, in vibrios, QS has been shown to have different effects on virulence in different species, including stimulation (e.g. V. cambellii; Noor et al., 2019), repression (e.g. V. cholerae; Miller et al., 2002; Zhu et al., 2002; and V. harveyi; Zhang et al., 2021), or no effect on virulence (e.g. V. tasmaniensis, V. crassostreae; Islam et al., 2022). The VanT-QS system is activated at high cell density in response to accumulation of several autoinducer molecules and regulates central behaviours in V. anguillarum such as biofilm formation, protease activity, and phage–host interactions (Croxatto et al., 2002; Milton, 2006; Tan et al., 2015, 2020). However, QS has so far not been directly linked to virulence in this species (Croxatto et al., 2002; Li et al., 2018; Milton et al., 1997), although the addition of specific QS inhibitors may reduce virulence (Rasch et al., 2004). Although, some of these traits were studied using single strains and thus the extent of traits that are regulated and the degree of variability in QS response between isolates are not known.

The QS systems identified in V. anguillarum include three described autoinducer synthase-receptor pairs (VanMN, VanSPQ, VanLR) (Milton, 2006) and two predicted autoinducer-receptor pairs (CqsAS, Tdh/VqmAR) that lead to the regulation of one predicted QS regulator (VqmA) and three described QS regulators (VanR, VanT, AphA) (Figure 1). Three autoinducer synthase-receptor pairs that activate a VanUO phosphorelay orchestrate the accumulation of VanT protein. At low autoinducer concentrations, phosphorylated VanO stimulates the expression of the sRNAs

**FIGURE 1** Quorum-sensing (QS) systems in Vibrio anguillarum. The central QS master regulator VanT accumulates in response to high autoinducer concentrations and regulates the expression of target genes. The accumulation of VanT is dependent on the VanMN and VanSPQ systems that are homologous to QS circuits of V. harveyi (Croxatto et al., 2004; Milton et al., 2001) and the CqsAS system that has been discovered in V. harveyi and predicted in V. anguillarum (Ng et al., 2011). VanM synthesizes C6-HSL and 3-hydroxy-C6-HSL that are detected by VanN (Milton, 2006). VanS synthesizes At-2, which is sensed by VanQ (Milton, 2006). CqsA synthesizes Ea-C8-CAI-1 that is recognized by CqsS (Henke & Bassler, 2004). At low autoinducer concentrations in V. harveyi, the QS receptors initiate a phosphorelay through LuxUO (homologous to VanO in V. anguillarum) that stimulates the expression of the sRNAs Qrr1-4 that destabilize luxR mRNA (homologous to vanT in V. anguillarum) and activate translation of aphA mRNA. At high autoinducer concentrations, the QS receptors act as phosphorylases, which dephosphorylate LuxO terminating the expression of Qrr1-4 and thereby allow the translation of vanT mRNA and repression of AphA (Papenfort & Bassler, 2016). The VanR/L system is homologous to the Lux/I system of V. fischeri (Milton et al., 1997). VanI synthesizes 3-oxo-C10-HSL that binds the regulator VanR. Moreover, the VqmAR/Tdh system present in V. cholerae has been predicted in V. anguillarum. Tdh is involved in the synthesis of 3,5-dimethylpyrazin-2-ol (DPO) that binds and activates VqmA leading to the synthesis of the sRNA VmqR that regulates target genes (Papenfort et al., 2015, 2017). Additionally, the regulator LuxT that represses qrr1 in V. harveyi is also predicted in V. anguillarum (Eickhoff et al., 2021). The degree of interaction between the QS systems in V. anguillarum is largely unknown. P indicates kinase activity or that the protein is phosphorylated.
Qrr1-4, which block translation and destabilize vanT mRNA, leading to low VanT protein levels. Oppositely, at high autoinducer concentrations, VanO is dephosphorylated, terminating the expression of qrr1-4 and thereby allowing increased translation of vanT mRNA. In parallel to the VanT pathway, a VanI/R QS system has been described in V. anguillarum, however its regulatory role, besides feedback regulation of vanI, is unknown (Milton et al., 1997). The VqmAR/Tdh QS system that plays a role in biofilm formation and toxin production in V. cholerae has been predicted in V. anguillarum (Papenfort et al., 2015, 2017). A homologue of the QS regulator LuxT is also present in V. anguillarum and appears to be widespread within vibrios, where it interferes with downstream QS regulation in V. harveyi by repressing qrr1 (Eickhoff et al., 2021).

Here, we investigate the QS-on/off response by comparing transcriptomes and phenotypes of two wild-type V. anguillarum strains with populations artificially locked in QS-on (ΔvanO) or QS-off (ΔvanT) states. Interestingly, while one strain had a weak QS response and primarily resembled the QS-off mutant, the other strain had a strong QS response and operated mainly in its QS-on state. Extending the analysis of strain-specific QS responses to seven additional V. anguillarum strains emphasized that there was a large diversity in QS responses among strains, and most strains exhibited a QS response similar to QS-on mutants. Furthermore, we identified shared and strain-specific VanT-QS regulons based on two V. anguillarum strains and observed that the strain with the weakest QS response had the largest QS regulon. Overall, these results demonstrated fundamental strain-specific differences in QS-responses and QS regulons that greatly influenced the functional characteristics of the strains. Moreover, we found that QS-repressed V. anguillarum populations caused lysis of fish cell lines and virulence against fish larvae, suggesting that the QS state is an important driver of V. anguillarum virulence potential, emphasizing the importance of understanding the diversity of QS systems in bacteria.

**EXPERIMENTAL PROCEDURES**

**Standard culture conditions**

Bacterial strains and plasmids are listed in Table S1. *Vibrio anguillarum* strains were routinely cultured in Marine Broth (MB; 0.5% tryptone, 0.1% yeast extract, 2% sea salts) with aeration at 30°C. *Escherichia coli* strains were grown in Lysogeny broth (LB; 1% tryptone, 0.5% yeast extract, 0.5% NaCl) with aeration at 37°C. Cell densities were measured by OD at wavelength 600 nm unless specified otherwise.

**Alignments and phylogenetic analysis**

Whole genome (Castillo et al., 2017; Rønneseth et al., 2017) alignment of V. anguillarum strains 90-11-287 and PF430-3 was performed using progressive MAUVE (Darling, 2004) in Geneious v. 11.0.5 (http://www.geneious.com). Global pairwise alignments were performed with cost matrices of 93% similarity for nucleotide sequences and BLOSUM80 for amino acid sequences. To reveal the phylogenetic relationships of the QS systems among the strains, 28 QS-affecting and -related genes (aphA, aphB, cqsA, cqsS, crp, crr, csrA, cyaA, cyaB, fis, hqf, luxT, ptsG, rpoN, tdh, vanI, vanM, vanN, vanO, VANP, vqmA, vanQ, vanR, vanS, vanT, vanU, varA, varS, vqmA) were concatenated, aligned and phylogenetic trees were built with the Neighbour-Joining algorithm with 1000 bootstrap using V. cholerae El Tor strain N16961 as outgroup (No vanI, vanM, vanN and vanR homologs were found in NC_002505 and NC_002506).

**Construction of spike-in strain for normalization of RNA-sequencing**

To construct the whole-cell spike-in strain, *E. coli* strain MG1655 pEVS143 was conjugated with V. anguillarum strain 90-11-287 resulting in spike-in strain 90-11-287 pEVS143. Transconjugants were selected on thiosulfate-chloride-iodide (TCI; 0.5% sodium thiosulfate, 3.9% Columbia blood agar base, 4.6% potassium iodide, 0.5% sodium chloride) (Beazley & Palmer, 1992) and Lysogeny broth Miller (LM; 1% tryptone, 0.5% yeast extract, 1% NaCl) agar plates containing 50 μg mL⁻¹ kanamycin.

**Strain construction**

Plasmids pES41 and pES42 were created by amplifying desired regions from each strain using primers vanT_O5/vanT_3xflag_15’ + vanT_O3/vanT_I3_3xflag containing desired modifications and overlapping regions (Table S2). The two fragments were then fused using PCR and cloned into vector pDM4. Construction of the chromosomal vanT::3XFLAG mutants in 90-11-287 and PF430-3 was performed using allelic exchange as described previously (Tan et al., 2020) with plasmids pES41 and pES42, respectively. Plasmid pCS42 was introduced to V. anguillarum strains by conjugating with E. coli MG1655 pCS42. Transconjugants were selected on thiosulfate-citrate-bile salts-sucrose agar (TCBS; 1.4% agar, 0.004% bromothymol blue, 0.1% ferric citrate, 1% mixed peptone, 0.5% ox-bile, 1% sodium chloride, 0.3% sodium chloride, 1% sodium citrate, 1% sodium thiosulfate, 2% sucrose, 0.004% thymol blue, 0.5% yeast extract).
(Kobayashi et al., 1963) and MB agar plates containing 100 μg mL⁻¹ gentamicin.

Whole-cell spike-in and RNA isolation

Overnight cultures of *V. anguillarum* strains were diluted to an OD₆₀₀ of 0.0001 in MB and cultured at 30°C with aeration. At OD₆₀₀ ~0.1 and ~1.8, cells were pelleted at 3200 g for 10 min at 4°C, resuspended in PBS and stored in stop solution on ice (20% ETOH and 1% phenol; Bernstein et al., 2002). In parallel, overnight cultures of the spike-in *V. anguillarum* strain 90-11-287 pEVS143 were diluted to an OD₆₀₀ of 0.01 in MB with kanamycin (50 μg mL⁻¹) and cultured at 30°C with aeration. To induce *gfp* expression from the plasmid of the spike-in cells, IPTG (1 mM final conc.) was added at OD₆₀₀ ~0.1, and culturing was continued for 2 h. Based on OD, 1% spike-in cells were added to the test cultures that were previously pelleted, resuspended in PBS and stored in stop solution. Total RNA was extracted using hot phenol (Gummesson et al., 2020) and DNase I treated (Roche) according to manufacturer's instructions. DNase I was inactivated using phenol purification and resuspended in water.

RT-qPCR analysis of relative mRNA levels normalized to spike-in cells

The relative levels of mRNA were calculated as the ratio between the vanT transcript and the spike-in derived *gfp* transcript from the pEVS143 plasmid unique to the spike-in cultures. DNase-treated RNA was reverse transcribed to cDNA with the RevertAid RT kit according to manufacturer’s instructions (Thermo Fisher). The relative amount of cDNA was determined by qPCR as previously described (Gummesson et al., 2020). Briefly, the ΔΔCₜ method (Schmittgen & Livak, 2008) was used to calculate the relative fold changes of mRNA quantity relative to the reference gene *gfp* from the spike-in plasmid. Primers are listed in Table S2.

RNA-seq, read mapping and counting

Total DNase-treated RNA was shipped to Genewiz (Leipzig, Germany) and subject to rRNA depletion using Illumina Ribo-Zero rRNA removal kit. Strand-specific libraries were prepared with the TruSeq stranded mRNA Library prep kit. Then, 150 bp paired-end sequencing was performed on Illumina NovaSeq and the reads were trimmed from adapter sequences prior to delivery.

Rockhopper v. 2.03 was used to predict novel transcripts and operons (McClure et al., 2013; Tjaden, 2015). Predicted RNAs and potential ORFs were searched against the rFam database (E-value <1 E-5) and the non-redundant database using blastp, respectively. In addition, QS-related regulatory RNAs Qrr1-4 (Weber et al., 2011), CsrBCD (Butz et al., 2019) and VqmR (Papenfort et al., 2015) were predicted using blastn.

Trimmed reads were mapped to a concatenated reference genome including the spike-in plasmid pEVS143 using Rsibread v.2.4.0 allowing multi-mapping reads, disallowing ambiguous reads (Liao et al., 2013). Mapped fragments (read pairs) were counted using featureCounts counting multi-mapping fragments fractionally (Liao et al., 2014).

To identify replicated genes in the bacterial genomes, all gene sequences were extracted and aligned against their native reference genome using blastn. To handle the replicated genes, ‘gene groups’ were defined as genes having a relative length of >80% and a complete nucleotide identity for >150 consecutive bp. The fragment counts of all genes within a gene group were summed and analysed as a gene group.

To analyse cross-strain transcript abundance differences, shared genes across the two strains were identified using a best-hit all versus all blastn analysis (E-value <1 E-6, relative length > 80%). As some genes across the strains were of different length, the counts of the longest gene were scaled according to their relative gene length between the strains. For example, if gene X had a length of 792 bp in strain PF430-3, but a length of 720 bp in strain 90-11-287, the fragment counts in strain PF430-3 would be divided by 1.1 (the gene length in PF430-3 relative to 90-11-287).

Differential expression and variance partition analysis

Ribosomal RNA and tRNA genes were removed from analysis and lowly expressed genes were filtered (genes with >10 counts per million in at least two replicates were kept) using the filterByExpr function with default settings in R package edgeR v. 3.30.3 (Robinson et al., 2010). Distribution plots of library size-normalized gene expression revealed that one replicate of each of strain PF430-3 ΔvanT at LCD and strain 90-11-287 ΔvanT at LCD were outliers and therefore excluded from further analysis (Figure S12). Fragment counts were normalized using the sum of spike-in counts as the library size or using the trimmed mean of M-values (TMM) (Robinson & Oshlack, 2010). To account for heteroscedasticity, precision weights were estimated using the function voomWithDreamWeights (Hoffman & Roussos, 2020; Law et al., 2014).
For each strain, linear mixed models were used to decompose the total variance of each gene into variance associated with cell density, genotype (i.e. WT, ΔvanT, ΔvanO), biological replicate, and residual variance using R package variancePartition v. 1.18.3 modelling parameters as categorical (Hoffman & Schadt, 2016). For cross-strain analysis, a similar approach was carried out, decomposing the total variance of each gene into variance linked with cell density, strain (i.e. PF430-3, 90-11-287), biological replicate, and residual variation.

For differential expression analysis, dream (Hoffman & Roussos, 2020), a limma-based method (Ritchie et al., 2015), was used to fit the counts to linear mixed models for each comparison, modelling genotype and cell density as a combined fixed effect and biological replicate as a random effect. Hypothesis testing was performed using the topTable function and p values were adjusted for multiple testing using the Benjamini–Hochberg approach. Genes with an adjusted p value < 0.05 were considered differentially expressed. Volcano plots were visualized using R package EnhancedVolcano v. 1.6.0 (Blighe et al., 2019). Heatmaps were constructed using R package pheatmap v. 1.0.12 (Kolde, 2015).

Genes were assigned Biological Process Gene Ontology terms using PANNZER (Törönen et al., 2018) and gene set enrichment analysis was performed using R package topGO v. 2.40.0 with adjusted p values from the differential expression analysis as the ranking metric with weight01 algorithm and Kolmogorov–Smirnov statistics (Alexa & Rahnenfuhrer, 2020).

To compare the transcriptome between LCD and HCD samples, we applied whole-cell spike-in normalization, allowing normalization of the sequencing read pair (fragment) counts to the number of cells (OD units) and thereby not assuming equal total mRNA content per cell (Gummesson et al., 2020). This was done by adding to each experimental culture an aliquot of spike-in cells, as measured in units of OD. The spike-in counts will therefore represent a larger fraction of the total fragment counts if added to an experimental culture with a low content of RNA per cell, than if added to an experimental culture with a high content of RNA per cell. In this experiment, fragments unique to the plasmid harboured by the spike-in cells represented a significantly larger fraction of the total fragment counts when cells were sampled at HCD compared to LCD (Figure S13), and the HCD population showed a lower median expression than the LCD population. These observations are in accordance with the expectation that the total mRNA pool is reduced as populations reach high cell density and transition from the exponential to the early stationary growth phase (Gummesson et al., 2020). Without the spike-in normalization for differential mRNA content between samples, RNA-seq counts will underestimate the expression levels of many genes in LCD populations compared to HCD populations (Figure S13). This ultimately leads to misinterpretation of downstream differential expression analysis (Figure S8). Analysing data without spike-in normalization results in an overestimation of the number of genes that are less expressed at LCD relative to HCD, and an underestimation of the number of genes that are overexpressed at LCD compared to cells at HCD (Figure S8). Therefore, we analysed spike-in normalized counts for analyses that included both LCD and HCD populations, while TMM normalization was sufficient for analyses that compared strains harvested at the same cell density.

**Western blot**

Overnight cultures of *V. anguillarum* strains were diluted to an OD600 of 0.0001 in MB and cultured at 30°C with aeration. At OD ~ 0.1 and ~ 1.8, cells were pelleted at 3200 g for 10 min at 4°C and resuspended in PBS. The resuspended pellet was subject to OD600 measurement, flash frozen in liquid nitrogen and stored at −80°C. Samples were adjusted to equal OD units in PBS and NuPAGE™ LDS loading buffer containing 100 mM dithiothreitol (DTT) and heat treated at 98°C for 8 min. The samples were centrifuged at 15,000g for 1 min, and proteins were separated using 12.5% SDS-PAGE with MES running buffer and transferred to a PVDF membrane (MSI). Visualization was performed using anti-FLAG-mouse and anti-mouse-HRP antibodies as well as anti-RpoB-rabbit and anti-rabbit-HRP, subject to chemiluminescence using the Pierce® ECL Western Blotting Substrate (Thermo Scientific) following manufacturer’s instructions and imaged using ImageQuant LAS4000 with LAS4000 software. Relative band intensities were quantified using imageJ v. 1.49.

**GFP-reporter assays**

To evaluate the QS response in the strains, overnight cultures of *V. anguillarum* strains containing pCS42 were diluted to an OD600 of 0.0001 in MB (200 μL final volume with gentamicin 100 μg mL⁻¹ final conc.), in black-well, clear-bottom 96-well plates. The plates were incubated with intermittent shaking at room temperature, and OD990 and fluorescence were measured dually using FLUOstar OPTIMA with OPTIMA software v. 2.20R2 in script mode.

**Fish larvae infection experiment**

In vivo fish larvae infection trials were performed using a previously described method (Rønneseth et al., 2017). Eggs of gilthead seabream, *Sparus*
of FBS was added to inactivate trypsin and cells were dislodged utilizing cell scrapers. The 1 mL of cell suspension was centrifuged at 400 g for 1 min at 5°C, and supernatant was removed. Cells were then washed with PBS and resuspended in 500 μL PBS and subsequently stained using Live/Dead Cell Double Staining Kit (catalogue no. 04511; Sigma-Aldrich). In brief, an assay solution was prepared with 10 μL solution A (calcein-AM) and 10 μL solution B (propidium iodide) in 4 mL PBS, and 100 μL assay solution added to cell suspension. After incubation at 37°C for 15 min in the dark, survival of CHSE-214 cells was assessed by fluorescence microscopy utilizing an Olympus BX51 microscope.

Quantification of virulence-associated phenotypes

Cod blood was sampled from wild Atlantic cod (Gadus morhua) by puncture of the caudal vein from the ventral side at a 45° angle with a heparin (5000 I.U. mL⁻¹, LEO Pharma) coated syringe and stored at 4°C. Dodefibrinated cod and sheep blood (SSI Diagnostica) was centrifuged at 1000 g for 5 min at 4°C and washed three times in PBS. Ten microliters of OD₆₀₀ = 1 adjusted overnight bacterial cultures were spotted onto MB agar plates containing either 0.2% elastin, 5% gelatin, or blood agar base (Sigma) containing 10% packed cod or sheep erythrocytes (with 0%–2% d-glucose) and incubated for 7, 2, 2 and 7 days, respectively. After incubation, saturated ammonium sulfate was added to the gelatin agar plates. Ammonium sulfate precipitates non-degraded gelatin and form white precipitates that are easily distinguishable on the plate (Smith & Goodner, 1958).

Biofilm assay

Measurements of biofilm formation capacity were conducted based on previous protocols (Tan et al., 2015). Briefly, overnight cultures were diluted to OD₆₀₀ = 0.01 in MB in a 96-well plate and incubated statically at room temperature. The wells were washed three times, stained with 0.1% crystal violet for 20 min, washed three times and destained with absolute ethanol. The absorbance was measured using FLUOstar OPTIMA with OPTIMA software v. 2.20R2. The experiment was repeated three independent times with eight replicates per experiment.

RESULTS

Differential QS responses in Vibrio anguillarum strains

To expand our knowledge of the shared and strain-specific QS regulons in V. anguillarum, we sequenced...
the transcriptome of batch cultures of two wildtype (WT) strains and their corresponding ΔvanT and ΔvanO mutants at low cell density (LCD; OD600 ~0.1) and at high cell density (HCD; OD600 ~1.8). We selected the strains PF430-3 and 90-11-287 as our previous studies revealed that biofilm formation and gene expression of the phage receptor ompK of wildtype strain PF430-3 resembled its QS-off state (ΔvanT) (Tan et al., 2015), while biofilm and protease secretion of wildtype strain 90-11-287 was most similar to its QS-on state (ΔvanO) (Tan et al., 2020). These observations made us suspect that the wildtype strains could navigate their QS regimes differently. Multidimensional scaling (MDS) and variance partition analysis revealed that cell density was the major driver of differential gene expression between samples accounting for 52%–55% of the average variance in gene expression (Figure 2A–D). Targeted mutation of the QS system, QS genotype, presented 18.5% and 7% of the average variance in gene expression in strain PF430-3 and 90-11-287, respectively, indicating that the global transcriptome of strain PF430-3 was more sensitive to the mutation of its QS genes than strain 90-11-287 (Figure 2A–D). This was supported by differential expression analysis, where the QS mutant versus WT comparisons for strain PF430-3 in general revealed more differentially expressed genes than for similar comparisons within strain 90-11-287, and the perturbations affected up to 43% (1453 genes) and 10% (348 genes) of the transcriptome, respectively (Figure 2E–L), of which 190 genes were shared in the VanT regulons. Interestingly, the two wildtype strains PF430-3 and 90-11-287 showed very different levels of QS regulation at both low and high cell density conditions. At LCD, WT strain PF430-3 resembled the gene expression pattern of its QS-off mutant ΔvanT, whereas the pattern of WT strain 90-11-287 at LCD was more similar to the expression profile of its QS-on mutant ΔvanO (Figure 2A,B). At HCD, the expression profile of WT strain 90-11-287 resembled the profile of ΔvanO mutant considerably, while strain PF430-3 WT resembled neither of its QS mutants, but revealed a gene expression pattern most similar to the ΔvanO mutant (Figure 2A,B). These patterns suggest that at an overall level, strain 90-11-287 operates mainly in the QS-on regime, even at LCD, while strain PF430-3 operates nearer to the QS-off regime defined by the ΔvanT mutation at low cell densities and does not reach its QS-on potential even at high cell densities. Remarkably, the gene expression profiles of wildtype strain 90-11-287 and the corresponding ΔvanO mutant overlapped almost entirely at HCD, suggesting that the 90-11-287 strain achieves full activation of the QS-on mode at HCD, similar to its ΔvanO counterpart, which is artificially locked in the QS-on state (Figure 2L; Supplementary dataset S1). The single differentially expressed gene in strain 90-11-287 ΔvanO versus WT at HCD was the vanU gene, which is encoded adjacent to the vanO gene and is likely to be differentially expressed as a direct consequence of the adjacent deletion of the vanO open reading frame. In contrast, strain PF430-3 did not reach the full QS-on mode at HCD, as 286 genes were differentially expressed for ΔvanO vs. WT at HCD. Notably, the absence of the vanT gene affected the expression of more genes in strain PF430-3 compared to 90-11-287 at HCD, while the opposite was evident at LCD. Finally, comparing the mutants lacking vanO with the WT at LCD showed only 22 differentially expressed genes in strain 90-11-287 while 886 genes were differentially expressed in strain PF430-3. Collectively, these data show that while the number of genes in the QS regulon, and thereby the potential effect of the QS response of strain PF430-3 is larger than that of 90-11-287, the WT PF430-3 strain only marginally exploited its potential for QS regulation at the selected cell densities, while populations of WT strain 90–11-287 switched their QS system on to a large degree at both the LCD and HCD conditions.

Intriguingly, a cross-strain analysis of the two wildtype strains revealed that strain specificity was a larger driver than cell density of transcript levels of several QS genes including the QS master regulators vanT and vanR along with the genes cqsA and tdh involved in autoinducer synthesis (Figure 3A). In addition, the expression of the phosphotransferase system (PTS) genes ptsG and crr and the sRNA CsrB were also highly strain dependent (Figure 3A). In more detail, the central genes vanR, vqmA, luxT, aphA and vanT of the V. anguillarum QS pathways were differentially expressed between the two strains with 0.7–6.9 fold change differences (Figure 3B). Further, the expression of the autoinducer synthase gene cqsA was increased by 16.1- and 5.4-fold at LCD and HCD, respectively, in strain 90-11-287 compared to strain PF430-3 (Figure 3B).

Similarly, the autoinducer synthase genes vanM and vanS, and the QS receptor gene vanN, which coordinate the VanT/AphA QS cascade, were overexpressed by 1.4- to 2.0-fold in strain 90-11-287 compared to strain PF430-3 (Figure 3B). Thus, strain 90-11-287 generally showed higher expression levels of its QS components, in particular the synthases that are responsible for producing the autoinducer signalling molecules. In concert, these differences were associated with an increase of vanT mRNA of ~7.5-fold at HCD and a decrease of aphA expression by 2.6-fold at LCD in strain 90-11-287 compared to PF430-3 (Figure 3B). Moreover, the data suggested that while vanT mRNA levels of strain 90-11-287 were elevated by ~2.1-fold at HCD compared to LCD, vanT mRNA levels did not change significantly in strain PF430-3 (Figures 3B and 4A). To determine the degree to which the vanT mRNA levels reflected VanT protein levels,
Figure 2  Differential QS responses in *Vibrio anguillarum*. Cultures were sampled at LCD (OD ~0.1) and HCD (OD ~1.8), spiked-in with 1% OD units of the spike-in strain and subject to RNA-seq as described in the Experimental Procedures section. The *vanO* and *vanT* genes were omitted from this analysis as the purpose was to explore the (dis)similarity of wild-type strains to their QS-off state. (A, B) Data were decomposed using multi-dimensional scaling (MDS) of Euclidean distances between samples. Distances between the samples reflect the similarity regarding the average absolute log2 fold change between all measured transcripts that passed the filtering criteria. (C, D) Violin plots of spike-in normalized genome-wide expression variance partitioned into cell density, genotype, biological replicate, and residuals. Each dot represents a gene. (E–L) Volcano plots of differentially expressed genes identified using TMM normalized counts. Each gene is plotted as the magnitude of fold change versus the adjusted p value. Dashed horizontal line represents the significance cutoff for differential expression (adj. p-value <0.05) and the dashed vertical line indicates an absolute log2FC >1. Numbers of genes significantly differentially expressed are listed within each plot.
we performed a Western blot analysis on strains carrying chromosomally tagged variants of the \textit{vanT} gene. For both strains, VanT protein levels increased significantly by \( \sim 2.5 \)-fold at HCD relative to LCD, showing that VanT protein levels are indeed responsive to cell density also in strain PF430-3. However, VanT protein levels were \( \sim 10 \)-fold higher in strain 90-11-287 \textit{vanT}:3x\text{FLAG} compared to strain PF430-3 \textit{vanT}:3x\text{FLAG} at both LCD and HCD (Figure 4B). Even at HCD, strain PF430-3 \textit{vanT}:3x\text{FLAG} did not reach levels of VanT protein that matched strain 90-11-287 \textit{vanT}:3x\text{FLAG} at LCD. Moreover, despite PF430-3 harbouring relatively low VanT levels, the VanT protein crucially affects the transcriptome; slightly at LCD and considerably at HCD, as seen when the wildtype strain is compared to the \textit{vanT} deletion mutant (Figures 2E–H and 4).

The QS circuit is affected by glucose concentration in \textit{V. cholerae}, as high glucose concentrations inhibit production of cAMP, which initiates catabolite repression leading to low expression of \textit{cqsA}, low production of the autoinducer molecule CAI-1 and low expression of \textit{hapR} (the \textit{vanT} homolog in \textit{V. cholerae}) (Liang et al., 2007, 2008). Consequently, high glucose concentration would result in a QS-off response in \textit{V. cholerae}. In the current study, we also observed a potential link between glucose uptake and the QS response of \textit{V. anguillarum} at the transcriptome level. The PTS genes, \textit{crp} and \textit{ptsG}, which are involved in the uptake of glucose (Presper et al., 1989) were 1.4- to 3.5-fold overexpressed in strain PF430-3 compared to 90-11-287 (Figure 3B).

Moreover, the PTS genes were 2.7- to 3.5-fold overexpressed, while \textit{cqsA} was 2.9-fold less expressed in WT strain PF430-3 at LCD compared to HCD (Figure 3B). These results suggest that an increased sugar uptake in strain PF430-3 at low cell density conditions could inhibit the QS response in this strain through repressed \textit{cqsA} expression and presumably reduced CAI-1 production. For strain 90-11-287, neither \textit{cqsA} nor the PTS genes were differentially expressed at LCD compared to HCD. In combination, these observations indicate that the differential expression of sugar uptake genes could contribute to the strain-specific differences in the expression of \textit{cqsA} and thus contribute to the differential QS response observed in the two strains.

To expand the analysis of differential QS responses beyond the two investigated strains, we evaluated the QS response of seven additional \textit{V. anguillarum} strains that have been previously isolated from diseased salmon and rainbow trout (Table S1). To accomplish this, we measured the relative \textit{vanT} mRNA levels using RT-qPCR, but we also introduced a plasmid containing a \textit{luc} promoter fused to \textit{gfp} into the strains. The \textit{luc} promoter is activated by LuxR-like proteins (homologous to VanT) in vibrios (Newman et al., 2021), thus this plasmid enables cells to produce GFP as a response to VanT-mediated activation of the promoter. These approaches further unveiled large differences in timing the onset of QS initiation (Figure S1) and revealed a general diversity in the extent of QS responses across cell densities (Figure 4A,C).
FIGURE 4  Diversity of QS responses in *Vibrio anguillarum*. (A) RT-qPCR. Cultures were sampled at LCD (OD ~0.1) and HCD (OD ~1.8), spiked-in with 1% OD units of spike-in culture as described in the Experimental Procedures section. Subsequent RT-qPCR values of *vanT* mRNA were normalized to *gfp* mRNA expressed from the inducible spike-in plasmid. The spike-in normalized *vanT* mRNA levels of all samples were compared to the levels in strain PF430-3 at LCD. Error bars represent standard deviations of biological triplicates and technical duplicates. * represents statistical significance within-strain across cell density (paired t-test, *p* value <0.05) and letters indicate statistical significance between groups across strains within LCD (letters a–d) or within HCD (letters x–z) comparisons (one-way ANOVA with post hoc Tukey; *p* value <0.05). (B) Western blot. Cultures were sampled at LCD and HCD and equal OD units were subject to Western blot analysis as described in the Experimental Procedures section. The relative band intensity of all samples was compared to the band intensity of strain PF430-3 at LCD. Error bars represent standard deviations of biological triplicates. * represents statistical significance within-strain across cell density (paired t-test, *p* value <0.05) and letters indicate statistical significance between groups across strains within LCD (letters a and b) or within HCD (letters x and y) comparisons (Kruskal–Wallis; *p* value <0.05). (C) QS response reporter P*luxC*-gfp. Cultures containing a plasmid with the QS reporter system, P*luxC*-gfp, were cultured as described in Experimental Procedures section and the area under the curve was calculated across OD590 values between 0.1 and 0.4 (Figure S1). Letters represent statistical significance between groups (ANOVA with post hoc Tukey; *p* value <0.05).
Specifically, most strains (90-11-287, VIB93, 775, NB10, 51/82/2) showed high vanT mRNA levels that did not differ significantly from the ΔvanO strains at both cell densities, as well as increased vanT levels at HCD relative to LCD. However, one strain (S2 2/9) revealed an intermediate vanT mRNA level that varied significantly from the ΔvanO strains at LCD, but not at HCD. Some strains (PF430-3, 87-9-11-6, 90-11-286) revealed low levels of vanT mRNA that differed significantly from the ΔvanO strains at LCD and 90-11-287 ΔvanO at HCD. Markedly, fluorescence produced from the $P_{\text{lux-gfp}}$ plasmid revealed that across growth curves, two strains (PF430-3 and 87-9-11-6) showed a low QS response that did not differ significantly from the vanT deletion mutants, and were significantly lower than the other strains including the vanO deficient mutants (Figure 4C). The seven other strains all activated their QS response to a high degree that was similar to the QS-on mutants (ΔvanO), yet still displayed diversity in their QS response as well as timing the onset of QS regulation (Figure 4C, Figure S1). Collectively, our data suggest that two strains exhibited a low QS response (PF430-3 and 87-9-11-6) and the seven other strains showed a high QS response.

QS systems are constantly under evolutionary pressure, and strains with dysfunctional QS systems are commonly isolated among pathogens, such as Pseudomonas aeruginosa (Smith et al., 2006) and V. cholerae (Joelsson et al., 2006). We conducted a phylogenetic analysis of concatenated amino acid sequences of 28 QS-related genes in the selected V. anguillarum strains to look for potential clustering of phylogenetic distances with the QS pathways. The analysis did not reveal a clear link between evolutionary distance of the QS genes and the observed QS response, as illustrated by the closely related strains 87-9-116 and NB10, which had a low and high QS response, respectively (Figure S2). In general, the phylogeny of the QS systems was similar to the phylogeny of core genes of the strains, and the growth rates of the phylogenetically related strains were more similar than those of distantly related strains (Figure S3) (Castillo et al., 2017). Next, we examined if the low QS response could be associated with either disruptive mutations or truncations of single QS genes or their putative promoters (500 bp upstream of ORF) by pairwise sequence alignments with strain 90-11-287, which had a high QS response. However, this approach did not reveal systematic sequence patterns or disruptions correlating with the QS responses (Figure S4–S6). Further investigation of vanT sequences in our V. anguillarum collection revealed that 3 out of 27 strains were QS negative as they contained VanT ORF frameshift mutations, likely disrupting the native function of VanT (strains Ba35, T265, and A023). Collectively, these data suggested that our V. anguillarum collection consisted of ~11% QS-negative strains and within the QS-positive strains, 2 of 9 had a low and 7 of 9 had a high QS response.

### The shared and strain-specific VanT regulons

Whole genome alignment revealed that the strains PF430-3 and 90-11-287 had 75.8% pairwise identity, and gene set enrichment analysis emphasized strain-specific differences in expression of genes involved in glycolysis and carbon metabolism, amino acid metabolism, polysaccharide and siderophore synthesis (Figure S7A,B; Supplementary dataset). These overall differences in genome sequence and gene expression between the two strains further underlined the fundamental differences in the functional properties of the strains. To unveil the biological processes that were affected by the VanT-QS system, a gene set enrichment analysis was conducted for the mutant versus WT comparisons that displayed >200 differentially expressed genes. These analyses revealed that the shared QS regulon of the two strains comprised ATP synthesis, amino acid, amide, and biotin metabolism (Figure S7C–F). On the other hand, some processes were regulated by the VanT regulon on a strain-specific basis, including chemotaxis, polysaccharide biosynthesis, carbon and fatty acid metabolism, cobalamin transport, and polyamine and thiamine biosynthesis (Figure S7C–F). Moreover, the data suggested that VanT stimulates the expression of biofilm genes, type IV pili genes, type VI secretion system-2 (T6SS-2) genes, and the toxin genes empA (hap homolog) and lasA, while repressing T1SS genes, T6SS-1 genes, iron uptake genes, chitinase genes, and the competence regulator tfoX (Figure 5; Supplementary dataset S1). In addition, we found that cell density-related factors other than the VanT-QS cascade play a major role in the regulation of biofilm, T6SS-2, and chitinase genes, and the expression of many of these (e.g. biofilm genes, T1SS genes, chitinase genes, toxin genes, and tfoX) was also strain dependent. We previously reported that the expression of the phage receptor gene ompK was repressed by QS as a strategy to reduce phage susceptibility at HCD (Tan et al., 2015). In the current study, there was also an increased expression of ompK in the mutants lacking vanT, however not significant (Supplementary dataset S1). Moreover, ompK was overexpressed at LCD compared to HCD in both WT and QS mutant strains, indicating that growth phase-related or cell density-related factors other than VanT are involved in the regulation of this phage receptor. In summary, the shared VanT regulon comprised genes involved in central metabolism, QS, biofilm, pili, T6SS, toxins, and competence. Thus, these data establish VanT as a central regulator in...
**V. anguillarum** and associate the differential QS response with differential cross-strain expression of the genes under the shared VanT regulon.

Populations at LCD compared to HCD differentially expressed 56% and 74% of the genes in 90-11-287 and PF430-3, respectively, underlining the fundamental difference in the functional properties of LCD and HCD populations that arise as a combined effect of differences in growth rate, QS autoinducer concentrations, and altered concentrations of other extracellular metabolites (Figure S8). Additionally, the differences in QS responses and regulons among the WT strains were reflected in divergent transcript levels of several VanT-controlled genes, as those transcripts were significantly differentially expressed between high and low cell densities in one strain, but not significantly different in the other strain (Figure S9). These genes were involved in processes such as biofilm, chemotaxis, autoinducer synthesis, T6SS-2, toxicity, and several metabolic genes (Figure S9).

**QS represses virulence in Vibrio anguillarum**

To investigate the virulence in relation to QS in both the poor and strong biofilm-forming *V. anguillarum* strains 90-11-287 and PF430-3, respectively, we tested the virulence towards CHSE-214 fish cells and gilt-head sea bream fish larvae of both WT strains and their corresponding QS mutants (ΔvanT and ΔvanO). The ΔvanT mutants caused higher mortality rates of fish larvae, though not significantly, and lysed significantly more fish cells than ΔvanO mutants (Figure 6). Further, the ΔvanT mutant exhibited significantly higher mortality rates towards fish larvae compared to WT for strain 90-11-287. Collectively, these data suggest that QS represses lysis of fish cells in both strains, whereas the direct effect of QS on virulence towards fish larvae was only observed in one of two strains (Figure 6). In agreement with this, the WT strain PF430-3, which had relatively low VanT levels, showed high virulence against fish cells and fish larvae, similarly to the ΔvanT mutant. Accordingly, the WT strain 90-11-287, with high VanT levels, had limited virulence towards fish cells and larvae, matching the low mortality rates of the ΔvanO mutant and was equivalent to the negative controls in most cases. Consequently, the higher virulence of the PF430-3 WT strain towards fish larvae and fish cells compared with 90-11-287 WT suggested that the differences in vanT expression between the strains were closely associated with their virulence properties. Thus, the observed difference in their QS-off and QS-on state
is an important factor of the strain-specific differences in virulence. Moreover, these data stress that despite strains PF430-3 and 90-11-287 exhibiting substantial VanT regulon differences, several virulence-associated properties were under QS control in both *V. anguillarum* strains (Figures 2 and 6). Another phenotypic difference between 90-11-287 and PF430-3 associated with virulence is biofilm formation. Hence, to investigate the potential role of biofilm formation for bacterial virulence, we measured biofilm formation of the seven selected *V. anguillarum* strains, allowing a cross-strain comparison of biofilm formation and QS response with virulence data (Rønneseth et al., 2017). However, this analysis did not reveal a clear link between virulence ranking towards fish larvae, QS response and biofilm formation (Table 1; Figure S10), though there was a tendency that strong biofilm formers were associated with higher virulence ranking. Thus, while it is evident that a QS-off state is important for fish cell lysis, an increase in virulence against fish larvae was only significant in one of two *V. anguillarum* strains, and additional parameters not considered in this study likely influence the virulence of the strains.

To assess the QS regulation of other virulence-associated phenotypes, we therefore also evaluated the ability of WT and QS mutants to degrade cod and sheep erythrocytes, gelatin, and elastin. Interestingly, opposite to the regulation of overall virulence against fish larvae and cell lines, these specific virulence properties were positively correlated with the VanT level of the bacteria. The results demonstrated that ΔvanO mutants had increased activity, while ΔvanT mutants displayed reduced or no degradation of erythrocytes, gelatin, and elastin, indicating that QS stimulates β-hemolysis, gelatinase, and elastase activity (Figure 6F–I). The absence of elastase activity in strain 90-11-287 is likely due to a premature stop codon in a *lasA*-like gene (QR76_09425), which is involved in
elastin degradation in P. aeruginosa (Toder et al., 1991). In agreement with the model of catabolite repression leading to lower cqsA and vanT expression discussed above, addition of glucose inhibited degradation of sheep erythrocytes in a dose-dependent manner in the high QS response strain 90-11-287 (Figure S11).

DISCUSSION

In this study, we found that the timing and degree of QS regulation can vary widely across strains, especially demonstrated by the QS state of the two model strains, which were opposite and most similar to their respective QS-off (ΔvanT) or QS-on (ΔvanO) mutants (Figures 2–4). More specifically, the transcriptome profile of the high-QS-response strain 90-11-287 was similar to its maximal QS-on mode at HCD (defined by the ΔvanO mutant), while it did not exploit the QS-off mode defined by the ΔvanT mutant at LCD. In contrast, the transcriptional response of the low QS response strain PF430-3 was comparable to its minimal QS regulation at LCD, but differed greatly from the maximal QS-on state at HCD. Thus, at the population level, the low QS responsive strain PF430-3 primarily engages in individual activities, whereas the high QS responsive strain 90-11-287 mainly employs collective behaviour across the range of cell densities examined in this study. In a broader view, measurements of relative vanT mRNA levels in addition to QS reporter measurements in a collection of V. anguillarum strains confirmed that diversity in strain-specific QS-responsiveness is a general pattern and that a high QS response was the most prevalent strategy in V. anguillarum. These results support previous studies of strain-specific differences in HapR protein levels in V. cholerae (Joelsson et al., 2006) and QS activation in Staphylococcus aureus (Geisinger et al., 2012).

We also demonstrate that strain-specific differences in QS regulation of V. anguillarum have large implications for the metabolic and virulence properties of this important pathogen (Figures 5–7; Figure S7 and S9). Regulons of Vibrio QS master regulators homologous to VanT have been reviewed elsewhere (Ball et al., 2017), and many central processes are under QS control in several Vibrio species. We found that QS is an important determinant of central metabolism, regulating genes involved in carbon, lipid, iron, chitin and amino acid metabolism, and the TCA cycle. Moreover, QS also regulates the expression of genes involved in chemotaxis, type IV pili, secretion systems, toxins, polysaccharide synthesis, and competence in V. anguillarum. Interestingly, we found that the low-QS-responsive strain PF430-3 harboured a larger VanT regulon, as defined by the genes that are differentially expressed between the QS-on vanO and QS-off vanT mutants compared to WT, than the high-QS-responsive strain 90-11-287. This could suggest a potential trade-off between engagement in cooperation (QS response) and investment of resources through coupling of traits to QS (QS regulon). Evolution experiments in V. campbellii showed that populations generated variants with a reduction of cooperative phenotypes under QS control, suggesting a contraction of the QS regulon (Bruger et al., 2021). A more radical approach to lower the burden of QS regulation is through mutation or omission of the QS master regulator as observed in P. aeruginosa during long term infections (Rossi et al., 2021; Smith et al., 2006), as well as in V. cholerae (Joelsson et al., 2006), and in our V. anguillarum collection. This was experimentally exemplified in V. campbellii, where cells locked in cooperative QS-on mode (ΔluxO and ΔluxU) were outcompeted by evolving QS-negative isolates (luxR mutants), while WT populations allowed for the stable maintenance of both QS-negative and QS-positive isolates (Bruger et al., 2021). It has been suggested that a decrease in traits regulated by QS or emergence of QS-negative strains are strategies to compensate for the metabolic burden to maintain cooperativity (Bruger et al., 2021; Heurlier et al., 2006; Kohler et al., 2009).
Some processes within vibrios appear to be universally QS controlled, for example, the activation of metalloproteases (hap-like genes) and the repression of hemolysins (hlyA-like genes) (Ball et al., 2017; Shao et al., 2011; Yildiz et al., 2004). Whereas other behaviours such as biofilm formation seem to vary between species, for example, QS stimulates the expression of biofilm genes in V. cholerae at LCD but represses at HCD, whereas biofilm genes are activated by QS in V. harveyi and V. vulnificus. Our study emphasizes that QS regulation can vary greatly even among isolates of the same species, here V. anguillarum, and that studies of LuxR-like regulons in single strains do not necessarily apply to more than the specific isolate investigated, so extrapolations and generalizations should be done with caution. For example, we found that expression of siderophore and T6SS genes are under strain-specific QS control, with the latter either stimulated or repressed depending on the strain of V. anguillarum (Figure 5; Figure S7). Moreover, the role of QS in biofilm formation varies between the investigated V. anguillarum strains: Biofilm formation was stimulated by QS in V. anguillarum strain NB10 (Croxatto et al., 2002) but repressed in strains 90-11-287 and PF430-3 (Tan et al., 2015; Tan et al., 2020). In contrast, we found that the vps genes involved in polysaccharide production in V. cholerae (Yildiz & Schoolnik, 1999) were stimulated by QS in 90-11-287 and PF430-3, and that other growth-phase or cell density-related factors play a major role in the regulation of those genes (Figure 5). However, biofilm formation is also known to be stimulated by prophage induction, which is regulated by QS in V. anguillarum strain 90-11-287 (Tan et al., 2020). A reduction in biofilm formation related to QS repression of prophage induction thus likely contributes to the observed strain-specific differences in QS-regulation of biofilm formation. Overall, these studies highlight strain-specific diversity in QS regulation of biofilm formation and emphasize the complexity of the biofilm regulation in vibrios.

The molecular differences underlying the differences in the QS response elicited by the two WT strains are still unknown. We propose that the very dissimilar expression levels of the autoinducer synthase cqsA, which produces the dominant autoinducer signal, CAI-1, in V. cholerae (Miller et al., 2002), is likely to play a key role (Figure 3). We further propose that the underlying cause for the difference in cqsA levels may be a consequence of discrepancies in the expression of sugar metabolic genes known to regulate cqsA expression in V. cholerae (Liang et al., 2008) (Figures 3 and 6F,G; Figure S11). Previous studies have linked shifting environments (Girard et al., 2019) and the metabolic state of the cell (Hoang et al., 2002; Liang et al., 2008) to the ability of bacteria to produce QS molecules and thus presumably activate QS pathways. It therefore seems plausible that the metabolic state of the cell, influenced by environmental conditions and strain-specific responses to these, dictates the rate of QS signalling and activation of QS pathways.

The destruction of blood cells by hemolysis is often described as a key virulence factor and expression of hlyA-like hemolysins are repressed by QS in both V. cholerae (Tsou & Zhu, 2010) and V. anguillarum (Figure 5). Accordingly, hemolysis of sheep erythrocytes was repressed by QS in V. cholerae (Gao et al., 2018). The observed stimulation of hemolysis by QS in our study thus contradicts previous studies and further underlines the previously discussed diversity in QS regulation of virulence properties in Vibrio pathogens (Figure 6F,G). However, our observation that the VanT QS system can repress overall virulence against gilthead seabream larvae and lysis of CHSE-214 fish cells emphasizes that virulence is driven by a combination of successive processes and interactions including both attachment, biofilm formation, tissue degradation, iron sequestration, hemolysis, and so on, which are under different QS control (Figures 5 and 6). Previous infection studies in V. anguillarum strain NB10 concluded that neither the VanI/R nor the VanT QS systems influenced virulence towards rainbow trout juveniles and sea bass larvae (Croxatto et al., 2002; Li et al., 2018; Milton et al., 1997). However, our results using V. anguillarum strains 90-11-287 and PF430-3 confirm that a Qs-off state was an important determinant for fish cell lysis in both strains, whereas QS only significantly affected virulence against fish larvae in one of two V. anguillarum strains (Figure 6; Table 1). It is, however, also clear that other strain-specific differences (e.g. biofilm formation) that are not solely controlled by VanT and/or different infection models may affect virulence in V. anguillarum.

Collectively, our data suggest that the virulence strategies between the two WT strains could be remarkably different. PF430-3 efficiently attaches and forms a strong biofilm at LCD, yet the population remains in a relatively low QS-response state (Figures 1 and 7; Figure S11). Consequently, a low QS response in this strain leads to decreased production of public goods, lower expression of T6SS-2, a more virulent phenotype, higher expression of the phage receptor ompK and possibly prophage induction, which could further stimulate biofilm/aggregate formation (Figures 5 and 6) (Tan et al., 2015, 2020). Moreover, as PF430-3 likely responds to signalling molecules secreted by other bacteria, this strain could exhibit more phenotypic plasticity through its relatively large QS regulon compared to 90-11-287, and upregulate its T6SS to combat neighbouring cells. On the other hand, strain 90-11-287 seems to employ an alternative virulence strategy: The QS-on state of this strain allocates resources to overexpress T1SS and toxins (rtxA, hlyA and empA) compared to PF430-3 (Figure 5). Strain 90-11-287 does not form a strong biofilm independently and may switch to
a QS-on state early during an infection. This would lead to repression of the phage receptor ompK, reduced prophage induction, lower expression of T6SS-1, and cooperative behaviour like secretion of proteolytic and hemolytic enzymes (Figures 5 and 6). Although this resulted in a less virulent phenotype in the larval and fish cell line infection experiments, it can be speculated that these phenotypes are efficient during later stages of fish infection or different pathogenesis strategies, and thus not quantified in the current challenge models.

In fact, the combined infection by strains with these different strategies may provide a more efficient exploitation of the animal host, if, for example, low QS response strains with strong biofilm capabilities (e.g. PF430-3) efficiently colonize the fish surface and initiate infection and strains overexpressing autoinducer synthases (e.g. 90-11-287) direct the population to a QS-on state and excrete toxins to destruct tissue and blood cells upon establishment of high cell densities in the biofilms. Overall, the observed strain-specific differences in QS regulation of phenotypic properties in V. anguillarum emphasized the role of QS as a driver of the functional diversity of the pathogen, likely supporting its ability to adapt to changing environmental conditions.

**AUTHOR CONTRIBUTIONS**

**Emilie Søndberg:** Conceptualization (equal); formal analysis (supporting); investigation (supporting); methodology (supporting); visualization (supporting); writing – review and editing (supporting).

**Panos Kalatzis:** Formal analysis (supporting); funding acquisition (supporting); investigation (supporting); methodology (supporting); writing – review and editing (supporting).

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**Sine Lo Svenningsen:** Conceptualization (equal); formal analysis (equal); funding acquisition (supporting); investigation (equal); methodology (equal); project administration (supporting); resources (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (equal).

**Mathias Middelboe:** Conceptualization (equal); formal analysis (equal); funding acquisition (lead); investigation (equal); methodology (supporting); project administration (lead); resources (lead); supervision (lead); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal).

**Jesper Juel Mauritzen:** Conceptualization (equal); data curation (lead); formal analysis (lead); funding acquisition (equal); investigation (lead); methodology (lead); project administration (equal); resources (equal); visualization (lead); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT
The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT
RNA-seq data is available at NCBI Gene Expression Omnibus (GEO) accession number GSE184853.

ETHICS STATEMENT
All animal experiments were performed at the facilities of Hellenic Center for Marine Research, Greece under their licence for fish experiments.

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