Supplemental Materials

Supplemental Figures

**Fig. S1.** The effect of wild type and constitutively active AphB on colonization. (Related to to Fig. 1). Approximately $10^6$ aerobically-grown or microaerophilically-grown bacterial cells of wildtype, $aphB^{C235S}$, and $\Delta aphB$ mutants were inoculated into 5-day-old CD-1 mice. After 20 hrs, bacteria were recovered from mouse small intestines and enumerated on LB agar containing appropriate antibiotics. CFU/mouse (B) was determined. Competitive index (A) was calculated by normalizing the output ratio of mutant/WT with the input ratio of mutant/WT.
**Fig. S2. Tn mutant colonization.** (Related to Fig. 1).
Wildtype and four Tn mutants were grown aerobically or microaerophilically. Infant mice were inoculated with $10^6$ 1:1 mixtures of wildtype and mutants and incubated for 20 hrs. Colonized bacteria were enumerated and competitive index (CI) was calculated by normalizing the output ratio of mutant/WT with the input ratio of mutant/WT. Ratio of CI of $O_2^-/O_2^+$ was then calculated. Horizontal lines represent average ratio from 3 mice.

**Fig. S3. Aerobic growth of ∆ohrR.** (Related to Fig. 1).
Wildtype and ∆ohrR were grown at 37ºC shaking and OD$_{600}$ was measured at the time points indicated. Results are the means and s.d. of three independent experiments.
Fig. S4. AphB gel-shift assays. (Related to Fig. 2).

100 nM purified AphB-His6 and 0.1 ng radiolabeled tcpP promoter DNA was used. Final concentration of DTT used was 1 mM.
A & B. Constitutively active OhrR (Related to Fig. 3). Approximately $10^6$ aerobically-grown or microaerophilically-grown bacterial cells of wildtype and $ohrR^{C128S}$ mutants were inoculated into 5-day-old CD-1 mice. After 20 hrs, bacteria were recovered from mouse small intestines and enumerated on LB agar containing appropriate antibiotics. CFU/mouse (B) was determined. Competitive index (A) was calculated by normalizing the output ratio of mutant/WT with the input ratio of mutant/WT.

C. Role of OhrR on colonization. (Related to Fig. 6). Approximately $10^6$ aerobically-grown or microaerophilically-grown bacterial cells of wildtype and $\Delta ohrR, \Delta ohrR\Delta aphB$ mutants were inoculated into 5-day-old CD-1 mice. After 20 hrs, bacteria were recovered from mouse small intestines and enumerated on LB agar containing appropriate antibiotics. CFU/mouse was determined.
Fig. S6. The effect of OhrR on AphB reduction. (Related to Fig. 4).
Early-log aerobically-grown cultures of ΔaphBΔohrR mutants carrying P_{BAD}aphB plasmids were shifted to the anaerobic chamber. At the time points indicated, trichloroacetic acid was added. The precipitated proteins were reacted with mPEG-Maleimide and detected by SDS-PAGE and Western blots.
**Supplemental Table**

**Tn-seq primer list (Related to Fig.1).**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZL 1.0 (Tn only)</td>
<td>CACCGTCATGGTCTTTGTAG</td>
</tr>
<tr>
<td>Olj376</td>
<td>GTGACTGGAGTTTACGATCTTTTCCCGATCTGGGCGGGGGGGGGGG</td>
</tr>
<tr>
<td>ZL 2.0 (Tn+ binding)</td>
<td>AATGATACGCGACCTACCTACCTTTTGCCGGGCCGGGACTTATCAGCCAACC</td>
</tr>
<tr>
<td>ZL 3.0 (Sequence primer):</td>
<td>AACTTTTTGGGCGGGGACTTATCAGCCAACCTGTTA</td>
</tr>
<tr>
<td>NEB index primer</td>
<td>CAAGCAGAAGACGGCATACGTATACNNNNNGGACTGGAGTTCCAG</td>
</tr>
</tbody>
</table>

**Supplemental Data Set S1: Complete set of Tn-seq analysis. (Related to Fig. 1).**
See Supplemental Data S1.
Supplemental Experimental Procedures

Strains, plasmids and culture conditions.
All *V. cholerae* strains used in this study were derived from El Tor C6706 (Joelsson et al., 2006), and were propagated in LB media containing appropriate antibiotics at 37°C unless otherwise noted. Cultures were grown aerobically (shaking at 250 rpm), anaerobically (Anaerobic chambers, Coy Laboratory Products), or microaerophilically (standing cultures). AKI medium was used to induce virulence gene expression (Iwanaga et al., 1986). Transcriptional lux reporters of promoter regions of *aphA*, *aphB*, *toxR*, *tcpP*, *tcpT*, and *tcpA* have been described previously (Liu et al., 2011). Plasmids containing P*BAD* inducible promoter for overexpressing *aphB* and *aphB* with cysteine mutants were constructed by cloning the PCR-amplified coding regions into pBAD24 (Guzman et al., 1995), or pACYC117 (Chang and Cohen, 1978). Plasmids harboring P*lac-ohrR*, P* lac-oehrR-FLAG* (both WT and cysteine mutants) were constructed by cloning *ohrR* or FLAG-*ohrR* coding sequence into pMalC2x (New England Biolabs). The plasmids overexpressing recombinant protein for the gel retardation assay were either described previously (pET-*aphB* and pET-* aphBC235S*) (Liu et al., 2011) or constructed by cloning the *ohrR* coding sequence into pET32a (EMD Biosciences). The resulting plasmids that produced OhrR His6 N-terminal fusion were then introduced into the *E. coli* strain BL21 (DE3) (Promega). In-frame deletion of *ohrR* was constructed by cloning the regions flanking the target gene into the suicide vector pWM91 containing a sacB counter-selectable marker (Metcalf et al., 1996). The resulting plasmid was introduced into *V. cholerae* by conjugation, and deletion mutants were selected for double homologous recombination events. Chromosomal *aphB* C235S was constructed as described previously (Liu et al., 2011). Chromosomal *ohrR*C128S was constructed by inserting *ohrR*C128S mutant copy (on pWM91) (Metcalf et al., 1996) into *ohrR* deletion strain by selection of double crossover events.

Measuring transcriptional expression of virulence genes.
For Lux-based reporters, bacterial cultures were grown aerobically and anaerobically in LB or AKI medium. The luminescence of cells was read using a BioTek Synergy H1 spectrophotometer and normalized by the OD at 600 nm. Lux expression is reported as light units/OD600. For quantitative real-time qPCR assay, total RNA was purified from bacterial cultures using TRIzol reagent (Invitrogen) and cleaned with RNeasy Kit (Qiagen). RNA reverse transcription was performed by using the SuperScript II Kit (Invitrogen). Quantitative real-time qPCR using primers specific for *tcpP* was then performed on a CFX96 Real-Time system (BioRad). The 16S rDNA and recA were used as internal controls in all reactions.

Gel Retardation Assays.
His6-OhrR and His6-AphB proteins were expressed and purified on nickel columns according to the manufacturer’s instructions (Qiagen) (Liu et al., 2011). PCR products containing *tcpP* promoter region were digested and end-labeled with α- 32P dATP. Binding reactions contained purified protein at the appropriate concentration, 0.1 ng of DNA in a buffer consisting of 20 mM Tris pH 7.0, 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 µg ml⁻¹ BSA, 5 µg ml⁻¹ calf thymus DNA, 0.8 µg ml⁻¹ polydl/dC, with or without 1 mM DTT. After 30 mins of incubation at 37 °C, samples were size-fractionated using 4% polyacrylamide gels in 0.5 X TAE buffer. The radio-activity of free DNA and protein-DNA complexes was visualized using a Typhoon 9410 Phosphoimager (Molecular Dynamics).

In vivo competition assays.
All animal experiments were carried out in strict accordance with animal protocols that were approved by the IACUC of the University of Pennsylvania. The infant mouse colonization assays were performed as previously described (Gardel and Mekalanos, 1994) with the following modifications. Briefly, early-log phase cultures of WT (*lacZ+*) and ΔohrR mutants (*lacZ−*) grown aerobically or anaerobically were mixed at a 1:1 ratio and approximately 10⁶ cells were intragastrically inoculated into 5-day-old CD-1 suckling mice. After a 20-hr period of incubation, the mice were sacrificed. Small intestines were harvested and homogenized and the ratio of mutants to WT bacteria was determined by plating on LB agar containing 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-Gal).

Recombinase-based in vivo expression technology (RIVET) assays.
*V. cholerae* WT and ΔohrR strains harboring lacZ::res1-tet-res1 and tcpP::tnpR or tcpA::tnpR (Lee et al., 1999) were grown aerobically at 37°C in LB broth containing appropriate antibiotics and approximately 10⁶ bacterial cells were intragastrically inoculated into 5-day-old CD-1 mice as previously described. At the time points indicated, mice were sacrificed and serial-diluted small intestine homogenates were plated on LB agar plates containing streptomycin. Colonies were then patched on LB + streptomycin and LB + tetracycline plates. For each data point, over 300 colonies were examined. The percentage of Tet^c CFU was then calculated.
Supplemental References


