

Supplementary Information for Upregulation of virulence genes promotes *Vibrio cholerae* biofilm hyperinfectivity.

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AUTHOR CONTRIBUTIONS

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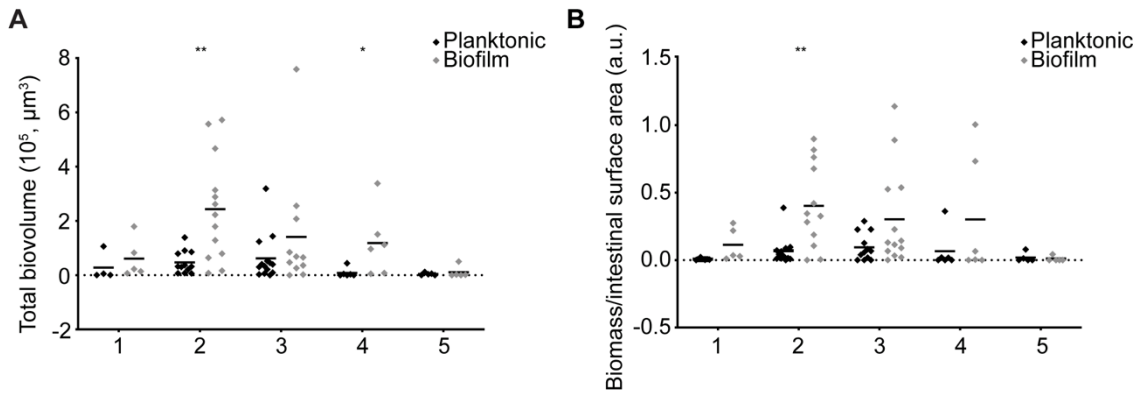
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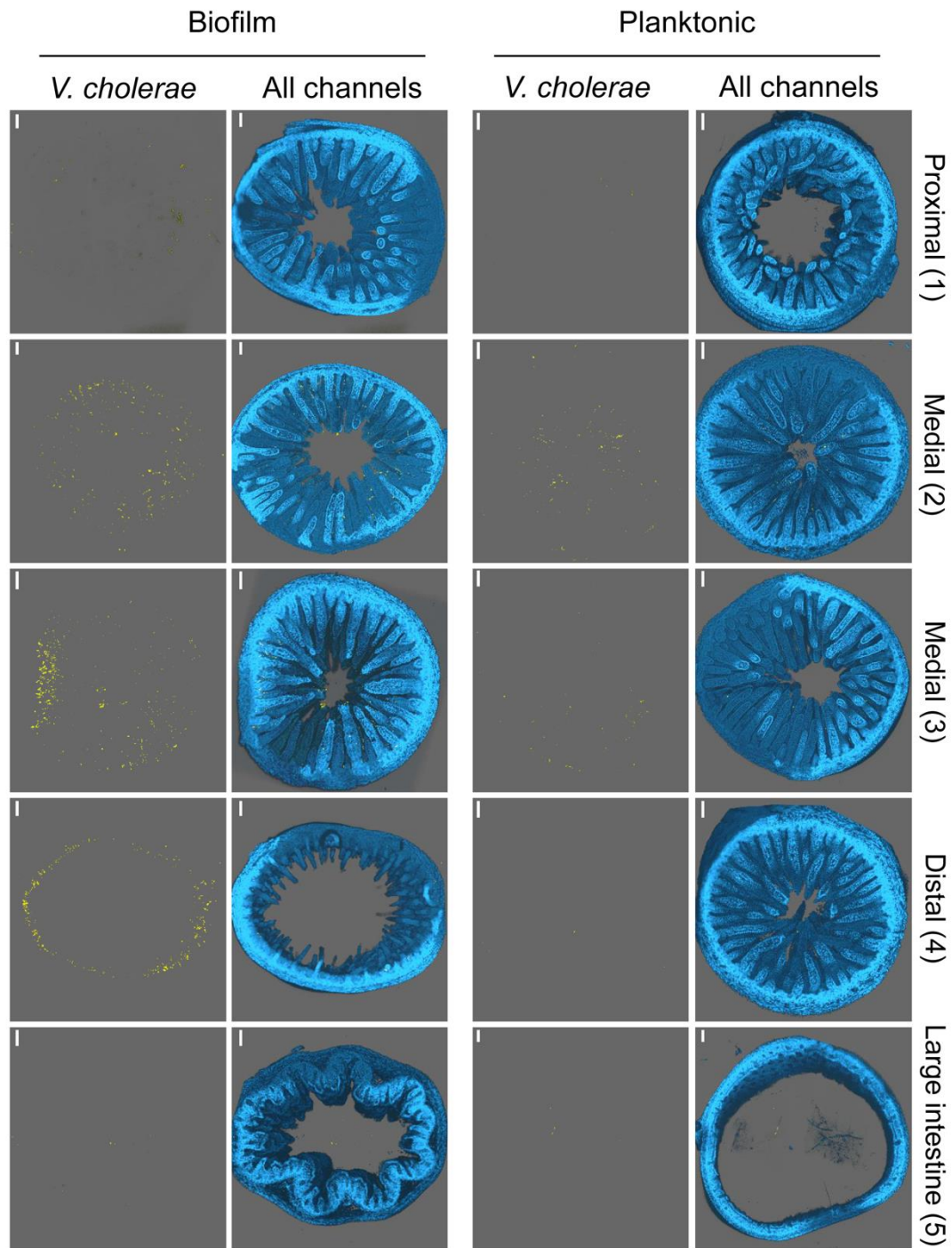
Figures S1 to S4
Legends for Movies S1 to S2
SI References

Other supplementary materials for this manuscript include the following:

Movies S1 to S2

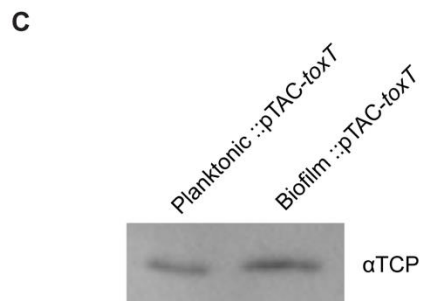
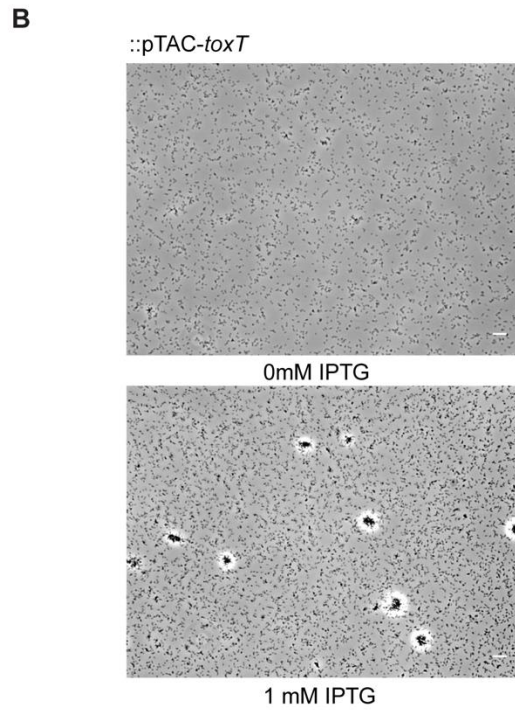
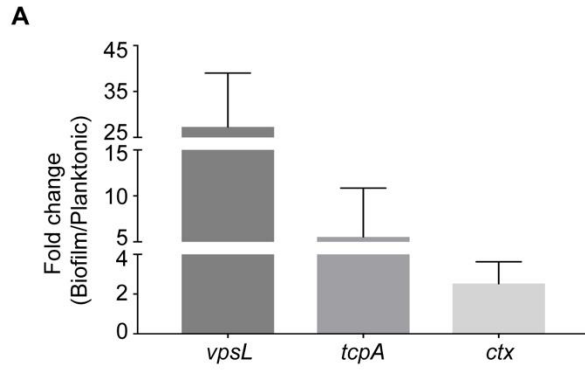


Supplementary Figure 1. Colonization patterns of planktonic and biofilm-grown cells along intestines reveal differences in bacterial abundance. A) Total biovolume of cells found in sections 1, 2, 3, 4, and 5 of the intestines infected with planktonic or biofilm-grown cells ($n \geq 4$). Statistical analysis was carried out using an unpaired two-sample t-test. (*, $p < 0.05$, **, $p < 0.005$). B) Bacterial biomass volume normalized by the intestinal surface area in sections 1, 2, 3, 4, and 5 of the intestines infected with planktonic or biofilm-grown cells ($n \geq 4$). Statistical analysis was carried out using an unpaired two-sample t-test. (**, $p < 0.005$).



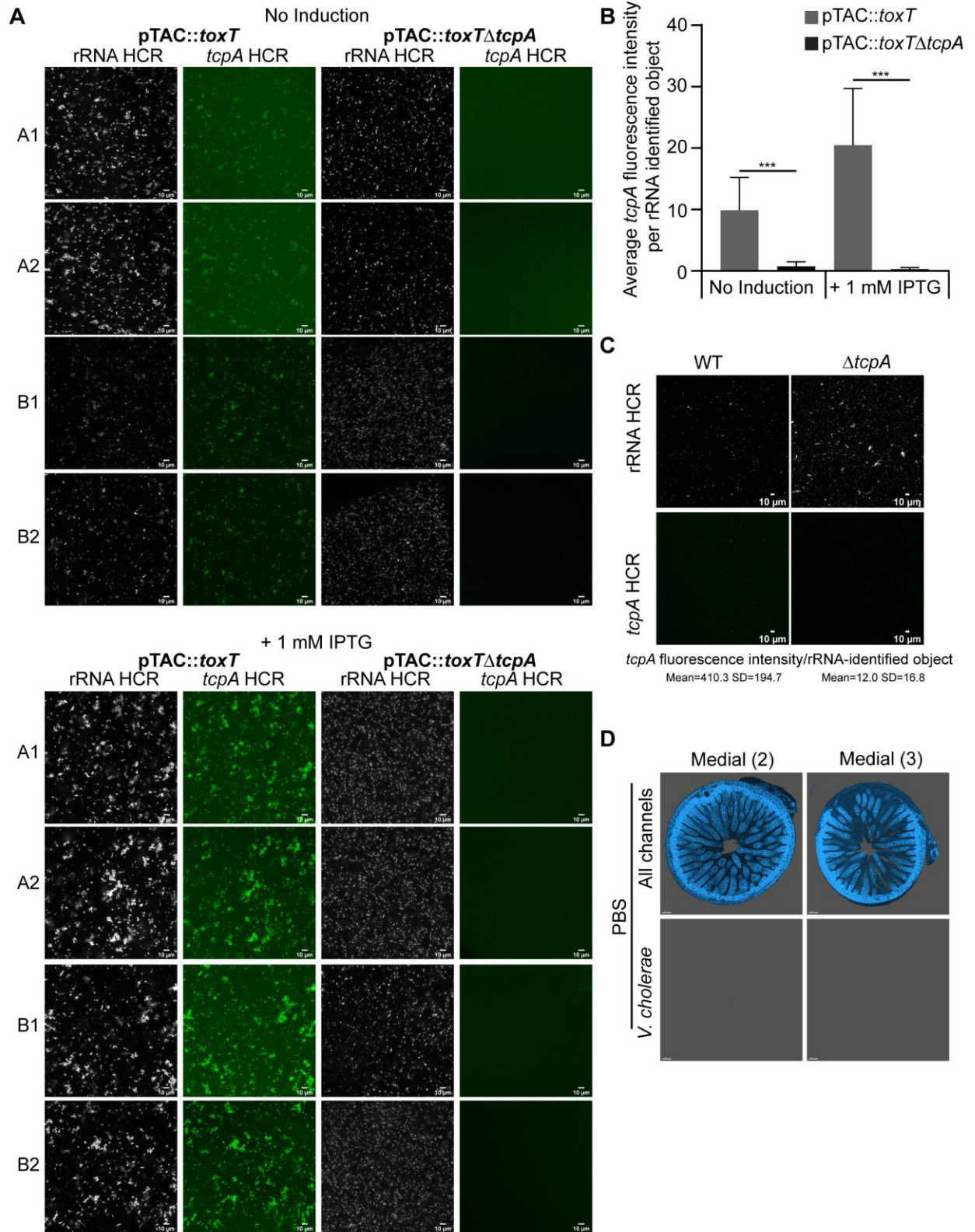
Supplementary Figure 2. MiPACT images of infected infant mouse intestines reveals differences in abundance and spatial patterns of colonization between planktonic and biofilm-grown cells. Images of biofilm and planktonic-grown cells

colonizing the intestine (showing representative images from $n \geq 3$). Projections of infected intestine show bacteria in yellow and intestines in blue. Scale bar is 100 μm .



Supplementary Figure 3. Biofilm-grown cells have enhanced expression of biofilm and virulence genes. A) Quantitative PCR was used to demonstrate fold-change in *vpsL*, *tcpA*, and *ctx* gene expression in biofilm-grown cells compared to planktonic-grown

cells ($n = 3$). B) TCP bundling upon induction of *toxT* using an inducible promoter was visualized using light microscopy ($n = 2$). C) Production of the TCP pilus upon induction of *toxT* in planktonic-grown and biofilm-grown cells was visualized using western blot ($n = 2$).



Supplementary Figure 4. HCR controls. (A) pTAC::*toxT* and pTAC::*toxT* Δ *tcpA* strains were grown at 30°C in the presence or absence of 1mM IPTG until they reached an OD₆₀₀ of approximately 1.0 before being spun down, resuspended in 1X phosphate-buffered saline (PBS) by pipetting, fixed, embedded and stained with HCR probes against rRNA (white, left panels) and *tcpA* mRNA (green, right panels). Maximum intensity projections of all Z-stacks from technical (1 vs. 2) and biological (A vs. B) replicates. Images were acquired in 8 bit mode (fluorescence intensity range of 0-255). (B) Using the 3D Objects Counter plugin in Fiji, the average fluorescence intensity of the *tcpA* channel within each rRNA identified object was calculated. Background signal for each Z-stack was determined by inverting the gray values of the rRNA channel and calculating the average *tcpA* fluorescence intensity within all non-object voxels. This Z-stack-specific background value was subtracted from the *tcpA* signal for each object in that respective Z-stack. Each bar represents the average of all objects in all four Z-stacks for each condition. Error bars represent standard deviation. Statistical analysis was carried out using an unpaired two-sample t-test. (***, $p < 0.0001$). (C) Wild type or Δ *tcpA* *V. cholerae* biofilm-grown cells were prepared by scraping into 1X phosphate-buffered saline (PBS) and resuspended by pipetting, fixed, embedded and stained with HCR probes against rRNA (white, top row) and *tcpA* mRNA (green, bottom row). Images show maximum intensity projections of Z-stacks. Images were acquired in 12 bit mode (fluorescence intensity range of 0-4095). The 3D Objects Counter plugin in Fiji was used to quantify the average fluorescence intensity of the *tcpA* signal in each rRNA probe identified object, and background was subtracted as described in B. (D) Images of intestine infected with PBS control (showing representative images from $n \geq 2$) and treated with specific HCR *V. cholerae* specific

rRNA probe. Projections of infected intestine show intestines in blue and any non-specific binding of the HCR probe in yellow. Scale bar is 30 μm .

Supplementary Movie 1 (separate file). Three-dimensional rendering of the medial sections of the small intestine (blue) infected with planktonic-grown cells (yellow).

Supplementary Movie 2 (separate file). Three-dimensional rendering of the medial sections of the small intestine (blue) infected with biofilm-grown cells (yellow).

Table S1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype	Source
<i>E. coli</i> strains		
CC118 λ pir	$\Delta(ara-leu) araD \Delta lacX74 galE galk$ <i>phoA20 thi-1 rpsE rpoB argE(Am) recA1</i> λ pir	(1)
S17-1 λ pir	Tp ^r Sm ^r <i>recA thi pro rK⁻ mK⁺</i> RP4::2- Tc::MuKm Tn7 λ pir	(2)
<i>V. cholerae</i> strains		
FY_VC_0001	<i>Vibrio cholerae</i> O1 El Tor A1552, wild type, Rif ^r	(3)
FY_VC_12506	<i>Vibrio cholerae</i> O1 El Tor A1552 $\Delta tcpA$	This study
FY_VC_14975	<i>Vibrio cholerae</i> O1 El Tor A1552 $\Delta VC1464$	This study
FY_VC_14629	<i>Vibrio cholerae</i> O1 El Tor A1552 pTAC:: <i>toxT</i> , Rif ^r ,	This study
FY_VC_14685	<i>Vibrio cholerae</i> O1 El Tor A1552 pTAC:: <i>toxT</i> $\Delta lacZ$, Rif ^r ,	This study
FY_VC_15844	<i>Vibrio cholerae</i> O1 El Tor A1552 pTAC:: <i>toxT</i> $\Delta tcpA$, Rif ^r ,	This study

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