Outer Membrane Vesiculation Facilitates Surface Exchange and In Vivo Adaptation of Vibrio cholerae

Graphical Abstract

Highlights
- Phospholipid transport of Vibrio cholerae is silenced upon host entry
- Reduced transporter activity triggers OMV release and increases colonization fitness
- Increased vesiculation accelerates modulation of cell surface composition
- Hypervesiculation leads to faster adaptation to host defense mediators

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In Brief
Upon infection, Vibrio cholerae must alter its surface profile to evade host defenses and adapt to the gastrointestinal environment. Zingl et al. show that increased release of outer membrane vesicles upon host entry allows the bacteria to rapidly modify their cell surface, thus increasing in vivo adaptation and colonization fitness.

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Outer Membrane Vesiculation Facilitates Surface Exchange and In Vivo Adaptation of *Vibrio cholerae*

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SUMMARY

Gram-negative bacteria release outer membrane vesicles into the external milieu to deliver effector molecules that alter the host and facilitate virulence. Vesicle formation is driven by phospholipid accumulation in the outer membrane and regulated by the phospholipid transporter VacJ/Yrb. We use the facultative human pathogen *Vibrio cholerae* to show that VacJ/Yrb is silenced early during mammalian infection, which stimulates vesiculation that expedites bacterial surface exchange and adaptation to the host environment. Hypervesiculating strains rapidly alter their bacterial membrane composition and exhibit enhanced intestinal colonization fitness. This adaptation is exemplified by faster accumulation of glycine-modified lipopolysaccharide (LPS) and depletion of outer membrane porin OmpT, which confers resistance to host-derived antimicrobial peptides and bile, respectively. The competitive advantage of hypervesiculation is lost upon pre-adaptation to bile and antimicrobial peptides, indicating the importance of these adaptive processes. Thus, bacteria use outer membrane vesiculation to exchange cell surface components, thereby increasing survival during mammalian infection.

INTRODUCTION

It is widely accepted that all domains of life produce membrane vesicles. Extracellular vesicles released from the outer membrane (OM) of gram-negative bacteria, generally referred to as outer membrane vesicles (OMVs), are one of the best-studied vesicles (Toyofuku et al., 2019). These spherical, non-living facsimiles of the bacterial donor cell have been mainly characterized as delivery vehicles for effector molecules, such as quorum-sensing signals, nucleic acids, degradative enzymes, inflammatory agents, or toxins (Jan, 2017; Schwechheimer and Kuehn, 2015). Consequently, OMVs have been attributed a role in intra- and inter-specific communication, horizontal gene transfer, nutrient acquisition, immunomodulation, and virulence (Kulp and Kuehn, 2010).

Recently, our group identified a conserved mechanism for OMV release based on phospholipid accumulation in the OM because of inactivation or transcriptional silencing of the VacJ/Yrb ATP-binding cassette (ABC) transporter, also known as the maintenance of OM lipid asymmetry (Mla) system (Figure 1A) (Roier et al., 2016). This highly conserved trafficking system of Gram-negative bacteria was originally reported to shuttle phospholipids from the outer to the inner membrane to maintain the lipid asymmetry of the OM (Malinverni and Silhavy, 2009). In contrast to this view, recent data suggest that the VacJ/Yrb transport system can also export phospholipids from the inner membrane (Hughes et al., 2019; Kamischke et al., 2019). Although mechanistic details remain to be elucidated, it is evident that inactivation or downregulation of the VacJ/Yrb transporter results in elevated OMV levels (Roier et al., 2016). Importantly, iron depletion in several Gram-negative bacteria (i.e., *Escherichia coli*, *Vibrio cholerae*, and *Haemophilus influenzae*) triggers a ferric uptake regulator (Fur)-dependent repression of the VacJ/Yrb transporter, leading to increased OMV release (Roier et al., 2016). Iron limitation is a common stressor for bacterial pathogens during host colonization; therefore, we predicted that transcriptional silencing of the vacJ and yrbF-B genes and the resulting boost in vesiculation should occur upon host entry. In support of this model, *H. influenzae* was shown to lower expression levels of the VacJ/Yrb transporter during nasopharyngeal colonization (Roier et al., 2016).

Benefits driving bacteria to alter vesiculation in vivo remain to be elucidated. In the case of *H. influenzae*, higher OMV levels correlated with increased serum resistance (Roier et al., 2016). Thus, increased vesiculation might facilitate bacterial proliferation in the nasopharynx.

Given that bacteria discharge components of the periplasm and OM into the extracellular milieu via OMVs, increased vesiculation could also represent a previously overlooked adaptation to modulate their cell surface composition. This strategy could be quite valuable as bacteria are frequently challenged to adapt to diverse conditions with different requirements along their life cycle (Conner et al., 2016; O’Connor and McClean, 2017). The need to change surface components upon host entry, e.g., capsule, flagella, fimbriae, pili, lipopolysaccharide (LPS)
modifications, metabolite uptake systems, and porins, is especially apparent for facultative bacterial pathogens (King and Roberts, 2016; Phillips et al., 2019).

Along its life cycle, the facultative human pathogen V. cholerae transits between the aquatic reservoir and the human intestinal tract. To assess the impact of vesiculation on surface modulation specifically upon host entry, we used V. cholerae as a model organism and focused on the initial stage of infection. Once ingested, V. cholerae modulates its surface profile to adapt to antimicrobial factors present in the gut. For example, the expression of two abundant OM porins OmpU and OmpT are inversely regulated upon host entry. This OmpU/T switch is directly controlled by the virulence regulator ToxR and is decisive for the bacterium to achieve bile resistance in vivo (Provenzano and Klose, 2000). Unlike OmpT, OmpU is an anion-selective porin that restricts the passage of negatively charged compounds (Simonet et al., 2003). Thus, depletion of OmpT in the OM makes V. cholerae less vulnerable to bile salts. Concordantly, upon host entry, V. cholerae activates transcription of ompU and represses transcription of ompT to achieve full colonization fitness (Provenzano and Klose, 2000). Another surface modulation of V. cholerae was described for this transitory step, wherein the addition of (di)glycine residues to the lipid A anode confers resistance to cationic antimicrobial peptides (Hanksins et al., 2012). Importantly, both OmpU overexpression and modification of the transporter results in phospholipid accumulation in the OM outer leaflet promoting vesiculation.

In this study, we used three scenarios—host entry, exposure to host defense mediators (bile and polymyxin B [PMB]), and medium-induced activation of ToxR and/or LPS modification—to model infection-relevant life cycle transitions for V. cholerae. These models were applied to monitor bacterial cell surface modulation under conditions where transporter activity and vesiculation could be controlled. We show that V. cholerae silences the expression of the VacJ/Yrb transporter inside the host and reduced transporter activity facilitates...
colonization fitness in the murine infection model. Our findings demonstrate that upregulated vesiculation aids adaptation to both the cationic antimicrobial peptide PMB, via fast accumulation of glycine-modified lipid A, and bile salts, via efficient removal of OmpT. Thus, this study defines a physiological role for OMV production during host colonization where cell surface variation driven by increased OM vesiculation promotes adaptation of a bacterial pathogen upon host entry.

RESULTS

Silencing of VacJ/Yrb ABC Transport System In Vivo Increases Colonization Fitness of V. cholerae

The recently established TetR-controlled recombination-based in vivo expression technology (TRIVET) was used to monitor transcriptional silencing of the yrb gene cluster in V. cholerae (Cakar et al., 2018). TRIVET is a highly sensitive, single-cell-based reporter technique to detect temporal gene repression by an irreversible switch of an antibiotic resistance phenotype. The system allows detection of gene repression in complex environments with a relatively low bacterial load, such as the intestinal tract. In this approach, a promoterless tetR allele is integrated in the V. cholerae chromosome under the sole control of the promoter of the gene of interest. Once produced, TetR, in turn, represses TnpR resolvase expression, which exerts reversibly an antibiotic resistance (res) cassette. In short, silencing of tetR will induce tnpR and cause an irreversible loss of the res cassette, which can be monitored by a phenotypic change of the antibiotic resistance profile. Hence, resolution frequencies inversely correlate with the expression level of the investigated gene.

Here, we integrated the tetR allele in the V. cholerae chromosome downstream of the yrbF-B operon promoter (Figure 1A). We then examined the resolution frequencies for the corresponding TRIVET strain Vc_res_TRIVET yrb::tpc grown for 22 h in Lura Bertani (LB) supplemented with either FeSO4 or 2,2'-bipyridyl to mimic high or low iron availability, respectively, as well as during colonization of the murine intestinal tract (Figure 1B). Relatively low-resolution frequencies were detected in regular LB broth, or in LB upon addition of FeSO4, indicating sufficient yrb-promoter activity to silence tnpR. In agreement with previous results (Roier et al., 2016), resolution levels were significantly increased in LB broth supplemented with the iron chelator 2,2'-bipyridyl, reflecting the silencing of the yrb genes under low iron conditions.

High-resolution frequencies in vivo were also observed during colonization of the mouse intestine 6 and 22 h post-infection (Figure 1B). In vivo repression of yrbE and vacJ was independently confirmed by quantitative real-time PCR (Figure S1). Overall, the results indicate a significant repression of the yrbF-B operon at early stages of the infection compared with growth in regular LB broth. Based on the current model, inactivation or downregulation of the VacJ/Yrb transporter system results in phospholipid accumulation in the OM promoting OMV release (Figure 1A) (Roier et al., 2016). Concordantly, deletion of yrbE, encoding the permease unit of the VacJ/Yrb transport system, resulted in a hypervesiculating ΔyrbE mutant (Figure 1C). Expression of yrbE in trans (ΔyrbE pyrbE) reduced the hypervesiculation compared with a ΔyrbE mutant carrying the empty vector (ΔyrbE p) (Figure 1C). In contrast, reduced vesiculation (hypovesiculation) was achieved upon overexpression of the yrbF-B operon from an isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible plasmid in WT (WT pyrbF-B) compared to the wild type (WT) carrying the empty vector (WT p) (Figure 1C). Notably, derivatives of this plasmid allow expression even in the absence of IPTG and have been used extensively for V. cholerae studies in vivo (Tamayo et al., 2008; Schild et al., 2007; Butler and Camilli, 2004; Osorio et al., 2004). However, stable plasmid maintenance requires antibiotic selection, which has been reported to elevate OMV levels in bacteria, including V. cholerae (Figure 1C) (Bauwens et al., 2017; Roier et al., 2016). To avoid this effect of antibiotic use, a complementation strain (yrbE*) was constructed by reconstitution of a slightly modified yrbE allele in ΔyrbE on the chromosome (described in STAR Methods). The yrbE* complementation strain not only showed significantly reduced vesiculation levels compared with ΔyrbE but also similar vesiculation levels compared with the WT (Figure 1C). Thus, yrbE* was chosen for complementation assays throughout this study.

We next investigated the impact of vesiculation on colonization fitness. The ΔyrbE mutant and WT overexpressing the yrbF-B operon (WT pyrbF-B) were challenged, respectively, in a competition assay with a fully virulent LacZ− derivative of the WT (WTlacZ−) or WT carrying the empty vector (WTlacZ− p), in LB broth (in vitro) and in vivo using the infant mouse model. Compared with the in vitro control assay, the hypervesiculating ΔyrbE mutant exhibited a significant advantage over the WT during intestinal colonization (Figure 1D). In contrast, the hypovesiculating WT pyrbF-B was significantly attenuated in vivo compared with the WTlacZ− p (Figure 1D). Differential loss of the empty vector (p) and expression vector (pyrbF−B) can be excluded as p and pyrbF−B are maintained at comparable levels during in vitro cultivation and in vivo colonization (Figure S1B). Thus, increased vesiculation correlates directly with enhanced colonization fitness in the murine model.

Hypervesiculation Facilitates Exchange of OM Structures during Transition Stages

A recent report indicated that OMV release allows for the selective loss of certain LPS species from the OM of Salmonella following pH shifts (Bonnington and Kuehn, 2016). Likewise, the observed fitness advantage of hypervesiculating V. cholerae strains might be at least partially explained by alteration of the cell surface via enhanced OMV release upon host entry. In V. cholerae, two adaptive surface modifications occurring in the host environment have been reported: (1) removal of the OM porin OmpT to confer bile resistance (Provenzano and Klose, 2000) and (2) accumulation of (dig)glycine-modified lipid A to increase resistance to cationic antimicrobial peptides (Hankins et al., 2012). We aimed to establish a system for inducing each of these surface modifications in vitro to subsequently analyze the impact of vesiculation on the remodeling efficacy. Fortunately, the regulatory pathways and key players for both surface modifications are known and include the virulence gene regulator ToxR, which inversely controls ompU and ompT expression, as well as the aminocyl lipid modification (Aim) pathway required for (dig)glycination of lipid A (Hankins et al., 2012; Provenzano and Klose, 2000).

Previous work indicated that cultivation in minimal media plus the four amino acids asparagine, arginine, glutamate, and serine
shifting dependent OmpU/T switch can also be activated as well as 1, 2, 4, and 8 h after the transition to M9(ToxR).

We measured alkaline phosphatase (PhoA) activities in validate differential regulation in our experimental setup, (NRES) significantly increases ToxR levels, resulting in the activation of ompU and repression of ompT (Mey et al., 2012). To validate differential regulation in our experimental setup, we measured alkaline phosphatase (PhoA) activities in ompU-phoA and ompT-phoA reporter strains harboring a transcriptional fusion of the promoterless phoA to the ompU or ompT promoters (Figures 2A and 2B). Upon transition from minimal medium M9 to M9 supplemented with NRES (M9(ToxR)) a significant increase in PhoA activity for ompU-phoA reporter strains was observed, whereas ompT-phoA reporter strains exhibited a significant decrease (Figures 2A and 2B). Thus, the ToxR-dependent OmpU/T switch can also be activated in vitro by shifting V. cholerae from M9 to M9(ToxR).

The LPS modification system responsible for the addition of (di)glycine residues to lipid A during de novo synthesis at the cytoplasmic side of the inner membrane is encoded by the operon almEFG (Hankins et al., 2012). The Alm pathway can be activated in the presence of sublethal concentrations of cationic antimicrobial peptides (Herrera et al., 2014). To validate almEFG induction in our setup, we constructed reporter strains harboring a transcriptional fusion of the promoterless phoA to the almEFG promoter. Concordant with previous reports (Herrera et al., 2014), we measured a significant increase in PhoA activity upon growth in M9(ToxR) with sub-growth inhibitory concentrations of PMB (M9(ToxR)/Alm) compared with M9 (Figure 2C). Notably, comparable temporal induction and maximum levels are observed with 0.3 and 3 μg/mL PMB, the two concentrations used throughout this study to activate the Alm pathway. This suggests that both PMB concentrations have a similar potential to activate the Alm pathway. We conclude that both surface modulations can be activated in vitro on the WT and ΔyrbE background using minimal medium M9 with supplements: (1) the OmpU/T switch is activated via cultivation in M9(ToxR) allowing bile adaptation, and (2) presence of PMB (M9(ToxR)/Alm) additionally activates the Alm pathway, allowing adaptation to cationic antimicrobial peptides.

To investigate whether differential vesiculation affects the dynamics of the surface exchange, regular and hypervesiculating V. cholerae strains were shifted from minimal medium M9 to M9(ToxR)/Alm, and samples were collected over time. Analysis of the transcriptional expression pattern of the almEFG operon along the shift experiment showed that induction takes approximately 4 h (Figure 2C). Therefore, we obtained lipid A extracts of the WT, hypervesiculating ΔyrbE, and yrbE* complementation strain for liquid chromatography-mass spectrometry (LC-MS) analyses at 0, 4, 6, and 8 h after the M9 to M9(ToxR)/Alm shift. The lipid A species were detected as doubly charged ions with m/z 877.7 for the unmodified lipid A (U), 906.2 for mono-glycinated lipid A (M1), and 934.7 for di-glycinated lipid A (M2) species. The peaks of these three species (U, M1, and M2) are shown in the corresponding extracted ion chromatograms (Figure 3). At 0 h, peaks reflecting the U species were readily detected in all strains, while the (di)glycine-modified lipid A species were below the limit of detection. At 4 h post-medium shift, peaks reflecting the M1 were already prevalent in hypervesiculating ΔyrbE mutant extracts but only a minor population in the WT or still below the limit of detection in the yrbE* complementation strain (Figure 3). At 6 h post-medium shift, peaks reflecting the (di)glycine-modified lipid A species dominated in the hypervesiculating ΔyrbE mutant extracts, whereas only a minor
Figure 3. Appearance of Lipid A Modification along the M9 to M9ToxR Alm Shift Analyzed by Mass Spectrometry

Lipid A extracted from WT, ΔyrbE, and yrbE* along the M9 to M9ToxR Alm transition at 0, 4, 6, and 8 h was compared. Extracted ion chromatograms of m/z 877.7 (unmodified lipid A (U)) in blue, m/z 906.2 (mono-glycinated lipid A (M1)) in orange, and m/z 934.7 (di-glycinated lipid A (M2)) in red are shown for the indicated times.

(legend continued on next page)
fraction was modified in WT and yrbE<sup>*</sup>. However, the extent of surface modulation in the WT still increased at later time points to finally reach a similar abundance of the unmodified and (di)glycine-modified lipid A species at 8 h after the medium shift. In general, the yrbE<sup>*</sup> complementation strain showed the lowest prevalence of (di)glycine-modified lipid A species with U species dominating even at the 8 h time point. Extracts from a ΔalmG mutant isolated at the same 8 h time point served as a negative control and lacked peaks reflecting (di)glycine-modified lipid A, as expected (Figure S2).

We conclude that exposure to sub-growth inhibitory concentrations of PMB caused ΔyrbE and WT to accumulate (di)glycine-modified lipid A in the OM in an Alm-dependent manner, but the hypervesiculating ΔyrbE displayed the surface modification more rapidly (Figure 3). A comparison of the activities obtained using the chromosomal almG-phoA reporter fusions generated in the ΔyrbE and WT backgrounds (Figure 2C) allowed us to exclude differential expression levels or altered regulation of alm genes during the induced transition as a possible explanation for this accelerated modulation of the hypervesiculating mutant. As expected, PhoA levels in the WT and ΔyrbE background were relatively low in M9 but increased comparably for both strains throughout the shift experiment (Figure 2C). Thus, the most likely scenario explaining the faster accumulation of modified lipid A in the hypervesiculating ΔyrbE is an enhanced surface turnover due to increased vesiculation, resulting in faster depletion of U from the OM.

We then monitored OmpT depletion in whole-cell lysates or OM preparations for the WT, hypervesiculating ΔyrbE, and ΔyrbE<sup>*</sup> complementation strain in response to the M9 to M9<sub>ToxR<sup>1</sup></sub> shift as a model for the adaptive transition to bile. Immunoblot analyses revealed that OmpU increased slightly over time in whole-cell lysates or remained at stable high levels at all time points sampled in OM preparations for all strains (Figure 4). The increase observed in whole-cell lysates follows the activation of ompU expression during the shift experiment (Figure 2A). Increasing OmpU might not be visible in OM preparations as these samples were normalized to protein equivalents, which can be seen as an adjustment to the most abundant protein, namely OmpU (Chakrabarti et al., 1996). In contrast to OmpU, OmpT was depleted in whole-cell lysates and from the OM preparations for all strains during the transition (Figure 4). We verified that equal amounts of protein were loaded in all lanes by Coomassie-stained SDS gels (Figure S3) and additionally by immunodetection of the α-subunit of the RNA Polymerase (RpoA) in whole-cell lysates (Figure 4). Notably, the rate of OmpT depletion differed between strains. In the WT and yrbE<sup>*</sup> complementation strain, a marked reduction of OmpT signal intensity was observed from 4 h onward, while OmpT loss was detected after 1 h in the hypervesiculating ΔyrbE strain (Figure 4). In line with the AlmEFG expression profile, the temporal regulation of ompT and OmpU expression during the shift experiment is similar in WT and ΔyrbE (Figures 2A and 2B), excluding differential regulation in the two strains.

To evaluate a direct role for vesiculation in the OmpU/T switch, OMVs were isolated from culture supernatants obtained at 0, 4, and 8 h of the transition experiment, and the relative abundance of these proteins was compared (Figure 4). OmpT appeared already at 4 h in OMVs released by ΔyrbE but only after 8 h in the OMVs derived from WT and yrbE<sup>*</sup>. Hence, the most likely explanation for the OM depletion of OmpT along the transition is a constant removal of OmpT and other components via vesiculation, combined with transcriptional repression of ompT and <i>de novo</i> synthesis. In summary, the adaptive depletion of OmpT from the OM of <i>V. cholerae</i> can be linked to its deposition into OMVs. The hypervesiculating ΔyrbE mutant is thus able to more rapidly discharge OmpT from its OM. In conclusion, both adaptive surface modifications tested here were implemented more rapidly in the hypervesiculating ΔyrbE mutant than in a normally vesiculating WT strain.

**Hypervesiculation Facilitates Faster Adaptation to the Cationic Antimicrobial Peptide PMB during Transition Stages**

As previously reported, accumulation of (di)glycine-modified lipid A facilitates bacterial survival in the presence of cationic antimicrobial peptides, while depletion of OmpT from the OM promotes resistance to bile (Hankins et al., 2012; Provenzano and Klose, 2000). We hypothesized that the faster accumulation of (di)glycine-modified lipid A as well as removal of OmpT observed in a hypervesiculating strain correlates with faster adaptation to these host defenses. To test this premise, we asked whether strains with differential vesiculation exhibit different sensitivity to PMB and bile.

First, the minimal inhibitory concentration (MIC) of PMB affecting growth of the hypervesiculating ΔyrbE mutant and regular vesiculating WT was assessed for the transition from M9 to M9<sub>ToxR<sup>1</sup>/Alm<sup>1</sup></sub> as a model for the adaptation phase early in infection. To this end, overnight cultures of the ΔyrbE mutant and WT grown in M9 were allowed to adapt for 2 h in M9<sub>ToxR<sup>1</sup>/Alm<sup>1</sup></sub> ((di)glycine-modified lipid A activating conditions using sub-minimal inhibitory concentrations [MIC] PMB [3 μg/mL]) before additional PMB was added to achieve inhibitory concentrations. A 2-h adaptation phase was chosen, as it closely reflects the dynamics of a natural <i>V. cholerae</i> infection, i.e., it reflects the time from oral ingestion to arrival of the bacteria at their primary colonization site in the small intestine (Angelichio et al., 1999). A significantly higher MIC was obtained for the hypervesiculating ΔyrbE mutant than WT, implying stronger resistance and better adaptation to PMB (Figure 5A). A transition of the same M9 overnight cultures to M9 failed to activate the Alm pathway. As expected under these conditions, ΔyrbE and WT exhibited comparably poor resistance to PMB indicated by equally low MIC values (Figure 5A). Conversely, overnight cultures grown in M9<sub>ToxR<sup>1</sup>/Alm<sup>1</sup></sub> shifted to M9<sub>ToxR<sup>1</sup>/Alm<sup>1</sup></sub> served as fully adapted controls. Accordingly, equally high MIC values were obtained.
for the ΔyrbE mutant and WT. Thus, the hypervesiculating strains have no advantage in non-adapted or fully adapted stages but only transiently during the adaptation phase (Figure 5A). To link the observed advantage to the lipid A modification, we performed the experiment in an ΔalmG background. Although deletion of yrbE in an ΔalmG background still resulted in an hypervesiculating phenotype (Figure S4A), the MICs of PMB were similar for the normally vesiculating ΔalmG and the hypervesiculating ΔalmGΔyrbE following the M9 to M9ToxR transition (Figure S4B). Notably, a lower adaptive PMB concentration (0.3 instead of 3 μg/mL) had to be used for ΔalmG and ΔalmGΔyrbE than for the other strains to accommodate the previously reported lower PMB resistance of alm mutants compared with WT (Hankins et al., 2012). Nonetheless, we observed earlier that induction of the Alm pathway was similar for 0.3 and 3 μg/mL PMB (Figure 2C), indicating that both PMB concentrations have a comparable potential to activate the Alm pathway. Thus, the transient advantage of the hypervesiculating strain in the PMB adaptation is AlmG dependent.

We next compared the survival fitness of WT, ΔyrbE, and yrbEΔ exposed to a sub-MIC PMB concentration (3 μg/mL) present in M9ToxR1/AlmT. Bacterial cultures were grown and shifted using the M9 and M9ToxR1/AlmT model as described above. Upon transition, cell viability dropped massively with equally low survival rates of approximately 1% obtained for all strains at 2 and 4 h (Figure 5B). At 8 h, average survival rates increased for all strains tested, suggesting adaptation to PMB. However, survival rates of ΔyrbE were significantly higher than that for WT and yrbEΔ, highlighting an advantage for the hypervesiculating strain. After 24 h, all strains had proliferated to a similar extent, exceeding the starting colony-forming unit (CFU). Moreover, growth upon transition from M9 to M9ToxR1/AlmT was analyzed to visualize the differential adaptation dynamics. After a relatively long lag phase, the hypervesiculating ΔyrbE entered into exponential growth about 2 h earlier than the WT (Figure 5C). Accordingly, the area under the curve (AUC), reflecting the overall biomass produced within the observed time, was significantly higher for the hypervesiculating ΔyrbE than that for WT (Figure 5D). Similar growth was observed for all strains used in these assays if bacteria were shifted from M9 to M9ToxR1 in the absence of PMB (Figures S5A and S5B). Thus, a general growth advantage of ΔyrbE can be excluded.

**Hypervesiculation Facilitates Faster Adaptation to Bile Salts during Transition Stages**

Next, we asked whether the faster removal of OmpT from the OM mediated by hypervesiculation promotes a more rapid adaptation to bile. Unexpectedly, the deletion mutant ΔyrbE was 3-fold more sensitive to bile compared with WT or yrbEΔ in M9 (Figure S6A). All three strains exhibited comparable sensitivity to PMB and SDS treatment, which excludes a general defect in the OM integrity upon hypervesiculation (Figures 5A and S6B). Characterization of the previous loss-of-function mutations of the VacJ/Yrb transporter, however, provided a possible explanation. The complete loss of the retrograde lipid trafficking resulted in excessive amounts of phospholipids in the OM (Roier et al., 2016). The normal lipid asymmetry of the OM was lost as a result, and large patches of phospholipid bilayers appeared (Malinverni and Silhavy, 2009; Roier et al., 2016). Importantly, bile penetrates such phospholipid bilayers more effectively than asymmetric OMVs composed of an LPS and phospholipid leaflet (Benz and Bauer, 1988; Hancock, 1984) and may therefore account for increased sensitivity of the ΔyrbE mutant to bile.

To overcome this limitation, a strain allowing finer control of the yrb genes was constructed. The chromosomal promoter of the yrb operon was replaced by an arabinose-inducible promoter strain, and the corresponding regulator araC was inserted into the lacZ locus generating the arabinose-inducible strain yrbF-B araA. This allowed a controllable expression of the yrb genes. Basal expression levels in the absence of arabinose and increased production in the presence of arabinose was confirmed by quantitative real-time PCR (Figure S6C). Accordingly, OMV production by a yrbF-B araA culture was low when grown in M9ToxR1 with arabinose and significantly higher in
addition to determine the MIC. Data presented are mean ± SD with the following number of biological replicates for yrbF-B<sup>ARA</sup> and yrbE<sup>ARA</sup>. (A–H) Significant differences are indicated by an asterisk (*p < 0.05). (H) Mean AUC values ± SD retrieved from the growth curves presented in (G) (n = 6).

See also Figures S4–S6.

M9<sup>ToxR</sup><sup>1</sup> lacking arabinose (Figure S6C). The yrbF-B<sup>ARA</sup> strain thus allowed us to assess the dynamics of vesiculation upon silencing of the yrb operon. Already, 4 h after the depletion of arabinose, a small but significant increase in vesiculation could be detected in comparison to a culture grown in the presence of arabinose (Figure S6C). Notably, the highest vesiculation levels achieved for yrbF-B<sup>ARA</sup> in the absence of arabinose were still 2-fold lower than that for the loss-of-function mutant ΔyrbE (Figures 1C and S6C), indicating a basal yrb operon expression level in yrbF-B<sup>ARA</sup> even in the absence of arabinose. Strain yrbF-B<sup>ARA</sup> followed the same principles as the regular vesiculating WT and hypervesiculating ΔyrbE<sup>ARA</sup> for the adaptation to PMB upon shift to M9<sup>ToxR<sup>1</sup>/Alm</sup> (Figures 5A and S6D). Consistent with the results obtained for WT and ΔyrbE, a higher MIC of PMB was observed for strain yrbF-B<sup>ARA</sup> under high vesiculation conditions (no arabinose) compared with low vesiculation cultivation (with arabinose). Most importantly, a similar sensitivity to bile was observed for yrbF-B<sup>ARA</sup> grown in M9 with and without arabinose (Figure 5E). Thus, the strain yrbF-B<sup>ARA</sup> is perfectly suited to analyze the impact of differential vesiculation on bile adaptation during transition events.

In general, bile adaptation experiments using the strain yrbF-B<sup>ARA</sup> in the presence of or absence of arabinose followed the design described above for the PMB resistance mediated by accumulation of (diglycine-modified lipid A (Figure 5). Overnight cultures of the strain yrbF-B<sup>ARA</sup> were grown in M9 or M9<sup>ToxR<sup>1</sup></sup> in the presence of arabinose to promote expression of the yrb genes, resulting in moderate vesiculation levels. Subsequently, overnight cultures were shifted from M9 to M9<sup>ToxR<sup>1</sup></sup> with or without arabinose (inducing ToxR-dependent ompT repression), from M9 to M9 with or without arabinose (no ompT repression) or M9<sup>ToxR<sup>1</sup></sup> to M9<sup>ToxR<sup>1</sup></sup> with or without arabinose.
Finally, growth curves in the presence of a sub-MIC bile concentration (0.1%) were created to visualize the differential adaptation dynamics (Figure 5G). Again, the hypervesiculating strain without arabinose entered earlier into the exponential phase and showed more robust proliferation compared with the lower vesiculating strain grown with arabinose. This advantage is also indicated by AUC values, which were significantly higher for the strain grown in the absence of arabinose than in the presence of arabinose (Figure 5H). Similar growth was observed in M9\textsubscript{ToxR\textsuperscript{R1}} with and without arabinose (Figures S5C and S5D), excluding an arabinose-dependent growth difference of strain \textit{yrBF-B\textsuperscript{PARA}}.

Pre-adaptation to Bile and PMB Negates In Vivo Advantage of a Hypervesiculating Strain

Our data support the model that the faster exchange of cell surface composition observed for hypervesiculating strains is associated with a faster adaptation to antimicrobial compounds. We therefore asked next whether this mechanism underlies the \textit{in vivo} fitness advantage of hypervesiculating strains. If true, the colonization advantage of a hypervesiculating strain should be diminished if fully adapted strains are used for the \textit{in vivo} competition experiments. We took advantage of the fact that growth overnight in M9\textsubscript{ToxR\textsuperscript{R1}} activates both infection-relevant surface modulations studied thus far. Equally high resistance levels to antibacterial challenge \textit{in vitro} indicated full adaptation of moderate and hypervesiculating strains (Figures 5A and 5E). The \textit{in vivo} competition assays were then performed using inocula of the hypervesiculating \textit{ΔyrBE} mutant and normally vesiculating WT that had been grown in M9 (no adaptation) or M9\textsubscript{ToxR\textsuperscript{R1}/Alm\textsuperscript{R1}} (full adaptation). As observed for LB cultures (Figure 1D), the hypervesiculating strain still outcompeted the WT \textit{in vivo} when the inoculum was derived from M9 cultures (Figure 6). In contrast, \textit{ΔyrBE} and WT competed equally well in the murine model when the inoculum was derived from M9\textsubscript{ToxR\textsuperscript{R1}/Alm\textsuperscript{R1}} cultures. Moreover, non-adapted WT (grown in M9) was approximately 4-fold attenuated \textit{in vivo} compared with an adapted WT (grown in M9\textsubscript{ToxR\textsuperscript{R1}}), whereas a non-adapted \textit{ΔyrBE} (grown in M9) was only 2-fold attenuated \textit{in vivo} compared with an adapted \textit{ΔyrBE} (grown in M9\textsubscript{ToxR\textsuperscript{R1}}). Thus, non-adapted strains are generally attenuated compared with their adapted counterparts, but hypervesiculation can partially compensate this disadvantage. Hence, we conclude that the \textit{in vivo} advantage of hypervesiculating strains at early stages of infection is due to accelerated surface modulation, thus promoting adaptation to antimicrobial stressors in the intestine.

DISCUSSION

This study provides insights into the adaptation strategies of the bacterial pathogen \textit{V. cholerae} along the environment to host transition. We characterize OMVs as a tool for OM exchange facilitating \textit{V. cholerae}'s adaptation to antimicrobial intestinal stressors upon host entry.

Upon ingestion by the host, \textit{V. cholerae} silences the VacJ/Yrb retrograde lipid trafficking system, which results in increased OMV release. In accordance with previous reports and data presented here, iron limitation is a likely signal for the repression of the well-conserved \textit{vacJ} and \textit{yrb} genes (Roier et al., 2016). Notably,
iron limitation—or nutrient limitation in general—could have been a driving evolutionary trigger for OMV release. OMV production generally increases during starvation conditions. Moreover, iron-scavenging proteins have been found in vesicles from several bacterial pathogens, which might imply a role of OMVs in iron acquisition (Gui et al., 2016; Lappann et al., 2013; Lin et al., 2017).

Coming from an aquatic lifestyle, *V. cholerae* needs to quickly change its expression profile in order to adapt to the conditions faced in the intestinal tract of the host. Besides activation of colonization factors and toxins, the adaptation to antimicrobial effectors is crucial for *V. cholerae* to achieve full virulence. The adaptation processes to cationic antimicrobial peptides and bile require changes in the OM profile, i.e., accumulation of (di)glycine-modified lipid A in LPS molecules and removal of OmpT (Hankins et al., 2011; Provenzano and Klose, 2000). Indeed, regulatory cascades driving both surface modulations are immediately activated upon host entry. Low levels of cationic antimicrobial peptides induce transcription of the AlmEFG system, which catalyzes the (di)glycine modification along de novo synthesis of LPS (Matson et al., 2017). Moreover, host entry activates TocR and downregulates *ompT* transcription (Li et al., 2000; Miller and Mekalanos, 1988). Nonetheless, the time elapsed until such transcriptional changes are manifest in OM alterations can be critical. This is especially true during early stages of the infection when bacterial proliferation is rather low. Besides preventing new OmpT production and activation of (di)glycine-modified LPS synthesis, it is crucial for *V. cholerae* to deplete disadvantageous structures from the surface. OMVs provide an efficient tool to discharge unfavorable OM compounds, including proteins such as OmpT and polysaccharides such as unmodified LPS. Increased vesiculation in *vivo* not only enhances the removal of factors detrimental for colonization but also creates new space for beneficial compounds. As demonstrated in this study, hypervesculation of *V. cholerae* allows an efficient exchange of the surface profile, facilitating the adaptation process to cationic antimicrobial peptides and bile along the environment to host transition. The importance of this adaption is highlighted by the observation that hypervesculation is associated with increased colonization fitness, whereas hypovesiculation results in reduced *vivo* fitness.

Notably, increased vesiculation provides a transient advantage during the adaptation process. Once the surface profile change is completed, the advantage of hypervesculation is lost. Previous reports indicate that increased OMV production in *E. coli* correlates with better bacterial survival in the presence of antimicrobial agents (Kulkarni et al., 2015; Manning and Kuehn, 2011; Urashima et al., 2017). This is likely due to the fact that OMVs can act as a sink for these substances. Notably, these reports rely on OMV amounts present in fully grown cultures. To initiate the transition processes studied here, the bacterial cultures are massively diluted. Thus, the initial OMV concentrations in the survival assays of this study are very low (approximately 100-fold below the OMV amounts described to be beneficial). This difference might explain why the advantage of the hypervesiculating strain in the present study is only observed during transitions activating the surface exchange and therefore relies on the OM adaptation rather than the OMV amount.

The continuous liberation of compounds of the OM and periplasm via OMVs is highly energy consuming. Thus, it is likely that bacteria have evolved to regulate OMV release in accordance with their needs, e.g., induction of vesiculation upon host entry. Notably, the regulation of vesiculation via transcriptional control of the VacuJ/Yrb lipid trafficking system in response to iron availability seems to be conserved among diverse Gram-negative bacteria (Roier et al., 2016). Moreover, several Gram-negative pathogens modulate the OM composition upon host entry to achieve full colonization fitness. In addition to the surface modifications analyzed in this study, prominent examples include modulation of OM protein abundance involved in serum resistance (e.g., reported for *Neisseria gonorrhoeae* and *Borrelia burgdorferi*) or iron uptake (e.g., reported for *Klebsiella pneumoniae*, *Pasteurella haemolytica*, and *H. influenzae*), OM-associated proteases of *E. coli* and *Salmonella typhimurium* that degrade antimicrobial peptides, LPS modifications such as the lipid A palmitoylation (e.g., reported for *S. typhimurium*, *E. coli*, and *Yersinia enterocolitica*), the lipid A aminoadeninabine modification (reported for *S. typhimurium*), and the aminolactylation of phospholipids reported for *Pseudomonas aeruginosa* contributing to antimicrobial peptide resistance (Gunne et al., 1998; Klein et al., 2009; Lin et al., 2002). Additionally, a recent *in vitro* study suggested that OMV production of *S. typhimurium* can facilitate infection-relevant LPS remodeling in the OM triggered by pH and magnesium availability (Bonnington and Kuehn, 2016). Based on these observations, we hypothesize that the surface exchange via increased vesiculation upon host entry could be a common principle of bacterial pathogens to quickly adapt to *in vivo* conditions.

Moreover, OMVs of pathogens have been reported to act as delivery vehicles of bacterial toxins and as a sink for host-derived antimicrobial factors, such as defensins and the complement system (Elluri et al., 2014; Kulp and Kuehn, 2010; Roier et al., 2016). Thus, it is becoming evident that enhanced bacterial vesicle production during host colonization can be linked to various beneficial roles, and OMVs can increase the colonization fitness of bacterial pathogens in multiple ways.

Notably, hypervesiculation is not only associated with iron availability. The VacuJ/Yrb lipid trafficking system might be controlled by additional regulatory elements, and alternative OMV biogenesis mechanisms have been reported (Kulp and Kuehn, 2010). Thus, other signals may induce vesiculation, which extend the observations beyond the environment to host transition performed by the facultative human pathogen *V. cholerae*. Because production of membrane vesicles is observed for Gram-negative and Gram-positive bacteria, it is tempting to speculate that varying vesiculation levels could represent a general and efficient adaptation strategy of bacteria during transitions demanding a surface profile modulation.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Housing Conditions for Experimental Animals
  - Competition Assay


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The authors declare no competing interests.

DECLARATION OF INTERESTS


G.N.R., M.J.K., and Z.G., Writing – Original Draft, F.G.Z.; Writing – Review &

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Conceptionalization, F.G.Z. and S.S.; Methodology, F.G.Z. and S.S.; Investiga-

tion, F.G.Z., P.K., F.C., D.R.L., F.M., K.E.B., G.N.R., and Z.G.; Resources,

G.N.R., M.J.K., and Z.G.; Writing – Original Draft, F.G.Z.; Writing – Review &

Editing, F.G.Z., F.C., K.E.B., J.R., and S.S.; Visualization, F.G.Z.; Supervision,


AUTHOR CONTRIBUTIONS

Conceptualization, F.G.Z. and S.S.; Methodology, F.G.Z. and S.S.; Investigation,


G.N.R., M.J.K., and Z.G.; Writing – Original Draft, F.G.Z.; Writing – Review &

Editing, F.G.Z., F.C., K.E.B., J.R., and S.S.; Visualization, F.G.Z.; Supervision,


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REFERENCES


## STAR★METHODS

### KEY RESOURCES TABLE

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<th>IDENTIFIER</th>
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#### Chemicals, Peptides, and Recombinant Proteins

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#### Experimental Models: Organisms/Strains

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</tr>
</tbody>
</table>

(Continued on next page)
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Stefan Schild (stefan.schild@uni-graz.at). All reagents generated in this study are available from the Lead Contact with a completed Materials Transfers Agreement.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Housing Conditions for Experimental Animals**

5 to 6 day old CD-1 mice (Crl:CD1, Charles River Laboratories) of both genders were used in all experiments in accordance with the rules of the ethics committee at the University of Graz and the corresponding animal protocol, which has been approved by the Austrian Federal Ministry of Science and Research Ref. II/10b. Mice were housed with food and water ad libitum and monitored under the care of full-time staff.

**Competition Assay**

Competition assays in infant mice (in vivo assay), LB broth or minimal media M9 were performed with WT, deletion mutants, or WT pyrbF-B (lacZ') competed for ~22 h against isogenic WT, deletion mutants, or WT strain carrying an empty vector (lacZ') essentially as previously described (Camilli and Mekalanos, 1995; Schild et al., 2007). The individual strains and conditions used for each competition are indicated along the presentation of the respective data set. Briefly, strains were grown on LB plates or in minimal media M9 (M9, M9ToxR<sup>+</sup> or M9ToxR<sup>−</sup>/Alm<sup>+</sup>) overnight (O/N), diluted to OD<sub>600</sub> = 0.002 in a 1:1 ratio, and used to intragastrically inoculate

---

**LEAD CONTACT AND MATERIALS AVAILABILITY**

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infant mice (5-day old CD-1 mice). Appropriate dilutions of the inoculum were plated on LB-Sm/X-Gal or on LB-Ap/X-Gal plates in case of *V. cholerae* strains harboring a plasmid to determine the exact input ratio. Approximately 22 h post-infection mice were euthanized, their small bowels were removed and homogenized in 1 ml of LB with 15% glycerol. *In vitro* competitions in LB or minimal media M9 were performed in parallel by inoculation of 2 ml liquid culture with ~\(10^5\) CFU from the inoculum and subsequent incubation for ~22 h at 37°C with aeration. CFU were determined by plating appropriate dilutions of the homogenized intestine or culture grown *in vitro* on LB-Sm/X-Gal or on LB-Ap/X-Gal plates in case of *V. cholerae* strains harboring a plasmid. Results are given by the competition index (CI), which is the ratio of lacZ^-CFU to lacZ^+CFU normalized for the input ratio. If applicable, plasmid maintenance was determined along the competitions *in vivo* and *in vitro* by calculating dividing the respective Ap^R^-CFU (LB-Ap/X-Gal plates) by the Sm^R^-CFU (LB-Sm/X-Gal).

**Resolution Assay**
Resolution assay was performed as previously described (Cakar et al., 2018). To quantify resolution, strain Vc_res1_TRIVET *yrbF*:\tpc was grown O/N on LB-Sm/Km/Ap plates and adjusted in LB-Sm/Km/Ap to OD\(_{600}=1\), which was used as inoculum. To determine *in vitro* resolution frequencies, the inoculum was diluted 1:100 in 5 ml of LB-Sm/Ap, LB-Sm/Ap supplemented with 75 \(\mu\)M of 2,2’-bipyridyl or 100 \(\mu\)M of FeSO\(_4\) and incubated for 8 h. To determine the *in vivo* resolution, the inoculum was diluted 1:500 in LB and anesthetized 5-day old CD-1 mice were intragastrically inoculated with 50 \(\mu\)l of this dilution (~\(10^5\) CFU per mouse). Mice were euthanized at 6 or 22 h post-infection time, and bacteria were recovered from intestine. At the given time point, the amount of resolution *in vitro* and *in vivo* was determined by plating appropriate dilutions on LB-Sm/Km and LB-Sm/Ap plates. Results were expressed as % resolution, calculated as the Sm^R//Km^Km^R CFU (Sm^R//Ap^K Ap^K CFU minus Sm^R//Km^K CFU) divided by Sm^R//Ap^K CFU.

**Infection Studies to Obtain Bacterial RNA**
For *in vivo* studies, anesthetized 5-day old CD-1 mice were intragastrically inoculated with 50 \(\mu\)l of WT adjusted to OD\(_{600}\) of 0.002 in LB, corresponding to an infection dose of ~\(10^6\) CFU per mouse. After 22h, mice were euthanized, and small intestine was removed and mechanically homogenized in 1.5 mL of TRIzol Reagent (Thermo Fisher Scientific).

**METHOD DETAILS**

**Bacterial Strains and Growth Conditions**
Bacterial strains, plasmids and oligonucleotides used in this study are listed in the “Key Resources Table”. The clinical isolate *V. cholerae* O1 El Tor E7946 (Miller et al., 1989) served as wild type (WT) strain in all experiments. Unless stated otherwise, all *V. cholerae* strains were grown with aeration in *E. coli* broth at 37°C. DH5alpha (pir and SM10pir) (Miller and Mekalanos, 1988) were used for genetic manipulations and grown with aeration in LB broth at 37°C. Unless stated otherwise, minimal media M9 compositions are abbreviated as follows: M9 for minimal media M9 supplemented with glucose; M9ToyR for minimal media M9 supplemented with glucose, asparagine, arginine, glutamate and serine; M9ToyR/M-IslamG for minimal media M9 supplemented with glucose, asparagine, arginine, glutamate, serine and sub-MIC concentrations of polymyxin B (PMB, 3 \(\mu\)g/ml or 0.3 \(\mu\)g/ml for strains with almG background, respectively). Antibiotics and other supplements were used in the following concentrations: streptomycin (Sm, 100 \(\mu\)g/ml), ampicillin (Ap, 50 \(\mu\)g/ml) in combination with other antibiotics, otherwise 100 \(\mu\)g/ml), sucrose (10%), glucose (0.2%), arabinose (Ara, 0.2%), asparagine (25 mM) arginine (25 mM), glutamate (25 mM), and serine (25 mM).

**Genetic Manipulations**
The isolation of chromosomal DNA, PCR reactions, the purification of plasmids or PCR products, the construction of suicide and expression plasmids as well as the subsequent generation of deletion mutants were carried out as described previously (Pressler et al., 2016; Seper et al., 2011). Qiagen plasmid kits were used for isolation of plasmid DNA. Qiaquick® Gel extraction and Qiaquick® PCR Purification kits (Qiagen) were used for purifying DNA fragments. PCR reactions for subcloning were carried out using the Q5® High-Fidelity DNA Polymerase (NEB), while Taq DNA Polymerase (NEB) was used for all other PCRs. Constructions of in-frame almG deletion mutants were carried out as described by Donnenberg and Kaper (Donnenberg and Kaper, 1991). Briefly, ~800 bp PCR fragments located up- and downstream of the gene of interest were amplified using the oligonucleotide pairs almG\(_{XbaI}\)\_1 and almG\(_{SacI}\)\_4. After digestion of the PCR fragments with the appropriate restriction enzyme (NEB) indicated by the name of the oligonucleotide, they were ligated into pCVD442, which was digested with the appropriate restriction enzymes.

Complementation of *yrbE* was achieved either in trans by using the expression plasmid pyrbE (Roier et al., 2016) or by re-insertion of a slightly modified *yrbE* allele (altered nucleotide sequence in the wobble position of some codons, resulting in identical amino acid sequence compared to the WT allele) in the original chromosomal gene locus to avoid the usage of antibiotics for plasmid maintenance. The latter was constructed via PCR fragments amplified using the oligonucleotide pairs VC2519\_Xbal\_up\_fw and comp_\_yrbE\_1 as well as comp_\_yrbE\_2 and VC2519\_Xmal\_down\_rv and subsequent splicing by overlap extension (SOE) PCR of the two purified PCR fragments introducing three silent point mutations changing AACTGATCA to AATTAAACA located around stop codon of *yrbE*. The SOE PCR fragment was digested with Xmal and Xbal (NEB), ligated into a similar digested pCVD442 resulting in pCVDyrbE*.

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In case of the suicide vector pCVDlacZ::araC used for araC insertion into the lacZ-locus of *V. cholerae* ~800 bp PCR fragments flanking lacZ were amplified using the oligonucleotide pairs lacZ_XbaI_1 and lacZ_BamHI_2 as well as lacZ_SphI_3 and lacZ_SacI_4, while araC was amplified from pBAD18 using the oligonucleotide pairs araC_BamHI_1 and araC_EcoRI_2. All fragments were digested using the restriction enzymes, indicated by the name of oligonucleotide. To construct the suicide vector pCVDyrbF-BpARA*, replacing the natural yrb-promoter with an arabinose-inducible promoter, ~800 bp PCR fragments flanking the yrb-promoter were amplified using the oligonucleotide pairs yrb_SacI_1 and yrb_SphI_2 as well as yrb_EcoRI_3 and yrb_XbaI_4, while the fragment harboring the arabinose-inducible promoter was amplified from pBAD18 using the oligonucleotide pairs pARA_SphI_1 and pARA_EcoRI_2. After digestion with the restriction enzymes, indicated by the name of oligonucleotide, PCR fragments were ligated into pCVD442, restricted with appropriate restriction enzymes (NEB).

The suicide vectors pGPphoA-almG, pGPphoA-ompU and pGPphoA-ompT for chromosomal insertion were constructed to obtain chromosomal transcriptional fusions of promoterless phoA to almG (almG::phoA), ompU (ompU::phoA) and ompT (ompT::phoA), as PhoA acts as a reporter for gene expression in *V. cholerae*. DNA fragments containing the upstream region of almG, ompU and ompT were amplified by PCR using oligonucleotide pair almG_pGPphoA_XbaI and almG_pGPphoA_KpnI, the PCR product was digested with the restriction enzymes indicated and ligated with similarly digested pGPphoA.

For construction of the expression plasmid pyrbF-B the entire yrb- operon was amplified with oligonucleotides yrb_operon_SacI_1, yrb_operon_XbaI_2, the PCR fragment was digested with the appropriate restriction enzymes and ligated into a similar digested pMMB67EH.

Unless noted otherwise, ligation products were transformed into DH5αλpir and ApR colonies were characterized for the correct constructs by PCR (and restriction analysis).

To obtain insertion and deletion strains generated derivatives of the pGPphoA or pCVD442 were transformed into *E. coli* Sm10:λpir and conjugated into *V. cholerae*. Exconjugants were purified by SmR/ApR selection. In the case of pCVD442 derivatives sucrose selection was used to obtain ApR colonies and chromosomal deletions/replacements were confirmed by PCR, respectively. To generate the complementation strain yrbE* the pCVDyrbE* was mobilized into ΔyrbE, exconjugants were purified by SmR/ApR selection and finally sucrose selection was used to obtain ApR colonies. Correct chromosomal insertion of the *yrbE* allele was confirmed via PCR using a discriminator oligonucleotide pair yrbE*_test and VC2519_XmaI_down_rv as well as via sequencing using yrbE*_seq.

**Resolution Assay**

Resolution assay was performed as previously described (Cakar et al., 2018). To quantify resolution, strain Vc_res1_TRIVET yrbF::tpc was grown O/N on LB-Sm/Km/Ap plates and adjusted in LB-Sm/Km/Ap to OD_{600}=1, which was used as inoculum. To determine in *vitro* resolution frequencies, the inoculum was diluted 1:100 in 5 ml of LB-Sm/Ap, LB-Sm/Ap supplemented with 75 µM of 2,2’-bipyridyl or 100 µM of FeSO_{4} and incubated for 8 h. To determine the in *vivo* resolution, the inoculum was diluted 1:500 in LB and anesthetized 5-day old CD-1 mice were intragastrically inoculated with 50 µl of this dilution (~10^{6} CFU per mouse). Mice were euthanized at 6 or 22 h post-infection time, and bacteria were recovered from intestine. At the given time point, the amount of resolution in *vitro* and in *vivo* was determined by plating appropriate dilutions on LB-Sm/Km and LB-Sm/Ap plates. Results were expressed as % resolution, calculated as the SmR/KmS CFU [SmR/ApR CFU – SmR/KmR CFU] divided by SmR/ApR CFU.

**Quantitative Real-time PCR**

Expression of *yrbE* and *vacA* was determined by quantitative real-time RT-PCR (qRT-PCR). For *in vitro* studies, WT was grown to an OD_{600} of 0.5 to 0.8 in LB, while the arabinose-inducible *yrbF*-B variant was grown to an OD_{600} of approximately 0.8 in M9ToxR with (w) or without (w/o) arabinose (ara). Bacterial RNA extraction, DNase digestion, cDNA synthesis, and qRT-PCR were performed as described previously (Lichtenegger et al., 2014). For *in vivo* studies, anesthetized 5-out 10 cd-1 mice were intragastrically inoculated with 50 µl of wt adjusted to OD_{600} of 0.002 in LB, corresponding to an infection dose of ~10^{6} CFU per mouse. After 22 h, mice were euthanized, and small intestine was removed and mechanically homogenized in 1.5 ml of TRizol Reagent (Thermo Fisher Scientific). RNA was isolated using either the Monarch Total RNA Miniprep Kit (New England Biolabs) or the RNA isolation Kit RNeasy (Quiagen) for *in vitro* samples grown in LB as well as *in vivo* samples or the PeqGOLD total RNA Kit (Peqlab) for *in vitro* samples grown in minimal media M9 according to the manufacturer’s protocols, and chromosomal DNA was digested by using RO1 RNase-Free DNase (Promega). Synthesis of cDNA and qRT-PCR were performed as described previously using the iScript Select cDNA Synthesis Kit (Bio-Rad) and the SYBR GreenER qPCR SuperMix for ABI PRISM ([Thermo Fisher Scientific]; [Seber et al., 2013]). Each cDNA sample was tested in triplicate. The sequences of the primers used for qRT-PCR starting with “qPCR,” are listed in the “Key Resources Table.” Results were analyzed using StepOne Software v2.1, and relative gene expression comparisons were calculated by the mean cycle threshold of samples, which were normalized to the housekeeping gene 16S RNA (VCr001) and to one randomly selected *in vitro* reference sample.

**Isolation of Lipid A**

The protocol for large scale lipid A extraction as described by Henderson and coworkers was used (Henderson et al., 2013). Briefly 500 ml of respective culture were harvested, washed with PBS and resuspended in 40 ml PBS. Subsequently 50 ml of chloroform and 100 ml of methanol were added to create a single-phase Bligh-Dyer mixture. Samples were washed with...
single-phase Bligh-Dyer mixture and resuspended in 54 ml of mild acid hydrolysis buffer. Samples were boiled for 1 h and then converted to a two-phase Bligh-Dyer mixture by adding 60 ml of chloroform and 60 ml of methanol. The lower phase was extracted and a second extraction was performed using the lower phase of a pre-equilibrated two-phase Bligh-Dyer mixture. Both lower phases were pooled and washed with new upper phase of a pre-equilibrated two-phase Bligh-Dyer mixture and dried for subsequent usage.

Analysis of Lipid A by LC-MS

Normal phase LC-MS was performed using an Agilent 1200 Quaternary LC system coupled to a high-resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). A Unison UK-Amino column (3 μm, 25 cm × 2 mm) (Imtakt USA, Portland, OR) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800/195/5, v/v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600/340/50/5, v/v/v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450/450/95/5, v/v/v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 μl/min. The MS settings are as follows: Ion spray voltage (IS) = -4500 V, Curtain gas (CUR) = 20 psi, Ion source gas 1 (GS1) = 20 psi, De-clustering potential (DP) = -55 V, and Focusing Potential (FP) = -150 V. Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using Analyst TF1.5 software (Sciex, Framingham, MA). High-resolution mass spectra and isotope patterns allowed a precise identification of the respective lipid A species. It should be noted that preparation of lipid A extracts derived from V. cholerae requires high culture volume and is labor-intensive. Thus, lipid A samples were prepared in batches and subjected to LC-MS analysis within two weeks to minimize artefact formation and sample degradation. Variations in LC retention times for the respective lipid A species can occur between different batches analyzed in different runs due to changes in column and solvent conditions.

Preparation of OMVs

In general, OMV were isolated as described previously with minor adaptations (Schild et al., 2009). O/N cultures of the respective strains were cultivated in M9, diluted 1:100 in M9ToxR or M9ToxR† with arabinose and grown for 4 or 8 h at 37°C and 180 rpm, before the cells were removed from the supernatant by centrifugation (9000 × g, 15 min). The supernatant was filtered through 0.22 μm pore size filters to remove intact cells. OD600 of each culture was determined by photometric measurements using a Beckman Coulter DU730 spectrophotometer for subsequent OMV quantification. To ensure that no bacteria were left in the supernatant, 1 ml of the filtrate was plated on LB-agar plates and incubated at 37°C O/N. The OMVs present in the supernatant were pelleted through subsequent ultracentrifugation (150,000 g, 4°C, 4 h) as previously described (Schild et al., 2009). Protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Protein Assay Dye Reagent) according to the manufacturer’s manual and normalized to the OD600 of the respective culture.

Preparation of Outer Membrane Proteins and Whole Cell Lysates

Proteins of the outer membrane were essentially prepared as previously published (Carlone et al., 1986; Roier et al., 2013; Salem et al., 2015). O/N cultures of the respective strains were cultivated in M9 and diluted 1:100 in M9ToxR†. At the indicated time point cultures of V. cholerae were harvested by centrifugation (3,200 g, 10 min, 4°C), washed once in HEPES buffer (10 mM, pH 7.4) and resuspended in 0.75 ml HEPES buffer (10 mM, pH 7.4). Then the suspension was transferred in a cryo-tube and cells were disrupted by homogenization with 0.1 mm glass beads in combination with a PowerLyzerTM 24 (MO BIO Laboratories, Inc.), applying three times, 1 min cycles at 3400 rpm with 1-min intervals on ice between each cycle. Unbroken cells were removed by centrifugation (13,000 g, 5 min, 4°C). The supernatant containing the outer membrane material was transferred into a new tube and centrifuged again (16,100 g, 30 min). The membrane pellet was re-suspended in 0.4 ml HEPES buffer (10 mM, pH 7.4). To solubilize the cytoplasmic membrane, 0.4 ml HEPES buffer (10 mM, pH 7.4) with 2% sarcosyl was added and incubated at room temperature and constant shaking for 30 min. After centrifugation (16,100 g, 30 min), the pellet containing proteins of the outer membrane was washed once with 0.5 ml HEPES buffer (10 mM, pH 7.4) and finally re-suspended in 50 μl HEPES buffer (10 mM, pH 7.4). Purified outer membrane preparations were stored at -20°C. The protein concentrations of outer membrane preparations were determined by photographic measurements of the absorbances at 260 nm and 280 nm using a Beckman Coulter DU730 spectrophotometer in combination with a TrayCell (Hellma) and the Warburg-Christian equation given as mg protein/ml = [(1.31 x A280) - (0.57 x A260)] x dilution factor (Warburg and Christian, 1941).

For whole cell lysates, equal amounts of cells (equivalent to 1.3 ml of an OD600=1) were harvested by centrifugation (3,200× g, 10 min, 4°C) from the respective V. cholerae cultures. Cell pellets were directly resuspended in 53 μl SDS-PAGE sample buffer (Laemmli, 1970), boiled for 10 min and subjected to SDS-PAGE.

SDS-PAGE and Immunoblot

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using polyacrylamide (15%) gels in combination with the Mini-PROTEAN Tetra cell system (Bio-Rad, Vienna) (Laemmli, 1970). As molecular mass standard Pre-stained Protein Marker Broad Range (New England Biolab) or PageRulerTM Prestained Protein Ladder 10 to 180 kDa (Thermo Fisher)
were used as indicated. Subsequently protein bands were visualized according to Kang et al. (Kang et al., 2002) or further processed for immunoblot analysis as previously described (Schild et al., 2008). As primary antibodies (anti-OmpU, anti-OmpT and anti-RpoA) generated in mice were used ([Leitner et al., 2013; Salem et al., 2015] and BioLegend). HRP-linked anti-mouse IgG was used as secondary antibody. Chemiluminescence detection was performed using the Immuno-Star™ WesternC^TM Kit (Bio-Rad Laboratories) and subsequent exposure in a ChemiDoc XRS system (Bio-Rad Laboratories) in combination with Quantity One software (Bio-Rad Laboratories).

Growth Kinetics

Growth kinetics were essentially performed as previously described in transparent 24-well plates (Greiner) with 1 ml culture volume (Gumpenberger et al., 2016; Moisi et al. 2013; Seper et al. 2011). Briefly, the respective strains were grown in a pre-culture for ~16 h in M9 with aeration and shaking at 37 °C. For growth assays of the WT, ΔyrbE mutant or yrbE<sup>e</sup> complementation strain, pre-cultures were adjusted to OD<sub>600</sub> = 0.05 in M9 or M<sub>GtoxR</sub>Tox<sub>R</sub> Mutant [[(di)glycine-modified lipid A activating conditions using sub-MIC PMB concentrations (3 μg/ml)] as well as ΔalmG or ΔalmGΔyrbE were grown in M9. O/N cultures were shifted into fresh M9 or M<sub>GtoxR</sub>Tox<sub>R</sub> Mutant [[(di)glycine-modified lipid A activating conditions using sub-MIC PMB concentrations (3 μg/ml or 0.3 μg/ml for strains with ΔalmG background, respectively)] to a final concentration of 10<sup>6</sup> CFU/ml. The arabinose-inducible yrbF-B variant was grown O/N in M9 with arabinose or M<sub>GtoxR</sub>Tox<sub>R</sub> Mutant with arabinose. To remove residual arabinose, the O/N cultures were pelleted by centrifugation (6000 x g, 10 min), resuspended in fresh M9 and then diluted into M9 with or without arabinose to a final concentration of 10<sup>6</sup> CFU/ml. Alternatively, O/N cultures were pelleted by centrifugation (6000 x g, 10 min), resuspended in fresh M9ToxR and then adjusted in 25 ml M9ToxR with or without arabinose to a final concentration of 10<sup>9</sup> CFU/ml.

In all cases, bacteria were allowed to adapt for ~2 h, before 100 μl of the bacterial culture were placed into 96-well plates and 10 μl of appropriate serial dilutions of the respective antimicrobial agent (bile salts, additional PMB, SDS) were added. Two-fold serial dilutions of antimicrobial agents were tested in the following final concentrations: 8% to 0.0078% for bile salts, 3,200 μg/ml to 0.39 μg/ml for PMB and 0.25% to 0.0078% for SDS. After 18 h incubation in a humid chamber at 37 °C OD<sub>600</sub> was measured using the Spectrostar<sup>Nano</sup> Microplate Reader (BMG Labtech). The MIC was defined as the lowest antimicrobial agent concentration, which inhibited bacterial growth. Growth was defined by an at least three-fold increase of the OD<sub>600</sub> compared to the respective sterile control (M9 with or without arabinose or M<sub>GtoxR</sub>Tox<sub>R</sub> Mutant with or without arabinose).

Survival Assays

<em>V. cholerae</em> WT, ΔyrbE mutant or the complementation strain yrbE<sup>e</sup> were grown O/N in M9, adjusted to an OD<sub>600</sub> = 0.001 in 25 ml M<sub>GtoxR</sub>Tox<sub>R</sub> before PMB was added to a final concentration of 3 μg/ml. For survival assays in presence of bile, the arabinose-inducible yrbF-B variant was grown O/N in M9 with arabinose, washed once in M<sub>GtoxR</sub>Tox<sub>R</sub> and then adjusted in 25 ml M<sub>GtoxR</sub>Tox<sub>R</sub> with or without arabinose to an OD<sub>600</sub> = 0.001. After an adaptation for 2 h in M<sub>GtoxR</sub>Tox<sub>R</sub> (activating OmpU/T switch) bile salts (0.1 %) were added. In general, samples were taken just before addition of PMB or bile salts (0 h), as well as 2, 4, 8 and 24 h after addition of the antimicrobial agent and appropriate dilutions were plated on LB plates. Percent survival for 2, 4, 8 and 24 h was calculated by the determined CFU at the respective time point divided by the determined CFU at 0 h multiplied by 100.

Alkaline Phosphatase Assays

To determine the enzymatic activities for the transcriptional ompU::phoA, ompT::phoA and almG::phoA fusion, alkaline phosphatase assays were performed as described previously (Manoil, 1991). Briefly, bacterial cultures were shifted from M9 to M<sub>GtoxR</sub>Tox<sub>R</sub> in case of ompU::phoA and ompT::phoA or shifted from M9 to M<sub>GtoxR</sub>Tox<sub>R</sub> Mutant in case of almG::phoA. Subsequently, bacterial cultures were harvested after 0, 1, 2, 4 and 8 h and subjected to the alkaline phosphatase assays. The activities were expressed in Miller units, given by (A<sub>405</sub> × 1,000)/(A<sub>600</sub> × ml × min).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data sets are presented as mean with standard deviation (SD) in case of normal distribution or as median with interquartile range (IQR) in case of non-gaussian distribution. Statistical analyses were performed using GraphPad Prism 5.0 as follows: unpaired t-test (single comparison) or one-way Anova followed by Bonferroni’s post test (multiple comparisons) in case of normal distribution.
(data sets presented as mean with SD) or Mann–Whitney U test (single comparison) or Kruskal–Wallis followed by Dunn’s post test (multiple comparisons) in case of non-gaussian distribution (data sets presented as median with IQR). A P value of less than 0.05 was considered significant and indicated by an asterisk (*P < 0.05). Unless stated otherwise, the number of biological replicates for each data set is given by “n” and is provided in the respective figure legend.

DATA AND CODE AVAILABILITY

This study did not generate/analyze datasets/codes.