Supplemental Material

Materials and Methods

Zebrafish studies. Wild type zebrafish (6-9 month old) were used for intestinal colonization and bacterial transmission experiments as described in a previous study with the following modifications (1). For euthanasia of zebrafish, the fish were placed in 100 mL of 168 µg/mL Tricaine solution (ethyl 3-aminobenzoate methane sulfonate salt; Sigma-Aldrich A5040) and fish remained in the solution for at least 25 min.

Inoculation of zebrafish via immersion. The wild-type and Δ*vchC* of *V. cholerae* Δ*vchC* 16961 were grown with aeration in LB broth at 37 °C for 16-18 h. Bacterial cells were subsequently washed once and then diluted to the correct concentration, both in sterile PBS. Bacterial cell densities ranged from 10^7 to 10^{10} per beaker (~5 x 10^4 to 5 x 10^7 cfu/mL). A group of 4-6 zebrafish was placed into a 400 mL beaker with a perforated lid containing 200 mL of sterile tank water. Bacterial inoculum (1 mL) was then added to the beaker with fish. Each beaker was placed into a glass-front incubator set at 28 °C for the duration of the experiment.

Intestinal colonization experiments. At designated time points, fish were removed from the beaker and euthanized as described above. Intestines were aseptically removed, placed into sterile beadbeater tubes (2.0 mL screw-cap vials with O-rings filled half-way with 1.0 mm glass beads) containing 1.0 mL of sterile PBS and homogenized using the Mini-Beadbeater-24 (BioSpec Products, Inc.). Serial

dilutions of the homogenate were plated on LB agar containing streptomycin and 40 μg/mL X-gal for enumeration.

Transmission experiments. A group of 4-6 zebrafish, anesthetized and fin-clipped for later identification, was placed in a 600 mL beaker with 300 mL tank water. A second group of 4-6 zebrafish was exposed to 10^7-10^9 *V. cholerae* in 200 ml tank water as described above. After 2-3 h, the exposed fish were moved to another beaker of fresh tank water twice to remove external *V. cholerae*. These infected fish were then placed in the beaker holding the fin-clipped naive zebrafish. After 24 h all of the fish were sacrificed and intestinal *V. cholerae* were enumerated as described above.

Infant mouse studies. Five-day-old CD-1 mouse pups were used for intestinal colonization assays. The pups were inoculated by gavage with 50 μL *V. cholerae* suspension containing approximately 10^6 total bacteria. Infected pups were then incubated at 30˚ C for 20 h. The mice were euthanized by decapitation, intestines were dissected aseptically and placed into sterile beadbeater tubes (2.0 mL screw-cap vials with O-rings filled half-way with 1.0 mm glass beads) containing 1.0 mL of sterile LB and homogenized using the Mini-Beadbeater-24 (BioSpec Products, Inc.). Serial dilutions of the intestinal homogenates were plated on LB agar containing 10 μg/mL streptomycin and 20 μg/ml X-Gal and incubated at 37 ˚ C for 24 hours before enumeration of bacterial colonies.

Ethics statement. All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal work was conducted according to the

relevant guidelines of the Public Health Service, Office of Laboratory Animal Welfare, Animal Assurance no. A3310-01, and was approved by the Wayne State University IACUC, protocol number A 01-14-10.

TABLES

Table S1. Strains and plasmids used in this study

Strain/plasmid	Description/relevant genotype[1]	Reference/source
***V. cholerae* strains**		
N16961	Wild-type El Tor O1 biotype, Sm^R	Laboratory collection
NΔ*eps*	N16961 Δ*epsC-N*, Cm^R	(2)
Δ*vchC*	N16961 Δ*vchC::cat*, Cm^R	This study
***E. coli* strains**		
MC1061	F^- *araD139* Δ(*ara-leu*)*7697* Δ(*lac*)*X74* *rpsL hsdR2 mcrA mcrB1*	(3)
MM294 (pRK2013)	Donor of transfer function for triparental conjugation	(4)
Plasmids		
pMMB67-EH	Expression vector, P_{TAC} promoter, Amp^R	(5)
pKD3	Expression vector, FRT-*cat*-FRT, R6K promoter, Amp^R, Cm^R	(6)
pPCR-Script	Cloning vector	Stratagene
pCVD442	*ori R6K mobRP4 sacB*, Amp^R	(7)
pPCR-Script-VchC	*vchC* cloned into pCRScript, Amp^R	This study
pPCR-*vchC::cat*	pPCR-Script-VchC containing *cat* cassette inserted into *vchC* gene	This study

p∆*vchC*	*vchC::cat* from cloned into pCVD442	This study
pVchC	pMMB67-EH carrying P_{TAC}-VchC	This study
pVchC-His	pMMB67-EH carrying recombinant VchC with C-terminal 6×His cloned under P_{TAC} control	This study
pVchC-H435A	pMMB67-EH carrying P_{TAC}-VchC-H435A	This study
pVchC-E436A	pMMB67-EH carrying P_{TAC}-VchC-E436A	This study
pVchC-H439A	pMMB67-EH carrying P_{TAC}-VchC-E439A	This study

[1]Sm^R, streptomycin resistant; Amp^R, ampicillin resistant; Cm^R, chloramphenicol resistant

Table S2. Oligonucleotide primers designed and used in this study

Name	Sequence (5' to 3')
Primers for cloning wild-type alleles[1]:	
VchCF	GAATTCCATCATAAATAGGTTTTGCAGTGGTC
VchCR	CTGCAGTCAGTCGAAATAGGCCACCAT
rVchCF	GAGCTCGAATTCCATCATAAATAGGTTTTGCAGTGTC
rVchCR	CTGCAGTCTAGACACCACCACCACCACCACTAAGAAGTCGAAA TAGGCCACCATTT
Primers for creating N∆*vchC* knockout strain:	
CmF	CATATGCCATGGTGTGTAGGCTGGAGCTGCTT
CmR	CATATGCCATGGCATATGAATATCCTCCTTAG
Primers for site-directed mutagenesis:	
H435F	ATTTGTCGATTCTCAATTTAGAGGCTGAGTACACTCATTATCTG

	GACG
H435R	CGTCCAGATAATGAGTGTACTCAGCCTCTAAATTGAGAATCGAC AAAT
E436F	GATTCTCAATTTAGAGCATGCGTACACTCATTATCTGGACG
E436R	CGTCCAGATAATGAGTGTACGCATGCTCTAAATTGAGAATC
H439F	TTTAGAGCATGAGTACACTGCTTATCTGGACGCGCGCTTC
H439R	GAAGCGCGCGTCCAGATAAGCAGTGTACTCATGCTCTAAA

[1]F, forward; R, reverse
[2]underlined nucleotides refer to restriction enzyme sites

Figures

Supplemental Figure 1.

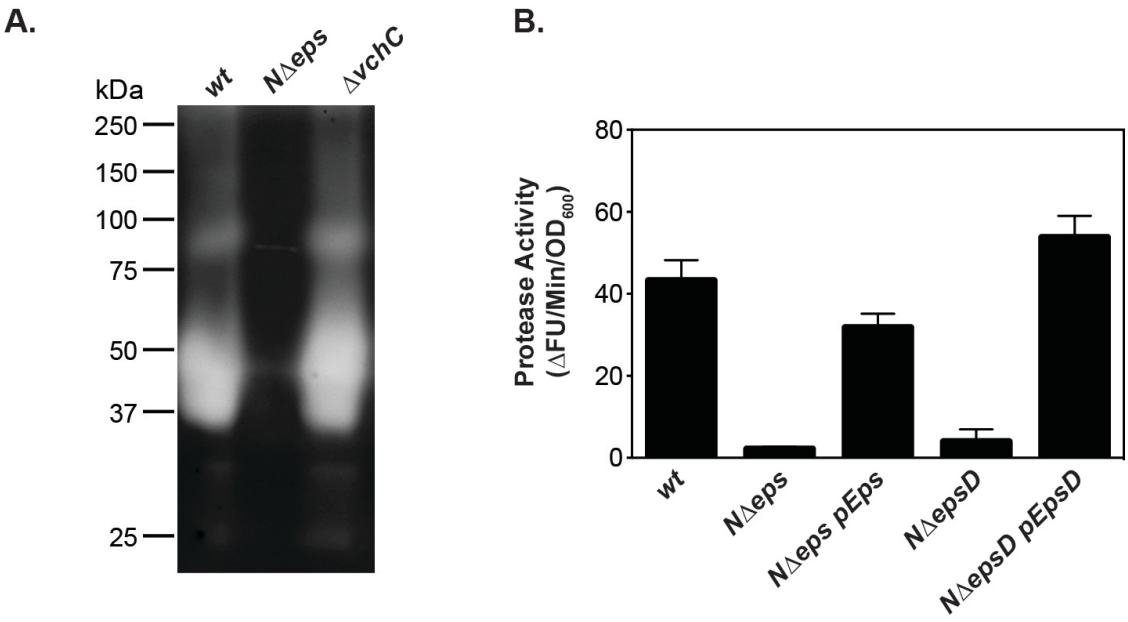
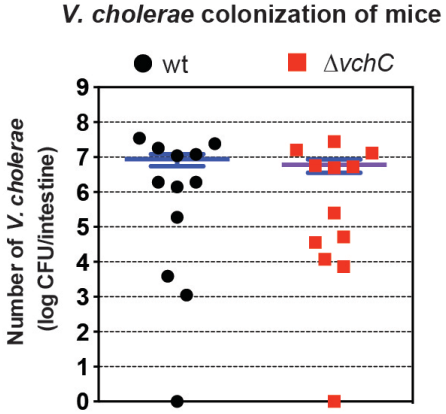


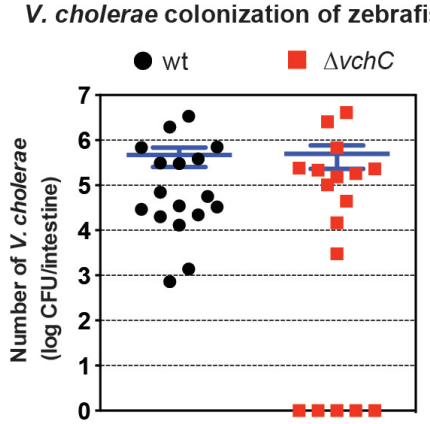
Figure S1. The extracellular collagenolytic/gelatinolytic activity is associated with a functional T2SS. (A) Detection of extracellular collagenolytic/gelatinolytic activity was performed by separating supernatants harvested at late stationary phase of growth (16 h) from wt *V. cholerae* N16961 and isogenic mutants NΔ*eps* as well as Δ*vchC* in 10% Tris-glycine gel copolymerized with 0.1% gelatin. Samples were matched by equivalent OD_{600} units. (B) The proteolytic activity against DQ gelatin was examined in supernatants isolated from late stationary cultures of wt *V. cholerae* N16961, isogenic T2S mutants: NΔ*eps* and NΔ*epsD*, as well as complemented strains NΔ*eps* pEps and NΔ*epsD* pEpsD. The mean ± SEM are presented (*n*=4).

Supplemental Figure 2.

A.



B.



C.

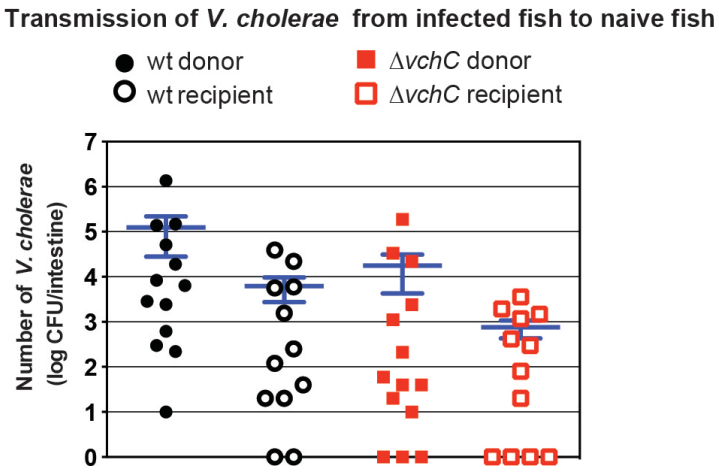
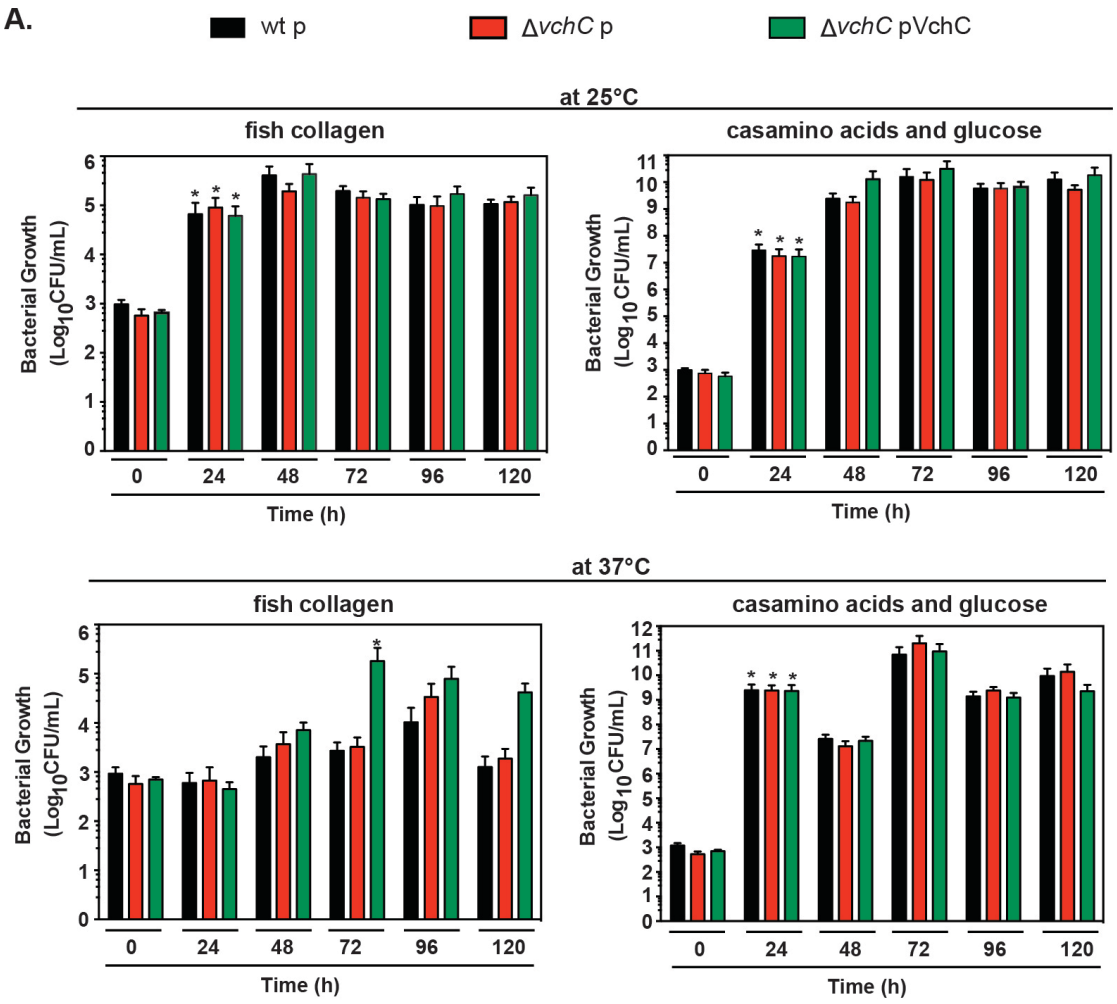


Figure S2. The lack of VchC does not significantly contribute to the *V. cholerae* N16961 ability to colonize mouse (A) and zebra fish (B) intestine nor to the transmission between infected and naive fish (C). (A) To study the impact of VchC on *V. cholerae* intestinal colonization in the mammalian host, an infant mouse model has been utilized. In the biological replicate experiments, groups of six CD-1 mouse pups were separately inoculated with either the wt N16961 strain or the isogenic Δ*vchC* mutant (10^6 of bacteria). Following 20 h, the mice were euthanized, their intestines were homogenized and the bacteria were recovered by plating the homogenates on LB agar containing streptomycin and X-gal. **(B, C)** A group of 4-6 wild type zebra fish was placed into a beaker followed by the addition of either wt *V. cholerae* N16961 or isogenic Δ*vchC* (5 $\times 10^4$ - 5 $\times 10^7$ CFU/mL). In the intestinal colonization studies, fish were removed from the beakers after 24 h and euthanized. Homogenates of their intestines were plated on LB agar to enumerate bacterial loads. In the transmission experiments, a group of 4-6 fin-clipped zebrafish was used as naive fish whereas a second group of 4-6 zebrafish was exposed to 10^7-10^9 of *V. cholerae* (wt or Δ*vchC*). After removal of the external *V. cholerae*, the infected fish were placed into the dish containing the naive group. The fish were sacrificed after 24 h and the bacteria were enumerated as described above.

Supplemental Figure 3.



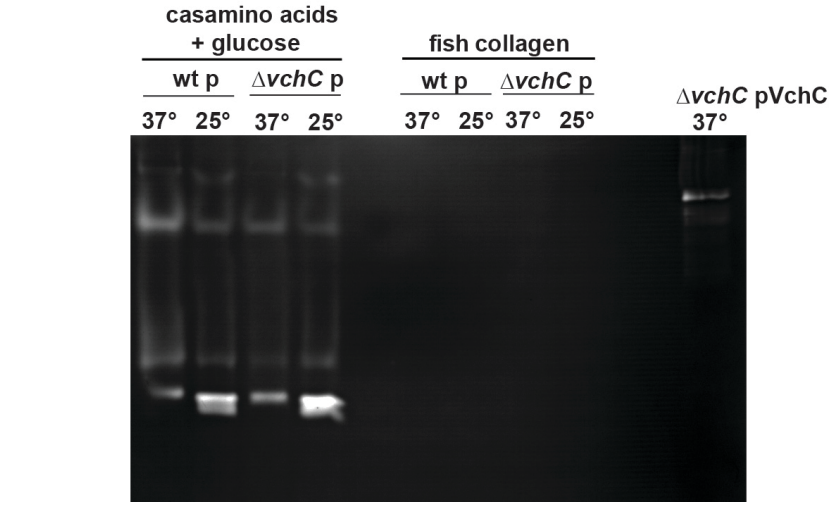


Figure S3. *V. cholerae* utilizes type I fish collagen as a source of nutrients for proliferation and growth. Suspensions (10^3 CFU/mL) of the parental wt p, Δ*vchC* p, and Δ*vchC* pVchC of *V. cholerae* N16961 were separately inoculated into M9 salts media supplemented with either 0.4% fish collagen or casamino acids and 0.4% glucose (as indicated). Bacterial cultures were maintained with aeration at either 25 °C or 37 °C and samples were withdrawn and plated onto LB agar every 24 h to assess CFU. Experiments were performed in biological quadruplicates and means with corresponding SEMs are presented. The statistical significance was assessed using Mann-Whitney test ($p<0.05$). **(B)** The supernatants were isolated from bacterial cultures at 48 h of growth, concentrated 40-fold using centrifugal 0.5-mL filter with a molecular weight cut-off of 10 kDa, and examined by *in-gel* zymography using 10% Tris-glycine gel copolymerized with 0.1% gelatin.

REFERENCES

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