Supporting Information

Liu *et al.* 10.1073/pnas.0802241105

SI Text

Strains, Plasmids, and Culture Conditions. All *V. cholerae* strains used in this study were derived from E1 Tor C6706 and were propagated in LB media containing appropriate antibiotics at 37°C, unless otherwise noted. AKI medium was used to induce virulence gene expression (1).

The *tcpA* promoter was fused to the *sh ble* gene, which had been amplified by PCR from pEM7/Zeo (Invitrogen), and cloned into the plasmid pCVD442 (2). The resulting construct was integrated into the *lacZ* locus within the chromosomes of various C6706-derivatived strains. The *aphA-lacZ* transcriptional reporter was constructed by cloning the *aphA* promoter sequence into a plasmid containing a promoterless *lacZ* and a pBBR322 origin (3). The construction details of the *hapR-lacZ* and *hapR-*K_m^r transcriptional fusions were described (4, 5). To construct FlgM-His_6 and FliA-His_6, *flgM* and *fliA* coding sequences were amplified with C-terminal His_6 tags by PCR and cloned into the plasmid pBAD24, which contains an arabinose-inducible promoter (3). Chromosomal in-frame deletions were constructed by cloning the two flanking regions of the target gene into the suicide vector pWM91, which also contains a *sacB* counterselectable gene. For each mutant, selection for double-cross-over homologous recombination as described (6) resulted in the desired in-frame deletion.

1. Iwanaga M, *et al.* (1986) Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. *Microbiol Immunol* 30:1075–1083.
2. Donnenberg MS, Kaper JB (1991) Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect Immun* 59:4310–4317.
3. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177:4121–4130.
4. Liu Z, Stirling FR, Zhu J (2007) Temporal quorum-sensing induction regulates *Vibrio cholerae* biofilm architecture. *Infect Immun* 75:122–126.
5. Liu Z, Hsiao A, Joelsson A, Zhu J (2006) The transcriptional regulator VqmA increases expression of the quorum-sensing activator HapR in *Vibrio cholerae*. *J Bacteriol* 188:2446–2453.
6. Metcalf WW, *et al.* (1996) Conditionally replicative and conjugative plasmids carrying lacZ alpha for cloning, mutagenesis, and allele replacement in bacteria. *Plasmid* 35:1–13.
7. Lenz DH, *et al.* (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* 118:69–82.
8. Miller MB, Skorupski K, Lenz DH, Taylor RK, Bassler BL (2002) Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell* 110:303–314.
9. Zhu J, *et al.* (2002) Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 99:3129–3134.
10. Gardel CL, Mekalanos JJ (1996) Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence factor expression. *Infect Immun* 64:2246–2255.
11. Miller, J. H (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).

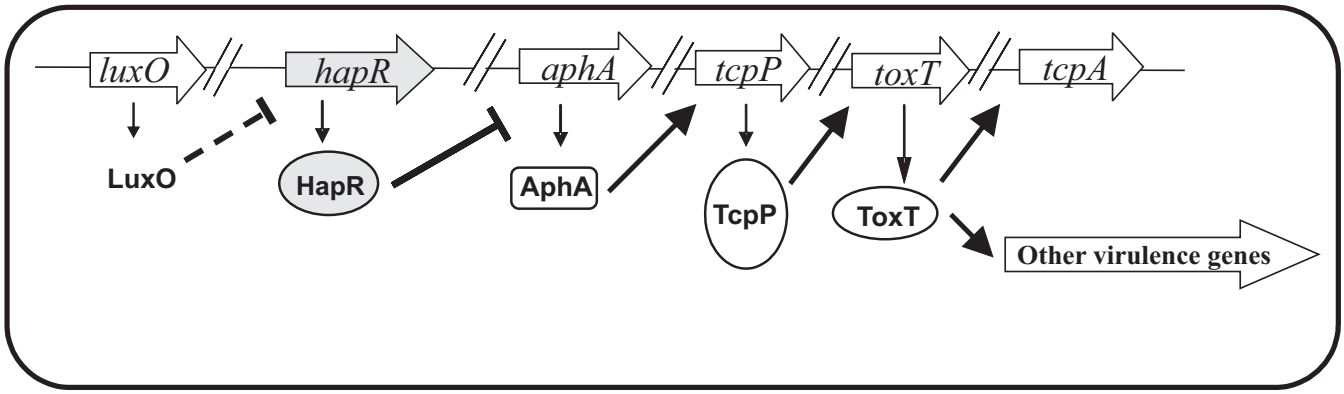


Fig. S1. Repression of *hapR* facilitates activation of a cascade of transcription factors that regulate the expression of virulence genes. HapR represses *aphA*, which encodes the transcription factor that up-regulates *tcpP* expression. The gene *tcpP* encodes the transcription factor TcpP, which in turn up-regulates *toxT* expression. ToxT regulates the expression of multiple virulence genes, including *tcpA*, which encodes the major TCP pilin. Also shown in the figure is LuxO, which is phosphorylated under conditions of low cell density. In its phosphorylated form, LuxO activates the expression of several small RNAs that repress *hapR* expression (7, 8). Thus, under conditions of low cell density, *hapR* expression is repressed, and virulence genes are transcribed.

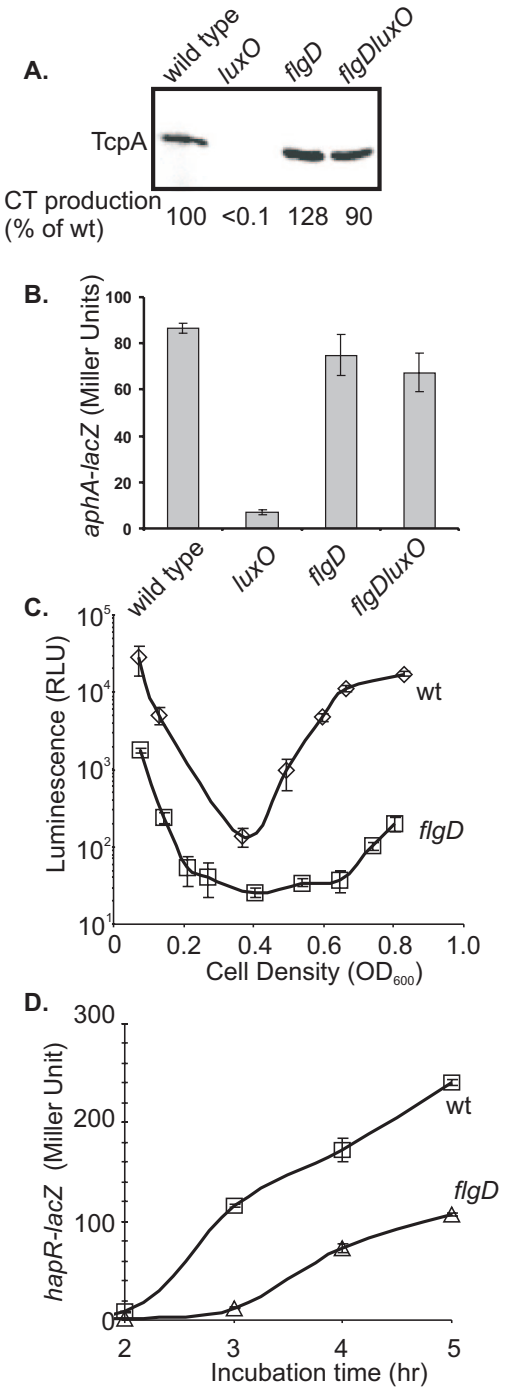


Fig. S2. The flagellar rod protein FlgD affects expression of virulence genes via quorum sensing. (*A*). The production of virulence factors in the *luxO* mutant is restored by a *flgD* mutation. Strains were grown in AKI medium (1), which induces virulence genes. TcpA production was assayed by Western blot analysis (9), and cholera toxin (CT) production was assayed by ELISA (10). All samples were normalized to contain 10^9 bacterial cells. (*B*). Expression of *aphA* in the *luxO* mutant is restored by mutation of *flgD*. Strains containing the *aphA-lacZ* transcriptional reporter plasmid were grown to midlog in AKI medium. β-Galactosidase activity was measured and reported as described (11). (*C*). Mutation of *flgD* attenuates regulation by quorum sensing. Strains harboring pBB1 from *V. harveyi* were diluted into fresh LB media and grown at 30°C. Luminescence and cell density were measured at various time points. Relative luminescence units (RLU) were calculated as light units/OD_{600} ml^{-1}. (*D*). Mutation of *flgD* reduces expression of *hapR*. Strains containing the *hapR-lacZ* chromosomal transcriptional fusion were diluted into LB and grown at 37°C. Samples were withdrawn at the time indicated and subjected to β-galactosidase assays. All results are means of three experiments $\pm$ standard deviations.

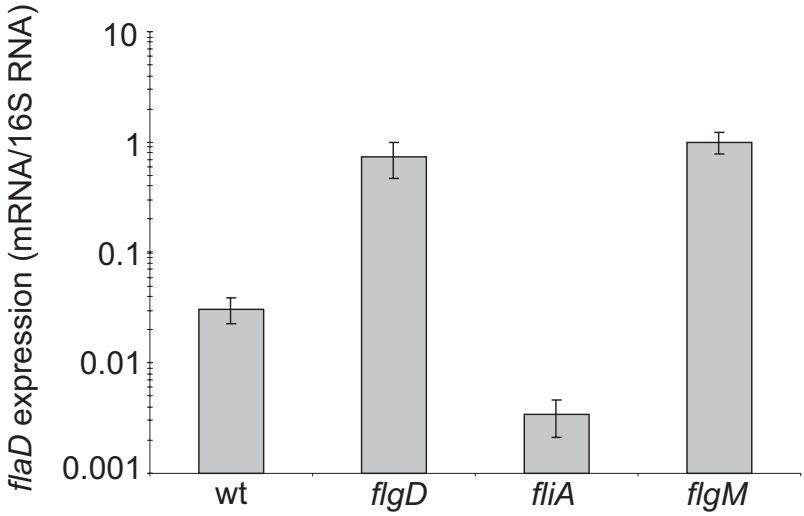


Fig. S3. Real-time RT-PCR analysis of *flaD* expression. RNA was extracted from wild-type and mutant strains grown to midlog phase in LB medium, and real-time RT-PCR was performed for *flaD* transcripts. Transcript levels were normalized by 16S RNA. Results are means of three experiments ± standard deviations.

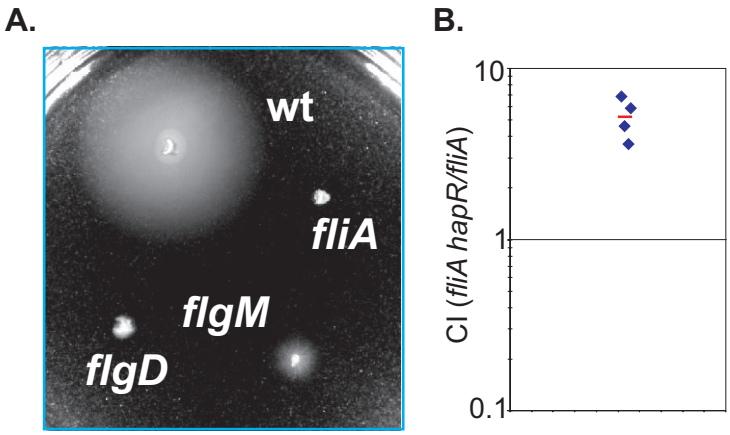


Fig. S4. Motility and mouse colonization of flagellar mutants. (*A*) Wild type and flagellar mutants were spotted on LB containing 0.3% agar and incubated at 37°C for 6 h. (*B*). Competitive index (CI) of *fliA hapR* and *fliA* mutants calculated from colonization of each infant mouse. Red line represents the average of CI.

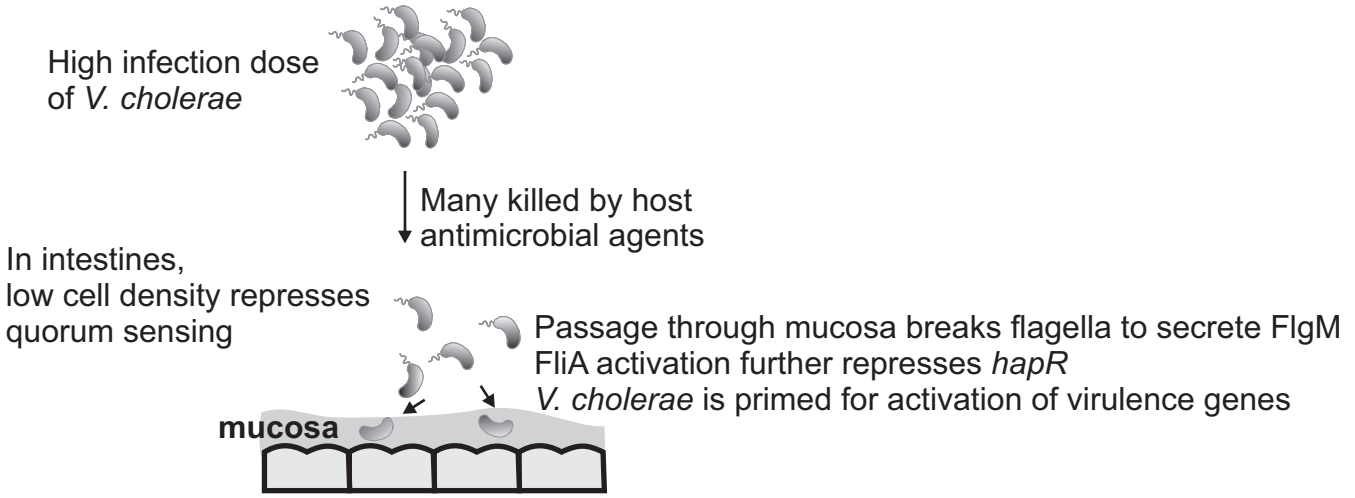
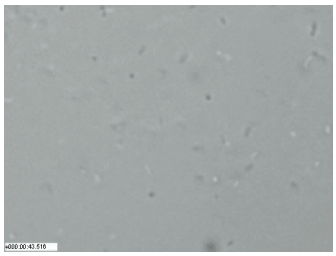
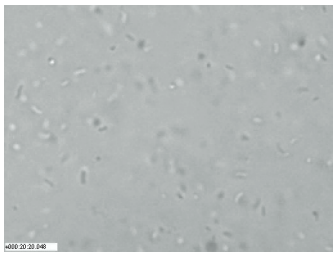


Fig. S5. The proposed model of enhanced expression of virulence genes due to passage through the mucosal layer by *V. cholerae* during host colonization. See *Discussion* in the text for details.



Movie S1. The motility of bacteria collected from transwell without 1% mucin.

Movie S1(MOV)



Movie S2. The motility of bacteria collected from transwell with 1% mucin.

Movie S2(MOV)

Table S1. Locations and relevant phenotypes of transposon insertions that activate *tcpA* expression in *luxO* mutants

	Gene name	Gene function	Zeocin-resistance*	CT/TcpA production†	Lux activity‡
Controls	Wild type		+	++	++
	luxO		−	−	+++
	hapR		+	++	−
Tn results in constitutive expression§	VC2338 (*lacZ*)	Express P_{tcpA}- *sh ble* directly	+	−	+++
	VC2647(*aphA*)	Virulence gene regulator	+	++	+++
	VC0826(*tcpP*)	Virulence gene regulator	+	++	+++
	VC0838 (*toxT*)	Virulence gene regulator	+	++	+++
	VC2066(*fliA*)	Late flagellar synthesis regulator	+	++	+
Tn results in disruption¶	VC0583(*hapR*)	Quorum sensing regulator	+	++	−
	VC1130(*vicH*)	H-NS-like protein	+	++	+++
	VC2305(*ompK*)	Outer membrane protein	+	++	+++
	VC2271(*ribD*)	Riboflavin biosynthesis	+	+	+++
	VC2198 (*flgD*)				
	VC2199 (*flgC*)	Flagellar biosynthesis, rod proteins	+	+	+
	VC2200 (*flgB*)				

All strains contain the P_{tcpA}-*sh ble* reporter in the *lacZ* locus and a cosmid harboring *luxCDABE* from *V. harveyi* (pBB1).
*Strains were grown in AKI and zeocin-resistant colonies were counted on LB plates.
†Strains were grown in AKI and cholera toxin ELISA and TcpA Western blots were performed.
‡Strains were grown in LB at 30°C to midlog and luminescence was measured.
§All phenotypes were dependent on arabinose induction.
¶All phenotypes were independent of arabinose.