

Direct activation of a phospholipase by cyclic GMP-AMP in El Tor *Vibrio cholerae*

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Sensing and responding to environmental changes is essential for bacteria to adapt and thrive, and nucleotide-derived second messengers are central signaling systems in this process. The most recently identified bacterial cyclic dinucleotide second messenger, 3', 3'-cyclic GMP-AMP (cGAMP), was first discovered in the El Tor biotype of *Vibrio cholerae*. The cGAMP synthase, DncV, is encoded on the VSP-1 pathogenicity island, which is found in all El Tor isolates that are responsible for the current seventh pandemic of cholera but not in the classical biotype. We determined that unregulated production of DncV inhibits growth in El Tor *V. cholerae* but has no effect on the classical biotype. This cGAMP-dependent phenotype can be suppressed by null mutations in *vc0178* immediately 5' of *dncV* in VSP-1. VC0178 [renamed as cGAMP-activated phospholipase in *Vibrio* (CapV)] is predicted to be a patatin-like phospholipase, and coexpression of *capV* and *dncV* is sufficient to induce growth inhibition in classical *V. cholerae* and *Escherichia coli*. Furthermore, cGAMP binds to CapV and directly activates its hydrolase activity in vitro. CapV activated by cGAMP in vivo degrades phospholipids in the cell membrane, releasing 16:1 and 18:1 free fatty acids. Together, we demonstrate that cGAMP activates CapV phospholipase activity to target the cell membrane and suggest that acquisition of this second messenger signaling pathway may contribute to the emergence of the El Tor biotype as the etiological agent behind the seventh cholera pandemic.

second messengers | cyclic dinucleotides | phospholipid metabolism | pathogenicity island | cGAMP

The molecular mechanisms and signal transduction pathways by which bacteria respond and adapt to a changing environment remain some of the most fundamental questions in microbiology. These processes are essential for all bacteria whether they are soil isolates, symbionts, or human and agricultural pathogens. Given the importance of environmental adaptation, it is not surprising that bacteria pursue an abundance of strategies to accomplish this task. Of primary importance to environmental adaptation are second messenger signaling pathways that leverage nucleotide-derived small molecules for the transduction of external signals. The intracellular concentration of these small molecule second messengers imparts global regulatory effects by altering transcription, translation, or even protein activity, leading to changes in bacterial physiology and behavior (1).

We have just begun to fully appreciate the breadth and central importance of nucleotide-derived second messengers to all aspects of bacterial life. The first second messenger system described, cyclic adenosine monophosphate (cAMP), is primarily associated with the regulation of carbon utilization (2, 3). However, cAMP is also a global regulator controlling biofilm formation, virulence, and central metabolism (4–6). Guanosine penta/tetraphosphate (p/ppGpp) has also been studied for decades and is the primary signal modulating the bacterial stringent response (7, 8). In response to nutrient limitation and other

stimuli, the level of p/ppGpp dramatically increases, resulting in the inhibition of macromolecular synthesis, a key adaptive behavior for survival under these stress conditions (9, 10). The last 15 y have witnessed the emergence of cyclic dinucleotide second messengers in both bacteria and eukaryotes. Initially, cyclic diguanosine monophosphate (c-di-GMP), the first cyclic dinucleotide identified, was not widely appreciated until the renewed interest in understanding the molecular mechanisms underpinning biofilm formation (11). The second cyclic dinucleotide discovered was cyclic diadenosine monophosphate (c-di-AMP) (12, 13). c-di-AMP signaling plays a major physiological role in the regulation of potassium uptake, as well as other essential cellular processes, such as fatty acid synthesis, cell wall homeostasis, detection of DNA damage, progression of sporulation, and regulation of cell division (12, 14–22).

In 2012, the most recently identified bacterial cyclic dinucleotide signaling molecule, the hybrid 3', 3'-cyclic GMP-AMP (cGAMP), was discovered in the El Tor biotype of the human pathogen *Vibrio cholerae* (23). This new class of second messenger is synthesized by the enzyme DncV, encoded at locus *vc0179* in the *Vibrio* seventh pandemic island-1 (VSP-1) pathogenicity island.

Significance

Second messengers are employed by all organisms to regulate fundamental behaviors, including biofilm formation, motility, metabolism, and pathogenesis in bacteria. We have identified a phospholipase in the El Tor *Vibrio cholerae* biotype, responsible for the current cholera pandemic, that is directly activated by the second messenger 3', 3'-cyclic GMP-AMP (cGAMP). Discovery of this proteinaceous bacterial cGAMP effector sheds light on the functions and basic principles of cGAMP signaling. Both this phospholipase and the cGAMP synthase are encoded within the VSP-1 pathogenicity island, unique to the El Tor biotype, and our findings assign a biochemical function to VSP-1 that may contribute to the epidemiological success of El Tor *V. cholerae*.

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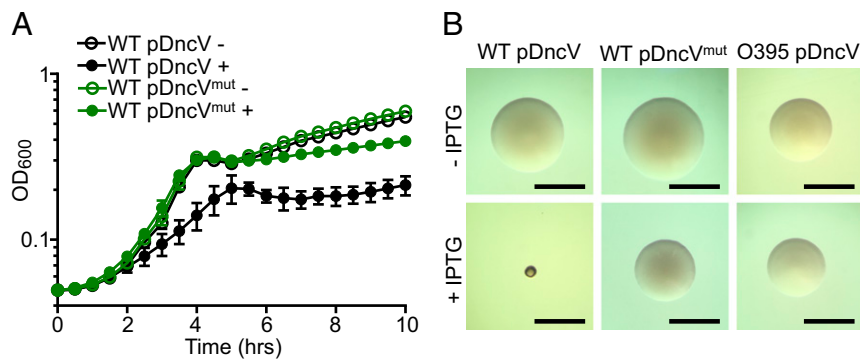


Fig. 1. Overproduction of the cGAMP synthase DncV induces planktonic growth arrest and a small colony phenotype in El Tor *V. cholerae*. (A) Growth curves of El Tor *V. cholerae* cultures carrying a P_{tac} -inducible plasmid encoding *dncV* (pDncV) or a catalytically inactive mutant (pDncV^{mut}), grown in the absence (–) or presence (+) of 100 μ M IPTG. Each data point represents the mean \pm SD of six biological replicates. (B) Colony morphologies of El Tor *V. cholerae* with pDncV (Left) or pDncV^{mut} (Middle), or classical *V. cholerae* strain O395 with pDncV (Right), grown on solid agar plates in the absence (Top) or presence (Bottom) of 100 μ M IPTG. (Scale bars: 1 mm.) Two independent plasmid constructs with different copy numbers were tested, and similar results were found (SI Appendix, Fig. S5 and Materials and Methods). Growth curves and colony images are representative of at least three independent experiments.

The expression of *dncV* is positively influenced by the master virulence regulator, ToxT. cGAMP signaling regulates virulence, chemotaxis, and fatty acid metabolic genes, yet there is no known cGAMP-binding effector in *V. cholerae* attributed to the control of these processes. Additionally, ectopic expression of *dncV* has been shown to enhance the transcription and accumulation of three phosphodiesterases capable of degrading cGAMP, named V-cGAP1-3 (24), but the mechanism for this regulation is also unknown. More recently, cGAMP-specific riboswitches were found to regulate the expression of genes mediating a variety of cellular processes, including adhesion and exoelectrogenesis in δ -proteobacteria, such as *Geobacter* species (25, 26). In addition, a GGDEF-encoding enzyme, which generally makes c-di-GMP, also synthesizes cGAMP and c-di-AMP in *Geobacter* (27), suggesting that cGAMP signaling has a broad impact on microbial physiology. Metazoans produce an isomer, 2',3'-cGAMP, that is synthesized by the DncV structural and functional homolog cGAS (28) to stimulate the innate immune system via the receptor STING in response to cytoplasmic double-stranded DNA (29, 30). Its regulatory mechanism has been intensively studied (31). In contrast, almost nothing is known about the proteinaceous receptors of bacterial cGAMP and how this signal regulates the activities of its interacting partners.

The VSP-1 island is only present in the circulating El Tor *V. cholerae* strains responsible for the seventh pandemic of cholera (1961 to the present) and is absent in the classical isolates that predominated in the previous six pandemics (32). This exclusive acquisition of VSP-1 by the El Tor biotype suggests that the functions encoded in this pathogenicity island are important for the displacement of the classical biotype as the etiological agent driving modern day cholera. A recent study that analyzed whole genome sequences to track the evolution of the El Tor biotype from a nonpathogenic bacterium to a pandemic pathogen found that there were six discrete stages in this process spanning the late 1800s to 1961 (33). During the fifth stage (1925 to 1954) paracholera El Tor strains, which could cause disease but lacked pandemicity, acquired VSP-1, as well as a second genomic island known as VSP-2 and an El Tor-specific variant of cholera toxin, CTX^{ET}. During the sixth stage (1954 to 1960) recombination and horizontal gene transfer in the El Tor biotype essentially stopped, and the final 12 SNPs, which have no conspicuous connection to pathogenicity, completed the pandemic maturation of the El Tor biotype. Thus, acquisition of the VSP islands was predicted to be the major event driving the evolution of pandemic El Tor *V. cholerae* from paracholera and led to the displacement of the classical biotype as the causative agent of cholera. While the ge-

nomic modifications in the evolution of El Tor *V. cholerae* have been identified and elegantly described, the evolutionary advantages that these changes provided are not known.

Here, we show that unregulated ectopic expression of the cGAMP synthase, DncV, in the *V. cholerae* El Tor biotype inhibits planktonic growth and produces an atypical colony morphology on solid agar. However, these phenotypes associated with overproduction of cGAMP were not observed in classical *V. cholerae* or *Escherichia coli*. Mutation of an uncharacterized gene *vc0178* [renamed herein as cGAMP-activated phospholipase in *Vibrio* (*capV*)], located immediately upstream of *dncV* in VSP-1, suppresses these cGAMP-induced phenotypes. Moreover, coexpression of *dncV* and *capV* in classical *V. cholerae* and *E. coli* sensitizes these species to cGAMP overproduction, leading to growth inhibition. Finally, in vitro biochemical assays, microscopy, and whole cell lipid analyses demonstrate that cGAMP directly binds to and activates the serine hydrolase/phospholipase activity of CapV, leading to degradation of cell membrane phospholipids and release of free fatty acids. Together, our results suggest that acquisition of this second messenger and its receptor, both present in the VSP-1 pathogenicity island, leads to changes in cell physiology that could be important in the pandemic evolution of the El Tor biotype. As homologs of *capV* and *dncV* are often linked to one another in a variety of bacteria from across the bacterial phylogenetic tree (34), cGAMP signaling and its connection to membrane lipid metabolism may be broadly conserved.

Results

High cGAMP Production Leads to Planktonic Growth Inhibition and Atypical Colony Morphology in El Tor *V. cholerae*. To understand the physiological functions of cGAMP in *V. cholerae*, we perturbed the intracellular concentration of this signal molecule by either deleting *dncV*, the gene encoding the cGAMP synthase, or overexpressing *dncV* from a plasmid. Although a Δ *dncV* mutant is moderately attenuated in infant mouse colonization (23), we did not observe any in vitro growth phenotype in this mutant. In contrast, when *dncV* expression was induced ectopically in El Tor *V. cholerae* WT C6706, under control of the P_{tac} promoter, planktonic growth was significantly arrested (Fig. 1A). Overexpression of *dncV*^{mut}, encoding a DncV active site mutant (D131A/D133A), did not lead to substantial growth arrest, suggesting that cGAMP synthesis is responsible for this growth inhibition (Fig. 1A). When grown on a solid agar surface, we also observed the development of a small colony morphology in response to DncV overproduction that was not observed in El Tor *V. cholerae* producing DncV^{mut} (Fig. 1B). To determine if

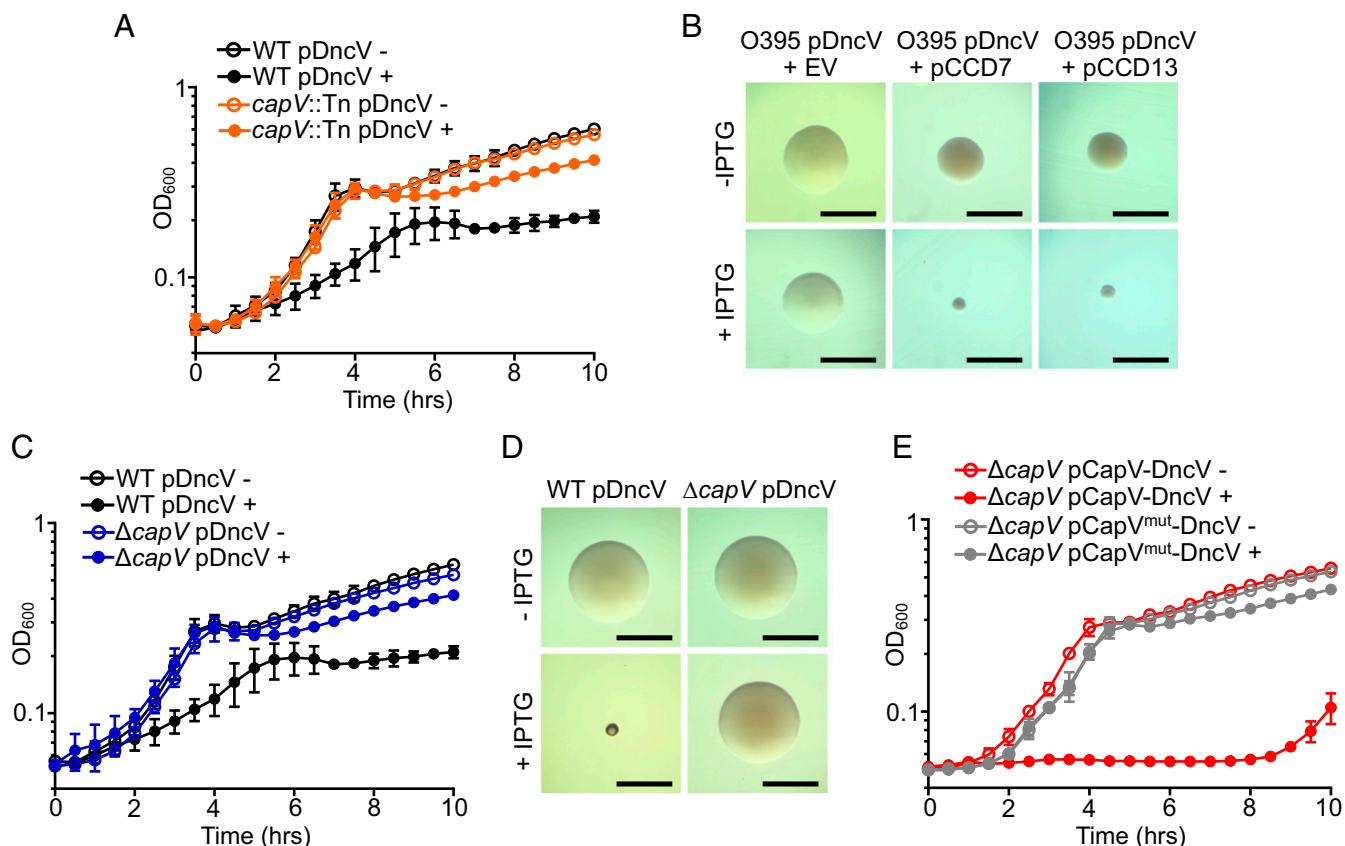


Fig. 2. The El Tor VSP-1 gene *capV* is necessary for cGAMP-induced growth arrest and small colony morphology. (A) Growth curves of El Tor *V. cholerae* WT and *capV::Tn*, each carrying pDncV, grown in the absence (–) or presence (+) of 100 μ M IPTG. (B) Colony morphologies of the classical *V. cholerae* strain O395 carrying both pDncV and either an empty vector (Left) or one of two unique El Tor genomic cosmids containing VSP-1 (pCCD7, Middle; pCCD13, Right). Strains were grown on solid agar plates in the absence (Top) or presence (Bottom) of 100 μ M IPTG. (Scale bars: 1 mm.) (C) Growth curves of El Tor *V. cholerae* WT and $\Delta capV$, each carrying pDncV, grown in the absence (–) or presence (+) of 100 μ M IPTG. (D) Colony morphologies of El Tor *V. cholerae* WT and $\Delta capV$, each carrying pDncV, grown on solid agar plates in the absence (Top) or presence (Bottom) of 100 μ M IPTG. (Scale bars: 1 mm.) (E) Growth curves of El Tor *V. cholerae* $\Delta capV$ mutants carrying a P_{tac} -inducible plasmid encoding both *dncV* and either *capV* (pCapV-DncV) or a catalytically inactive mutant (pCapV^{mut}-DncV), grown in the absence (–) or presence (+) of 100 μ M IPTG to induce expression of the polycistronic transcript. Each data point in the growth curves represents the mean \pm SD of six biological replicates. Growth curves and colony images are representative of at least three independent experiments.

abnormal accumulation of cGAMP is generally toxic, we overproduced DncV in a classical strain of *V. cholerae* (O395) and a laboratory strain of *E. coli* using the same P_{tac} -inducible plasmid. In both cases, DncV overproduction alone had no impact on planktonic growth or colony morphology, indicating that the observed cGAMP-induced phenotypes were specific to El Tor *V. cholerae* (Fig. 1B and SI Appendix, Fig. S1).

Both cGAMP-Induced Growth Arrest and Atypical Colony Morphology Require CapV. We then performed two distinct genetic screens to identify the gene/s necessary for cGAMP-induced growth arrest and atypical colony morphology. Because the *V. cholerae* El Tor strain overexpressing *dncV* forms tiny colonies on agar plates containing isopropyl β -D-1-thiogalactopyranoside (IPTG), we reasoned that, under the same inducing conditions, suppressor mutations would restore colony morphology and planktonic growth despite the presence of excessive cGAMP. Thus, an mTn10 transposon library was constructed from a *dncV* merodiploid strain and screened for clones that developed as regular-sized colonies on plates containing IPTG. We screened over 50,000 transposon mutants and obtained dozens of suppressors; half of these mutants carried mutations in the *dncV*-overexpression plasmid and were not further studied. The vast majority (>90%) of the remaining suppressors carried a transposon insertion (with different insertion

sites and orientations) in *vc0178* (*capV*), a gene immediately 5' of *dncV* (gene designation *vc0179*) in VSP-1. The growth rates and yields of these *capV* transposon mutants were nearly identical when they were grown with and without IPTG (a representative mutant is shown in Fig. 2A).

As a complementary approach to identify the cGAMP receptor/s that mediate growth arrest and small colony morphology, we screened for genes from the El Tor strain that sensitized classical *V. cholerae* to DncV overproduction. This approach would allow us to identify genes in this pathway that may not be amenable to transposon mutagenesis, such as essential genes. To perform this screen, a random cosmid library that encoded \sim 20 kb El Tor genomic fragments was introduced into the classical strain O395 expressing *dncV* from a plasmid. We identified two unique cosmids that sensitized classical *V. cholerae* to growth inhibition by cGAMP overproduction and determined that each contained the VSP-1 pathogenicity island harboring *capV*. Small colony morphologies were also observed in these classical *V. cholerae* strains maintaining either of the VSP-1-containing cosmids upon overexpression of *dncV* on agar plates (Fig. 2B). These results further suggest that *capV* is the gene necessary for cGAMP-induced growth arrest and small colony morphology in El Tor *V. cholerae*.

capV is the first ORF in a putative four-gene operon that also includes *dncV* and downstream genes *vc0180* and *vc0181*, both of

which encode putative proteins of unknown function. VC0180 contains E1 and E2 domains similar to eukaryotic ubiquitin-ligase enzymes while VC0181 contains a JAB domain similar to eukaryotic isopeptidases. To clarify the role of *capV* in cGAMP-dependent growth inhibition and test for polarity, we introduced single in-frame deletions of *capV*, *dncV*, *vc0180*, and *vc0181* on the chromosome of El Tor *V. cholerae*. Only deletion of *capV* suppressed cGAMP-dependent growth phenotypes resulting from ectopic expression of *dncV* (Fig. 2 C and D and *SI Appendix*, Fig. S2), indicating that the ability of mutations in *capV* to suppress cGAMP-induced growth arrest is not due to polar effects on downstream genes. We also observed that coexpression of *capV* and *dncV* from a common plasmid restored cGAMP-dependent growth arrest in the $\Delta capV$ mutant (Fig. 2E). Likewise, coexpression of *capV* and *dncV* was sufficient to induce planktonic growth arrest in classical *V. cholerae* and *E. coli*, which are otherwise unperturbed by the independent expression of either gene alone (*SI Appendix*, Fig. S1).

Sequence analysis suggested that CapV carries a canonical patatin domain, appearing to belong to a class of PLA2 phospholipases containing a conserved serine residue in the active site (35). We tested if the putative serine hydrolase activity of CapV is required for growth arrest caused by cGAMP overproduction by generating a catalytically inactive S62A variant of the protein (CapV^{mut}). Coexpression of *capV^{mut}* and *dncV* in a $\Delta capV$ El Tor strain did not elicit cGAMP-induced growth arrest, demonstrating that CapV catalytic activity is required for this cGAMP-dependent phenotype (Fig. 2E). Together, these results indicate that DncV and CapV are necessary and sufficient to cause growth inhibition. Our data are consistent with a model that the current El Tor *V. cholerae* pandemic strains employ cGAMP as a second messenger to regulate the serine hydrolase/phospholipase activity of CapV.

cGAMP Binds CapV and Stimulates Its Activity. Our genetic evidence suggested that the phospholipase activity of CapV might be directly activated by binding to cGAMP as we identified no additional proteins necessary for cGAMP-induced growth arrest by either genetic screen. We tested this hypothesis in vitro by determining if the CapV active-site serine can be covalently labeled by a reactive rhodamine-labeled fluorophosphonate probe (FP-Rh), a commonly used suicide substrate to monitor serine hydrolase activity, in the presence of cGAMP or other related nucleotides (36, 37). Purified His₆-tagged CapV was covalently labeled with this serine hydrolase probe in the presence of as low as 50 nM cGAMP, and the labeling increased in a dose-dependent manner (Fig. 3A). While DncV was previously shown to synthesize all three known cyclic dinucleotides in vitro (23), it preferentially catalyzed the production of cGAMP over c-di-GMP and c-di-AMP. Under our standard growth conditions (LB, 37 °C, OD ≈ 1), we determined that both cGAMP and c-di-AMP were below the level of detection before induction of *dncV* expression. After 30 min of induction in a $\Delta capV$ mutant, the average intracellular concentrations of cGAMP and c-di-AMP increased to 1.2 and 0.2 μM, respectively (*SI Appendix*, Fig. S3A). Induction of *dncV* expression, however, did not change the concentration of c-di-GMP in the cell, which decreased from ~0.3 to ~0.1 μM over the course of the experiment in both conditions (*SI Appendix*, Fig. S3A). We also assayed CapV labeling with FP-Rh in the presence of both c-di-GMP and c-di-AMP and found that they were about 1,000-fold less active than cGAMP (Fig. 3 B and C). Labeling of CapV by FP-Rh was only observed with at least 25 μM c-di-GMP or 50 μM c-di-AMP. This level of c-di-GMP has been observed when *V. cholerae* is grown in M9 medium to late-exponential phase, but not in rich medium such as LB (38), and c-di-AMP is not regularly detected in *V. cholerae*. Therefore, c-di-GMP and c-di-AMP are both unlikely to be a physiologically relevant signal for CapV activation under

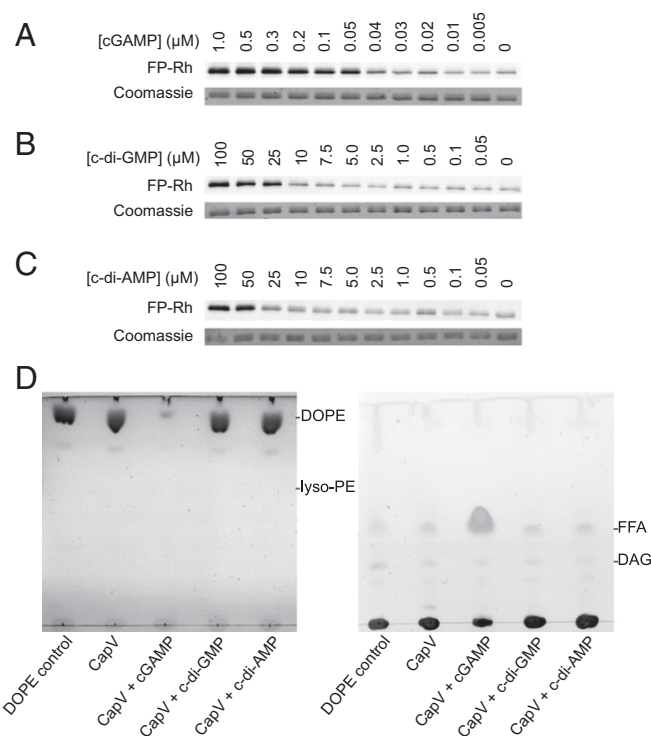


Fig. 3. cGAMP binding directly induces CapV phospholipase activity. (A–C) Covalent labeling (Top) of the CapV active-site serine by a reactive rhodamine-labeled fluorophosphonate probe (FP-Rh). 1.6 μM His₆-tagged CapV was mixed with both FP-Rh and (A) different concentrations of cGAMP or (B) c-di-GMP or (C) c-di-AMP. (Bottom) Coomassie blue staining of CapV. (D) Thin-layer chromatographic analysis of polar (Left) and neutral (Right) lipids released from the in vitro degradation of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) by 500 nM purified CapV in the presence of 1 μM cGAMP or other cyclic dinucleotides. DAG, diacylglyceride; FFA, free fatty acid; lyso-PE, lysophosphatidylethanolamine.

our tested growth conditions. Moreover, CapV labeling is specific for cGAMP as other related nucleotides (cAMP, GTP, ATP, dGTP, and dATP) did not increase CapV labeling when present at 1.75 μM or 1 mM (*SI Appendix*, Fig. S3B).

We then tested if purified CapV displayed bona fide phospholipase activity by providing a phospholipid substrate, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), in the presence and absence of different cyclic dinucleotides. Only the addition of 1 μM cGAMP, but not equimolar concentrations of c-di-GMP or c-di-AMP, was capable of stimulating CapV to catalyze the degradation of DOPE (Fig. 3D, Left) resulting in the release of free fatty acids (FFAs) (Fig. 3D, Right), but not of lyso-PE, suggesting that both acyl groups of the lipid are susceptible to the lipase. This result not only further demonstrates CapV's capacity to distinguish between cyclic dinucleotide ligands but also shows that the enzyme is a phospholipase capable of degrading a biologically relevant phospholipid in a cGAMP-dependent manner. Finally, we used microscale thermophoresis (MST) to confirm that purified CapV directly bound to cGAMP with a K_d of 8.6 ± 1.9 μM (*SI Appendix*, Fig. S3C). Together, our genetic and biochemical data support a model whereby cGAMP functions as a specific activator of CapV catalytic activity. Thus, DncV, cGAMP, and CapV constitute a functional cGAMP signaling pathway in *V. cholerae*.

High cGAMP Production Results in the Degradation of Phosphatidylethanolamine and Phosphatidylglycerol in El Tor *V. cholerae*. To test if cGAMP regulates the activity of CapV in vivo, total

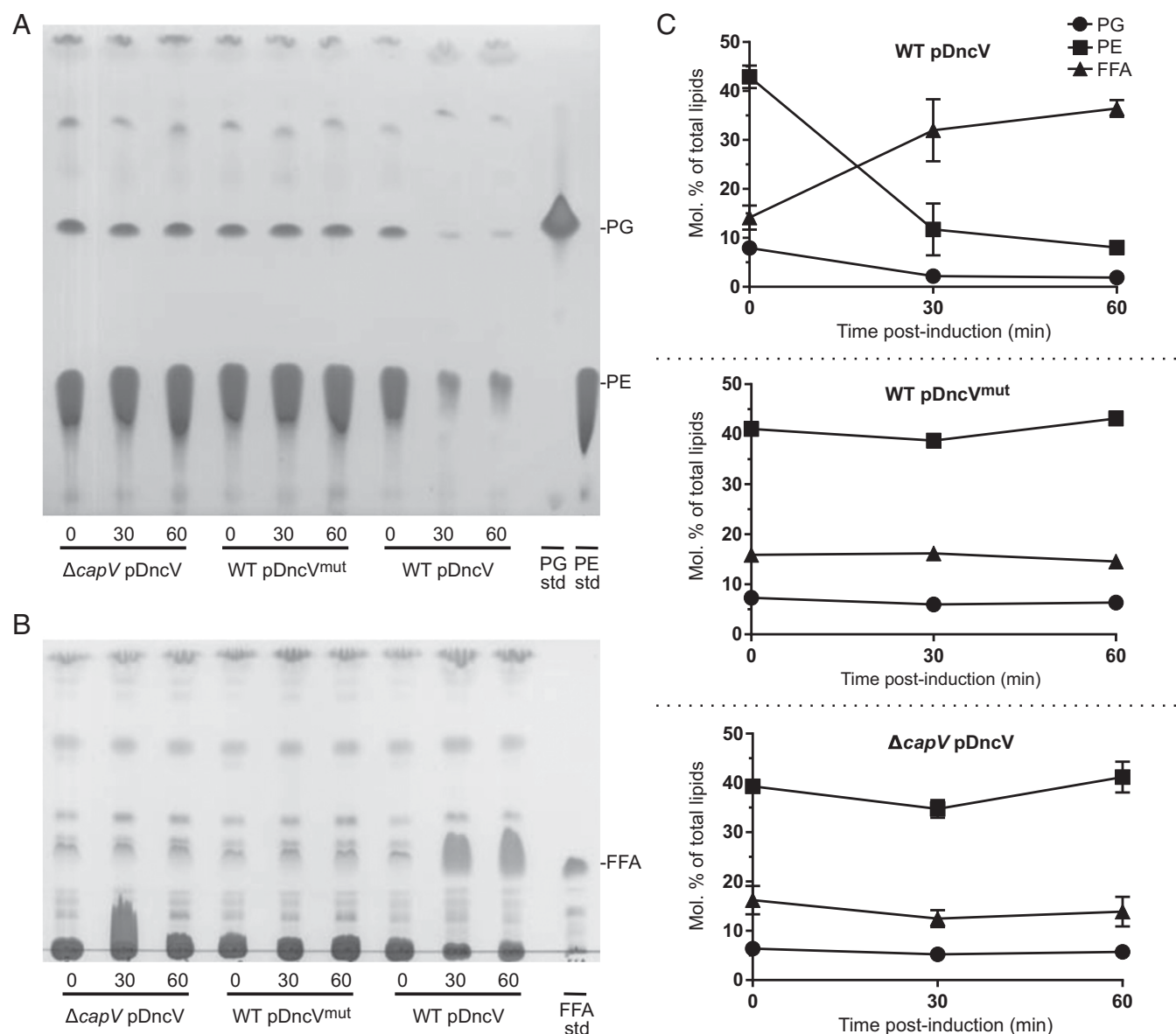


Fig. 4. Overproduction of the cGAMP synthase DncV results in degradation of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) and release of free fatty acids (FFAs) in a CapV-dependent fashion. TLC plates of (A) polar lipids and (B) neutral lipids extracted at 0, 30, and 60 min postinduction with 1 mM IPTG from El Tor *V. cholerae* WT carrying pDncV or pDncV^{mut} and from the El Tor *V. cholerae* ΔcapV mutant carrying pDncV. Plates are representative of three independent experiments. The PE, PG, and FFA standards are dioleoyl-PE, dioleoyl-PG, and linoleic acid, respectively. (C) Quantification (molar percentage of total lipids) by GC of PG, PE, and free fatty acids (FFAs) from A and B. Graphs show the mean of three independent experiments.

lipids were extracted from WT El Tor *V. cholerae* overexpressing *dncV* and *dncV*^{mut} and the ΔcapV mutant overexpressing *dncV* (SI Appendix, Fig. S4). Extracted lipids were then resolved by TLC and stained with iodine. We observed that phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), the major phospholipids in *V. cholerae* (39), were rapidly degraded when DncV production was induced in the presence of CapV (Fig. 4 A and C and SI Appendix, Fig. S4B). Overproduction of DncV^{mut} in WT or of DncV in ΔcapV had no effect on gross phospholipid pools, indicating that the observed phospholipid degradation in the WT El Tor *V. cholerae* strain was dependent on both the production of cGAMP and CapV activity.

PE and PG represent two broad classes of phospholipids characterized by the presence of a common head group and a phosphoglycerol moiety with two acyl groups of variable lengths and degrees of saturation giving rise to different molecular

species. The degradation of PE and PG by cGAMP-activated CapV significantly increased the FFAs in the afflicted cells (Fig. 4 B and C). To understand whether activated CapV preferentially degraded specific molecular species of PE and PG, we used gas chromatography to quantify the total lipid extracts and the acyl composition of TLC-resolved PE, PG, and FFAs from all three strains. Ectopic expression of *dncV* in the WT El Tor *V. cholerae* strain led to a profound change in the composition of the FFA pool. Before *dncV* induction, the FFA pool was mainly composed of species with 16:0 (carbons:double bonds) and 18:0 tails. However, after 60 min of *dncV* overexpression, 16:1 and 18:1 FFAs, which were originally undetectable, made up ~30% of the FFA pool (Fig. 5). Moreover, overproduction of DncV in the WT El Tor *V. cholerae* resulted in a decrease in the molar ratio of PE and PG phospholipids containing 16:1 unsaturated FFAs that was not seen in the ΔcapV or *dncV*^{mut}

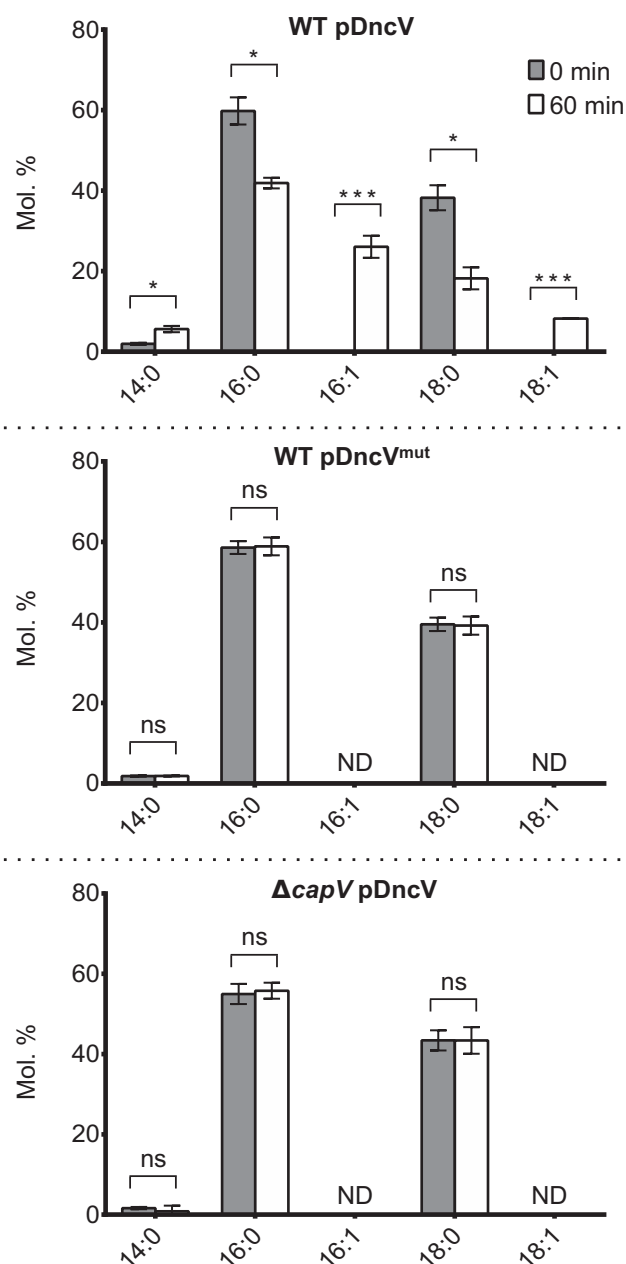


Fig. 5. Overproduction of the cGAMP synthase DncV results in accumulation of 16:1 and 18:1 free fatty acids (FFAs) in the cell. Shown is the proportion (molar percentage) of fatty acid species present in the FFA fractions collected from El Tor *V. cholerae* WT carrying pDncV (Top) or pDncV^{mut} (Middle) and from El Tor *V. cholerae* ΔcapV carrying pDncV (Bottom). Each data point represents the mean ± SD of three independent experiments (* $P < 0.01$, *** $P < 0.0001$). ND, not detected; ns, not significant.

overexpression strains (SI Appendix, Fig. S4B). These results suggest that CapV preferentially degrades PE and PG species containing unsaturated acyl groups in a cGAMP-dependent manner leading to the accumulation of cytosolic 16:1 and 18:1 FFAs.

Finally, we used fluorescence microscopy to visualize the effect of *dncV* overexpression and CapV activation on El Tor *V. cholerae* cell membranes. Rapid degradation of phospholipids from the membrane could compromise membrane integrity and increase permeability. Using the lipophilic dye FM 4-64, which selectively stains cell membranes, we assessed the impact on the

overall cell membrane structure of WT El Tor *V. cholerae* cells following *dncV* overexpression (40, 41). Uninduced cells appeared as normal comma-shaped *V. cholerae* under bright-field microscopy, and the FM 4-64 staining was localized to the cell periphery (Fig. 6, Left). Following ectopic expression of *dncV*, “ghost cells,” which are more translucent than normal cells with bright-field microscopy, appeared within 30 min of *dncV* overexpression and became a substantial proportion of the visualized population 2 h after induction (Fig. 6, Right). While the shape of most of these ghost cells remained normal, the interior of the cells was flooded with FM 4-64 dye, indicating that cell membrane integrity had been compromised following *dncV* overexpression. This microscopy study further implicates the cell membrane is the target of the DncV, cGAMP, CapV signaling pathway in El Tor *V. cholerae*.

Discussion

The hybrid dinucleotide second messenger, cGAMP, was first discovered in El Tor *V. cholerae* and is involved in the regulation of a number of processes, including virulence, chemotaxis, and fatty acid metabolism (23). There is very little understanding of the direct targets of bacterial cGAMP or how this signal regulates its interacting partners. Here, we identify CapV, a previously uncharacterized phospholipase, as a proteinaceous cGAMP receptor in *V. cholerae*, demonstrate that this small molecule interaction activates the enzyme, and show that this activity leads to the degradation of specific phospholipid components from the cell membrane.

Results from our biochemical and whole-cell assays (Figs. 3–6) show that cGAMP directly and specifically activates CapV phospholipase activity. We note that it is possible that the $8.6 \pm 1.9 \mu\text{M}$ K_d for cGAMP binding to CapV, as determined in vitro using MST, underestimates the in vivo binding affinity for numerous

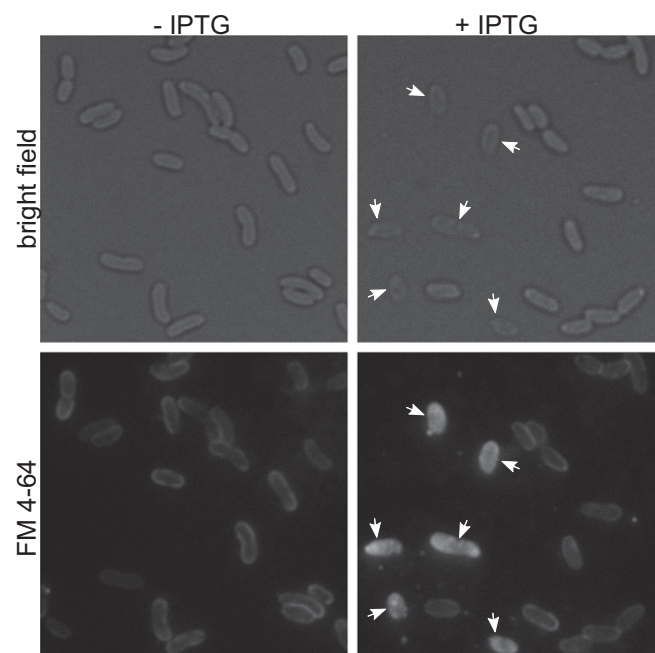


Fig. 6. Overproduction of the cGAMP synthase DncV is detrimental to membrane integrity. Images depict the cell morphology (Top, bright field) and membrane integrity (Bottom, FM 4-64) of El Tor *V. cholerae* WT carrying pDncV 2 h postinduction with (Right) or without (Left) 100 μM IPTG. Expression of *dncV* leads to a loss of cellular integrity (Top Right) and altered fluorescent labeling of the cell membrane (Bottom Right). Arrows indicate affected ghost cells. Images are representative of six independent experiments.

reasons, including, among other possibilities, the absence of phospholipid substrate. However, we found that cGAMP concentration reached 1.2 μM in *dncV*-expressing cells (*SI Appendix, Fig. S34*), and the K_d determined by MST is in line with previous in vitro measurements of cyclic dinucleotide (e.g., c-diGMP) binding to some target receptors (42–45). While additional studies are required to measure the effects of phospholipid substrate on CapV-cGAMP binding affinity, it is clear that CapV phospholipase activity is directly and specifically triggered by cGAMP.

Both DncV and CapV are encoded in the VSP-1 genomic island that was acquired by the current El Tor *V. cholerae* strains (32). Our results and others' (23) suggest that the acquisition of DncV and cGAMP signaling provides El Tor with additional biochemical capacities that are likely important in the evolution of pandemic El Tor *V. cholerae*. While we now understand that cGAMP interacts with CapV to form a functional cGAMP signaling pathway, it is still unclear how this contributes to the in vitro and in vivo fitness of the current *V. cholerae* pandemic strains. Our current study demonstrates that full activation of CapV by cGAMP results in growth inhibition due to phospholipid degradation; however, it is very likely that CapV is activated to various extents during the *V. cholerae* life cycle. We hypothesize that, in part, cGAMP regulation of CapV remodels the cell membrane of El Tor *V. cholerae* to adapt to different membrane stresses.

Moreover, unsaturated fatty acids (USFAs) are key regulators of membrane fluidity (46), and their abundance plays a role in homeoviscous adaptation (47). We speculate that the specific removal of USFAs bound to PE and PG would make the cell membrane more rigid and this cGAMP-CapV degradative process may provide El Tor *V. cholerae* with a fitness advantage when confronted with fluctuations in ambient temperature (48). USFAs have also been shown to negatively impact the transcriptional activity of the master virulence transcriptional activator, ToxT (49–51). The cGAMP-CapV signaling network is part of the ToxT regulon (23), and this signal transduction system may have contributed to the emergence and persistence of pandemic El Tor by releasing USFAs to alter the dynamics of virulence gene expression. Clinical manifestations of El Tor during human infections are reported to be less severe than those of classical strains, consistent with cGAMP-CapV negatively regulating virulence factor expression (52, 53).

Giles et al. (39) showed that polyunsaturated fatty acids from bile and other natural sources can be incorporated into the *V. cholerae* membrane, resulting in alterations of the membrane phospholipid profile in this bacterium (39). These findings suggest that *V. cholerae* undergoes membrane remodeling during infection of the small intestine. The expression of both *capV* and *dncV* are indirectly induced by ToxT in *V. cholerae* (23), suggesting that CapV and DncV might participate in membrane

remodeling in the presence of bile during infection. However, it should be noted that incorporation of exogenous FFAs occurs in both El Tor and classical strains; thus, cGAMP signaling must provide additional advantages to the El Tor biotype.

Burroughs et al. (34) recently performed a comprehensive bioinformatic analysis of the genes associated with members of the DncV nucleotide synthase family. They determined that these synthases are frequently associated with specific classes of effector proteins, including patatin-like phospholipases homologous to CapV. The phylogenetic distribution of these gene elements suggested that they moved by horizontal gene transfer, and their analysis found a DncV ortholog linked to a phospholipase in *Verrucomicrobia*, α -, β -, δ -, and γ -proteobacteria, *Paenibacillus*, cyanobacteria, bacteroidetes, and actinobacteria (34). These authors predicted that the synthases might directly control the activity of the associated phospholipases, and our results in El Tor *V. cholerae* support this prediction. Thus, cGAMP regulation of a phospholipase to modify bacterial membranes may be widespread.

Materials and Methods

Strains and Plasmids. All bacterial strains and plasmids used in this study are listed in *SI Appendix, Table S1*. PCR primers used are listed in *SI Appendix, Table S2*. *V. cholerae* mutants were constructed with allelic exchange using plasmid pKAS32 as described previously (54). Details of strain construction, growth analysis, and suppressor screens can be found in *SI Appendix*.

Serine Hydrolase Labeling Assay. The serine hydrolase activity of His₆-tagged CapV purified from *E. coli* was probed with a fluorescently labeled suicide substrate FP-Rh based on a procedure previously described (55). Details of assays can be found in *SI Appendix*.

Phospholipase Assay and Lipid Analysis. In vitro lipase assays using 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (dioleoyl-PE) (Avanti Polar Lipids) as the lipase substrate with His₆-tagged CapV were assembled similar to a procedure previously described (56). Extraction and analysis of *V. cholerae* lipids was based on a previously published protocol (57). Details of these assays can be found in *SI Appendix*.

Detailed information of all other experimental procedures is provided in *SI Appendix*.

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