SUPPLEMENTAL INFORMATION

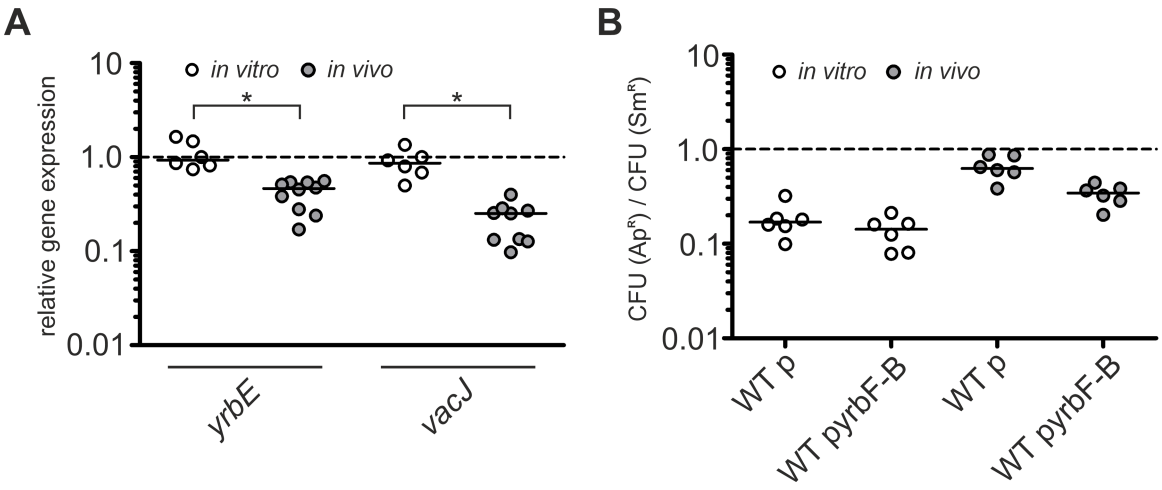

Figure S1. *In vivo* repression of *yrbE* and *vacJ* as well as plasmid maintenance of p and pyrbF-B in absence of antibiotic selection. Related to Figure 1.

(A) Relative expression levels of *yrbE* and *vacJ* were determined by quantitative real time PCR *in vitro* (n = 6) during growth in LB broth (open circles) and *in vivo* (n = 10 for *yrbE* and n = 9 for *vacJ*) during intestinal colonization (22 h post-infection, grey circles).

(B) Plasmid maintenance of the empty pMMB67EH vector (p, Ap^R) and the *yrb* operon expression vector (pyrbF-B, Ap^R) in *V. cholerae* is indicated as the ratio of CFU (Ap^R)/ CFU (Sm^R) after 22 h growth in LB broth (open circles) and *in vitro* during intestinal colonization (22 h post-infection, grey circles). A ratio of 1 (dashed line) indicates optimal plasmid stability. Horizontal bars indicate the median of each data set (n = 6).

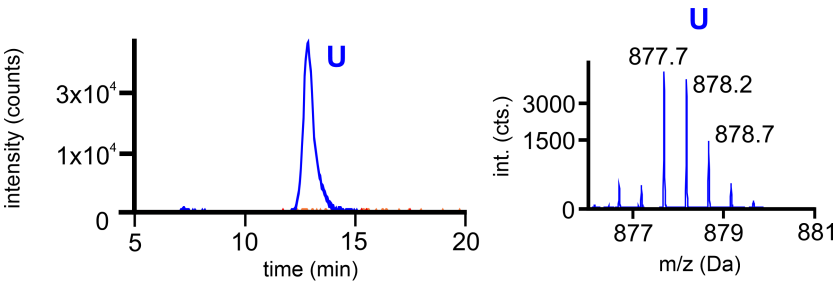

Figure S2. Mass spectrometry analysis of lipid A extracted from the Δ*almG* mutant. Related to Figure 3.

Lipid A extracted after the M9 to M9$^{ToxR\uparrow/Alm\uparrow}$ transition from Δ*almG* after 8 h was analyzed. Comparable to figure 3 the left graph shows the extracted ion chromatogram of m/z 877.7 (U, representing unmodified lipid A) in blue, m/z 906.2 (M1, representing mono-glycinated lipid A) in orange, and m/z 934.7 (M2, representing di-glycinated lipid A) in red. Only the unmodified lipid A species at m/z 877.7 could be detected, the mono- and di-glycinated lipid A species were below limit of detection. In addition, the high-resolution mass spectrum of the unmodified doubly charged lipid A species with its isotope pattern is displayed in the right graph.

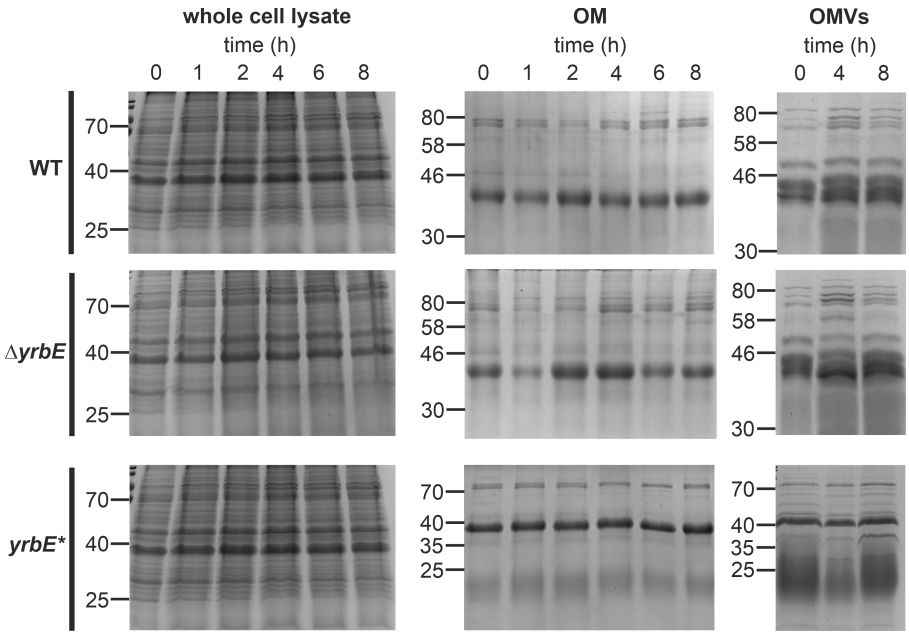

Figure S3. Coomassie-stained gels of whole cell lysates, OM preparations and isolated OMVs derived from WT, Δ*yrbE* and *yrbE. Related to Figure 4.**

Shown are Coomassie-stained gels loaded with cell equivalents of whole cell lysates or protein equivalents (5 µg) of OM preparations and OMVs from WT, Δ*yrbE* and *yrbE**. Samples were taken at time points 0, 1, 2, 4, 6 and 8 h for whole cell lysates and OM as well as 0, 4 and 8 h for OMVs after transition from M9 to M9$^{ToxR\uparrow}$. Molecular mass standards are indicated on the left using either Prestained Protein Marker Broad Range (New England Biolab) or PageRuler^{TM} Prestained Protein Ladder 10 - 180 kDa (Thermo Fisher).

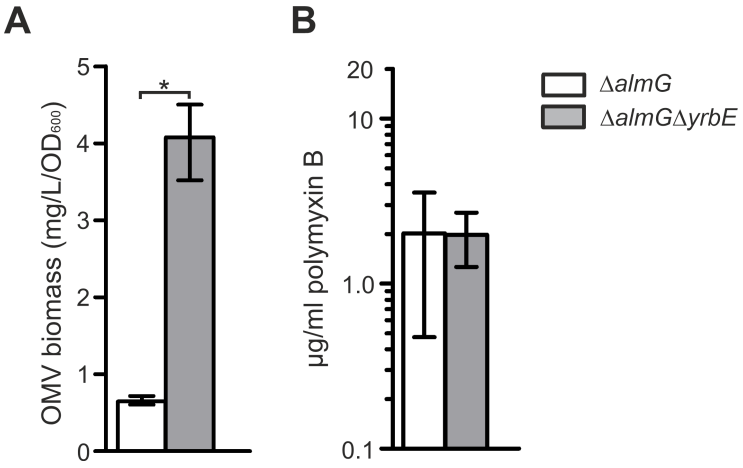

Figure S4. OMV production and minimal inhibitory concentrations (MIC) of polymyxin B (PMB) determined for Δ*almG* and Δ*almG*Δ*yrbE*. Related to Figure 5.

(A) Quantification of OMV biomass (Bradford) prepared from equivalent OD_{600} units of Δ*almG* and Δ*almG*Δ*yrbE* after 8 h cultivation in minimal media $M9^{ToxR\uparrow}$. Data is presented as the median with interquartile range (n = 6; **P* < 0.05).

(B) Shown are minimal inhibitory concentrations (MIC) of polymyxin B (PMB) for the Δ*almG* (n = 12) and Δ*almG*Δ*yrbE* mutants (n = 15) along a transition from M9 to $M9^{ToxR\uparrow/Alm\uparrow}$ [(di)glycine-modified lipid A activating conditions using sub-MIC PMB concentrations (0.3 µg/ml)]. Bacteria were allowed to adapt for 2 h after transition into the fresh medium before additional PMB was added in diverse concentrations to determine the MIC. Results are presented as mean ± SD (**P* < 0.05).

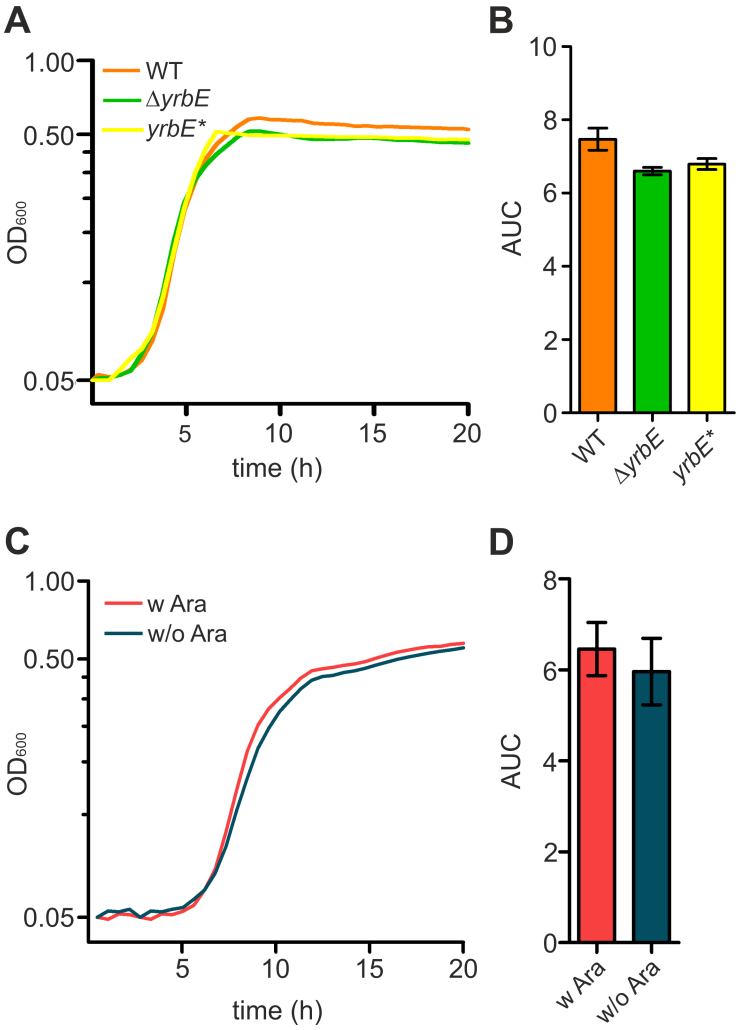

Figure S5. Hyper- or hypo-vesiculation by deletion of *yrbE* or overexpression of *yrbF-B* does not affect growth in minimal media M9$^{ToxR\uparrow}$. Related to Figure 5.

Shown are growth curves (A, C) and the retrieved area under curve (AUC) values (B, D) for the *V. cholerae* WT, Δ*yrbE* and the complementation strain *yrbE** (A, B) shifted from M9 to M9$^{ToxR\uparrow}$ or the arabinose-inducible strain *yrbF-B*^{pARA} shifted from M9 to M9$^{ToxR\uparrow}$ with (w) or without (w/o) arabinose (Ara) (C, D). Data is presented as mean values ± SD with the following number of biological replicates: n = 4 for WT and Δ*yrbE* as well as n = 8 for *yrbE**, *yrbF-B*^{pARA} w Ara and *yrbF-B*^{pARA} w/o Ara, respectively.

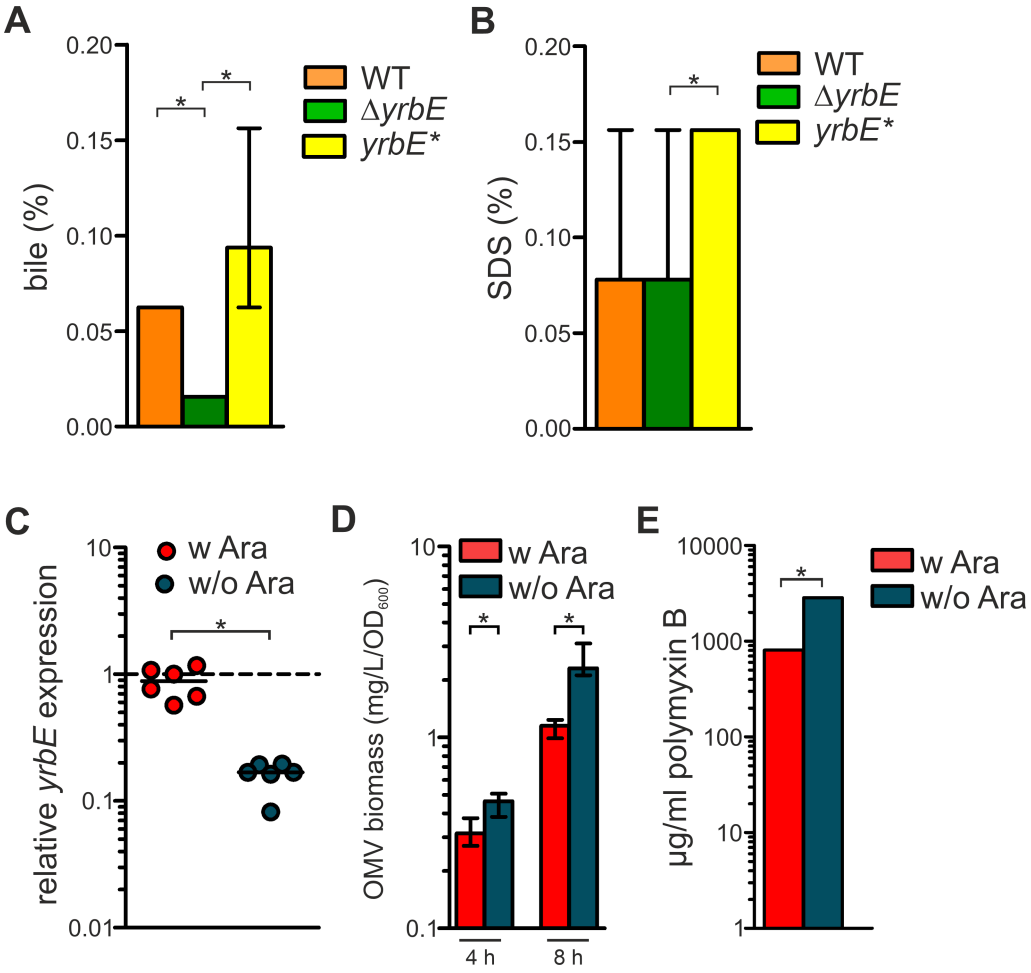

Figure S6. Minimal inhibitory concentrations (MIC) of antimicrobial substances and OMV production for various *V. cholerae* strains used in this study. Related to Figure 5.

(A and B) Shown are minimal inhibitory concentrations (MIC) of bile (A) and SDS (B) for WT, Δ*yrbE* and the complementation strain *yrbE** grown in M9. Data is presented as the median with interquartile range (**P* < 0.05) with the following number of biological replicates: MIC of bile for WT (n = 22), for Δ*yrbE* (n = 8) and for *yrbE** (n = 18) as well as n = 12 for the MIC of SDS for all strains tested.

(C) Relative expression levels of *yrbE* in the arabinose-inducible *yrbF-B* variant were determined by quantitative real time PCR *in vitro* during growth in $M9^{ToxR\uparrow}$ with (w) or without (w/o) arabinose (Ara) (n = 6; **P* < 0.05).

(D) Quantification of OMV biomass (Bradford) prepared from equivalent OD_{600} units of the arabinose-inducible *yrbF-B* variant. Values were obtained after 4 and 8 h cultivation in minimal

media $M9^{ToxR\uparrow}$ with (w) or without (w/o) arabinose (Ara). Data is presented as the median with interquartile range (n = 6; *$P < 0.05$).

(E) Shown are the minimal inhibitory concentrations (MIC) of the arabinose-inducible strain *yrbF-B*^{pARA} against polymyxin B (PMB) along a transition from M9 to $M9^{ToxR\uparrow/Alm\uparrow}$ [(di)glycine-modified lipid A activating conditions using sub-MIC PMB concentrations (3 µg/ml)] with (w) or without (w/o) arabinose (Ara). Bacteria were allowed to adapt for 2 h after transition into the fresh medium before additional PMB was added in diverse concentrations to determine the MIC. Results are presented as mean ± SD (n = 6; *$P < 0.05$).

Table S1. List of oligonucleotides used in this study, related to STAR Methods

Oligonucleotides	Source
almG_XbaI_1 AATTCTAGAGGCAAACAGTTAGTGAAGGG	This paper
almG_BamHI_2 TATGGATCCTTTTTTCCGACTTATCGGCTTA	This paper
almG_BamHI_3 AAAGGATCCAAGCAATTCGGTGTATCTAAGA	This paper
almG_SacI_4 AATGAGCTCTCCACAATAAGTTGTGCCAAAG	This paper
lacZ_XbaI_1 TATCTAGAATGTACGCCGTAGAGCAAAG	This paper
lacZ _BamHI_2 TAGGATCCATGGCGAGTCACTTGGCTAA	This paper
lacZ _SphI_3 ATGCATGCGTGTGGAATGTGACGAT	This paper
lacZ _SacI_4 ATGAGCTCTTATTGTGGGGATGACCTTTAAAG	This paper
araC_BamHI_1 AAAGGATCCAGACACTTTTGTTACGCGTTTT	This paper
araC_SphI_2 ATTGCATGCTTATGACAACTTGACGGCTAC	This paper
yrb_SacI_1 AAAGAGCTCGCGATATTGGCAATGTTTGAAC	This paper
yrb_SphI_2 AAAGCATGCGATAAGGATAATTAATTGGAATC	This paper
yrb _EcoRI_3 AAAGAATTCTCAGGATGTCGACCTAACAG	This paper
yrb _XbaI_4 TTTTCTAGAGATTAAGGTTACGGATCAGTTC	This paper
para_SphI_1 AAAGCATGCAAAGCCATGACAAAAACGCG	This paper
para_EcoRI_2 GAATTCGCTAGCCCAAAAAAAC	This paper
almG_pGPphoA_SacI TTGAGCTCGTTAACCTCAGGTTTATTTTATTT	This paper
almG_pGPphoA_KpnI ATTGGTACCTTACTTAAAACGCCGATAAAGC	This paper
yrb_operon_SacI_1 TTTGAGCTCTTTCAGGATGTCGACCTAA	This paper
yrb_operon_XbaI_2 AAATCTAGACTATCCACAATTCACCTCTGC	This paper
VC2519_XbaI_up_fw AAATCTAGACTTAGTCACCATCAAAAATT	(Roier et al., 2016)
VC2519_XmaI_down_rv AAACCCGGGCTCCGCCACATCTTCACGTT	This paper
comp_yrbE*_1 GTTGCATGTTTAATTCCCAAACATCAATGCGG	This paper
comp_yrbE*_2 TTTGGGAATTAAACATGCAACAAACACGAAAAATTG	This paper