Published in final edited form as:

Microb Pathog. 2017 December ; 113: 17–24. doi:10.1016/j.micpath.2017.10.016.

Flagellar motility, extracellular proteases and *Vibrio cholerae* detachment from abiotic and biotic surfaces

Loree Mewborn, Jorge A. Benitez, and Anisia J. Silva*

Morehouse School of Medicine Department of Microbiology, Biochemistry and Immunology, 720 Westview Dr., SW Atlanta, Georgia, 30310

Abstract

Vibrio cholerae of serogroups O1 and O139, the causative agent of Asiatic cholera, continues to be a major global health threat. This pathogen utilizes substratum-specific pili to attach to distinct surfaces in the aquatic environment and the human small intestine and detaches when conditions become unfavorable. Both attachment and detachment are critical to bacterial environmental survival, pathogenesis and disease transmission. However, the factors that promote detachment are less understood. In this study, we examine the role of flagellar motility and hemagglutinin/protease (HapA) in *vibrio* detachment from a non-degradable abiotic surface and from the suckling mouse intestine. Flagellar motility facilitated *V. cholerae* detachment from abiotic surfaces. HapA had no effect on the stability of biofilms formed on abiotic surfaces despite representing > 50 % of the proteolytic activity present in the extracellular matrix. We developed a balanced lethal plasmid system to increase the bacterial cyclic diguanylate (c-di-GMP) pool late in infection, a condition that represses motility and HapA expression. Increasing the c-di-GMP pool enhanced *V. cholerae* colonization of the suckling mouse intestine. The c-di-GMP effect was fully abolished in *hapA* isogenic mutants. These results suggest that motility facilitates detachment in a substratum-independent manner. Instead, HapA appears to function as a substratum-specific detachment factor.

Keywords

Vibrio cholerae; cholera; cyclic diguanylate; motility; proteases; detachment; mucosal escape

Introduction

Bacterial adhesion to abiotic and biotic surfaces is mediated by hair-like appendages known as pili. *Vibrio cholerae* of serogroup O1 and O139, the causative agent of the diarrheal disease cholera, expresses multiple pili, which mediate its adherence to different substrata in the aquatic environment and the human small intestine (1). For instance, the mannose-

*Correspondence: Phone 1 404 7566660; asilva-benitez@msm.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest. The authors declare no conflict of interest

sensitive hemagglutinin (MSHA) participates in *V. cholerae* attachment to borosilicate (2, 3) and to the exoskeleton of planktonic crustacean (4); the N-acetylglucosamine binding protein GbpA mediates attachment of vibrios to chitin and intestinal mucin (5, 6); the chitin-regulated pilus ChiRP promotes attachment to chitinous surfaces (7) and the toxin-coregulated pilus (TCP) mediates attachment to cultured intestinal cells, the intestinal microvilli and microcolony formation (8–10). This latter type IV pilus is essential for *V. cholerae* colonization of the suckling mouse and human intestine (11–13).

V. cholerae attachment to chitinous surfaces in aquatic ecosystems can progress to the formation of biofilm communities that can persist for longer periods in the environment (1). In the small intestine, initial attachment of vibrios to the protective mucus layer can be followed by multiple cycles of mucus gel penetration, adherence to the microvilli, microcolony formation and detachment [reviewed in (14, 15)]. Vibrios have also been suggested to form biofilm-like structures during infection, which can be excreted in a hyperinfective stage that promotes cholera fecal-oral transmission (10, 16, 17). Flagellar motility plays an important role in surface attachment wherein bacterial near-surface motility allows vibrios to scan surfaces for regions favoring strong pili-surface interactions (18). Following attachment, the intracellular level of cyclic diguanylic acid (c-di-GMP) regulates the transition between the motile and sessile lifestyles by (i) binding to and inhibiting the activity of FlrA to suppress the transcription of the flagellar biosynthesis regulon (19) and (ii) binding to receptor proteins VpsR and VpsT to activate the biosynthesis of the biofilm exopolysaccharide and protein extracellular matrix (20).

The ability of *V. cholerae* to detach from a substratum when the environmental conditions are unfavorable is equally critical to its persistence in the environment, pathogenesis and disease transmission. Detachment of vibrios late in infection returns $\approx 10^9$ virulent bacteria per ml of cholera stool to the aquatic environment and facilitates their transmission to secondary hosts. Detachment could result from the cleavage of an adhesin anchoring cells to a substratum and/or degradation of the substratum itself. For example, the *V. cholerae* Zn-metalloprotease HapA has been shown to degrade mucin (21) and cleave the mucin binding adhesin GbpA (6). As a consequence, *V. cholerae* *hapA* mutants remain attached for longer periods to mucin-coated polystyrene plates and cultured mucin-secreting HT29-18N2 goblet cells (22, 23). Detachment could also result from the expression of hydrolytic enzymes that degrade the extracellular matrix that hold vibrios together in a biofilm. For example, *V. cholerae* mutants lacking the extracellular DNases Dns and Xds make enhanced biofilms *in vitro* presumably because they fail to detach (24). Finally, derepression of genes encoding the polar flagellum system could provide vibrios with momentum to detach and swim away toward an unspent substratum. Studies conducted in ligated rabbit ileal loops suggested that activation of motility in the stationary phase by the general stress response regulator RpoS contributes to *V. cholerae* detachment from the intestinal mucosa into the lumen (25). Flagellar motility, however, has also been reported to enhance the capacity of *V. cholerae* to establish infection (22, 26). This effect appears to be dependent on strain and assay conditions. A recent study showed that motility is required for colonization of the proximal but not the distal small intestine (10).

In this study, we focus on the role of flagellar motility and HapA in *V. cholerae* detachment from abiotic and biotic surfaces. HapA, the major soluble protease produced by the cholera bacterium, is a member of the M4 neutral peptidase family that displays significant amino acid sequence homology to *P. aeruginosa* elastase and *Bacillus thermoproteolyticus* thermolysin (27). This soluble protease is present in the matrix of biofilms formed under laboratory conditions (28). Further, HapA is expressed during infection and *hapA* mutants exhibit a modest increase in colonization of the suckling mouse intestine (22, 29). Similar to motility, the expression of HapA is negatively regulated by c-di-GMP (30) and activated by RpoS (25, 31, 32). Studies on the role of motility and HapA on *V. cholerae* detachment have been hindered by the fact that both activities also influence bacterial adherence (3). To circumvent this obstacle, (i) we examined the role of motility and HapA in the stability of biofilms formed under conditions in which these activities are repressed by artificially increasing the c-di-GMP pool and (ii) tested the effect of diminishing *V. cholerae* motility and HapA late in infection on bacterial detachment from the small intestine. We used the suckling mouse model to examine the effect of increasing the c-di-GMP pool late in infection on bacterial exit from the host. We provide evidence that motility, but not HapA, is required for *V. cholerae* detachment from biofilms formed on an abiotic surface. We also show that increasing the c-di-GMP pool late in infection enhances intestinal colonization and that this effect is fully abrogated in a *hapA* mutant.

Materials and methods

Strains and media

Escherichia coli and *V. cholerae* strains, plasmids and oligonucleotide primers used in this study are described in Table 1. *V. cholerae* mutants and transformants were all derived from the O1 El Tor biotype strain C7258. *E. coli* TOP10 (Life Technologies, Carlsbad, CA) and S17-1 λ pir (33) were used for cloning purposes. *V. cholerae* strains were grown in Luria-Bertani (LB) medium at 30°C. Swarm agar consisted in LB medium containing 0.3 % agar. For measuring the expression of HapA, vibrios were cultivated until stationary phase in tryptic soy broth (TSB) at 37°C. When necessary, culture media were supplemented with thymidine (200 μ g /mL), ampicillin (100 μ g/mL), kanamycin (25 μ g/mL), polymyxin B (100 units/mL), isopropyl- β -D-thiogalactopyranoside (IPTG; 0.5 mM) or 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; 20 μ g/mL).

Construction of plasmids and mutants

The construction of isogenic *thyA*, *lacZ*, *hapA::celA*, *pomAB* and *cqsA* mutants of strain C7258 has been described previously (29, 31, 34–36). Double and triple mutants combining the above alleles were constructed by conjugal transfer of a pCVD442-based suicide vectors (37) bearing the particular mutation from the permissive host *E. coli* S17-1 λ pir to the corresponding *V. cholerae* receptor strain followed by sucrose selection as described previously (35). To this end, suicide vector pCVD *thyA* (34) was used for introducing *thyA* deletions; pCVD *lacZ* (31) for *lacZ*; pGPH6 (29) for *hapA::celA*; pCVD *cqsA* (36) for *cqsA*, and pCVD *pomAB* (35) for *pomAB*. All deletion mutants were confirmed by PCR and DNA sequencing. To study the effect of enhancing the c-di-GMP pool late in infection, we developed a balanced lethal plasmid system consisting of vectors

pAJAIthyA-8 and pAJAIthyA-8*. The active vector pAJAIthyA-8 contains a complementing *thyA* gene and VCA0956 encoding a diguanylate cyclase (DGC) expressed from the *hapR* promoter, which is activated when vibrios reach high cell density ($> 2 \times 10^8$ cells/ml) (38). We selected VCA0956 based on its proven high DGC activity that correlates with suppression of motility (39). To construct this vector, we started with plasmid pTT3 containing the *rrnBT₁T₂* transcription terminator in pUC19 (40). Then, we amplified a DNA fragment containing *hapR* transcription and translation initiation signals using primers HapR39 and HapR493 from strain C7258 genomic DNA and cloned the resulting PCR product as an *XbaI*-*BamHI* fragment downstream from the transcription terminator in plasmid pTT3. A promoterless VCA0956 gene was amplified using primers VCA0956-15 and VCA0956-1043 and inserted as a *BamHI*-*EcoRI* fragment downstream from the *hapR* promoter. Next, the DNA fragment containing the *rrnBT₁T₂*-*hapR* promoter-VCA0956 cassette was cloned in pBR322 to yield the plasmid pAJAI (Table 1). Finally, an additional DNA fragment encoding a complementing *thyA* gene was amplified from strain C7258 genomic DNA using primers ThyA703 and ThyA2045 and inserted as an *EcoRI* fragment in pAJAI to yield pAJAIthyA-8. PCR was conducted using the Advantage 2 PCR kit (Takara Bio USA, Inc.) following the provider's instructions and all PCR products were confirmed by DNA sequencing. As a control vector, we constructed plasmid pAJAIthyA-8* encoding an active site mutant of VCA0956. Site-directed mutagenesis was conducted using the QuickChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technology) and primer VCA0956mut following the provider's protocol. The GGDEF active site motif of VCA0956 in pAJAIthyA-8 was changed to AAEEF in pAJAIthyA-8*, a modification known to abolish enzyme activity (41).

Manipulation of the c-di-GMP pool

To enhance the c-di-GMP pool *in vitro*, strains C7258 and its mutants were transformed with plasmids pCMW75 which encodes *V. harveyi* DGC QrgB expressed from the Tac promoter (42). Induction of QrgB in *V. cholerae* with IPTG results in a 10-fold increase in intracellular c-di-GMP that suppresses motility and activates matrix exopolysaccharide expression (30, 42).

In vitro adherence and detachment assay

Strains containing plasmid pCMW75 were grown in LB medium overnight and diluted 1:20 in fresh medium containing 0.5 mM IPTG. Bacterial adherence was measured in 96-well polystyrene microtiter plates incubated 8 to 64 h at 30°C using the crystal violet staining method as described previously (43). The crystal violet readings were normalized for growth and attachment was expressed as the optical density (OD) ratio OD₅₇₀/OD₆₀₀. For the differential enumeration of vibrios in mixed biofilms containing strain C7258 (motile) and C7258 ABL (non-motile), the biofilms were developed in borosilicate glass tubes and disrupted by vortexing in the presence of 1.0 mm glass beads. Dispersal was confirmed by light microscopy and the ratio of motile to non-motile cells was determined by dilution plating in LB agar containing X-gal. Strain C7258 T containing plasmid pAJAIthyA-8 or pAJAIthyA-8* were also allowed to form biofilms in 96-well polystyrene microtiter plates previously coated with porcine gastric mucin (1 mg/mL) (type III; Sigma Chemical Co.).

Determination of protease activity

Proteolytic activity in the biofilm matrix was determined using the EnzChek Protease Assay Kit (Molecular Probes) following the provider's protocol. The biofilms were developed for different time periods in black wall 96-well microtiter plates, the planktonic cells were discarded, and the wells were washed three times with phosphate-buffered saline (pH 7.4) (PBS). Then, the intact biofilm was incubated with the BODIPY FL labeled peptide for 60 min and fluorescence was read using excitation/emission wavelengths 505/589 nm. The fluorescence readout was normalized by the quantity of adherent cells estimated by crystal violet staining and the results expressed as fluorescence units (FI) per OD₅₇₀. To determine protease activity in disrupted biofilms, single colonies were inoculated into 2 mL of LB medium and the biofilms were formed in borosilicate tubes for different time periods at 30°C. Triplicate tubes were used for biofilm disruption and crystal violet staining, respectively. In both cases, planktonic cells were discarded and the biofilms were washed three times with PBS. To estimate the biofilm mass, adherent bacteria were stained with crystal violet (0.1 %), washed with PBS and the stained biomass was resuspended in 1 mL dimethylsulfoxide for OD₅₇₀ measurement. For protease activity, adherent bacteria were vortexed for 1 min in 1 mL PBS containing 1 g of glass beads (1 mm Ø). Then, 0.1 mL aliquots were transferred to microtiter plate wells containing 0.1 mL of BODIPY FL labeled peptide (10 µg/mL) and the reaction was incubated 60 min. As above, the fluorescence readout was normalized by the quantity of adherent cells estimated by crystal violet staining (OD₅₇₀). The presence of HapA in *V. cholerae* cell-free supernatants was measured using an azocasein-degradation assay as described previously (44). One azocasein unit was defined as the amount of enzyme producing an increase of 0.01 optical density units at 442 nM per h.

Competitive colonization assays

Animal studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and a protocol approved by the Morehouse School of Medicine Institutional Animal Care and Use Committee. Competitive colonization assays using the suckling mouse model were performed as described previously (22). Briefly, a minimum of six 4–5 days old CD-1 mice were starved for 6 h and inoculated orally through a 21-gauge disposable feeding needle with 10⁶ colony forming units consisting of a 1:1 mixture of each challenge strain in 50 µL of PBS. Mice were maintained for 16 h in a cage with bedding at 30°C. The animals were sacrificed by decapitation and the entire small intestine was removed and homogenized in 5 mL of PBS in an Ultraturrax T8 homogenizer. For differential counting, one challenge strain contained a deletion of the *lacZ* gene that does not affect intestinal colonization. Colonizing vibrios were enumerated by dilution plating in LB agar containing X-gal. Finally, the competitive index (CI) was calculated as the output ratio of the competing strains after intrainestinal growth normalized by the input ratio.

Results

Motility enhances *V. cholerae* detachment from an abiotic surface

Flagellar motility has been shown to facilitate bacterial attachment (3, 18). However, the role of motility in detachment has remained unexplored. Since non-motile vibrios can still form

robust biofilms, we took a novel approach to address the role of motility in detachment. Specifically, we allowed biofilms to develop under conditions in which the c-di-GMP pool is artificially enhanced to inhibit motility. Then, we examined the effect of preventing vibrios from returning to the planktonic lifestyle with the inhibitor Q24DA, which blocks flagellar motility by interacting with PomB, a component of the inner membrane sodium-conducting channel that drives flagellar rotation (35, 45). In Fig. 1, we show that the amount of crystal violet-stained material declines with time in the wild type strain treated with vehicle only. However, in the presence of Q24DA the mass of adherent bacteria continued to increase rather than declining. Addition of Q24DA phenotypically mimicked the effect of deleting *pomAB*, though the mutant as expected adhered less at the early time point (Fig. 1). Q24DA did not significantly affect the stability of the biofilm in the *pomAB* mutant (Fig. 1). These results suggested that, although motility favors initial adhesion, it also plays a fundamental role in the detachment phase. To further support this conclusion, we developed mixed biofilms consisting of a 1:1 mixture of strain C7258 (motile) and C7258 ABL (non-motile) that form blue and white colonies in X-gal agar, respectively. Then, we disrupted the biofilm by vortexing in the presence of glass beads and enumerated the number of motile and non-motile cells at different time points by dilution plating. As shown in Fig. 1, motile vibrios predominated in the biofilm at the early time points. On the contrary, non-motile vibrios predominated at the late time points. These findings indicate that while motile vibrios have an attachment advantage, non-motile cells have a detachment disadvantage.

HapA does not promote detachment of vibrios from an abiotic surface

We next examined the role of HapA in bacterial detachment from an abiotic surface. HapA is the major soluble protease secreted by *V. cholerae* through the type II secretion system together with other proteins including cholera toxin (46). Furthermore, HapA has been detected in the biofilm matrix (28) though its abundance relative to other proteases present in this compartment is unknown. Thus, we first examined if the activity of HapA accounts for a significant fraction of the proteolytic activity exhibited by *V. cholerae* biofilms. As shown in Fig. 2a, comparison of the proteolytic activity in biofilms formed by strain C7258 and C7258H (*hapA::celA*) showed that HapA activity accounted for a significant fraction ($\approx 50\%$) of the total proteolytic activity of the biofilm matrix. To rule out the possibility of the lower protease activity detected in the *hapA* biofilm being due to structural differences affecting diffusion of the fluorescent peptide probe, we compared the protease activity present in wild type and *hapA* disrupted biofilms. The results shown in Fig 2b, confirm that HapA accounts for a significant fraction of the proteolytic activity present in the biofilm matrix.

HapA has been suggested to play an early role in biofilm growth through limited proteolysis of the matrix protein RbmA (47). To dissect the role of HapA in detachment from its participation in biofilm growth, we allowed biofilms to develop under conditions of high c-di-GMP to repress *hapA*. Then, we examined if permanent loss of HapA by mutation had any effect on biofilm stability. To accentuate the stability of the biofilms formed by wild type and *hapA* strains incubated for different times, the OD₅₇₀ measurements were normalized by the quantity of bacteria attached at 8 h (relative attachment). As shown in Fig. 3, we could not demonstrate differences in the decline of crystal violet-stainable material

over time between the wild type and *hapA* strain. We conclude that HapA does not act on the *V. cholerae* biofilm matrix to promote detachment under the conditions tested. However, addition of the protease inhibitor cocktail cOmplete significantly increased biofilm stability in the wild type and *hapA* mutant (Fig. 3) suggesting that other proteases or hydrolases inhibited by cOmplete could function as detachment factors.

HapA facilitates vibrio detachment from the suckling mouse intestine

HapA exhibits mucinase activity (21) and has been shown to promote *V. cholerae* detachment from mucin-secreting goblet cells in cell culture (23). Thus, we considered the possibility of HapA acting as a substratum-specific detachment factor in infection. We developed the balanced lethal plasmid system shown in Fig. 4 to enhance the c-di-GMP pool late in infection, a condition that represses the expression of HapA and motility. It has been reported that increasing the c-di-GMP pool early in infection diminishes *V. cholerae* motility and ToxT-dependent transcription of *tcpA* encoding the TCP pilus, thereby inhibiting intestinal colonization (48). Thus, we engineered our balanced lethal plasmid system to enhance the c-di-GMP pool after vibrios reaching a high cell density in the small intestine. We hypothesized that, if HapA and/or motility is required for detachment, increasing the c-di-GMP pool late in infection to suppress these activities should enhance intestinal colonization. In plasmid pAJA1thyA-8, the DGC VCA0956 is expressed from *hapR* transcription and translation initiation signals previously proven to be activated at high cell density due to the accumulation of cholera autoinducer 1 (CAI-1) (40). We confirmed that strain C7258 T containing the active plasmid pAJA1thyA-8 made enhanced biofilms on polystyrene and mucin-coated surfaces compared to the transformants harboring the VCA0956 active site mutant (Fig. 5a). In Fig. 5c we show that enhancing the c-di-GMP by expressing VCA0956 suppressed motility in the swarm agar test and inhibited the expression of HapA (Fig. 5b), a phenotype with a tight requirement for the quorum sensing regulator HapR (49). As expected, the above experiment showed that deletion of *pomAB* does not impact quorum sensing-dependent *hapA* expression neither deletion of *hapA* impinges on motility.

Wild type *V. cholerae* containing plasmid pAJA1thyA-8 effectively colonized the suckling mouse intestine confirming that activation of VCA0956 expression to increase the c-di-GMP pool is delayed until vibrios reach a high cell density in the gut. Increasing the c-di-GMP pool late in infection in wild type *V. cholerae* augmented colonization 3.6-fold, indicating that under these conditions vibrios tend to remain associated to the suckling mouse intestinal tissue (Fig. 6). No effect was observed in competing strains containing a deletion of *cqsA* that does not produce the CAI-1 signaling molecule required to activate the *hapR*-VCA0956 promoter fusion (Fig. 6). This result corroborates that under these experimental conditions, vibrios increase their c-di-GMP pool upon entering quorum sensing mode to activate the plasmid-encoded *hapR*-VCA0956 fusion. To determine if the effect of enhancing the c-di-GMP pool late in infection was due to inhibition of motility and/or HapA production, we repeated the same competition in *pomAB* (non-motile) and *hapA* genetic backgrounds. In Fig. 6 we show that increasing the c-di-GMP pool had a smaller effect on intestinal colonization in the *pomAB* genetic background. Similarly, the effect of enhancing the c-di-GMP pool was fully abrogated in the *hapA* genetic background (Fig. 6). To rule out the

possibility of the *pomAB* and *hapA* mutants not reaching the cell density required to activate the *hapR*-VCA0956 fusion, we determined the titer of vibrios recovered from the intestinal tissue. *V. cholerae* strains containing pAJAthyA-8 were recovered from the small intestine of mice with mean titers of 6.8 ± 0.3 (Wt), 6.6 ± 0.6 (*pomAB*) and 6.6 ± 0.5 (*hapA*) log units per g of tissue.

Discussion

V. cholerae detachment from biofilms and the intestinal mucosa is a critical step in cholera dissemination, which remains poorly understood. In this study, we examined the role of motility and *V. cholerae*'s major secreted protease HapA in this process. It has been shown that non-motile vibrios use a distinct developmental pathway in biofilm formation that entails formation of aggregates in solution that could settle onto surfaces as microcolonies to further develop into mature biofilms (50, 51). We took advantage of the above behavior and the motility inhibitor Q24DA to dissect the role of motility in surface detachment. To this end, we allowed vibrios to (i) initiate biofilm formation under conditions in which vibrio exopolysaccharide expression is exacerbated and motility inhibited and (ii) examined the effect of blocking their capacity to regain motility on biofilm stability. Following this approach, we provide the first direct evidence that flagellar motility promotes the detachment of vibrios from biofilms formed on abiotic surfaces. In an aging biofilm, activation of motility could result from entry of cells into quorum sensing (high cell density) mode, a condition that lowers the c-di-GMP pool (42). Furthermore, lowering of the c-di-GMP diminishes the assembly of the MSHA pili required for surface attachment (19, 52). We hypothesize that late in the biofilm cycle, low c-di-GMP enhances motility and simultaneously disrupts the flagellum-MSHA cooperation that favors permanent attachment providing cells with momentum to detach and swim toward an unspent substratum.

Biofilm dispersal can also result from disruption of the biofilm matrix by hydrolytic enzymes. HapA is the major protease secreted by *V. cholerae* into the culture medium. For instance, negligible proteolytic activity can be detected in milk agar plates or using an azocasein assay in the cell-free supernatants of *hapA* mutants (44). Furthermore, HapA is activated at high cell density (49, 53). However, *V. cholerae* secretes additional proteolytic enzymes. Thus, we used a more sensitive protease fluorescence-based assay, to show that HapA accounts for a significant fraction of the total proteolytic activity present in the *V. cholerae* biofilm matrix. Nevertheless, the biofilms formed by a *hapA* mutant were not significantly more stable than wild type biofilms. A broad-spectrum protease inhibitor cocktail enhanced biofilm stability suggesting the participation of other hydrolases in biofilm dispersal. Other proteases present in the *V. cholerae* secretome include a collagenase (VchC) (54); amino peptidases (Lap, LapX); serine proteases VesA, VesB, VesC (55) and IvaP (56) and the metalloprotease PrtV (57). In addition, inhibition of Dns and Xds DNases activities by the cComplete cocktail could contribute to biofilm stability.

We used the suckling mouse cholera model to determine the role of c-di-GMP, HapA and/or motility in detachment *in vivo*. In this model, vibrios that fail to attach to the intestinal mucosa are cleared resulting in diminished bacterial recovery post infection (58). Compared to ligated ileal loops, this model lends itself to examining the conditions that favor bacterial

exit from the host. We designed a balanced lethal plasmid system to enhance the c-di-GMP pool late in infection to inhibit the expression of motility and HapA. Here we demonstrate that enhancing the c-di-GMP pool augmented colonization and that this increase was dependent on the expression of HapA and motility late in infection. We show that at the time mice were sacrificed (16 h), the wild type, *pomAB* and *hapA* strains were recovered from the small intestine with similar titers. Therefore, the lack of response of the *pomAB* and *hapA* mutants to an increase in c-di-GMP in the competitive colonization assay cannot be explained by a failure of these mutants to reach high cell density in the suckling mouse intestine. However, since non-motile vibrios may require more time to establish infection, we cannot rule out the possibility of the *pomAB* mutant entering quorum sensing mode later than the wild type strain resulting in a lower expression of the *hapR-VCA0956* fusion.

The above results are in agreement with a previous study using the rabbit ileal loop model proposing that detachment of vibrios from the intestinal epithelium into the lumen was attributable to activation of motility in the stationary phase (25). Our findings also concur with studies showing that HapA promotes detachment of vibrios from cultured intestinal cells (22, 23, 59) and with the observation that *hapA* mutants exhibit a modest increase in intestinal colonization in the suckling mouse model (22, 29). A *hapA* detachment phenotype was not observed, however, in the rabbit ileal loop model (25). This discrepancy could be due to the nature of the animal models used. Contrary to the suckling mouse model, a rabbit ileal loop is a closed system in which the normal flow of intestinal content is disrupted, a condition that could alter the physiology of the test animal and the host-pathogen interaction. Possible mechanisms by which HapA could contribute to detachment include the degradation of the protective mucus barrier to which vibrios are known to attach (60) or by cleaving the GbpA adhesin that mediates attachment of vibrios to mucin (6). It is likely that both motility and HapA synergistically promote detachment from the intestinal mucosa since the polar flagellum of vibrios is not an efficient locomotion organelle in a highly viscous media such as the protective mucus gel (61). Furthermore, it has been reported that the vibrio polar flagellum tends to break in the viscous mucus gel (62).

In sum, we compared the role of flagellar motility and HapA in *V. cholerae* detachment from two extreme surfaces: the non-degradable abiotic polystyrene or borosilicate surfaces and the degradable suckling mouse intestinal mucosa. Flagellar motility but not HapA appeared to facilitate detachment from an abiotic surface. The finding that HapA could facilitate detachment from the suckling mouse intestine but not from a polystyrene surface suggests that HapA could function as a substratum-specific detachment factor. We note that *V. cholerae* may also hijack host hydrolytic enzymes to detach late in infection. This would explain the finding that inactivation of *hapA* has a smaller effect on intestinal colonization compared to other colonization factors such as the toxin coregulated pilus (22).

Acknowledgments

This study was supported PHS Grant AI104993 from the National Institutes of Health to AJS. We are grateful for the continuous support provided by the Morehouse School of Medicine Center for Laboratory Animal Research.

References

1. Conner JG, Teschler JK, Jones CJ, Yildiz FH. Staying Alive: *Vibrio cholerae*'s cycle of environmental survival, transmission, and dissemination. *Microbiology spectrum*. 2016; 4:2. Epub 2016/05/27.
2. Watnick PI, Fullner KJ, Kolter R. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *Journal of bacteriology*. 1999; 181(11):3606–9. Epub 1999/05/29. [PubMed: 10348878]
3. Watnick PI, Kolter R. Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Molecular microbiology*. 1999; 34(3):586–95. Epub 1999/11/17. [PubMed: 10564499]
4. Chiavelli DA, Marsh JW, Taylor RK. The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. *Applied and environmental microbiology*. 2001; 67(7):3220–5. Epub 2001/06/27. [PubMed: 11425745]
5. Bhowmick R, Ghosal A, Das B, Koley H, Saha DR, Ganguly S, et al. Intestinal adherence of *Vibrio cholerae* involves a coordinated interaction between colonization factor GbpA and mucin. *Infection and immunity*. 2008; 76(11):4968–77. Epub 2008/09/04. [PubMed: 18765724]
6. Jude BA, Martinez RM, Skorupski K, Taylor RK. Levels of the secreted *Vibrio cholerae* attachment factor GbpA are modulated by quorum-sensing-induced proteolysis. *Journal of bacteriology*. 2009; 191(22):6911–7. Epub 2009/09/08. [PubMed: 19734310]
7. Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK. The *Vibrio cholerae* chitin utilization program. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101(8):2524–9. Epub 2004/02/26. [PubMed: 14983042]
8. Jude BA, Taylor RK. The physical basis of type 4 pilus-mediated microcolony formation by *Vibrio cholerae* O1. *Journal of structural biology*. 2011; 175(1):1–9. Epub 2011/04/30. [PubMed: 21527347]
9. Krebs SJ, Taylor RK. Protection and attachment of *Vibrio cholerae* mediated by the toxin-coregulated pilus in the infant mouse model. *Journal of bacteriology*. 2011; 193(19):5260–70. Epub 2011/08/02. [PubMed: 21804008]
10. Millet YA, Alvarez D, Ringgaard S, von Andrian UH, Davis BM, Waldor MK. Insights into *Vibrio cholerae* intestinal colonization from monitoring fluorescently labeled bacteria. *PLoS pathogens*. 2014; 10(10):e1004405. Epub 2014/10/03. [PubMed: 25275396]
11. Thelin KH, Taylor RK. Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. *Infection and immunity*. 1996; 64(7):2853–6. Epub 1996/07/01. [PubMed: 8698524]
12. Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin-coregulated pili, and the *toxR* regulon are essential for *Vibrio cholerae* pathogenesis in humans. *The Journal of experimental medicine*. 1988; 168(4):1487–92. Epub 1988/10/01. [PubMed: 2902187]
13. Tacket CO, Taylor RK, Losonsky G, Lim Y, Nataro JP, Kaper JB, et al. Investigation of the roles of toxin-coregulated pili and mannose-sensitive hemagglutinin pili in the pathogenesis of *Vibrio cholerae* O139 infection. *Infection and immunity*. 1998; 66(2):692–5. Epub 1998/02/07. [PubMed: 9453628]
14. Almagro-Moreno S, Pruss K, Taylor RK. Intestinal colonization dynamics of *Vibrio cholerae*. *PLoS pathogens*. 2015; 11(5):e1004787. Epub 2015/05/23. [PubMed: 25996593]
15. Silva AJ, Benitez JA. *Vibrio cholerae* biofilms and cholera pathogenesis. *PLoS neglected tropical diseases*. 2016; 10(2):e0004330. Epub 2016/02/06. [PubMed: 26845681]
16. Faruque SM, Biswas K, Udden SM, Ahmad QS, Sack DA, Nair GB, et al. Transmissibility of cholera: in vivo-formed biofilms and their relationship to infectivity and persistence in the environment. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103(16):6350–5. Epub 2006/04/08. [PubMed: 16601099]
17. Tamayo R, Patimalla B, Camilli A. Growth in a biofilm induces a hyperinfectious phenotype in *Vibrio cholerae*. *Infection and immunity*. 2010; 78(8):3560–9. Epub 2010/06/03. [PubMed: 20515927]

18. Utada AS, Bennett RR, Fong JC, Gibiansky ML, Yildiz FH, Golestanian R, et al. *Vibrio cholerae* use pili and flagella synergistically to effect motility switching and conditional surface attachment. *Nature communications*. 2014; 5:4913. Epub 2014/09/23.
19. Srivastava D, Hsieh ML, Khataokar A, Neiditch MB, Waters CM. Cyclic di-GMP inhibits *Vibrio cholerae* motility by repressing induction of transcription and inducing extracellular polysaccharide production. *Molecular microbiology*. 2013; 90(6):1262–76. Epub 2013/10/19. [PubMed: 24134710]
20. Teschler JK, Zamorano-Sanchez D, Utada AS, Warner CJ, Wong GC, Linington RG, et al. Living in the matrix: assembly and control of *Vibrio cholerae* biofilms. *Nature reviews Microbiology*. 2015; 13(5):255–68. Epub 2015/04/22. [PubMed: 25895940]
21. Finkelstein RA, Boesman-Finkelstein M, Holt P. *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin: F.M. Burnet revisited. *Proceedings of the National Academy of Sciences of the United States of America*. 1983; 80(4):1092–5. Epub 1983/02/01. [PubMed: 6341990]
22. Silva AJ, Leitch GJ, Camilli A, Benitez JA. Contribution of hemagglutinin/protease and motility to the pathogenesis of El Tor biotype cholera. *Infection and immunity*. 2006; 74(4):2072–9. Epub 2006/03/23. [PubMed: 16552036]
23. Benitez JA, Spelbrink RG, Silva A, Phillips TE, Stanley CM, Boesman-Finkelstein M, et al. Adherence of *Vibrio cholerae* to cultured differentiated human intestinal cells: an in vitro colonization model. *Infection and immunity*. 1997; 65(8):3474–7. Epub 1997/08/01. [PubMed: 9234816]
24. Seper A, Fengler VH, Roier S, Wolinski H, Kohlwein SD, Bishop AL, et al. Extracellular nucleases and extracellular DNA play important roles in *Vibrio cholerae* biofilm formation. *Molecular microbiology*. 2011; 82(4):1015–37. Epub 2011/10/29. [PubMed: 22032623]
25. Nielsen AT, Dolganov NA, Otto G, Miller MC, Wu CY, Schoolnik GK. RpoS controls the *Vibrio cholerae* mucosal escape response. *PLoS pathogens*. 2006; 2(10):e109. Epub 2006/10/24. [PubMed: 17054394]
26. Lee SH, Butler SM, Camilli A. Selection for in vivo regulators of bacterial virulence. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98(12):6889–94. Epub 2001/06/08. [PubMed: 11391007]
27. Benitez JA, Silva AJ. *Vibrio cholerae* hemagglutinin(HA)/protease: An extracellular metalloprotease with multiple pathogenic activities. *Toxicon : official journal of the International Society on Toxinology*. 2016; 115:55–62. Epub 2016/03/10. [PubMed: 26952544]
28. Absalon C, Van Dellen K, Watnick PI. A communal bacterial adhesin anchors biofilm and bystander cells to surfaces. *PLoS pathogens*. 2011; 7(8):e1002210. Epub 2011/09/09. [PubMed: 21901100]
29. Robert A, Silva A, Benitez JA, Rodriguez BL, Fando R, Campos J, et al. Tagging a *Vibrio cholerae* El Tor candidate vaccine strain by disruption of its hemagglutinin/protease gene using a novel reporter enzyme: *Clostridium thermocellum* endoglucanase A. *Vaccine*. 1996; 14(16):1517–22. Epub 1996/11/01. [PubMed: 9014293]
30. Wang H, Wu JH, Ayala JC, Benitez JA, Silva AJ. Interplay among cyclic diguanylate, HapR, and the general stress response regulator (RpoS) in the regulation of *Vibrio cholerae* hemagglutinin/protease. *Journal of bacteriology*. 2011; 193(23):6529–38. Epub 2011/10/04. [PubMed: 21965573]
31. Silva AJ, Sultan SZ, Liang W, Benitez JA. Role of the histone-like nucleoid structuring protein in the regulation of *rpoS* and RpoS-dependent genes in *Vibrio cholerae*. *Journal of bacteriology*. 2008; 190(22):7335–45. Epub 2008/09/16. [PubMed: 18790865]
32. Wang H, Ayala JC, Benitez JA, Silva AJ. Interaction of the histone-like nucleoid structuring protein and the general stress response regulator RpoS at *Vibrio cholerae* promoters that regulate motility and hemagglutinin/protease expression. *Journal of bacteriology*. 2012; 194(5):1205–15. Epub 2011/12/24. [PubMed: 22194453]
33. de Lorenzo V, Eltis L, Kessler B, Timmis KN. Analysis of *Pseudomonas* gene products using lacI^q/ptp-lac plasmids and transposons that confer conditional phenotypes. *Gene*. 1993; 123(1):17–24. Epub 1993/01/15. [PubMed: 8380783]

34. Silva AJ, Benitez JA, Wu JH. Attenuation of bacterial virulence by quorum sensing-regulated lysis. *Journal of biotechnology*. 2010; 150(1):22–30. Epub 2010/08/03. [PubMed: 20673838]
35. Wang H, Zhang L, Silva AJ, Benitez JA. A quinazoline-2,4-diamino analog suppresses *Vibrio cholerae* flagellar motility by interacting with motor protein PomB and induces envelope stress. *Antimicrobial agents and chemotherapy*. 2013; 57(8):3950–9. Epub 2013/06/05. [PubMed: 23733460]
36. Liang W, Pascual-Montano A, Silva AJ, Benitez JA. The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. *Microbiology*. 2007; 153(Pt 9):2964–75. Epub 2007/09/05. [PubMed: 17768239]
37. Donnenberg MS, Kaper JB. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infection and immunity*. 1991; 59(12):4310–7. Epub 1991/12/01. [PubMed: 1937792]
38. Miller MB, Skorupski K, Lenz DH, Taylor RK, Bassler BL. Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell*. 2002; 110(3):303–14. Epub 2002/08/15. [PubMed: 12176318]
39. Massie JP, Reynolds EL, Koestler BJ, Cong JP, Agostoni M, Waters CM. Quantification of high-specificity cyclic diguanylate signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2012; 109(31):12746–51. Epub 2012/07/18. [PubMed: 22802636]
40. Silva AJ, Pham K, Benitez JA. Haemagglutinin/protease expression and mucin gel penetration in El Tor biotype *Vibrio cholerae*. *Microbiology*. 2003; 149(Pt 7):1883–91. Epub 2003/07/12. [PubMed: 12855739]
41. Simm R, Morr M, Kader A, Nimtz M, Romling U. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Molecular microbiology*. 2004; 53(4):1123–34. Epub 2004/08/13. [PubMed: 15306016]
42. Waters CM, Lu W, Rabinowitz JD, Bassler BL. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of *vpsT*. *Journal of bacteriology*. 2008; 190(7):2527–36. Epub 2008/01/29. [PubMed: 18223081]
43. Wang H, Ayala JC, Silva AJ, Benitez JA. The histone-like nucleoid structuring protein (H-NS) is a repressor of *Vibrio cholerae* exopolysaccharide biosynthesis (*vps*) genes. *Applied and environmental microbiology*. 2012; 78(7):2482–8. Epub 2012/01/31. [PubMed: 22287003]
44. Benitez JA, Silva AJ, Finkelstein RA. Environmental signals controlling production of hemagglutinin/protease in *Vibrio cholerae*. *Infection and immunity*. 2001; 69(10):6549–53. Epub 2001/09/13. [PubMed: 11553605]
45. Rasmussen L, White EL, Pathak A, Ayala JC, Wang H, Wu JH, et al. A high-throughput screening assay for inhibitors of bacterial motility identifies a novel inhibitor of the Na⁺-driven flagellar motor and virulence gene expression in *Vibrio cholerae*. *Antimicrobial agents and chemotherapy*. 2011; 55(9):4134–43. Epub 2011/06/29. [PubMed: 21709090]
46. Johnson TL, Fong JC, Rule C, Rogers A, Yildiz FH, Sandkvist M. The Type II secretion system delivers matrix proteins for biofilm formation by *Vibrio cholerae*. *Journal of bacteriology*. 2014; 196(24):4245–52. Epub 2014/10/01. [PubMed: 25266381]
47. Smith DR, Maestre-Reyna M, Lee G, Gerard H, Wang AH, Watnick PI. In situ proteolysis of the *Vibrio cholerae* matrix protein RbmA promotes biofilm recruitment. *Proceedings of the National Academy of Sciences of the United States of America*. 2015 Epub 2015/08/05.
48. Tamayo R, Schild S, Pratt JT, Camilli A. Role of cyclic di-GMP during El Tor biotype *Vibrio cholerae* infection: characterization of the in vivo-induced cyclic di-GMP phosphodiesterase CdpA. *Infection and immunity*. 2008; 76(4):1617–27. Epub 2008/01/30. [PubMed: 18227161]
49. Jobling MG, Holmes RK. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Molecular microbiology*. 1997; 26(5):1023–34. Epub 1998/01/13. [PubMed: 9426139]
50. Watnick PI, Lauriano CM, Klose KE, Croal L, Kolter R. The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139. *Molecular microbiology*. 2001; 39(2):223–35. Epub 2001/01/03. [PubMed: 11136445]

51. Lauriano CM, Ghosh C, Correa NE, Klose KE. The sodium-driven flagellar motor controls exopolysaccharide expression in *Vibrio cholerae*. *Journal of bacteriology*. 2004; 186(15):4864–74. Epub 2004/07/21. [PubMed: 15262923]
52. Jones CJ, Utada A, Davis KR, Thongsomboon W, Zamorano Sanchez D, Banakar V, et al. C-di-GMP regulates motile to sessile transition by modulating MshA pili biogenesis and near-surface motility behavior in *Vibrio cholerae*. *PLoS pathogens*. 2015; 11(10):e1005068. Epub 2015/10/28. [PubMed: 26505896]
53. Silva AJ, Benitez JA. Transcriptional regulation of *Vibrio cholerae* hemagglutinin/protease by the cyclic AMP receptor protein and RpoS. *Journal of bacteriology*. 2004; 186(19):6374–82. Epub 2004/09/18. [PubMed: 15375117]
54. Park BR, Zielke RA, Wierzbicki IH, Mitchell KC, Withey JH, Sikora AE. A metalloprotease secreted by the type II secretion system links *Vibrio cholerae* with collagen. *Journal of bacteriology*. 2015; 197(6):1051–64. Epub 2015/01/07. [PubMed: 25561716]
55. Sikora AE, Zielke RA, Lawrence DA, Andrews PC, Sandkvist M. Proteomic analysis of the *Vibrio cholerae* type II secretome reveals new proteins, including three related serine proteases. *The Journal of biological chemistry*. 2011; 286(19):16555–66. Epub 2011/03/10. [PubMed: 21385872]
56. Hatzios SK, Abel S, Martell J, Hubbard T, Sasabe J, Munera D, et al. Chemoproteomic profiling of host and pathogen enzymes active in cholera. *Nature chemical biology*. 2016; 12(4):268–74. Epub 2016/02/24. [PubMed: 26900865]
57. Ogierman MA, Fallarino A, Riess T, Williams SG, Attridge SR, Manning PA. Characterization of the *Vibrio cholerae* El Tor lipase operon *lipAB* and a protease gene downstream of the *hly* region. *Journal of bacteriology*. 1997; 179(22):7072–80. Epub 1997/11/26. [PubMed: 9371455]
58. Klose KE. The suckling mouse model of cholera. *Trends in microbiology*. 2000; 8(4):189–91. Epub 2001/02/07. [PubMed: 10754579]
59. Finkelstein RA, Boesman-Finkelstein M, Chang Y, Hase CC. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. *Infection and immunity*. 1992; 60(2):472–8. Epub 1992/02/01. [PubMed: 1730478]
60. Yamamoto T, Yokota T. Electron microscopic study of *Vibrio cholerae* O1 adherence to the mucus coat and villus surface in the human small intestine. *Infection and immunity*. 1988; 56(10):2753–9. Epub 1988/10/01. [PubMed: 3417355]
61. Atsumi T, Maekawa Y, Yamada T, Kawagishi I, Imae Y, Homma M. Effect of viscosity on swimming by the lateral and polar flagella of *Vibrio alginolyticus*. *Journal of bacteriology*. 1996; 178(16):5024–6. Epub 1996/08/01. [PubMed: 8759871]
62. Liu Z, Miyashiro T, Tsou A, Hsiao A, Goulian M, Zhu J. Mucosal penetration primes *Vibrio cholerae* for host colonization by repressing quorum sensing. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(28):9769–74. Epub 2008/07/09. [PubMed: 18606988]

Highlights

- Motility facilitates detachment of *Vibrio cholerae* O1 from abiotic and biotic surfaces.
- Hemagglutinin/protease (HapA) is the major protease present in the biofilm extracellular matrix.
- An increase in the intracellular pool of cyclic diguanylic acid late in infection diminishes *V. cholerae* detachment from the intestinal mucosa.
- HapA contributes to *V. cholerae* detachment from the suckling mouse small intestine.

Fig. 1. Role of motility in *V. cholerae* detachments and biofilm dispersal

Left and middle panels. Strain C7258 (Wt) and C7258 AB (*pomAB*) containing plasmid pCMW75 were allowed to form biofilms in LB medium containing IPTG for 8 h at 30°C. At this time point, the preformed biofilms were treated with Q24DA (10 µg/mL) in dimethyl sulfoxide (DMSO) or DMSO (vehicle control) and further incubated up to 64 h. Then, biofilm formation was determined as described in materials and methods. **Rightmost panel.** Mixtures of strain C7258 (Wt) and C7258 ABL (*pomAB*) at a 1:1 ratio (based on colony forming units) were allowed to form biofilms for different time periods, the biofilms were disrupted with glass beads and the fraction of motile to non-motile cells determined by dilution plating in LB agar containing X-gal. The trendline indicates the decline in motile cells in the aging biofilm. Each bar represents the average of at least three independent experiments. Error bars indicate the standard deviation (STDEV). Symbols: * $p < 0.05$, unpaired T test.

Fig. 2. Proteolytic activity of *V. cholerae* biofilms

a. Strains C7258 (Wt) and C7258H (*hapA*) were allowed to form biofilms for different time periods. Planktonic cells were discarded and the total proteolytic activity of the intact biofilm determined as described in materials and methods using a fluorescent probe. The biofilm mass was determined by crystal violet staining and the results expressed as fluorescence units (FI) normalized by the biofilm mass (OD₅₇₀). **b.** Strains C7258 (Wt) and C7258H (*hapA*) were allowed to form biofilms in borosilicate tubes. Biofilm mass was estimated by crystal violet staining and the proteolytic activity present in the disrupted biofilms was measured using a fluorescent probe as described in materials and methods. Each bar represents the average of at least three independent experiments. Error bars indicate the STDEV. Symbols: ** p < 0.01, unpaired T test.

Fig. 3. Role of proteases in the stability of *V. cholerae* biofilms

Strains C7258 (Wt) and C7258H (*hapA*) transformed with plasmid pCMW75 were allowed to form biofilms in the presence of IPTG to enhance the c-di-GMP pool for different time periods in microtiter plates. The amount of crystal violet stained material was measured at OD₅₇₀. The quantity of bacteria attached at each time point was normalized by the amount present at 8 h (relative attachment). The cComplete protease inhibitor cocktail was added as recommended by the provider to the Wt and *hapA* strain at 8 h. Each bar represents the average of at least three independent experiments. Error bars indicate the STDEV. Statistical significance was determined using an unpaired T test (* $p < 0.05$; ** $p < 0.01$).

Fig. 4. Balanced lethal plasmid system designed to increase the c-di-GMP pool late in infection
 The vector pAJA1thyA-8 consists of (i) a wild type *thyA* (thymidylate synthase) gene for positive selection *in vivo* when transformed into a *V. cholerae* strain harboring a *thyA* deletion and (ii) a gene encoding the DGC VCA0956 expressed from the *hapR* promoter, which is activated at high cell density. The *rrnBT₁T₂* transcription terminator was inserted upstream the *hapR* promoter to prevent spurious activation of VCA0956 transcription from vector sequences. The control plasmid pAJA1thyA-8* harbors a mutation in the active site of VCA0956 abolishing enzyme activity. Symbols: bla, β -lactamase gene; *hapR_p*, *hapR* promoter.

Fig. 5. Validation of balanced lethal plasmid system to increase c-di-GMP pool late in infection
a. Strain C7258 T containing plasmid pAJA1thyA-8* (open bar) or pAJA1thyA-8 (filled bar) were allowed to form biofilms in polystyrene and mucin-coated polystyrene microtiter plates as described in materials and methods. **b.** Strains C7258 T, C7258 TAB and C7258H T containing plasmid pAJA1thyA-8* (open bars) or pAJA1thyA-8 (filled bars) were grown to stationary phase in TSB medium and production of HapA measured as described in methods. Each bar represents the average of three independent cultures and error bars denote the STDEV (** $p < 0.01$, unpaired T test). **c.** The above stationary cultures were stabbed into 3 mL of swarm agar in 6-well tissue culture plates and incubated 16 h at 30°C. The plate shown is representative of three experiments.

Fig. 6. Competition between strains exhibiting enhanced versus basal c-di-GMP pool

The following strains were transformed with plasmids pAJA1thyA-8 or pAJA1thyA-8*, respectively: C7258 T and C7258 TL (Wt); C7258 T C and C7258 TL C (*cqsA*); C7258 TAB and C7258 TLAB (*pomAB*), C7258H T and C7258H TL (*hapA*). Paired strains containing pAJA1thyA-8 or pAJA1thyA-8* were mixed at 1:1 ratio and inoculated to newborn mice by oral gavage. The competitive index (CI) was calculated by normalizing the output ratio of strains containing pAJA1thyA-8 over pAJA1thyA-8* after intestinal colonization by their input ratio. The lines through each data set denote the median CI. Symbols: * $p < 0.01$ (unpaired T test).

Table 1

Strains, plasmids and oligonucleotide primers

Strain	Description	Reference
<i>E. coli</i>		
TOP10	<i>F-mcrA (mrr-hsdRMS-mcrB C) 80dlacZ M15 lacX74 recA1 araD139 (ara leu)7697galUgalK rpsL(Str) endA1 nupG</i>	Invitrogen
S17-1λpir	F ⁻ <i>recA hsdR</i> RP4-2 (Tc::Mu) (Km::Tn7) lysogenized with λpir	(33)
<i>V. cholerae</i>		
C7258	O1 El Tor, Ogawa	C linical isolate, Perú 1991
C7258 AB	C7258 <i>pomA pomB</i>	(35)
C7258 ABL	C7258 <i>pomA pomB lacZ</i>	This study
C7258H	C7258 <i>hapA::celA</i>	This study
C7258 T	C7258 <i>thyA</i>	This study
C7258 T C	C7258 <i>thyA cqsA</i>	This study
C7258 TL	C7258 <i>thyA lacZ</i>	This study
C7258 TLC	C7258 <i>thyA lacZ cqsA</i>	This study
C7258 TAB	C7258 <i>thyA pomA pomB</i>	This study
C7258 TLAB	C7258 <i>thyA lacZ pomA pomB</i>	This study
C7258HT	C7258 <i>thyA hapA::celA</i>	This study
C7258HTL	C7258 <i>thyA lacZ hapA::celA</i>	This study
Plasmids		
pTT3	<i>rrnB</i> T ₁ T ₂ transcription terminator in pUC19	(40)
pCMW75	<i>V. harveyi</i> IPTG-inducible QrgB expression vector	(42)
pCVD <i>thyA</i>	Suicide vector with genomic DNA flanking <i>thyA</i> deletion	(34)
pCVD <i>lacZ</i>	Suicide vector with genomic DNA flanking <i>lacZ</i> deletion	(31)
pGPH6	Suicide vector with <i>hapA::celA</i> insertion	(29)
pCVD <i>pomAB</i>	Suicide vector with genomic DNA flanking <i>pomAB</i> deletion	(35)
pCVD <i>cqsA</i>	Suicide vector with genomic DNA flanking <i>cqsA</i> deletion	(36)
pAJA1	<i>rrnB</i> T ₁ T ₂ -hapR promoter VCA0956 cassette in pBR322	This study
pAJA1 <i>thyA</i> -8	Complementing <i>thyA</i> gene cloned in pAJA1	This study
pAJA1 <i>thyA</i> -8*	pAJA1 <i>thyA</i> -8 containing GGDEF → AADEF mutation in VCA0956	This study
Primer		
Sequence (5'–3')		
HapR39	GCCTCTAGATGAATTTGACGAGCAA	
HapR493	TAAGGATCCGCGTCCATAGGGGTA	
VCA0956-15	GCCGGATCCATGACAACCTGAAGATT	
VCA0956-1043	GGAGAATTCGGGTACGTATAGCAGA	
ThyA703	GCCAGCTTTATGAATTCGCCTTAGA	
ThyA2045	GGCGAATTCCTAAAGGTGCTGAGCT	
VCA0956mut	CGTGACGCGTGACAGCTTATCGTTATGCCGCTGAAGAGTTGCATGATTGCTCCG	