Supplementary Material for

High cell densities favor lysogeny: Induction of an H20 prophage is repressed by quorum sensing and enhances biofilm formation in *Vibrio anguillarum*

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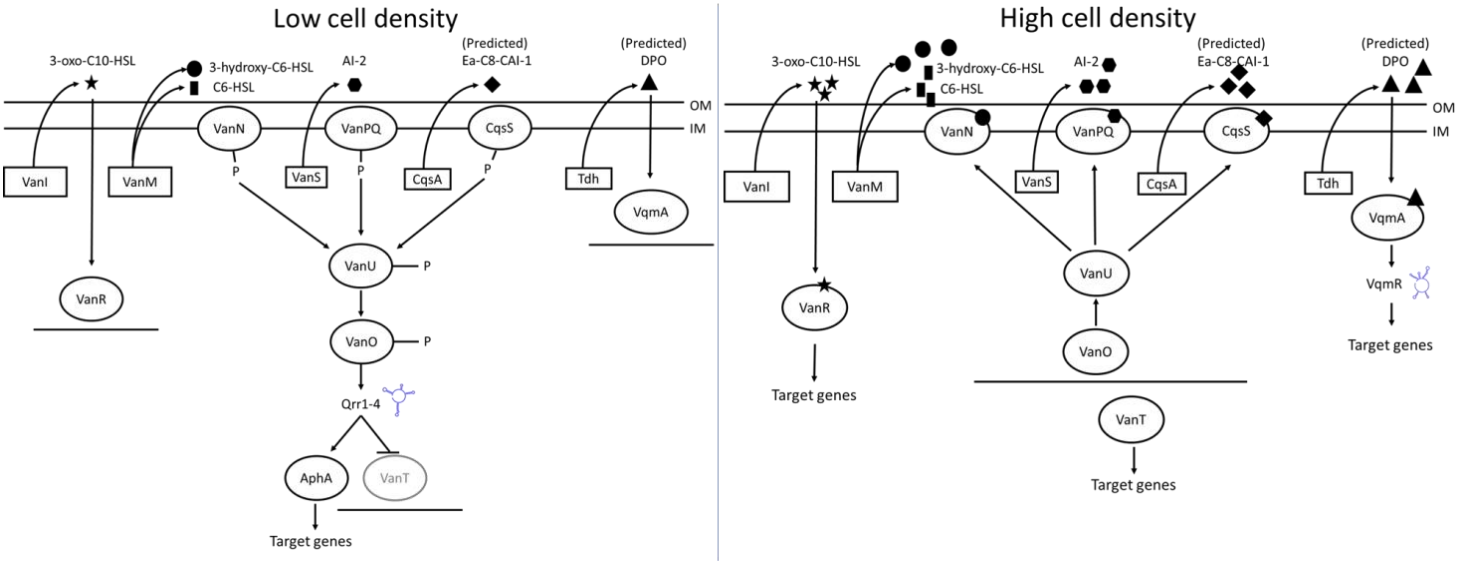
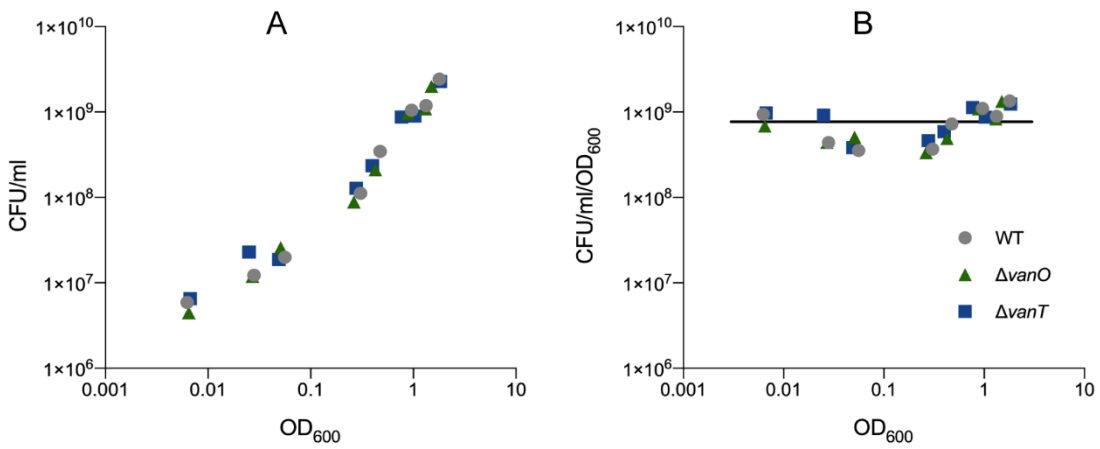


Supplementary Figure 1. Schematic of known and predicted components of the *V. anguillarum* QS circuits at low and high cell densities.

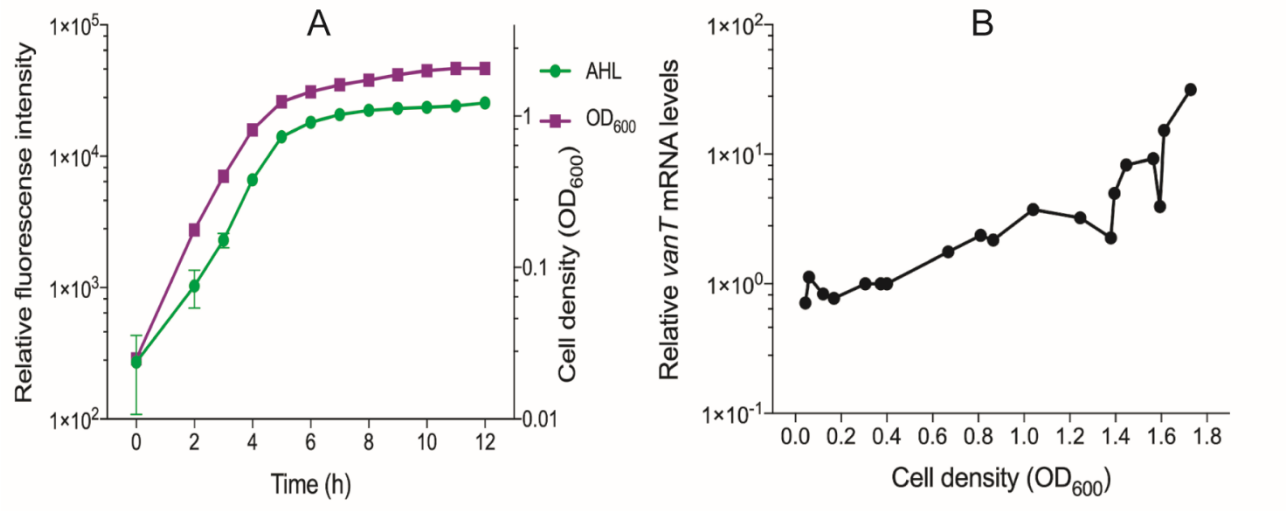
The central transcription factor VanT is activated upon accumulation of the indicated autoinducers via a phosphorelay. This pathway is analogous to the well-described QS phosphorelays of *V. harveyi* and *V. cholerae*[1]. In *V. harveyi*, the histidine kinase receptors initiate a phosphorylation cascade that activates the response regulator LuxO (homologous to VanO in *V. anguillarum*) at low cell density, when the concentrations of extracellular autoinducers are low. Phosphorylated LuxO then activates the expression of small RNAs (*qrr*1–4) which destabilize the *luxR* mRNA (homolous to *vanT* in V. anguillarum) and activate translation of the *aphA* mRNA. At high cell density, when the concentration of the extracellular signaling molecules reaches a threshold level, the autoinducer-bound receptors function as phosphatases, rendering LuxO in an inactive, dephosphorylated state, which terminates sRNA synthesis and allows for accumulation of the quorum-sensing transcription factor LuxR, and repression of AphA synthesis[1]. The *V. anguillarum* VanM/VanN and VanS/VanPQ are homologous to the LuxM/LuxN and LuxS/LuxPQ autoinducer synthase-receptor pairs of *V. harveyi*. VanM synthesizes N-(3-hydroxyhexanoyl) homoserine lactone (3-hydroxy-C6-HSL) and N-hexanoyl homoserine lactone (C6-HSL), while VanS synthesizes an Autoinducer-2 (AI-2) signal[41,59]. These two systems control QS-regulated genes via the transcription factor VanT[2,3]. *V. anguillarum* also harbors *aphA*[4], but its role in the QS network has not yet been investigated. A third quorum-sensing synthase-receptor pair, CqsA/CqsS, is predicted to exist in *V. anguillarum*, and cell-free spent medium of *V. anguillarum* contains (*Z*)-3-aminoundec-2-en-4-one (Ea-C8-CAI-1), which in *V. harveyi* is produced by the CqsA synthase[5,6]. Based on homology to other *Vibrio* quorum-sensing systems, the CqsA/CqsS system is also predicted to feed into the signalling pathway controlling VanT[7]. In addition, genes homologous to the VqmA quorum-sensing system of *V. cholerae* are also present in *V. anguillarum*. The VqmA receptor binds 3,5-dimethylpyrazin-2-ol (DPO), an autoinducer that depends on threonine dehydrogenase (Tdh) for its synthesis[8,9]. Finally, *V. anguillarum* utilizes the VanI/VanR synthase-receptor pair, which is homologous to the classical LuxI/LuxR system of *V. fischeri*[10]. VanI produces the autoinducer N-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), which is detected by the VanR transcription factor[11]. The extent to which

the quorum-sensing signal transduction pathways of *V. anguillarum* are interdependent has not been determined, but there are clear indications of some degree of cross-regulation. For example, VanI activity seems to be dependent on VanM, since none of the AHL autoinducers are produced in a *vanM* mutant[12]. OM and IM indicate outer membrane and inner membrane, respectively. -P indicates that the protein is phosphorylated.



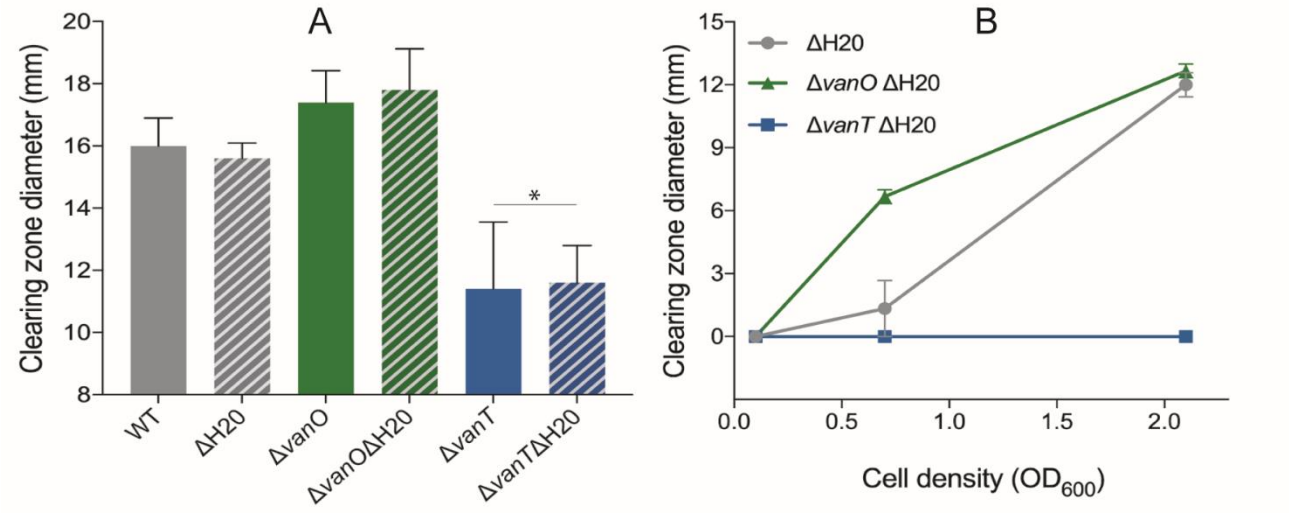
Supplementary Figure 2. Conversion from OD_{600} units to cells per milliliter of culture (CFU/ml).

The conversion factor from OD_{600} units to cells per milliliter of culture (CFU/ml) was estimated at $7.7*10^8 \pm 3.3*10^8$ CFU/ml/OD_{600} by parallel measurements of the OD_{600} and plating of cultures of the *V. anguillarum* 90-11-287 wildtype, *ΔvanT*, and *ΔvanO* strains at selected time points during two of the three experiments shown in Figure 1C of the main text. Culture aliqouts were rapidly diluted to appropriate cell densities in sterile LM medium supplemented with 10 mM $MgSO_4$ and 5 mM $CaCl_2$ and immediately plated in dublicate on LM plates. As no systematic variation in the CFU/ml/OD_{600} was observed at different cell densities or between the three strains, the conversion factor 1 OD_{600} unit = $7.7*10^8 \pm 3.3*10^8$ CFU/ml was simply determined as the average value of the 24 measurements shown in panel B. The error indicates one standard deviation from the mean.



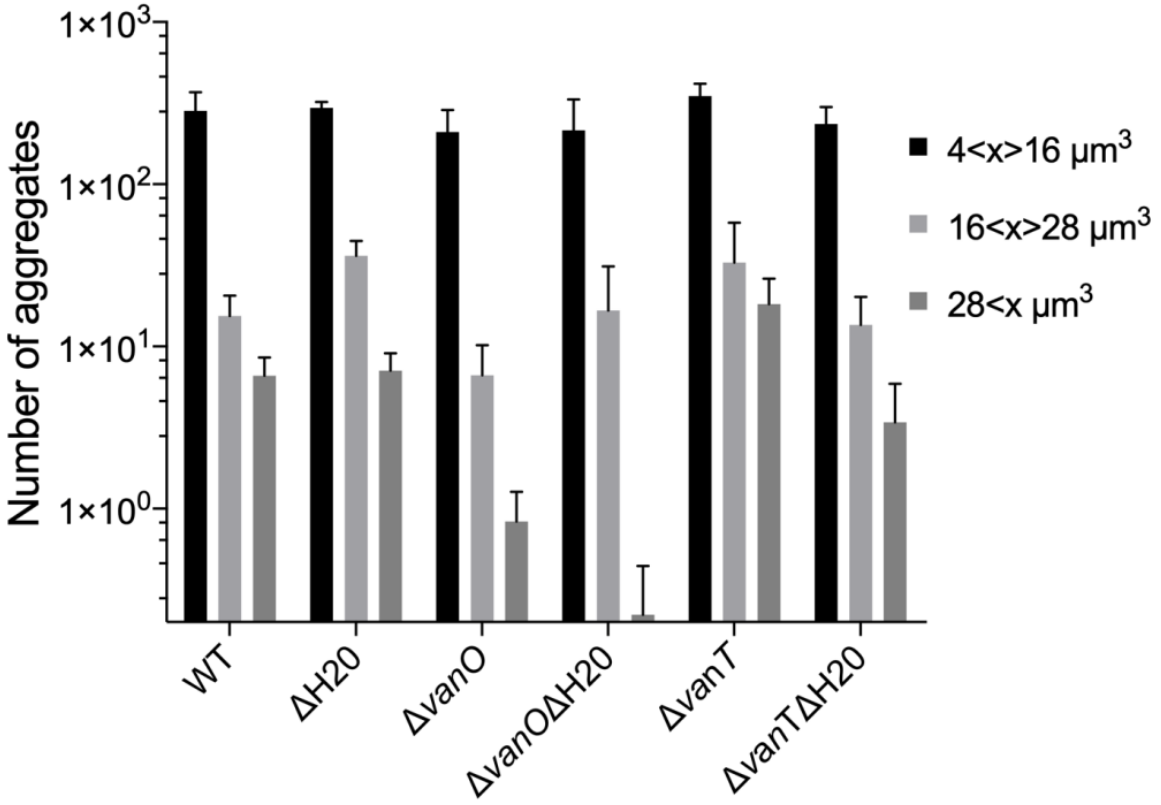
Supplementary Figure 3. AHL autoinducers and *vanT* mRNA accumulate in *V. anguillarum* strain 90-11-287 during growth from low to high cell density.

Aliquots of batch cultures were withdrawn at the indicated time points for measurements of AHL production, OD_{600}, and *vanT* mRNA levels as described in Materials & Methods. The AHL assay was repeated three times with similar results. Error bars indicate standard deviation of triplicate technical replicates. The relative *vanT* mRNA levels are shown as the mean value obtained from RNA harvested from three independent cultures.



Supplementary Figure 4. Extracellular proteolytic activity is QS-induced.

(A) Diameter (in mm) of clearing zones produced by bacteria spotted on 2% skim milk agar. (B) Diameter of clearing zones from cell-free spent supernatant, filtered at different bacterial concentrations. Asterisks indicate significance (*ANOVA and Tukey's multiple comparison test, α=0.05*) and error bars represent standard error of the mean (N=6).



Supplementary Figure 5. Quantification of aggregates formed in stationary cultures of wildtype (WT), *ΔvanO and ΔvanT* mutants with or without the ϕH20-like prophage.

The volume of aggregates formed by the wildtype and various mutants was quantified and subsequently categorized according to size based on LIVE/DEAD staining and CLSM z-stack image acquisition (see Materials and Methods). Images were acquired for three distinct areas of the bottom of a well for averaging and the experiment was performed four times. Error bars represent standard error of the mean.

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

Strains, phage or plasmids	Genotype or relevant markers	Reference
E. coli		
S17-1	*thi pro hsdR hsdM*$^{+}$*recA RP4-2-Tc::Mu-Km::Tn7 λpir*	[13]
V. anguillarum		
BA35	wt, isolated from USA	[14]
90-11-287	wt, isolated from Denmark	[15]
Δ*vanT*	90-11-287 Δ*vanT*	this study
Δ*vanO*	90-11-287 Δ*vanO*	this study
ΔH20	90-11-287 with a 45929 bp φH20 prophage deletion	this study
ΔH20 Δ*vanT*	90-11-287 ΔH20 Δ*vanT*	this study
ΔH20 Δ*vanO*	90-11-287 ΔH20 Δ*vanO*	this study
PF430 Δ*vanT*	Strain PF430 Δ*vanT*	[16]
Plasmids		
pDM4	Cm^{r}; suicide vector with an R6K origin (pir requiring) and *sacBR*	[13]
pDTvanT	Cm^{r}; pDM4 derivative containing *vanT* flanking regions fused in-frame	[16]
pDTvanO	Cm^{r}; pDM4 derivative containing *vanO* flanking regions fused in-frame	[16]
pDT-H20	Cm^{r}; pDM4 derivative containing H20 flanking regions fused in-frame	this study
pAHL-GFP	Ap^{r}, *luxR*-P$_{luxI}$-*gfp*mut3	[17]
Bacteriophage		
ϕVa-90-11-287_p41	Genome 53 kbp, φH20-like *Siphoviridae*, isolated from Denmark	[18]
ϕH20	Genome 53 kbp, *Siphoviridae*, isolated from Denmark	[18]
KVP40	Genome 245 kbp, *Myoviridae,* isolated from Japan	[19,20]

Supplementary Table 2. Oligonucleotides used in this study.

Name	Primers (5'-3)
qRT-PCR (gene)	
recA	Forward: CTAGTCGAAATTTTTTCGACACAGC
	Reverse: GGCTCGTTAAATTTTTTATCGACTCTT
vanT	Forward: GTCTCCACCAACCGTACTAATC
	Reverse: GAGCGTAGCAAGATGTTCTGG
H20 deletion:	
H20_1	Forward: TTTAGATCTCATAGTCAGCACCACCGATG
H20_2	Reverse: CAGATCGTAGCTTCGGATCTGATCATGAGCTCTCTACG
H20_3	Forward: GAAGCTACGATCTGCTGTAACGCGTAGGCCTCC
H20_4	Reverse: TTTCTCGAGCGAACTCGATAACCCAAACG

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