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***Vibrio cholerae* residing in food vacuoles expelled by protozoa are more infectious in vivo**

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Abstract

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Author contributions

G.E.-V., P.N., A.C. and D.M. designed the study and planned the experiments. G.E.-V., P.N., C.A.S.-V., B.B.A.R., M.M.H., C.A. and A.C. carried out the experiments. G.E.-V., B.B.A.R. and M.S.J. performed the microscopy experiments. G.E.-V., P.N. and C.A. carried out the biological assays. G.E.-V., C.A.S.-V. and A.C. performed the infection assay. M.P. assisted with TEM imaging. G.E.-V., P.N., S.S., A.C., M.L. and D.M. contributed to interpretation of the results. G.E.-V. and D.M. took the lead in writing the manuscript. A.C., S.K., S.P.D., M.L. and D.M. provided funding. All authors provided critical feedback and helped shape the manuscript.

Competing interests

The authors declare no competing interests.

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Vibrio cholerae interacts with many organisms in the environment, including heterotrophic protists (protozoa). Several species of protozoa have been reported to release undigested bacteria in expelled food vacuoles (EFVs) when feeding on some pathogens. While the production of EFVs has been reported, their biological role as a vector for the transmission of pathogens remains unknown. Here we report that ciliated protozoa release EFVs containing *V. cholerae*. The EFVs are stable, the cells inside them are protected from multiple stresses, and large numbers of cells escape when incubated at 37 °C or in the presence of nutrients. We show that OmpU, a major outer membrane protein positively regulated by ToxR, has a role in the production of EFVs. Notably, cells released from EFVs have growth and colonization advantages over planktonic cells both in vitro and in vivo. Our results suggest that EFVs facilitate *V. cholerae* survival in the environment, enhancing their infectious potential and may contribute to the dissemination of epidemic *V. cholerae* strains. These results improve our understanding of the mechanisms of persistence and the modes of transmission of *V. cholerae* and may further apply to other opportunistic pathogens that have been shown to be released by protists in EFVs.

The aquatic bacterium *Vibrio cholerae* is the aetiological agent of the acute diarrheal disease cholera, which is endemic in many countries. Outbreaks are linked to inadequate access to clean water and sanitation and it is estimated that there are 1.3 to 4.0 million cases and 21,000 to 143,000 deaths annually worldwide¹. Both toxigenic (producing cholera toxin (CT)) and non-toxigenic (CT-negative) *V. cholerae* are globally-distributed aquatic bacteria. Despite strong evidence that the primary habitat of *V. cholerae* is the marine environment² (for example, estuarine and coastal waters³), there are also reports showing the persistence of *V. cholerae* in freshwater systems⁴. In the aquatic environment, *V. cholerae* interacts with sediments and many organisms, including protozoa, aquatic plants, phytoplankton and zooplankton, all of which may act as reservoirs. These natural reservoirs may have a critical role in survival of *V. cholerae* in inter-epidemic periods and may be responsible for the development of virulence⁵. For example, it has been shown that *V. cholerae* colonizes and reproduces in copepods, and copepod blooms might result in the numbers of *V. cholerae* required for an infective dose⁶. Furthermore, cholera outbreaks have been linked to ingestion of fresh fish⁷, shellfish, crabs and oysters⁸.

Protozoa take up bacterial prey into phagosomes that become acidified and filled with enzymes, resulting in digestion. However, several species of ciliates and amoebae can package and release undigested cells when feeding on certain species of bacterial pathogens. For example, the amoebae *Acanthamoeba* spp. and *Dictyostelium discoideum* and ciliates such as *Tetrahymena* spp., *Colpodia* spp. and *Glaucom* spp. release food vacuoles containing live bacterial cells when feeding on *Salmonella enterica*, *Legionella pneumophila*, *Mycobacterium smegmatis*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Pseudomonas aeruginosa* and *Helicobacter pylori*^{9–17}. Notably, bacteria inside EFVs are more resistant to acidic environments¹⁸, freeze–thaw, sonication and 24 h exposure to cooling-tower biocides¹⁹. Cells within EFVs show enhanced survival under starvation conditions and may remain viable for at least six months¹⁰. Such resistance to stress and long-term starvation may facilitate the subsequent uptake by and infection of a host¹¹, although this has not yet been demonstrated. This represents a major gap in our understanding of epidemiology of many infectious diseases.

V. cholerae produces defences against *Tetrahymena pyriformis*, including the PrtV protease²⁰, chitin-induced production of ammonia²¹ and the pigment pyomelanin²². However, to our knowledge, the production of *V. cholerae* EFVs has not previously been demonstrated. Here we report that co-incubation of *V. cholerae* with different ciliates results in the release of *V. cholerae* in EFVs to the extracellular environment. We also demonstrate that *V. cholerae* EFVs survive better than planktonic free-living cells under different stresses and show an increased infectious potential. Taken together, our results suggest that *V. cholerae* EFVs lead to increased survival of *V. cholerae* epidemic strains in both the natural and the host environment, contributing to the dissemination and infection of *V. cholerae*.

Results

The production of *V. cholerae* EFVs by *T. pyriformis* is dependent on prey cell number and enhanced by bacterial protein synthesis.

This study shows that when *V. cholerae* and *T. pyriformis* are co-incubated, EFVs containing live undigested bacteria are released into the environment through the ciliate cytoproct (anus) (Fig. 1a, Supplementary Fig. 1 and Supplementary Video 1). Transmission electron microscopy (TEM) of the EFVs reveals that *V. cholerae* is packaged into multilamellar vacuoles, similar to previously reported EFVs containing *L. pneumophila*^{9,19} (Fig. 1b and Supplementary Fig. 2a). The production of *V. cholerae* EFVs increases as the bacterial concentration increases, showing that EFV production is dependent on prey cell number (Fig. 1c). EFVs are also produced when *V. cholerae* is co-incubated with *Acanthamoeba castellanii* (Supplementary Fig. 2b).

To determine whether active *V. cholerae* is necessary for EFV production, bacteria were pre-treated with sublethal concentrations of gentamicin to inhibit protein synthesis. Inhibition of protein synthesis resulted in a significant decrease in production of EFVs by *T. pyriformis* (Fig. 1d and Supplementary Fig. 3a). Furthermore, there was a significant reduction in EFV production with heat-killed *V. cholerae* (Supplementary Fig. 3b). These results indicate that *V. cholerae* produces one or more specific factors when inside the protozoan phagosome, contributing to release of EFVs.

Co-incubation of different *V. cholerae* strains with several ciliated protozoa also results in the production of EFVs.

To further determine whether the production of EFVs is a general response, different *V. cholerae* and wild-type ciliate strains were tested for EFV production. Results showed that the co-incubation of *V. cholerae* O1 N16961, A1552, C6706 and HC1037 with *T. pyriformis*, *Tetrahymena malaccensis*, *Tetrahymena* sp., *Uronema marinum* and *Tetrahymena thermophila* led to the release of *V. cholerae* EFVs to the extracellular space (Fig. 2a–e). Higher numbers of EFVs were observed in the co-incubations with *T. thermophila* at 30 °C (Fig. 2e), suggesting that increased temperature might enhance EFV production. These results show that predation of *V. cholerae* by ciliated protozoa at both room temperature and 30 °C results in the production of EFVs. We also tested different *Vibrio* spp. (Fig. 2f), demonstrating that there are strain-dependent differences in EFV production.

The *V. cholerae* outer membrane protein OmpU is involved in the release of EFVs.

To identify the potential factor(s) responsible for the production of EFVs, various *V. cholerae* A1552 mutants with deletions in genes related to grazing resistance and biofilm formation^{22–24}, transcriptional regulation^{25–28}, motility²⁹, acid resistance³⁰, outer membrane proteins^{31,32}, aminoacyl lipid modification³³, type I³⁴, II^{35–37} and VI³⁸ secretion systems and intracellular survival and multiplication³⁹ were tested. None of the mutants used in this study showed a growth defect in LB medium. Before each co-incubation, bacteria were adjusted to an OD_{600 nm} of 1.00 to 1.04 (approximately 10⁹ c.f.u. per ml) in 0.55× NSS (see Methods) and serially diluted to the desired concentration. Compared to the wild type, a significant decrease in the number of EFVs was observed when *toxR* or *ompU* mutants were used as prey (Table 1).

ToxR is the transcriptional regulator of *ompU*. Thus, to determine whether the defect in EFV production in the *toxR* strain is due to loss of *ompU* expression, or if other genes in the virulence operon regulated by ToxR are involved, *ompU* and *toxR* strains were complemented with *ompU*. In addition, as the operon that encodes *ompU* includes *dacB* (a carboxypeptidase located downstream of *ompU*), a *dacB*-deletion mutant was also constructed and tested. Deletion of *dacB* did not affect EFV production; however, complementation of the *ompU* gene in both *ompU* and *toxR* strains restored the number of EFVs back to wild-type levels (Fig. 3). These results indicate that OmpU, an outer membrane protein involved in resistance to antimicrobial peptides⁴⁰, bile salts⁴¹ and organic acids⁴², which is positively regulated by the master regulator of virulence, ToxR, has an important role in the production of EFVs.

EFVs protect cells from stress.

Bacterial cells inside the EFVs are potentially protected from various environmental and host stresses, such as acid stress, antimicrobials and starvation. To test this, we purified *V. cholerae* EFVs by filtration and washed and exposed them to pH stress (pH 3.4, the pH of the human stomach⁴³) alongside planktonic *V. cholerae* cells as controls. The viability of the cells in the EFVs was only slightly affected by the treatment (less than 1-log reduction), whereas planktonic *V. cholerae* were completely killed after 40 min of incubation (Fig. 4a). Thus, EFVs can protect *V. cholerae* from low pH conditions that would be encountered on entering a human host gut. Another common stress encountered by bacteria is exposure to biocides. Thus, the experiment was repeated using gentamicin at a bactericidal concentration (300 µg ml⁻¹) at room temperature. Again, whereas planktonic *V. cholerae* cells were completely eradicated, the cells in EFVs showed no loss of viability (Fig. 4b). These results therefore show that EFVs act as a protective barrier against different *V. cholerae* stressors.

Starvation is a common environmental stress for bacteria in aquatic environments⁴⁴. Many marine bacteria can survive long periods under starvation conditions, whereas others decline in number over time. To determine whether cells in the EFVs can survive long-term starvation, EFVs were collected, resuspended in artificial seawater (0.55× NSS) and stored at room temperature. Viability was assessed and compared with that of planktonic *V. cholerae* maintained under the same conditions. After one week, there was an approximate 2.5-log decrease in the viability of the planktonic cells (Fig. 4c). By contrast, cells in EFVs

maintained viability for at least three months (less than 0.5-log reduction). This result confirms that EFVs confer a fitness advantage to *V. cholerae* and increase their viability in seawater, thus contributing to their persistence in the environment.

The escape of *V. cholerae* from EFVs is mediated by temperature and the presence of nutrients.

For EFVs to be an ecologically relevant mechanism of protection and transmission for pathogens in the environment, the cells inside EFVs must be able to escape and propagate. EFVs that were incubated in LB broth at 37 °C escaped quickly (in 15–30 min) and began dividing (Fig. 5a and Supplementary Videos 4 and 5). At 37 °C in 0.55× NSS without carbon or nutrient sources, motility of cells in EFVs increased and they escaped the EFVs within 4 h, but at a slower rate than in LB medium (Fig. 5b). This experiment was repeated with EFVs that had been stored in 0.55× NSS at room temperature (about 22 °C) for two months. Cell escape and propagation from EFVs in LB broth was observed within 3 h of incubation (Fig. 5c), but no EFV escape was observed during the preceding 2 months (Fig. 5d). Thus, the escape of *V. cholerae* from EFVs is triggered by increased temperature and the presence of nutrients.

Cells in EFVs have a fitness advantage in vitro.

We next tested the fitness of *V. cholerae* cells contained in EFVs and of planktonic cells for growth in nutrient medium (LB). The *V. cholerae* A1552 wild-type strain was used to produce 24 h-old EFVs and competed against a *lacZ* isogenic strain that had been grown in vitro and acclimatized in 0.55× NSS before inoculation. The in vitro competition was performed by inoculating 50 µl of a 0.55× NSS suspension containing purified EFVs (approximately 6×10^4 EFVs ml⁻¹) and *lacZ* isogenic strain planktonic cells (approximately 6×10^5 cells per ml; to differentiate planktonic cells from cells originating from EFVs by growth in the presence of X-gal for blue–white screening) in LB broth and incubating at 37 °C overnight with agitation. The competition index was calculated as the ratio of colony-forming units (c.f.u.) of EFVs to the c.f.u. of *lacZ* wild type corrected by the number of viable *V. cholerae* cells in EFVs (Supplementary Fig. 5a–c and Supplementary Files 1 and 2). The in vitro competition index of *V. cholerae* in EFVs over planktonic *V. cholerae* (Fig. 6a, median value 6.5) suggests that the EFVs confer a growth advantage for *V. cholerae* when nutrients are encountered.

Purified *V. cholerae* EFVs are primed for infection in vivo.

Since EFVs are produced in large numbers under intense predation, and cells in the EFVs are protected against a range of stresses and can maintain long-term viability under environmental conditions, it follows that these EFVs may be infective when consumed by a host. To assess the infectivity of *V. cholerae* EFVs, an infant-mouse model of colonization was employed. For this, 50 µl of the same inoculum used for in vitro competition was used to infect the mice (Methods). After 24 h of infection, the competition index was calculated from cells obtained from the small intestine of each animal. Despite considerable variability in the results, *V. cholerae* EFVs outcompeted the in vitro grown bacteria in vivo, with median competition index significantly higher than 1.0 ($P < 0.0001$, Wilcoxon signed-rank

test). The in vivo competition index (Fig. 6b, median value 14.7) demonstrates that *V. cholerae* in EFVs have a significant colonization advantage compared with planktonic cells.

***V. cholerae* EFVs maintain in vivo hyperinfectivity for six weeks.**

The incubation of *V. cholerae* within EFVs in the environment might result in long periods before they are ingested by a host. To test whether aged EFVs maintain the hyperinfective phenotype, purified EFVs were incubated in 0.55× NSS for six weeks at room temperature and used for in vitro and in vivo competition assays as described above. Contrary to the earlier results, the six-week-old EFVs showed an in vitro growth disadvantage (median value 0.07) compared to the control (planktonic six-week-old *V. cholerae*, median value 1.43) (Fig. 6c). However, many aggregates were detected after overnight growth in LB broth, suggesting that *V. cholerae* from EFVs grew as aggregated bacteria, which could have affected the c.f.u. calculation for the escaped *V. cholerae*. Nevertheless, the six-week-old EFV *V. cholerae* cells still showed a colonization advantage (median value 1.74) over the control cells (median value 0.56) (Fig. 6d), confirming that long-term incubation did not affect the hyperinfective capability of *V. cholerae* from EFVs.

***V. cholerae* EFVs are not degraded at 37 °C and low pH but are digested in the presence of deoxycholic acid.**

To assess whether the EFVs might be degraded, either in the stomach or the small intestine, EFVs were incubated in two conditions. First, purified EFVs were resuspended in 0.55× NSS at pH 3.4 and incubated at 37 °C for 4 h. Imaging showed that there was no escape of *V. cholerae* from EFVs (Fig. 6e, left). However, exposure of the EFVs to 0.4% deoxycholic acid resulted in immediate digestion of EFVs (Fig. 6e, right). Together, these results suggest that *V. cholerae* would remain inside EFVs when transiting through the stomach, but would be released at the site of colonization (in the small intestine) in the presence of bile.

Discussion

Our results suggest that when numbers of *V. cholerae* are high in the environment, such as during disease outbreaks, there would be intense predation pressure and some of these protist predators release EFVs into the water column. Although the production of EFVs has been shown for other pathogens, it has not been demonstrated whether this process is mediated by the protist or the bacteria. Here we show that OmpU has a key role in the production of EFVs containing *V. cholerae*, demonstrating that bacterial factors positively contribute to this process. After ingestion by *T. pyriformis*, *V. cholerae* in phagosomes encounter an adverse environment characterized by the presence of low pH and cationic antimicrobial peptides^{45,46}. As previously shown in *V. cholerae*, OmpU enables resistance to such environments. For example, reports have shown that OmpU protects *V. cholerae* from antimicrobial peptides^{40,47,48}, low pH⁴² and bile⁴⁹. In addition, it has been shown that OmpU is involved in intestinal colonization by *V. cholerae*³¹ and is essential for invasion and infection of oysters by other *Vibrio* species^{47,50}. In sum, the egestion of *V. cholerae* from EFVs is promoted by an outer membrane protein that is essential for the pathogenesis of this bacterium.

The function of OmpU in protecting *V. cholerae* cells from low pH and antimicrobial peptides indicates that once inside the phagosome, OmpU probably acts to resist digestion of the bacterial cells. This results in a large number of undigested cells in the vacuole. The undigested cells that remain in the phagosome may trigger the expulsion of vacuoles containing bacteria from *T. pyriformis*, as previously demonstrated⁵¹.

Since EFVs confer a survival advantage to *V. cholerae* under stressful conditions, the cells in the EFVs are protected from various environmental stresses and pH stress that would be encountered following ingestion. The EFVs would enhance survival of cells passing through the stomach, and as the EFVs contain numerous cells, would increase numbers of *V. cholerae* that reach the small intestine (Fig. 4). Our mouse colonization data shows that *V. cholerae* in EFVs can outcompete planktonic cells, suggesting that EFVs might protect cells and may enhance efficient infection, possibly through improved survival on exposure to gastric acid and increased resistance to host antimicrobial defences through active expression of *ompU*. Furthermore, as stated above, OmpU is critical for intestinal colonization³¹, suggesting that the expression of OmpU in EFVs might be responsible for the in vivo colonization advantage. We suggest that the findings reported here establish a novel understanding of the mechanisms of persistence and the modes of transmission of *V. cholerae* and may further apply to other opportunistic pathogens that have been shown to be released by protists via EFVs. Hence, protozoan EFVs may constitute a mechanism for transmission and infection more broadly, as has been previously speculated^{4,19}.

Methods

Strains and growth conditions.

Organisms used in this study are listed in Supplementary Table 1. Bacterial strains were routinely grown in LB and on LB agar plates. *V. cholerae* mutants were constructed by splicing using overlap extension PCR⁵² and natural transformation⁵³. Complementation was done using the expression vector pBAD24. Bacteria carrying the vector were grown in LB broth at 37 °C containing 100 µg ml⁻¹ ampicillin and 0.2% arabinose for gene expression. Environmental isolates of *Vibrio* spp. were routinely grown in LB broth and LB agar plates supplemented with 2% NaCl and incubated at 28 °C.

Tetrahymena spp. were routinely passaged in 15 ml growth medium containing peptone–yeast–glucose (20 g l⁻¹ proteose peptone and 1 g l⁻¹ yeast extract) supplemented with 1 l of 0.1× M9 minimal medium (6 g l⁻¹ NaH₂PO₄, 3 g l⁻¹ K₂PO₄, 0.5 g l⁻¹ NaCl and 1 g l⁻¹ NH₄Cl) and 0.1 M sterile-filtered glucose in 25 cm² tissue culture flasks with ventilated caps (Sarstedt) and incubated statically at room temperature. *U. marinum* was routinely grown in 0.55× NSS medium (8.8 g l⁻¹ NaCl, 0.735 g l⁻¹ Na₂SO₄, 0.04 g l⁻¹ NaHCO₃, 0.125 g l⁻¹ KCl, 0.02 g l⁻¹ KBr, 0.935 g l⁻¹ MgCl₂·6H₂O, 0.205 g l⁻¹ CaCl₂·2H₂O, 0.004 g l⁻¹ SrCl₂·6H₂O and 0.004 g l⁻¹ H₃BO₃) supplemented with 1% heat-killed *P. aeruginosa* PAO1 in a 25 cm² tissue culture flask, and further incubated at room temperature statically for 2 d before enumeration and use.

Before experiments, 500 µl of *Tetrahymena* spp. were passaged in 20 ml of 0.55× NSS medium supplemented with 1% heat-killed *P. aeruginosa* in a 25 cm² tissue culture flask,

and further incubated at room temperature statically for 2 d before enumeration and use. This process is necessary to remove the nutrient medium and to acclimatize the ciliate to phagotrophic feeding.

To prepare heat-killed bacteria, *P. aeruginosa* or *V. cholerae* were grown overnight in LB at 37 °C with shaking at 200 r.p.m. and adjusted to ($OD_{600\text{ nm}} = 1.0$; 10^9 cells ml^{-1}) in $0.55\times$ NSS. The cultures were then transferred to a water bath at 65 °C for 2 h, and then tested for viability by plating on LB agar plates at 37 °C for 2 d. Heat-killed bacteria stocks were stored at -20°C .

Production of EFVs containing *V. cholerae*.

To produce EFVs, *V. cholerae* A1552 was co-incubated with *T. pyriformis* in $0.55\times$ NSS. In brief, *T. pyriformis* were enumerated by microscopy and adjusted to 10^3 cells ml^{-1} and added to co-cultures of *V. cholerae* A1552 adjusted to 10^8 cells ml^{-1} in $0.55\times$ NSS using a spectrophotometer ($OD_{600\text{ nm}}$). After overnight incubation at room temperature, samples were analysed using an inverted epifluorescence microscope (Nikon Eclipse Ti inverted microscope) to detect the presence of EFVs in the supernatant. To purify *V. cholerae* EFVs, supernatants were filtered (by gravity) several times through $8\text{ }\mu\text{m}$ filters (Millipore) and the filters containing EFVs were suspended in 1 ml $0.55\times$ NSS. The EFVs were incubated for 1 h with $300\text{ }\mu\text{g ml}^{-1}$ gentamicin at room temperature to kill any remaining extracellular bacteria. After gentamicin treatment, *V. cholerae*-EFV pellets were collected by centrifugation ($3,220g$ for 20 min), washed three times in $0.55\times$ NSS and suspended in 1 ml of $0.55\times$ NSS. Finally, the number of *V. cholerae* EFVs was determined by microscopy after 48 h of co-incubation (the time needed for the eradication of all extracellular bacteria).

Enumeration of live/dead *V. cholerae* in EFVs.

To establish the number of viable *V. cholerae* in EFVs, a genomic staining assay was conducted. In brief, EFVs were produced and collected as above and suspended in 1 ml of $0.55\times$ NSS. The EFVs were stained with LIVE/DEAD BacLight Bacterial Viability Kit for microscopy (Invitrogen) following the manufacturer's instructions. After staining, the sample was centrifuged ($7,607g$ for 5 min) to remove the staining solution and resuspended in 1 ml of $0.55\times$ NSS. Eight microlitres of sample were placed on a glass slide, covered with a coverslip (1.5 mm thickness) and sealed with nail polish. Stained EFVs were immediately analysed by confocal microscopy (Nikon A1 confocal laser scanning microscope) to assess the number of live (green) and dead (red) bacterial cells.

Survival of *V. cholerae* EFVs under stress conditions.

To assess the effect of stress conditions on the viability of *V. cholerae* in EFVs, two treatments were performed independently. For the acid-tolerance experiments, *V. cholerae* EFVs were obtained as described above and suspended in either $0.55\times$ NSS or NSS adjusted to pH 3.4 (with 1 N HCl). Incubation of the *V. cholerae* EFVs was carried out in triplicate for 60 min in a 96-well plate at room temperature with agitation (60 r.p.m.). The number of viable bacteria was determined at different time points by adding 1% Triton-X100 (Sigma) to each well at 0, 20, 40 and 60 min (to release the *V. cholerae* cells from the EFVs, Supplementary Fig. 4a–c) and plating serial dilutions on LB plates. For the gentamicin

assay, *V. cholerae* EFVs were exposed to 300 $\mu\text{g ml}^{-1}$ gentamicin in 0.55 $\times$ NSS at room temperature with agitation at 60 r.p.m. in a 96-well plate. After 1 h incubation, 1% Triton-X100 (Sigma) was added to each well and serial dilutions were plated on LB. As a control, planktonic *V. cholerae* adjusted to $\sim 10^6$ cells ml^{-1} in 0.55 $\times$ NSS was used for each of the three conditions.

Escape of *V. cholerae* from EFVs.

To obtain images and videos of *V. cholerae* cells escaping from EFVs, the EFVs were collected as described above, suspended in LB broth or 0.55 $\times$ NSS and 1 ml of the suspension was added to a 24-well glass-bottom microtitre plate. Plates were incubated at 37 °C or room temperature under a confocal microscope (Nikon A1 confocal laser scanning microscope) and videos or still images were taken.

Incubation of EFVs at low pH and in the presence of deoxycholic acid.

Purified *V. cholerae* EFVs were incubated at 37 °C for 4 h in 0.55 $\times$ NSS at pH 3.4. To test the effect of deoxycholic acid (a component of bile) on the EFVs, treatments with 0.4% deoxycholic acid were performed at 37 °C after 4 h of incubation in 0.55 $\times$ NSS at pH 3.4.

Infant mouse colonization experiments.

Five-day-old litters of CD1 mice were inoculated orogastrically as described⁵⁴ with 50 μl of inoculum containing $\sim 10^6$ rifampicin-resistant *V. cholerae* A1552 in EFVs (24 h old) and $\sim 10^6$ c.f.u. of an isogenic competing strain, *V. cholerae* A1552 *lacZ*, which was prepared by culturing in vitro to stationary phase in LB broth at 37 °C with aeration. In parallel, 2 μl of inoculum was diluted into 2 ml of LB broth in culture tubes and competed in vitro for 18 h with aeration at 37 °C. After 24 h, mice were euthanized, and the small intestine was removed and homogenized in 1 ml of LB broth supplemented with 20% glycerol.

For the experiments with six-week-old EFVs, in vitro growth and in vivo infections were performed as described above. As a control, planktonic *V. cholerae* in 0.55 $\times$ NSS that was starved for six weeks at room temperature was used. The ratios of wild-type to *lacZ V. cholerae* at the input (inoculum) and outputs were determined by plating serial dilutions on LB agar supplemented with 100 $\mu\text{g ml}^{-1}$ rifampicin and 80 $\mu\text{g ml}^{-1}$ X-Gal. The competition index was calculated as the output ratio divided by the input ratio corrected by the number of *V. cholerae* in EFVs.

All animal procedures were conducted in accordance with the rules of the Department of Laboratory Animal Medicine at Tufts University School of Medicine. Five-day-old CD-1 infant mice (both male and female) were used for the infection experiments to obtain an accurate median for statistical analyses. For all experiments, mice were randomly allocated to each treatment group before inoculation of samples. All mice were obtained from Charles River Laboratories.

Transmission electron microscopy.

Cell cultures were fixed for 24 h at 4 °C by immersion in a fixative solution containing 3% glutaraldehyde in PBS buffer (0.1 M phosphate, pH 7.5) and then stored in PBS buffer at 4

°C until further processing. Samples were subsequently post-fixed for 1 h in a solution containing 1% osmium tetroxide in PBS (1×, final pH 7.5), washed with MilliQ water and dehydrated in an increasing gradient of ethanol before infiltration and embedding in Spurr resin (ProsciTech). Resin blocks were then cut into 90 nm sections using an Ultracut UC6 microtome (Leica Microsystems). Selected sections containing cells and EFVs were stained on finder grids (Electron Microscopy Sciences) with uranyl acetate and lead citrate. Stained sections on finder grids were viewed at 200 kV accelerating voltage using a FEI Tecnai G2 20 transmission electron microscope at the Mark Wainwright Analytical Centre: Electron Microscope Unit (University of New South Wales).

Data analysis.

Statistical analysis was performed using GraphPad Prism v.7.01 for Windows (www.graphpad.com). Data that did not follow a Gaussian distribution was determined by analysing the frequency distribution graphs and was transformed using natural logs. Two-tailed student's *t*-tests were used to compare means between experimental samples and controls. For experiments including multiple samples, one-way or two-way analysis of variance (ANOVA) was used for the analysis and Dunnett's multiple comparison test provided the post hoc comparisons of means. For the mouse colonization experiments, the data were analysed using a non-parametric test for medians that follow Gaussian distribution (Wilcoxon signed-rank test or Mann–Whitney test) for non-normally distributed data.

Reporting Summary.

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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Fig. 1 |. Production of EFVs containing *V. cholerae*.

a, Fixed samples of green fluorescent protein (GFP)-tagged (green) *V. cholerae* co-incubated with *T. pyriformis* in 0.55× NSS. *T. pyriformis* was stained with 4,6-diamidino-2-phenylindole (DAPI, blue) and FM 4–64 FX (red) was used to stain the EFV membranes. Intracellular food vacuoles containing GFP-tagged *V. cholerae* (left; scale bar, 15 μm; Supplementary Video 2) and an expelled EFV (right; scale bar, 5 μm; Supplementary Video 3). Images are representative of three independent experiments. **b**, TEM of fixed samples of *T. pyriformis* and *V. cholerae* EFVs. Left, *V. cholerae* (white arrows) in vacuoles of *T. pyriformis* after overnight incubation in 0.55× NSS at room temperature. The presence of many mitochondria around the vacuole is observed. Middle, a magnified view of the bounded area in the left panel. Right, a single bacterial cell in an EFV containing multiple

cells to show the presence of multiple layers of membrane surrounding *V. cholerae*. Scale bars: 2 μm (left), 500 nm (middle) and 500 nm (right) (see also Supplementary Fig. 2). Images are representative of three independent experiments. **c**, Number of EFVs after co-incubation of *T. pyriformis* with different numbers of *V. cholerae*. Data are from four independent biological replicates and are shown as the mean $\pm$ s.d. **d**, Numbers of EFVs produced when protein synthesis is inhibited before co-incubation. Data are from three independent biological replicates and are shown as the mean $\pm$ s.d. Significant differences were determined using one-way ANOVA with Dunnett's multiple comparisons test. ** $P<0.01$ and *** $P<0.001$.

Fig. 2 |. Production of EFVs by different *Vibrio* spp. and ciliate wild-type strains.

a–e, EFV production when *V. cholerae* O1 strains N16961, A1552, C6706 and HC1037 were co-incubated in $0.55 \times \text{NSS}$ with *T. pyriformis* (**a**), *T. malaccensis* (**b**), *Tetrahymena* sp. (**c**) and *U. marinum* (**d**) at room temperature, and with *T. thermophila* at 30°C (**e**). **f**, Different *Vibrio* spp. incubated with *T. pyriformis*. Data are from three (**a–e**) or four (**f**) independent biological replicates and are shown as the mean $\pm$ s.d. Significant differences were determined using one-way ANOVA with Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Fig. 3 |. Number of EFVs produced by different *V. cholerae* mutants.

Number of EFVs produced by wild-type, *toxR*, *dacB*, *ompU* and the *ompU*-complemented *ompU* and *toxR* strains (*ompU* pBAD24::*ompU* and *toxR* pBAD24::*ompU*, respectively). Strains were grown in LB broth at 37°C, with agitation at 200 r.p.m. overnight except for the *ompU*-complemented mutants, which were supplemented with 100 μgml^{-1} carbenicillin and with (for *ompU* expression) or without (control) 0.2% arabinose. Data are from three independent biological replicates and are shown as the mean $\pm$ s.d. Significant differences were determined using one-way ANOVA with Dunnett's multiple comparisons test. **** $P < 0.0001$.

Fig. 4 |. Survival of *V. cholerae* cells in EFVs under stress and starvation conditions.

a, Planktonic cells (open bars) and *V. cholerae* EFVs (closed bars) were incubated for 60 min in 0.55× NSS adjusted to pH 3.4. The number of colony-forming units was determined every 20 min by treating the samples with 1% Triton X-100 (Supplementary Fig. 4a–c), serial dilution and plating on LB agar plates. Data are from three independent biological replicates and are shown as the mean±s.d. **b**, *V. cholerae* EFVs and planktonic cells were treated (closed bars) or not (open bars) with gentamicin ($300 \mu\text{g ml}^{-1}$) for 1 h at room temperature and the number of colonies was determined. Data are from three independent biological replicates and are shown as the mean±s.d. ($P > 0.999$). Significant differences were determined using two-way ANOVA with Sidak's multiple comparisons test. NS, not significant; **** $P < 0.0001$. **c**, Number of cells in EFVs (red) and number of planktonic cells (blue) after incubation in 0.55× NSS for 12 weeks. Data are from three independent biological replicates and are shown as the mean ± 95% confidence intervals (shaded area).

Fig. 5 |. Escape of *V. cholerae* from EFVs under different nutrient and temperature conditions. **a**, Escape of *V. cholerae* from EFVs. *V. cholerae* EFVs were incubated in LB broth at 37°C and were observed using time-lapse imaging for 3 h. Numbers show image time stamps (h:min:s.ms). Top left, a single *V. cholerae* EFV (white arrow) suspended in LB broth at the beginning of the incubation. Top right: rupture of the EFV membrane after approximately 10 min (indicated by white arrows) with the subsequent release of *V. cholerae* cells (indicated by a black arrow). Bottom left, *V. cholerae* cells actively dividing and escaping from EFVs (indicated by white arrows) with more extracellular bacteria present (indicated by a black arrow). Bottom right, dense growth from EFVs (indicated by white arrows) and many extracellular *V. cholerae* cells, showing active division (indicated by a black arrow). **b**, Incubation of EFVs at 37°C without carbon or nutrient source (suspended in 0.55× NSS). Left, white arrows show two EFVs at time 0. Middle, a single EFV (indicated by a white arrow) and many *V. cholerae* cells after 4 h of incubation. Right, a single EFV (indicated by a white arrow) and extracellular bacteria (indicated by black arrows) are observed after 24 h of incubation. **c**, EFVs incubated in LB broth at room temperature. The video started recording after 2 h of incubation. Left, a single *V. cholerae* EFV. Middle, rupture of the EFV membrane (indicated by a white arrow). Right, *V. cholerae* cells showing active growth from

the EFV (indicated by a white arrow). **d**, EFVs (indicated by white arrows) suspended in 0.55× NSS at room temperature for two months. Intact EFVs are observed without extracellular bacteria. Scale bars in **a–d**, 10 µm. Images are representative of three independent experiments.

Fig. 6 |. Competition index for in vitro and in vivo assays of *V. cholerae* EFVs versus planktonic *V. cholerae* and working model.

a,b, Competition index of in vitro (**a**) and in vivo (**b**) assays calculated by the output ratio after incubation (in vitro: overnight, 37°C; in vivo: 24 h, 24°C) corrected by the input ratio. Number of colony-forming units was assessed by plating on LB10 agar plates supplemented with 100 $\mu\text{g ml}^{-1}$ rifampicin to inhibit other intestinal bacteria and 80 $\mu\text{g ml}^{-1}$ X-gal. Data in **a** and **b** are from 16 independent biological replicates and are shown as the median. The competition index of *V. cholerae* EFVs compared to planktonic cells in in vitro and in vivo assays is significantly higher than a hypothetical median of 1.0 (two-tailed, non-parametric, Wilcoxon signed-rank test; **** $P < 0.0001$). **c,d,** Competition index of in vitro (**c**) and in vivo (**d**) assays performed with either six-week-old EFVs (incubated in 0.55 $\times$ NSS at room temperature) or six-week-old planktonic cells (control, incubated in 0.55 $\times$ NSS at room

temperature) compared with *lacZ* wild type, calculated by the output ratio after incubation (in vitro: overnight, 37°C; in vivo: 24 h, 24°C) corrected by the input ratio. Colony-forming units were assessed by plating on LB10 agar plates supplemented with 100 µg ml⁻¹ rifampicin to inhibit other intestinal bacteria and 80 µg ml⁻¹ X-Gal. Data in **c** and **d** are from five independent biological replicates and are shown as the median. The competition index of the six-week-old *V. cholerae* EFVs compared with the six-week-old planktonic cells (control) was significantly different in both in vitro and in vivo conditions (two-tailed, non-parametric Mann–Whitney test; ***P*<0.01). **e**, *V. cholerae* EFVs incubated at 37°C for 4h in 0.55× NSS at pH 3.4 without (left) or with (right) 0.4% deoxycholic acid treatment. Images show intact *V. cholerae* EFVs in the untreated condition (left), with arrows showing digested EFVs after deoxycholic acid treatment. Scale bars, 10 µm. Images are representative of three independent experiments.

Table 1 |Numbers of EFVs produced by different *V. cholerae* A1552 mutants

Classification	A1552 mutants tested ^a	Number of EFVs per ml (mean $\pm$ s.d.)
Grazing resistance or biofilm formation	<i>vpsA</i> ^b	$4.15 \times 10^5 \pm 6.81 \times 10^4$
	<i>rpoS</i> ^b	$3.93 \times 10^5 \pm 1.30 \times 10^5$
	<i>hmgA</i> ^b	$3.78 \times 10^5 \pm 1.34 \times 10^5$
	<i>vpsR</i> ^b	$2.73 \times 10^5 \pm 1.30 \times 10^5$
Transcriptional regulators	<i>hapR</i> ^b	$4.50 \times 10^5 \pm 1.44 \times 10^5$
	<i>phoB</i> ^b	$2.75 \times 10^5 \pm 1.02 \times 10^5$
	<i>chiS</i> ^b	$5.40 \times 10^5 \pm 9.76 \times 10^4$
	<i>toxR</i> ^c	$1.28 \times 10^4 \pm 3.95 \times 10^3$
Motility	<i>flaA</i> ^b	$3.20 \times 10^5 \pm 1.83 \times 10^5$
Acid resistance	<i>cadC</i> ^b	$3.50 \times 10^5 \pm 1.96 \times 10^5$
Outer membrane proteins	<i>ompU</i>	$4.25 \times 10^3 \pm 5.00 \times 10^2$
	<i>ompV</i> ^b	$4.03 \times 10^5 \pm 1.44 \times 10^5$
Aminoacyl lipid modification	<i>almEFG</i> ^b	$2.97 \times 10^5 \pm 1.15 \times 10^5$
Type I secretion system	<i>rtxA</i> ^b	$4.00 \times 10^5 \pm 1.67 \times 10^5$
Type II secretion system	<i>CTXΦ</i> ^b	$2.85 \times 10^5 \pm 1.98 \times 10^5$
	<i>gbpA</i> ^b	$3.90 \times 10^5 \pm 1.95 \times 10^5$
	<i>lapA</i> ^b	$3.20 \times 10^5 \pm 1.68 \times 10^5$
Type VI secretion system	<i>hcpI</i> ^b	$3.85 \times 10^5 \pm 1.55 \times 10^5$
	<i>hcp2</i> ^b	$3.65 \times 10^5 \pm 6.19 \times 10^4$
	<i>hcp1,2</i> ^b	$3.80 \times 10^5 \pm 1.76 \times 10^5$
Intracellular survival and multiplication in other bacteria	<i>ankB</i> ^b	$2.98 \times 10^5 \pm 2.08 \times 10^5$

^aSample size, $n = 4$.^bNo significant reduction compared with wild type.^cSignificant reduction compared with wild type.