Supplementary Information

Vibrio cholerae filamentation promotes chitin surface attachment at the expense of competition in biofilms

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Supplementary Materials and Methods

Strains and media:

Supplementary Table S1 includes a full strain and plasmid list for this study. CVD112 and NT330 were generously given by the Skorupski lab in the Geisel School of Medicine at Dartmouth College. MO10 was the gift of Matt Waldor; H1C1 was provided by Jesse Shapiro via collection by Jason Harris, Jacques Boncy, and Josiane Buteau; A1552 was generously provided by Fitnat Yildiz. Strain AI-1837 was the gift of Joachim Reidl. Strain AI-1837 was found to contain a frameshift mutation in the quorum-sensing locus *luxO* that was not present in the avirulent CVD112 derivative of AI-1837 (see supplementary Table S2). This mutation was thus corrected in AI-1837 to return *luxO* to the wild type state. All modifications to N16961 were made using Escherichia coli S17-λpir carrying the suicide vector pKAS32 for allelic exchange, and all other strains were made using pBW1 with SacB counterselection for allelic exchange (1). All strains were grown in LB, M9 minimal media with 0.5% glucose, or artificial sea water with either 0.5% GlcNAc or chitin as in (2). Antibiotics and reagents were used in the following concentrations: 100 μg/ml ampicillin, 50 μg/ml kanamycin, 50 μg/ml polymyxin B, 1000 μg/ml streptomycin, 5% sucrose, 40 μg/ml X-Gal and 5 μg/ml cefalexin. All chemicals and reagents were purchased from Millipore Sigma unless otherwise stated.

Plasmid Construction:

All restriction enzymes and ligase were purchased from New England Biolabs, and PCR reagents were purchased from BioRad. Codon optimized versions of *mKO-κ* and *mKate2* were purchased from Invitrogen. pCN764 (for insertion of m*KO*-κ to the *lacZ* locus) and pCN765 (for insertion of *mKate2* to the *lacZ* locus) were constructed by amplification of the flanking regions upstream and downstream of the lacZ open reading frame and fusing the respective genes for these fluorescent proteins to a synthetic P_{tac} promoter for high expression from a single chromosomal locus. The *lacZ*-flanking fragments and fluorescent protein expression constructs were then combined using overlap extension PCR. These conjoined fragments were cloned into the pKAS32 vector backbone of pCN251 using the enzymes BsrG1 and BspE1 and ligated with T4 DNA ligase. These constructs were then introduced into *E. coli* S17-λpir by electroporation and conjugated into *V. cholerae*. Deletions of *vpsL*, *rbmA*, *tcpA*, *pilA*, *flaA*, *gbpA*, *mshA*, and *wbfR* were made by cloning 1kb sequences up- and downstream of the respective reading frames, joining these by PCR overlap extension, and cloning these fused products into the pKAS32 or pBW backbone. Insertion of the 3xFLAG epitope to the C-terminus of *rbmA* was performed using pKAS32-based or pBW-based allelic exchange as described previously (3).

Liquid growth curve experiments

V. cholerae strains were grown at 37° C shaking in LB overnight prior to the experiment. Overnight cultures were then back diluted to an OD_{600} of 0.01 in LB, M9 minimal medium with 0.5% glucose, or artificial sea water with 0.5% GlcNAc. OD_{600} readings were monitored every hour and once overnight. To measure viable cell count, serial dilutions were also performed at each timepoint and plated on LB agar for colony forming units. Each growth curve was performed with three biological replicates from independent overnight cultures and three technical replicates per biological replicate. Measurements for optical density were taken with a CO8000 cell density meter (Biochrom, Cambridge UK).

Dry biomass measurements

Strains were grown in 20ml LB or artificial seawater with 0.5% GlcNAc for 16 h at 37° C with shaking. Following incubation, biomass was separated from the media by centrifugation (3200 rcf, 15 minutes) and the supernatant was discarded. Cell pellets were then placed in a lyophilizer (Labconco) and dried overnight. Dry cell pellets were then weighed in milligrams.

Swim assay and motility video

Swim motility plates were comprised of 0.3% agar and either LB, or artificial sea water with 0.5% GlcNAc. Agar was inoculated via pipette tip stabbed into the center of the plate. Swim distance was measured via the diameter of

colonies after 16 h incubation at room temperature. Motility of CVD112 was captured on a Nikon 90i at 100x/ N.A. 0.9 oil objective. 15 µl of culture was placed on a glass slide and topped with a cover slip. Videos were then acquired at 30 frames per second.

Membrane and nucleoid staining

CVD 112 was cultured overnight in DSW with 0.5% GlcNAc to induce filamentation. Culture was then diluted 10-fold and treated sequentially with 0.5 µM syto9 DNA stain (Invitrogen) and 1µg/ml FM4-64 membrane stain (Invitrogen). Samples were exposed to stain no longer than 30 seconds prior to imaging. After staining, 15µl of sample was placed under an agar pad and imaged using 488 and 543 nm laser lines on a Zeiss 880 laser scanning confocal microscope.

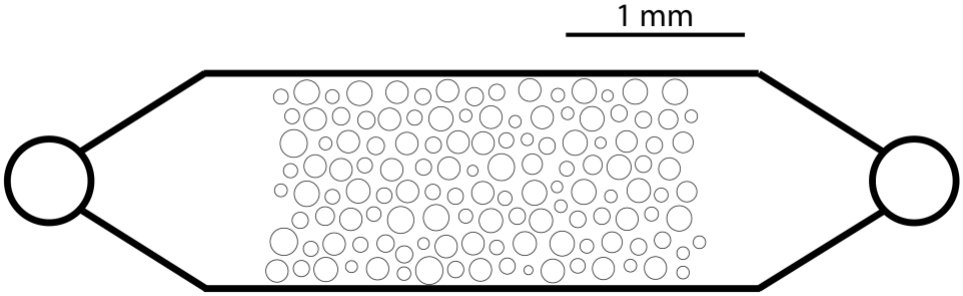
CVD 112 septation imaging

CVD 112 was grown overnight in sea water with 0.5% GlcNAc. Thirty minutes prior to imaging, 1 ml of overnight culture was spun down, resuspended in fresh LB, and incubated at 37° C to encourage septation. 10 µl of this culture was then spotted onto a #1.5 cover glass and covered with an LB agar pad. Phase contrast images were taken every 5 minutes at 100x magnification and a video was rendered using Nikon NIS elements (Tokyo, Japan).

Microfluidic device assembly

The microfluidic devices used consist of poly-dimethylsiloxane (PDMS) bonded to size # 1.5 36mm X 60mm cover glass (ThermoFisher, Waltham MA) using standard soft lithography techniques (4, 5). Two designs of chamber were used, planar and columnar. To establish flow in these chambers, media was loaded into 1ml BD plastic syringes with 25-gauge needles. These syringes were joined to #30 Cole palmer PTFE tubing (inner diameter 0.3mm), which was connected to pre-bored holes in the microfluidic device. Tubing was also placed on the opposite end of the chamber to direct the effluent to a waste container. Syringes were mounted to syringe pumps (Pico Plus Elite, Harvard Apparatus), and flow was maintained at 0.2 µl min^{-1} for all experiments.

For simple straight-chamber flow experiments, we used devices that created tunnels measuring 3000 x 500 x 75 µm (LxWxD). For experiments in which chitin particles were trapped for colonization and biofilm growth, we use a chamber containing column obstacles to hold chitin particles in places for the duration of the experiment. The layout of this chamber is depicted below. These chambers were ~ 4000 x 1500 x 75 µm (LxWxD). These dimensions, which are 1 order of magnitude larger than the typical chitin granule size and CVD112 filament length, and 2 orders of magnitude larger than the typical spacing between granule, where most of the bacterial transport is occurring. Given these experiments are in a low Reynolds number regime, the flow field near the granules, which ultimately rules the encounter rates of single cells with chitin, is largely unaffected by the chamber walls.



Biofilm growth and matrix staining on chitin

Prior to bacterial inoculation, chitin flakes were be sterilized with 70% ethanol and washed in sea water (as in Drescher *et al.* (2)). These flakes were suspended in sea water and flowed into a columnar chamber at high speed using a 1 ml syringe attached to a small length of PTFE tubing. After 30 minutes, sea water was introduced into the device at a rate of 0.2 µl/min. Overnight cultures of each strain were normalized to an OD_{600} of 1.0, inoculated into

a microfluidic chamber previously filled with chitin, and allowed to rest for 30 minutes. The devices were then run at room temperature for different periods of time depending on the experimental design. For experiments in which RbmA matrix protein was stained for localization and quantification: twelve hours prior to imaging, the influent media was replaced with sea water supplemented with 1 µg/ml anti-FLAG antibody conjugated to the fluorescent dye Cy3 (Millipore-Sigma) to stain FLAG-tagged RbmA protein *in situ*.

Chitin colonization experiments

Strains were grown overnight at 37ºC in sea water so that CVD112 would be filamented prior to the experiment. In Figure 2B, strain N16961 was sub-cultured, grown to mid log phase and exposed to a sub inhibitory concentration of cefalexin (5 µg/ml) to induce filamentation as in Bartlett *et al.* (6). Cultures were then diluted to an OD_{600} of 0.1. Equal amounts of each strain were mixed 1:1 in a BD 1ml plastic syringe (for experiments in Figure S6, strains were loaded at an OD_{600} of 0.1 to syringes in monoculture). The syringes were connected to a new chamber filled with chitin flakes. These chambers were then perfused with the corresponding culture preparations at a flow rate of 0.2 µl/min and imaged every 30 minutes for 3 hours.

Chitin competition assays

Microfluidic devices were prepared with chitin as described above. Fluorescent protein expressing strains of CVD112 and N16961 (producing mKate2 and mKO-κ, respectively) were grown in LB overnight at 37º C. These cultures were then equalized to an OD_{600} of 1, mixed 1:1 and inoculated into the microfluidic device. After 30 minutes, flow of fresh sea water lacking any carbon or nitrogen source was introduced at 0.2 µl/min. Under the undisrupted condition, the devices were run at room temperature for 12 days and imaged daily. In conditions with disruption events, new microfluidic devices containing chitin were prepared, and the effluent from the currently incubating device was flowed into the new devices for 30 min. To do this, new lengths of sterile tubing, as short as possible to minimize the time the cells spent in the liquid phase, were used to transfer effluent from one chamber to the next. This method ensures that cells colonizing the new chambers were dispersed members of the biofilms from the previous chamber. After colonization of the new device, the previous device was discarded. Between these disruption events, chambers were incubated at room temperature and imaged daily (see Figure S11).

Microscopy and image analysis

Biofilms inside microfluidic chambers were imaged using a Zeiss LSM 880 confocal microscope with a 40x / 1.2 N.A. water objective. A 543-nm laser line was used to excite mKO-κ, and a 594-nm laser line was used to excite mKate2. A 458-nm laser line was used to excite chitin autofluorescence. For biomass accumulation and competition experiments, images were acquired as a wide view tile scan, so that all chitin in each chamber would be present for analysis. In experiments involving RbmA-FLAG staining, representative images were taken at several locations within the chamber and averaged per biological replicate. Figure panels were rendered using Zeiss ZEN blue software. To quantify bacterial or matrix biomass per area, we used customized scripts in MATLAB (MathWorks, Natick, MA) as in Drescher *et al.*, 2014 (2) and Nadell *et al.* 2015 (3).

RNA extraction and quantitative RT- PCR

Strains were grown in shaking culture of DSW with 0.25g of chitin flakes for 48 hours. After growth cultures were vortexed with glass beads to shear biomass from the chitin. Biomass was then equalized by OD prior to RNA extraction. Whole RNA was extracted using the Quick-RNA Fungal/Bacterial Microprep Kit (Zymo Research). cDNA libraries were assembled using superscript IV first strand synthesis (Invitrogen). qPCR was performed using SYBR green qPCR master mix and a CFX-96 thermocycler (Biorad). *mshA* transcript counts were normalized to those of *hfq*.

DNA extraction and whole genome sequencing

DNA was extracted by first pelleting 1ml of overnight culture and adding 300 µl of cell lysis solution (Qiagen). Cells were then incubated at 80^{O} C for 5 minutes. Following incubation, lysate was treated with 10 µg/ml RNaseA for 1 hour. The lysate was then spun down and the supernatant was added to 300 µl of isopropanol. Mixture was

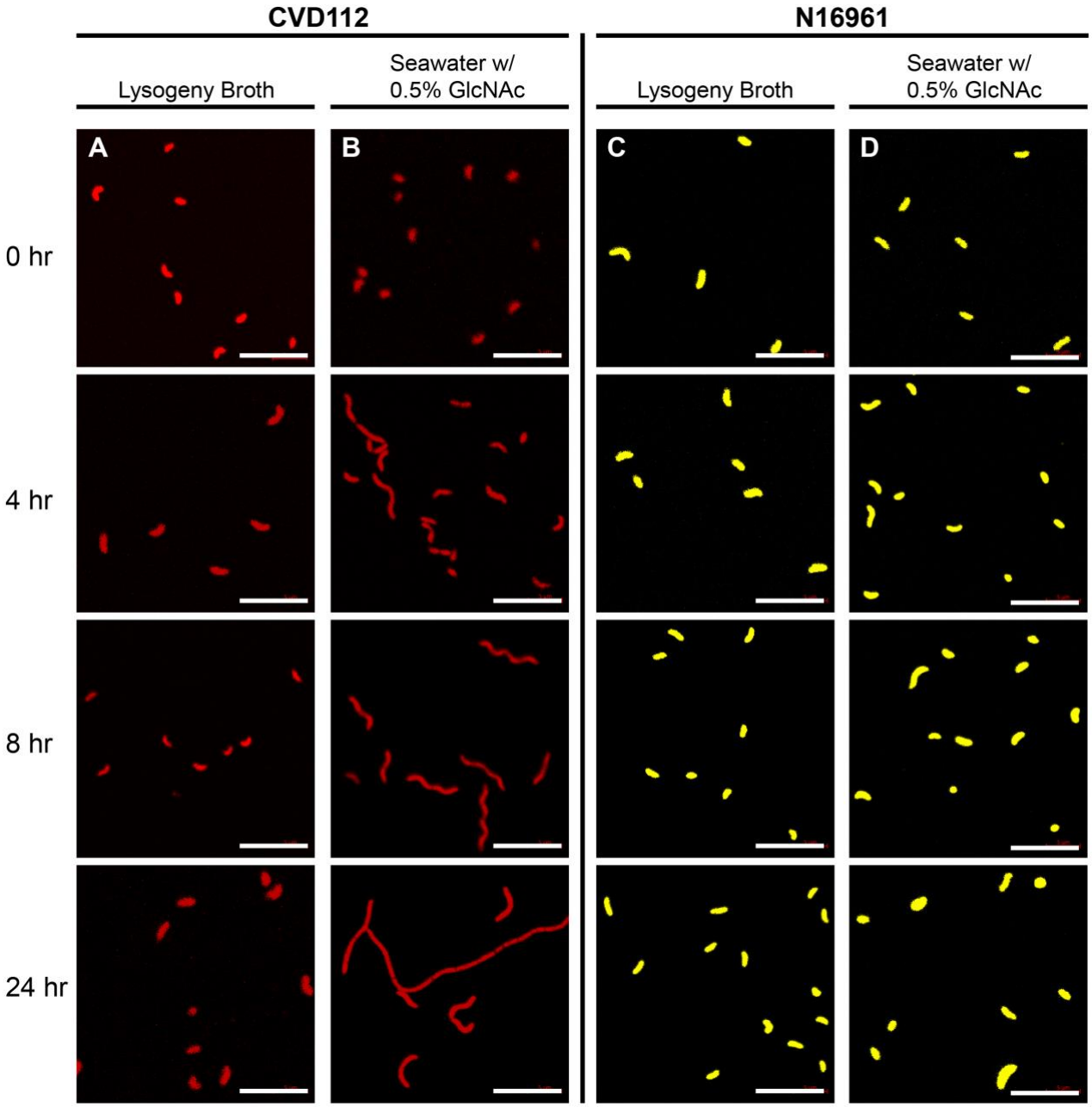
spun down and DNA pellet was washed several times with 70% EtOH and eluted with 100 µl dH_2O. cDNA libraries were prepared using the Nextera DNA Flex Library Prep Kit (NEB). The libraries were sequenced using a MiSeq in 150 bp paired-end mode. Reads were trimmed for quality and adaptors.

The trimmed read files in Fastq format were imported into CLC Genomics Workbench v11 (Qiagen). Short-read sequencing for H1C1 (7) was obtained from SRA. Reads were mapped against two closed, annotated *V. cholerae* genomes using the "Map Reads to Reference" tool with default settings. N16961 (8) was used as reference genome for O1 strains (N16961, A1552 and H1C1), while 48853_H01 (9) was used for O139 strains (MO10, NT330, AI-1837 and CVD112). See Supplementary Table S2 for accession numbers of sequencing data and reference genomes. Any larger deviations from the reference genomes like missing genes or regions with a high fraction of variable sites were determined by manual inspection of the read mappings. SNVs and smaller indels were detected with the "Basic Variant Detection" tool requiring a minimum frequency of 35% and a minimum coverage of 10, followed by manual filtering for sequencing or mapping errors. Finally, differences between NT330, AI-1837 and CVD112 were detected with the "Compare Sample Variants Tracks" tool in pairwise comparisons.

Statistics

All statistical analyses were performed in GraphPad prism. All reported comparisons are Wilcoxon signed ranks tests with Bonferroni correction. For biomass accumulation experiments reported in Figure 2A-B, individual slopes were determined via a linear regression for each biological replicate. These slopes were used as data points for the corresponding comparison tests.

Supplementary Figures



195 **Figure S1. Snapshots of CVD112 cells (red, left) and N16961 cells (yellow, right)**, over the course of planktonic growth curve experiments in LB or artificial sea water with 0.5% GlcNAc. CVD112 filaments in sea water, but not LB, while N16961 does not filament in either condition. Scale bars denote 10 μm.

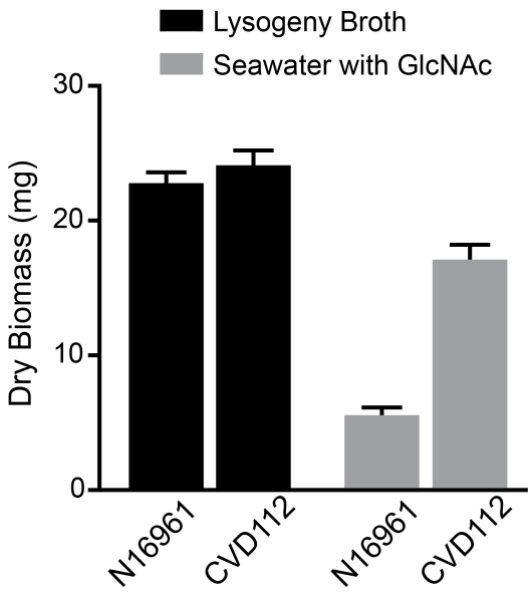


Figure S2. **Dry biomass of 20mL shaken liquid cultures of *V. cholerae* N16961 and CVD112**, grown overnight in LB (in which CVD112 is not filamentous) and artificial seawater with 0.5% GlcNAc (in which CVD112 produces filaments). Measurements were taken after 16 h of growth. n = 3 biological replicates; error bars denote the SEM

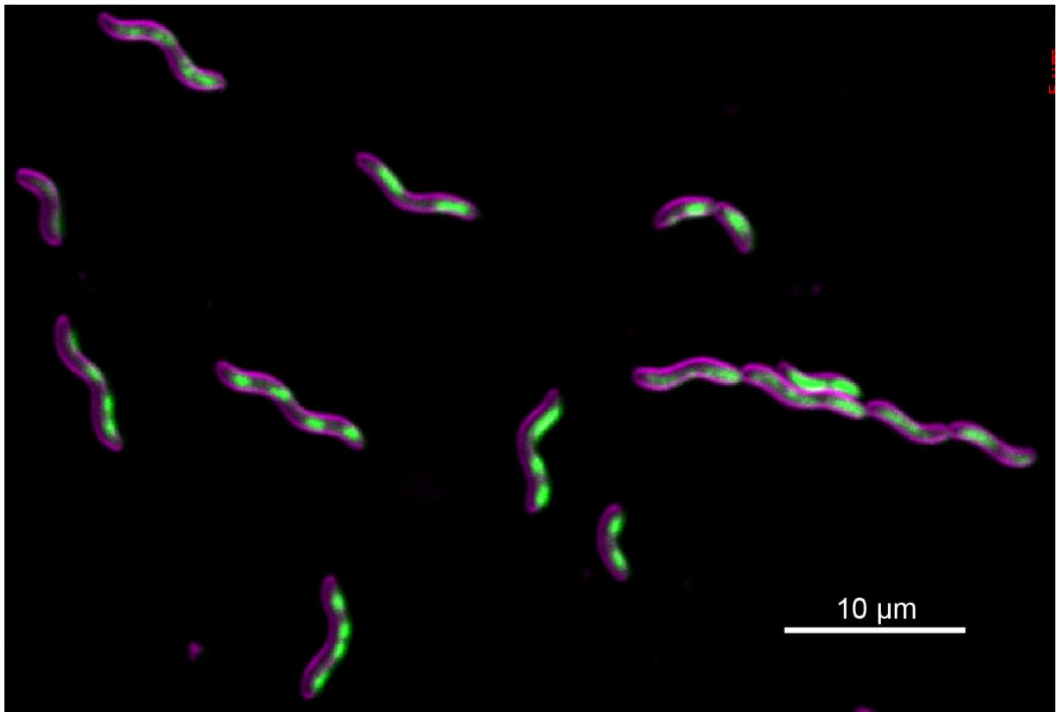


Figure S3. A filamentous cell of *V. cholerae* CVD112, after staining nucleoids with Syto9 (green) and membrane with FM4-64 (magenta). CVD112 cultures were grown overnight in artificial seawater with 0.5% GlcNAc. Syto9 and FM4-64 were then added to an aliquot of this culture, spotted onto a coverslip under an agar pad prior to imaging.

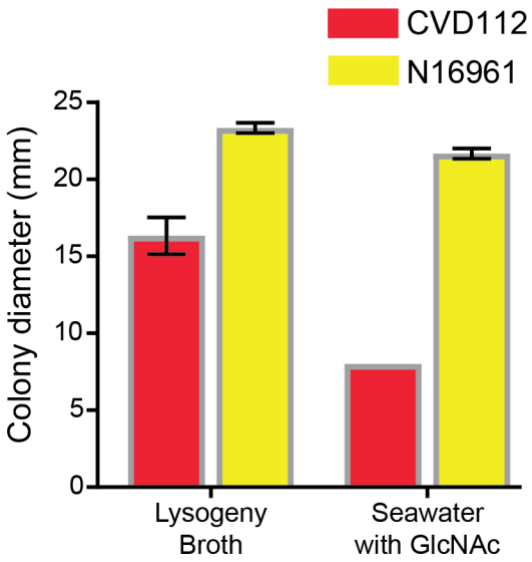


Figure S4. Low agar concentration plate motility assays for *V. cholerae* CVD112 and N16961. Swim motility plates were comprised of 0.3% agar and either LB, or seawater with 0.5% GlcNAc. Agar was inoculated via pipette tip stabbed into the center of the plate. Swim distance was measured as the diameter of colonies after 16 h incubation at room temperature. *n* = 5 biological replicates and error bars denote the SEM.

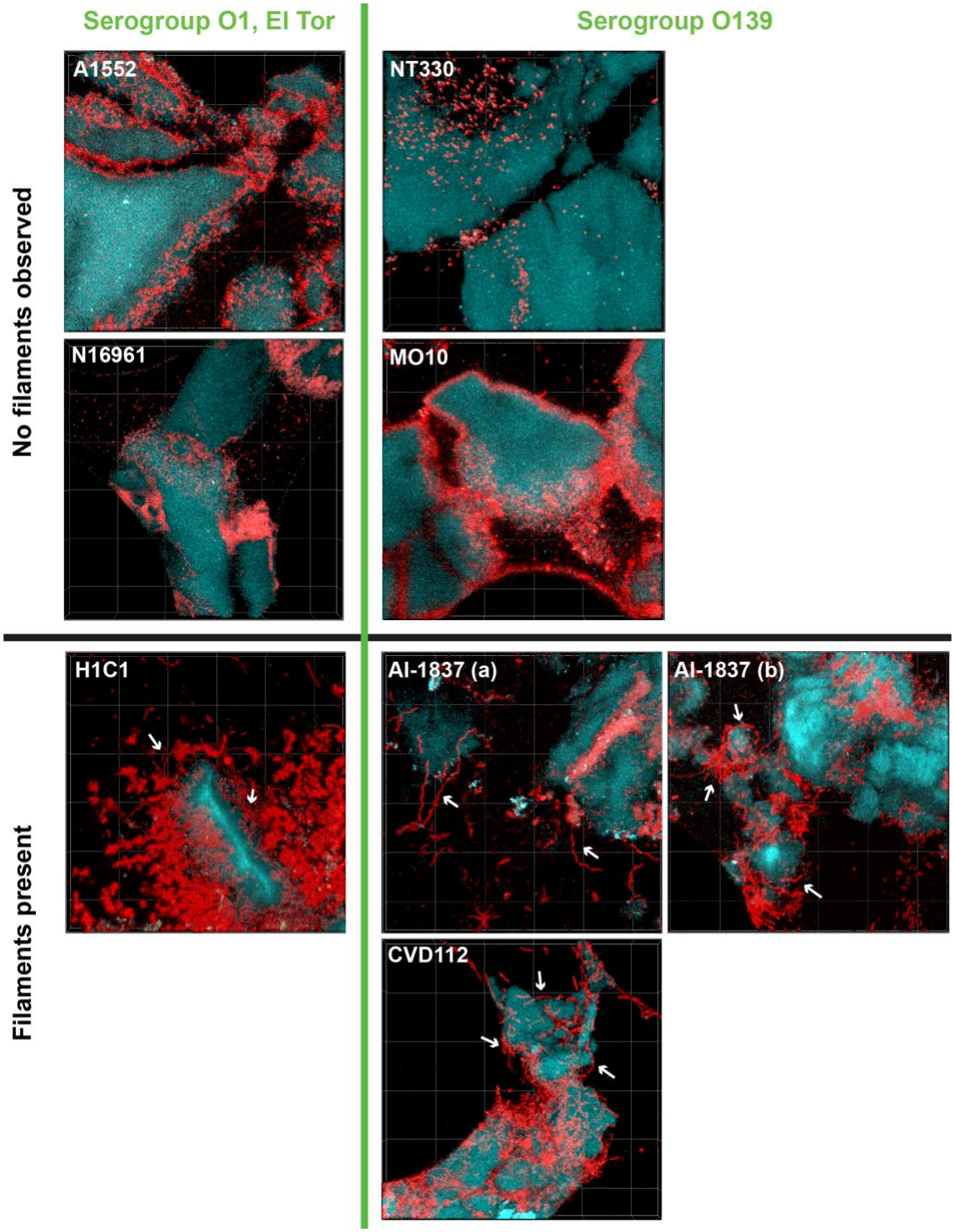


Figure S5. Representative images of different O1-El Tor and O139 (red) growing biofilms on chitin (blue) in artificial sea water. Images to the left of the green line are O1-El Tor isolates, while images to the right of the green line are O139 isolates. Above the black line are strains for which we did not observe subpopulations of filamentous cells. Below the black line are strains for which we did observe filamentous subpopulations. CVD112 was the focal filamenting strain of the study – it is an avirulent derivative of AI-1937, for which there are two images illustrating sparse (AI1837-a) and dense (AI1837-b) filamentous biofilms. All 3-D renders are 110x110x6μm [LxWxD].

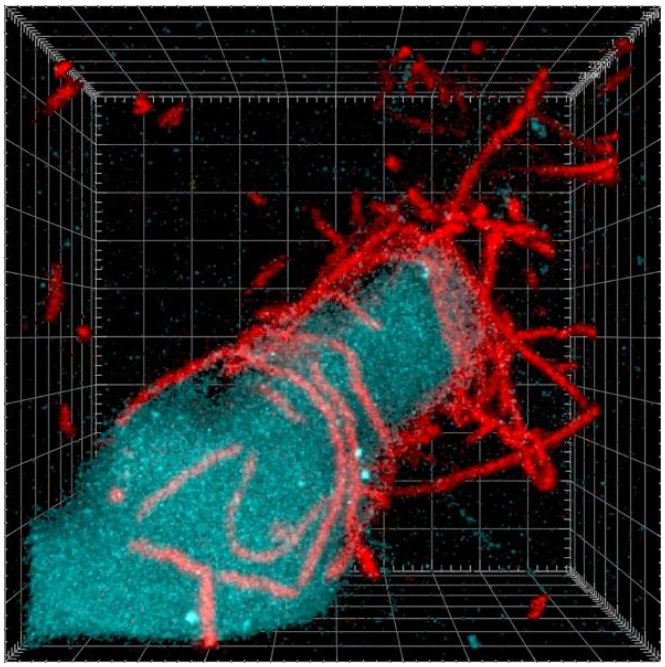


Figure S6. **Filamentous cells of *V. cholerae* CVD112 (red) wrapped around the contours of a chitin particle (blue) in artificial sea water.** This 3-D rendering is 35x35x60μm [LxWxD].

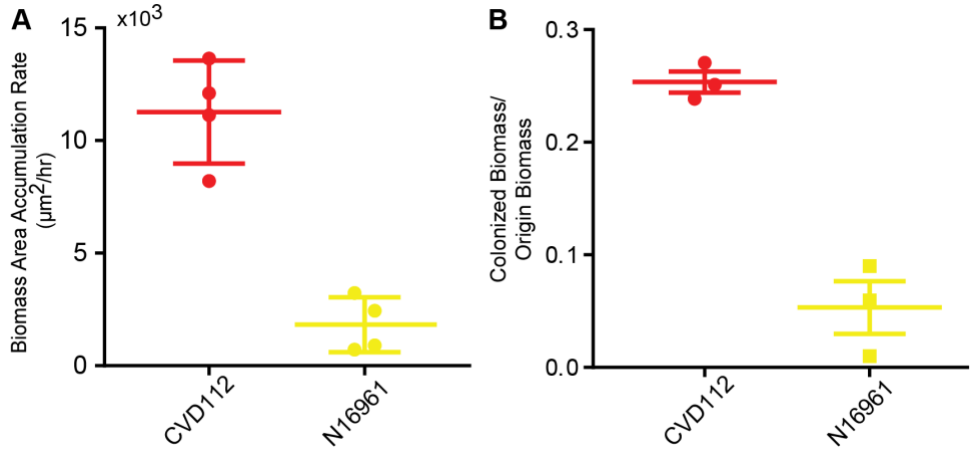


Figure S7. Chitin colonization rates of filamentous *V. cholerae* CVD112 and non-filamentous N16961. (**A**) CVD112 and N16961 were flowed in monoculture into chambers containing fresh chitin particles, and their biomass accumulation rates (biomass area per hour) were measured (n = 4 biological replicates, error bars denote the SEM). (**B**) Chambers containing monoculture biofilms of CVD112 or N16961 growing on chitin particles were allowed to grow for 48 h, after which the effluent from these chambers was connected to the fluid inlet of chambers containing fresh chitin for 1 h (n = 3 biological replicates, error bars denote the SEM). The total biomass area of cells colonized on the fresh chitin particles was measured and normalized to the biomass of cells present in the previous chamber from which effluent was collected for the colonization experiment.

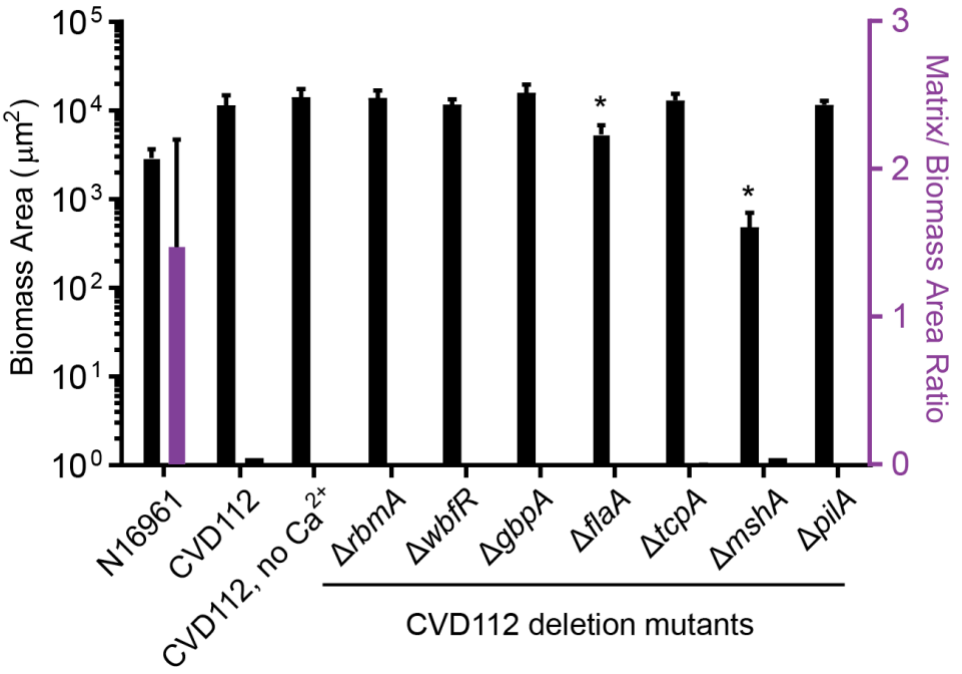


Figure S8. **Filamentous biofilms of CVD112 were grown on chitin, with the parental wild type shown at left for comparison.** Biofilm biomass (black bars) and matrix production (purple) were quantified after 48 h of growth at room temperature on chitin particles in artificial sea water lacking other sources of carbon or nitrogen. *n* = 5 replicates for all data groups, and error bars denote SEM. Note that little or no RbmA matrix stain was detectable for any CVD112 strain on chitin, hence the apparent absence of purple bars for those strains. The biomass of CVD112 in seawater without $^{Ca2+}$, and the deletion mutants of CVD112, were compared to the CVD112 parental strain's biomass using Wilcoxon signed-ranks tests with a Bonferroni correction for 8 pairwise comparisons. Asterisks indicate statistical significance at $p < 0.05$; all other comparison tests indicated no significant differences to the CVD112 parental strain.

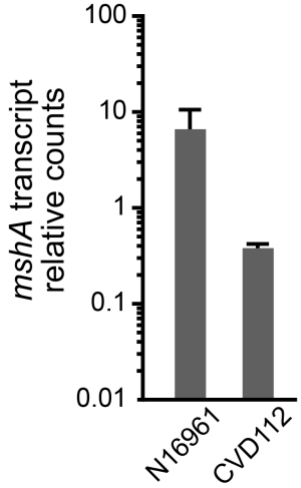


Figure S9. Relative transcript counts for the *mshA* locus encoding MSHA pili. RNA was extracted from 48-h shaken liquid cultures of N16961 and CVD112, grown in artificial seawater with chitin flakes as the sole course of carbon and nitrogen. Transcript counts for *mshA* were normalized to those of the *hfq* housekeeping locus. *n* = 4 replicates and error bards denote the SEM.

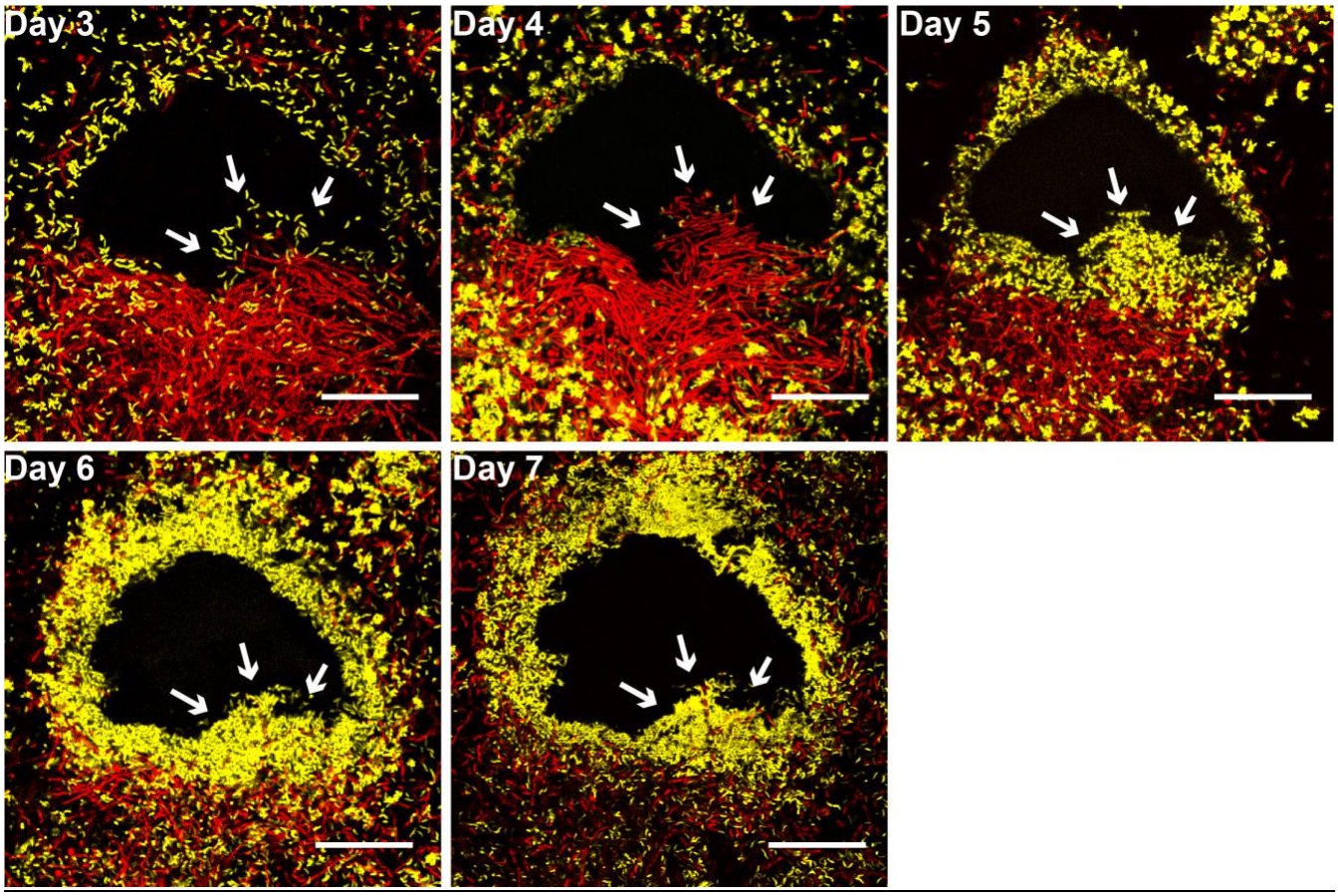


Figure S10. Filamentous biofilms of CVD112 (red) can be invaded and physically displaced by cells of N16961 (yellow). The central region of each panel is the space occupied by a chitin particle, whose color here is not shown in order to aid visualization of the two strains in competition. Filamentous meshes of CVD112 can be found occupying the lower surface of this particle (indicated by arrows) in the early period of this competition experiment. Over time, the more compact, adhesive, and matrix-replete biofilm clusters of N16961 displace CVD112 on the chitin surface. Scale bars denote 20 μm.

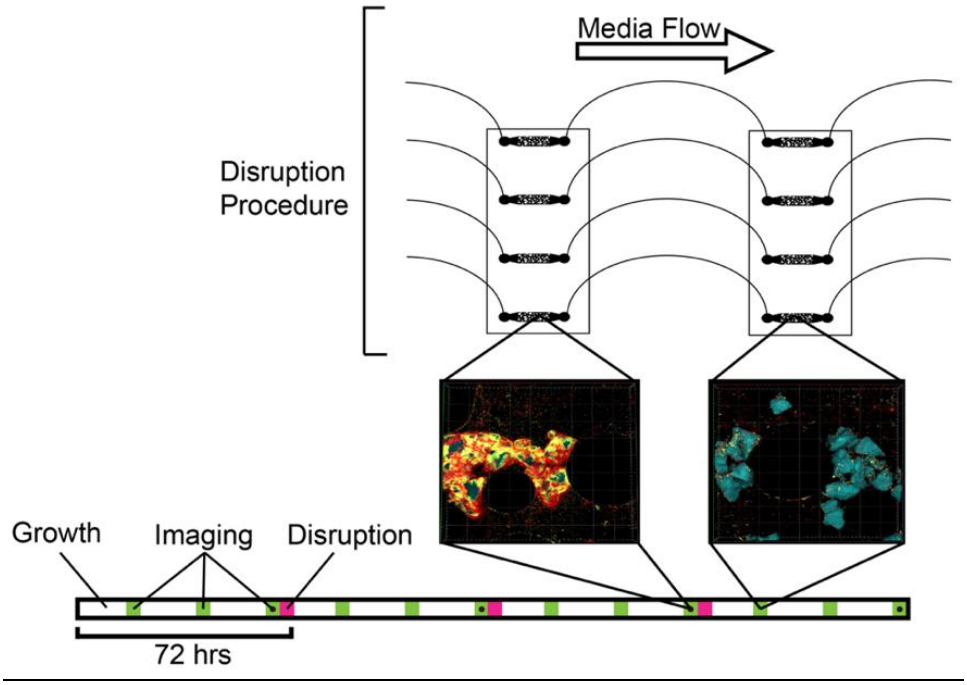


Figure S11. An illustration of the regime for competition experiments with disturbance/recolonization. *V. cholerae* CVD112 and N16961 were introduced in co-culture to microfluidic devices containing chitin and imaged daily (imaging points are indicated by green square events in the timeline). Every 72 hours (magenta square events), the effluent from the current chamber was used to inoculate a new chamber containing fresh chitin, which was then monitored daily until the next disturbance event. In another treatment (see Figure 3, main text), disturbance/recolonization events were performed every 24 hours.

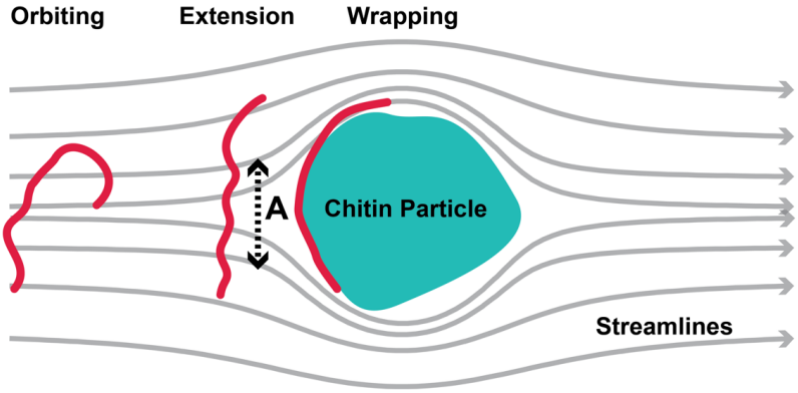


Figure S12. A fluid dynamics model for cell filament stretching in flow near chitin particles. The flow profile near the surface of a cylinder can be described as an extensional flow. This description is most accurate close the particle surface at the stagnation point (where streamlines split on either side of the particle, point A above). Elastic filaments such as polymers experiencing this extensional flow align in the direction of extension. We therefore anticipate that single filamentous *V. cholerae* cells align with approaching chitin particles in flow. In addition, extended cells span either side of the stagnation point, effectively increasing their residence time near the particle, while short cells ballistically follow streamlines. Altogether, stretching and increased residence time may participate to the improved ability of filamentous cells to encounter and attach to chitin particles.

Supplementary Table 1: *V. cholerae* strains and plasmids

Strain	Relevant markers/ genotypes	Source	
E. coli			
S17-1	λpir	(10)	
V. cholerae			
CNV180	*V. cholerae* CVD112, wild type (avirulent deriv. of AI-1837)	(11)	*V. cholerae* Serogroup O139
CNV184	CVD112 *lacZ*:P_{tac}-*mKate2*	This study	
CNV229	CVD112 *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV205	CVD112 Δ*vpsL*, *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV206	CVD112 Δ*wbfR*, *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV207	CVD112 Δ*rbmA lacZ*:P_{tac}-*mKate2*	This study	
CNV239	CVD112 Δ*pilA rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV240	CVD112 Δ*tcpA*, *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV241	CVD112 Δ*mshA*, *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV242	CVD112 Δ*gbpA*, *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV243	CVD112 Δ*flaA*, *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV246	V. cholerae AI-1837, wild type	Joachim Reidl Group	
CNV247	AI-1837 *lacZ*:P_{tac}-*mKate2* (*luxO* frameshift corrected)	This study	
CNV208	V. cholerae NT330, wild type	Karen Skorupsi Group	
CNV244	NT330 *lacZ*:Ptac-mKate2	This study	
CNV209	V. cholerae M010, wild type	Matt Waldor Group	
CNV245	MO10 *lacZ*:Ptac-mKate2	This study	
CNV049	*V. cholerae* N16961, wild type	(12)	*V. cholerae* Serogroup O1, El Tor
CNV116	N16961 *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV121	N16961 *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKO-κ*	This study	
CNV203	N16961 Δ*vpsL*, *rbmA*-3xFLAG, *lacZ*:P_{tac}-*mKate2*	This study	
CNV185	*V. cholerae* H1C1, wild type	Jesse Shapiro Group	
CNV224	H1C1 *lacZ*:P_{tac}-*mKate2*	This study	
CNV219	*V. cholerae* A1552, wild type	Fitnat Yildiz Group	
CNV236	A1552 *lacZ*:P_{tac}-*mKate2*	This study	

Plasmid	origin, marker	comments	templates, primers	source
pCN251	pR6K, Amp, Kan	pKAS32		(1)
pCN768	pR6K, Amp, Kan	pBW1 for insertion of *lacZ*:P_{tac}-*mKate2* with SacB counterselection	cno 437-440; pCN 765	This study
pCN764	pR6K, Amp, Kan	PKAS32 for insertion of *lacZ*:P_{tac}-*mKO-κ*	cno233-236	This study
pCN765	pR6K, Amp, Kan	pKAS32 for insertion of *lacZ*:P_{tac}-*mKate2*	cno233-236	This study
pCN006	pR6K, Amp	pKAS32 Δ*vpsL*		C. M. Waters, Michigan State University, East Lansing, MI
pCN778	pR6K, Amp	pBW Δ*rbmA*	cno 459-464	This study
pCN769	pR6K, Amp	pBW *rbmA*-3xFLAG insertion	cno 437-440; pCN189 (3)	This study
pCN784	pR6K, Amp, Kan	pBW Δ*vpsL*	cno 465-470	This study
pCN791	pR6K, Amp, Kan	pBW Δ*wbfR*	cno 302-305	This study
pCN792	pR6K, Amp, Kan	pBW Δ*gbpA*	cno 523-526	This study
pCN793	pR6K, Amp, Kan	pBW Δ*tcpA*	cno 527-530	This study
pCN794	pR6K, Amp, Kan	pBW Δ*pilA*	cno 531-534	This study
pCN795	pR6K, Amp, Kan	pBW Δ*mshA*	cno 535-538	This study
pCN800	pR6K, Amp, Kan	pBW Δ*flaA*	cno 557-560	This study

Primer Name	Sequence	Description
cno211	CCGTTGATTTAAATAATGGCCGC	Forward sequencing primer for checking 3xFLAG insertion to *rbmA*
cno212	CAGTGAGTGAATGGAAGAAGAATAGA	Reverse sequencing primer for checking 3xFLAG insertion to *rbmA*
cno233	GCTCCGGACACCAATGGATCCTTTGACAA	Forward primer for insertion of *mKO-κ* or *mKate2* into KAS32
cno234	CGTGTACATTGAATTCTTTTCTAGAAAGGAGCTC	Reverse primer for insertion of *mKO-κ* or *mKate2* into pKAS32

cno235	GCTGTACAAGAGGCCTGCTAGAGCTA	Forward primer for backbone amplification of pCN251
cno236	CGTCCGGAGACTCTGGATCCCCGG	Reverse primer for backbone amplification of pCN251
cno237	GGTGGTTAATAAGTGAGTCTCAAGG	Forward primer for checking deletion of *vpsL* in *V. cholerae*
cno238	GCGAAAACGGTATCCCAATTG	Reverse primer for checking deletion of *vpsL* in *V. cholerae*
cno239	CGTATTGCGGGCTATCAAC	Forward primer for *lacZ* insertion check
cno240	GGGAAGGGTAAAACCGAGA	Reverse primer for *lacZ* insertion check
cno241	TGGCTCTTGCTTTGGCAAC	Upstream sequencing primer for *mKO-κ* and *mKate2*
cno244	GGTCATCAAGAAATGACCCTG	Sequencing primer 1 for *mKO-κ* insert
cno245	ACCTATAAAGCGGCGAAAGA	Sequencing primer 2 for *mKO-κ* insert
cno246	CACTGCCATTTGCGTTTGA	Sequencing primer 1 for *mKate2* insert
cno247	CTATGTGGAACAACATGAAGTGG	Sequencing primer 2 for *mKate2* insert
cno437	ggcaggtatatgtggtcgacgtcccatggagat	Reverse amplification of pCN 38 for replacing *rpsL* with sacB
cno438	ggacgtcgaccacatatacctgccgttcactat	forward amplification of sacB for instertion into pCN38
cno439	tacctcaggtcgacttatttgttaactgttaattgtccttgttcaag	reverse amplification of sacB for instertion into pCN38
cno440	ggacaattaacagttaacaaataagtcgacctgaggtaattataaccc	forward amplification of pCN 38 for replacing *rpsL* with sacB
cno441	acaaataggggttccgcg	sequencing primer for SacB replacement of *rpsL* on pCN38
cno442	actcaagcgtttgcgaaag	sequencing primer for SacB replacement of *rpsL* on pCN38

cno459	gtccaTTTCCTAGGAAAgttatccgctcacaattccacat	reverse primer for backbone amp of *rbmA* deletion gibson
cno460	aattgtgagcggataacTTTCCTAGGAAAtggacacaggcg	forward primer for upstream *rbmA* deletion gibson
cno461	tacatacgactaagtgactaggtaaatccatttgtttttacaactggcgc	reverse primer for upstream *rbmA* deletion gibson
cno462	ttgtaaaaacaaatggatttacctagtcacttagtcgtatgtataaaaaacc	forward primer for downstream *rbmA* deletion gibson
cno463	gcgcgcgatcaaaggtaccttagtggttcaaaatcaatt	reverse primer for downstream *rbmA* deletion gibson
cno464	ctaaggtacctttgatcgcgcgcagatct	forward primer for backbone amp of *rbmA* deletion gibson
cno465	CGGCAGGTATgttatccgctcacaattccacat	reverse primer for backbone amp of *vpsL* deletion gibson
cno466	ttgtgagcggataacATACCTGCCGCGGTTG	forward primer for upstream *vpsL* deletion gibson
cno467	AGTGCCCTGTCTAGACGCTCCTAACCGATGTAAGAT	reverse primer for upstream *vpsL* deletion gibson
cno468	TAGGAGCGTCTAGACAGGGCACTTGCGTC	forward primer for downstream *vpsL* deletion gibson
cno469	gcgcgatcCGCGTCACCATCGCC	reverse primer for downstream *vpsL* deletion gibson
cno470	TGGTGACGCGgatcgcgcgcagatct	forward primer for backbone amp of *vpsL* deletion gibson
cno519	aattgtgagcggataacctgtttgtttttgtttttataataagctatccct	upstream for gibson amp of *wbfR* deletion into pBW1
cno520	attataaaaacaaaaacaaacaggttatccgctcacaattccacat	downstream backbone amp of pBW1 for *wbfR* deletion
cno521	gcgcgatcccggttctggtaagtaaggataaacc	downstream for gibson amp of *wbfR* deletion into pBW1
cno522	cttaccagaaccgggatcgcgcgcagatct	upstream backbone amp of pBW1 for *wbfR* deletion
cno523	gagcggataacCGCATTCGTCGATAAAAGTGTTC	upstream for gibson amp of *gbpA* deletion into pBW1

cno524	TTTATCGACGAATGCGgttatccgctcacaattccacat	downstream backbone amp of pBW1 for *gbpA* deletion
cno525	cgcgcgatcCGGTGCTCAATAATTCATTACCCAT	downstream for gibson amp of *gbpA* deletion into pBW1
cno526	ATTATTGAGCACCGgatcgcgcgcagatctg	upstream backbone amp of pBW1 for *gbpA* deletion
cno527	tgtgagcggataacCTCACAAAGTAATGCTAGCCGTG	upstream for gibson amp of *tcpA* deletion into pBW1
cno528	GCATTACTTTGTGAGgttatccgctcacaattccacatg	downstream backbone amp of pBW1 for *tcpA* deletion
cno529	gcgcgatcCATCACGTTGCATTGCTATTTCTCC	downstream for gibson amp of *tcpA* deletion into pBW1
cno530	CAATGCAACGTGATGgatcgcgcgcagatctg	upstream backbone amp of pBW1 for *tcpA* deletion
cno531	tgagcggataacTGGCATGTGCAAGATGGTGA	upstream for gibson amp of *pilA* deletion into pBW1
cno532	TGCACATGCCAgttatccgctcacaattccacatg	downstream backbone amp of pBW1 for *pilA* deletion
cno533	tgcgcgcgatcGAATTTTAATCCGCGCAGCTAAAC	downstream for gibson amp of *pilA* deletion into pBW1
cno534	CGGATTAAAATTCgatcgcgcgcagatctg	upstream backbone amp of pBW1 for *pilA* deletion
cno535	agcggataacCGTTACGTAAGTGAGTTGAAAAATATGAAGA	upstream for gibson amp of *mshA* deletion into pBW1
cno536	CAACTCACTTACGTAACGgttatccgctcacaattccacatg	downstream backbone amp of pBW1 for *mshA* deletion
cno537	cgcgcgatcGATGATCATCTCAACCAAGGTAAAACCAC	downstream for gibson amp of *mshA* deletion into pBW1
cno538	GTTGAGATGATCATCgatcgcgcgcagatctg	upstream backbone amp of pBW1 for *mshA* deletion
cno555	AAGTATCAAACACACTAgttatccgctcacaattccacat	downstream backbone amp for pBW *flaA* backbone
cno556	TAGGTGTCGCCGTTCgatcgcgcgcagatctg	upstream backbone amp for pBW *flaA* backbone

cno557	attgtgagcggataacTAGTGTGTTTGATACTTTCCGTGATGC	upstream amp of upstream *flaA* deletion pBW fragment
cno558	TGAAGGTACCGTGAAAGTTTGCTCTCCTATCGAGTTCG	downstream amp of upstream *flaA* deletion pBW fragment
cno559	ATAGGAGAGCAAACTTTCACGGTACCTTCATTAATGAGCT	upstream amp of downstream *flaA* deletion pBW fragment
cno560	gcgcgcgatcGAACGGCGACACCTAAACCTC	downstream amp of downsteam *flaA* deletion pBW fragment
cno349	GCTGGTGCAATGGGTATCT	*mshA* fwd qPCR
cno350	ACCGTTGCAGGATTATTAGCGG	*mshA* rev qPCR
cno353	CGTGAACGTATCCTTGTTTCT	*hfq* fwd qPCR
cno354	CTTCAGACTTCTCTGCTGGAC	*hfq* rev qPCR

Table S2. Whole-genome sequencing of O1 and O139 *V. cholerae* strains

**Note: The full Table S2 content is formatted as a separate Excel file for clarity. Below is a list of contents:

S2A	Strains analyzed by whole-genome sequencing, the reference genomes used for mapping and the respective accession numbers for sequencing reads and references
S2B	List of undetectable or highly variable genomic regions in the sequenced isolates compared to the respective reference genomes
S2C	Cross-comparison of mutations between CDV112, its parent AI-1837 and non-filamenting NT330
S2D	Mutations detected in the sequenced N16961 isolate compared to the reference genome
S2E	Mutations detected in the sequenced A1552 isolate compared to the reference genome
S2F	Mutations detected in the sequenced H1C1 isolate compared to the reference genome
S2G	Mutations detected in the sequenced MO10 isolate compared to the reference genome
S2H	Mutations detected in the sequenced NT330 isolate compared to the reference genome
S2I	Mutations detected in the sequenced AI-1837 isolate compared to the reference genome
S2J	Mutations detected in the sequenced CVD112 isolate compared to the reference genome

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