1-1-2019

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Recommended Citation

Sperling, L., Mulero Alegría, M. D., Kaever, V., & Curtis, P. D. (2019). Analysis of Brevundimonas subvibrioides Developmental Signaling Systems Reveals Inconsistencies between Phenotypes and c-di-GMP Levels. Journal of Bacteriology, 201(20), e00447-19, /jb/201/20/JB.00447-19.atom. https://doi.org/10.1128/JB.00447-19

Analysis of *Brevundimonas subvibrioides* developmental signaling systems reveals inconsistencies between phenotypes and c-di-GMP levels

Running title: *Brevundimonas* developmental signaling and c-di-GMP

Keywords: Brevundimonas, Caulobacter, development, signaling, DivK, c-di-GMP

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Abstract

The DivJ-DivK-PleC signaling system of *Caulobacter crescentus* is a signaling network that regulates polar development and the cell cycle. This system is conserved in related bacteria, including the sister genus *Brevundimonas*. Previous studies had shown unexpected phenotypic differences between the *C. crescentus divK* mutant and the analogous mutant of *Brevundimonas subvibrioides*, but further characterization was not performed. Here, phenotypic assays analyzing motility, adhesion, and pilus production (the latter characterized by a newly discovered bacteriophage) revealed that *divJ* and *pleC* mutants have mostly similar phenotypes as their *C. crescentus* homologs, but *divK* mutants maintain largely opposite phenotypes than expected. Suppressor mutations of the *B. subvibrioides divK* motility defect were involved in cyclic-di-GMP (c-di-GMP) signaling, including the diguanylate cyclase *dgcB*, and *cleD* which is hypothesized to affect flagellar function in a c-di-GMP dependent fashion. However, the screen did not identify the diguanylate cyclase *pleD*. Disruption of *pleD* in *B. subvibrioides* caused no change in *divK* or *pleC* phenotypes, but did reduce adhesion and increase motility of the *divJ* strain. Analysis of c-di-GMP levels in these strains revealed incongruities between c-di-GMP levels and displayed phenotypes with a notable result that suppressor mutations altered phenotypes but had little impact on c-di-GMP levels in the *divK* background. Conversely, when c-di-GMP levels were artificially manipulated, alterations of c-di-GMP levels in the *divK* strain had minimal impact on phenotypes. These results suggest that DivK performs a critical function in the integration of c-di-GMP signaling into the *B. subvibrioides* cell cycle.

Importance

Cyclic-di-GMP and associated signaling proteins are widespread in bacteria, but its role in physiology is often complex and difficult to predict through genomic level analyses. In *C. crescentus*, c-di-GMP has been integrated into the developmental cell cycle, but there is increasing evidence that environmental factors can impact this system as well. The research presented here suggests that the integration of these signaling networks could be more complex than previously hypothesized, which could have a bearing on the larger field of c-di-GMP signaling. In addition, this work further reveals similarities and differences in a conserved regulatory network between organisms in the same taxonomic family, and the results show that gene conservation does not necessarily imply close functional conservation in genetic pathways.

Introduction

Though model organisms represent a small portion of the biodiversity found on Earth, the research that has resulted from their study shapes much of what we know about biology today. The more closely related species are to a model organism, the more that theoretically can be inferred about them using the information from the model organism. Modern genomic studies have given this research an enlightening new perspective. Researchers can now compare the conservation of particular systems genetically. Using model organisms can be a very efficient and useful means of research, but the question still remains of how much of the information gained from the study of a model can be extrapolated unto other organisms. Though genomic comparison shows high levels of conservation between genes of different organisms, this does not necessarily mean the function of those genes or systems has been conserved. This phenomenon seems to be evident in the *Caulobacter crescentus* system.

C. crescentus is a Gram-negative alphaproteobacterium that lives a dimorphic lifestyle. It has been used as a model organism for the study of cell cycle regulation, intracellular signaling, and polar localization of proteins and structures in bacteria. The *C. crescentus* life cycle begins with the presynthetic (G_1) phase in which the cell is a motile "swarmer cell" which contains a single flagellum and multiple pili at one of the cell's poles [for review, see (1)]. During this period of the life cycle, the cell cannot replicate its chromosome or perform cell division. Upon differentiation, the cell dismantles its pili and ejects its flagellum. It also begins to produce holdfast, an adhesive polysaccharide, at the same pole from which the flagellum was ejected. The cell then develops a stalk, projecting the holdfast away from the cell at the tip of the stalk. The differentiation of the swarmer cell to the "stalked cell" marks the beginning of the synthesis (S) phase of the cell life cycle as chromosome replication is initiated. As the stalked cell

replicates its chromosome and increases its biomass in preparation for cell division, it is referred to as a predivisional cell. Toward the late predivisional stage, it again becomes replication incompetent and enters the postsynthetic (G_2) phase of development. At the end of the G_2 phase, the cell completes division forming two different cell types. The stalked cell can immediately reenter the S phase, while the swarmer cell moves once again through the G_1 phase.

Brevundimonas subvibrioides is another Gram-negative alphaproteobacterium found in oligotrophic environments that lives a dimorphic lifestyle like that of *C. crescentus*. *Brevundimonas* is the next closest genus phylogenetically to *Caulobacter*. According to a Pairwise Average Nucleotide Identity (ANI) test, their genomes are approximately 74% identical. Bioinformatic analyses showed that all developmental signaling proteins found in the *C. crescentus* cell cycle are conserved *B. subvibrioides* (2, 3). All the known developmental regulators found in *C. crescentus* are also present in *B. subvibrioides*, and these regulators are orthologs as they are bi-directional best hits when searched against each genome, and amino acid identity is extremely high (3)(Supplementary Table S1). Conversely, no other proteins thought to interact with DivK in more distantly related Alphaproteobacteria, such as PdhS1 or CbrA, are apparent in the *B. subvibrioides* genome. However, little physiological characterization has been performed. Conservation of genes does not necessarily mean conservation of function or properties (3). Essential gene studies within the Alphaproteobacteria have shown that gene essentiality/non-essentiality in one organism does not always correspond with that in another organism (3-6). Analyses that have been performed on *C. crescentus* and *B. subvibrioides* have shown many similarities in gene essentiality between the two, but have shown several surprising differences as well (3).

In *C. crescentus*, the DivJ-DivK-PleC system controls the spatial activation of one of the master regulators in *C. crescentus*, CtrA (1, 7). This system is a prime example of how *C. crescentus* has evolved traditional two-component proteins into a more complex signaling pathway and, as a result, has developed a more complex life cycle. The DivJ-DivK-PleC pathway consists of two histidine kinases (PleC and DivJ) and a single response regulator (DivK) (8, 9). DivJ is absent in swarmer cells but is produced during swarmer cell differentiation. It then localizes to the stalked pole (8). DivJ is required for, among other things, proper stalk placement and regulation of stalk length. *C. crescentus divJ* mutants display filamentous shape, a lack of motility, and holdfast overproduction (8, 9).

PleC localizes to the flagellar pole during the predivisional cell stage (10). Though structurally a histidine kinase PleC acts as a phosphatase, constitutively de-phosphorylating DivK (8, 9). *C. crescentus pleC* mutants display a lack of pili, holdfast, and stalks, and have paralyzed flagella leading to a loss of motility (11-13). DivK is a single-domain response regulator (it lacks an output domain) whose location is dynamic throughout the cell cycle (9, 14). DivK remains predominantly unphosphorylated in the swarmer cell, while it is found mostly in its phosphorylated form in stalked cells. Photobleaching and FRET analysis show that DivK shuttles rapidly back and forth from pole to pole in the pre-divisional cell depending on its phosphorylation state (9). Previous studies have shown that phosphorylated DivK localizes bipolarly while primarily unphosphorylated DivK is delocalized throughout the cell (9). A *divK* cold-sensitive mutant suppresses the non-motile phenotype of *pleC* at 37°C. However, at 25°C, it displays extensive filamentation much like the *divJ* mutant (15). Additionally, filamentous *divK* mutants sometimes had multiple stalks, though the second stalk was not necessarily polar.

121 Furthermore, electron microscopy of *divK* disruption mutants led to the discovery that they lack
122 flagella.

123 Upon completion of cytokinesis, PleC and DivJ are segregated into different
124 compartments, thus DivK phosphorylation levels in each compartment are dramatically different.
125 This leads to differential activation of CtrA in the different compartments (9, 16). In the
126 swarmer cell, the de-phosphorylated DivK leads to the downstream activation of CtrA. CtrA in
127 its active form binds the chromosome at the origin of replication and prevents DNA replication
128 (17, 18). The opposite effect is seen in stalked cells where highly phosphorylated DivK results
129 in the inactivation of CtrA and, therefore, permits DNA replication (19).

130 Gene essentiality studies in *B. subvibrioides* led to the discovery of a discrepancy in the
131 essentiality of DivK. In *C. crescentus* DivK is essential for growth, while in *B. subvibrioides*
132 DivK is dispensable for growth (3, 15). Further characterization found dramatic differences in
133 the phenotypic consequences of disruption. Through the use of a cold-sensitive DivK allele or
134 by ectopic depletion, *C. crescentus divK* disruption largely phenocopies *divJ* disruption in cell
135 size and motility effects (8, 9, 15). This is to be expected as DivK~P is the active form and both
136 *divJ* or *divK* disruption reduce DivK~P levels. In *B. subvibrioides*, disruption of *divJ* leads to the
137 same effects in cell size, motility, and adhesion (3). However, *divK* disruption leads to opposite
138 phenotypes of cell size and adhesion, and while motility is impacted it is likely by a different
139 mechanism.

140 While the previous study revealed important differences between the organisms, it did not
141 analyze the impact of PleC disruption, nor did it examine pilus production or subcellular protein
142 localization. The work presented here further characterizes the DivJ-DivK-PleC signaling

143 system in *B. subvibrioides* and begins to address the mechanistic reasons for the unusual
144 phenotypes displayed by the *B. subvibrioides divK* mutant.
145
146 ## Materials and Methods
147 *Strains and growth conditions*
148 A complete list of strains used in this study is presented in the appendix (see
149 Supplementary Table S2). *Brevundimonas* strains were cultured at 30°C on PYE medium (2 g
150 peptone, 1 g yeast extract, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.735 $CaCl_2$) (20). Kanamycin was used at 20
151 µg/ml, gentamycin at 5 µg/ml, and tetracycline at 2 µg/ml when necessary. PYE plates
152 containing 3% sucrose were used for counter-selection. *Escherichia coli* was cultured on Luria-
153 Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) at 37°C. Kanamycin
154 was used at 50 µg/ml, gentamycin at 20 µg/ml, and tetracycline at 12 µg/ml when necessary.
155
156 *Mutant generation*
157 The *B. subvibrioides ΔdivJ, ΔdivK,* and *ΔdivJΔdivK* mutants were used from a previous
158 study (3). The *B. subvibrioides ΔpleC* construct was made by PCR amplifying an upstream
159 fragment of ~650 bps using primers PleC138Fwd
160 (attgaagccggctggcgccaCCAGATCGAAAAGGTGCAGCCC) and PleCdwRev
161 (tctaggccgcGCCCCGCAAGGCGCTCTC) and a downstream fragment of ~550 bps using
162 primers PleCupFwd (cttgcggggcGCGGCCTAGAGCCGGTCA) and PleC138Rev
163 (cgtcacggccgaagctagcgGGTGCTGGGATGAAGACACG). The primers were designed using the
164 NEBuilder for Gibson Assembly tool online (New England Biolabs) and were constructed to be
165 used with the pNPTS138 vector (MRK Alley, unpublished). Following a digestion of the vector

using HindIII and EcoRI the vector along with both fragments were added to Gibson Assembly Master Mix (New England Biolabs) and allowed to incubate for an hour at 50°C. Reactions were then transformed into *E. coli* and correct plasmid construction verified by sequencing to create plasmid pLAS1. This plasmid was used to delete *pleC* in *B. subvibrioides* as previously described (3).

To create insertional mutations in genes, internal fragments from each gene were PCR amplified. A fragment from gene *cpaF* was amplified using primers cpaFF (GCGAACAGAGCGACTACTACCACG) and cpaFR (CCACCAGGTTCTTCATCGTCAGC). A fragment from gene *pleD* was amplified using primers PleDF (CCGGCATGGACGGGTTC) and PleDR (CGTTGACGCCCAGTTCCAG). A fragment from gene *dgcB* was amplified using primers DgcBF (GAGATGCTGGCGGCTGAATA) and DgcBR (CGAACTCTTCGCCACCGTAG). A fragment from gene *cleD* was amplified using primers Bresu1276F (ATCGCCGATCCGAACATGG) and Bresu1276R (TTCTCGACCCGCTTGAACAG). The fragments were then cloned into the pCR vector using the Zero Blunt cloning kit (Thermo Fisher), creating plasmids pPDC17 (*cpaF*), pLAS1 (*pleD*), pLAS2 (*dgcB*), and pLAS3 (*cleD*). These plasmids were then transformed into *B. subvibrioides* strains as previously published (3). The pCR plasmid is a non-replicating plasmid in *B. subvibrioides* that facilitates insertion of the vector into the gene of interest via recombination, thereby disrupting the gene.

To create a C-terminal *B. subvibrioides* DivJ fusion, ~50% of the *divJ* gene covering the 3' end was amplified by PCR using primers BSdivJgfpF (CCTCATATGGGTTTACGGGGCCTACGGG) and BSdivJgfpR (CGAGAATTCGAGACGGTCGGCGACGGTCCTG), and cloned into the pGFPC-2 plasmid

(21), creating plasmid pPDC11. To create a C-terminal *B. subvibrioides* PleC fusion, ~50% of the *pleC* gene covering the 3' end was amplified by PCR using primers BSpleCgfpF (CAACATATGCCAGAAGGACGAGCTGAACCGC) and BSpleCgfpR (TTTGAATTCGAGGCCGCCCGCGCCTGTTGTTG), and cloned into the pGFPC-2 plasmid, creating plasmid pPDC8. These plasmids are non-replicative in *B. subvibrioides* and therefore integrate into the chromosome by homologous recombination at the site of each targeted gene. The resulting integration creates a full copy of gene under the native promoter that produces a protein with C-terminal GFP tag, and a ~50% 5' truncated copy with no promoter. This effectively creates a strain where the tagged gene is the only functional copy.

Due to the small size of the *divK* gene, a region including the *divK* gene and ~500 bp of sequence upstream of *divK* was amplified using primers BSdivKgfpF (AGGCATATGCCAGCGACAGGGTCTGCACC) and BSdivKgfpR (CGGGAATTCGATCCCGCCAGTACCGGAACGC) and cloned into pGFPC-2, creating plasmid pPDC27. After homologous recombination into the *B. subvibrioides* genome, two copies of the *divK* gene are produced, both under the native promoter, one of which encodes a protein C-terminally fused to GFP.

Constructs expressing *E. coli ydeH* under IPTG induction on a medium copy (pTB4) and low copy (pSA280) plasmids were originally published in (22). Constructs expressing *Pseudomonas aeruginosa pchP* under vanillate induction (pBV-5295) as well as an active site mutant (pBV-5295$_{E328A}$) were originally published in (23).

Transposon mutagenesis

211 Transposon mutagenesis was performed on the *B. subvibrioides ΔdivK* mutant using the
212 EZ-Tn5 <KAN-2> TNP transposome (Epicentre). *B. subvibrioides ΔdivK* was grown overnight
213 in PYE to an OD_{600} of about 0.07 [quantified with a Themo Nanodrop 2000 (Themo Scientific)].
214 Cells (1.5 ml) were centrifuged 15,000 x g for 3 min at room temperature. The cell pellet was
215 then resuspended in 1 ml of water before being centrifuged again. This process was repeated.
216 Cells were resuspended in 50 µl of nuclease free water, to which 0.2 µl of transposome was
217 added. The mixture was incubated at room temperature for 10 minutes. The mixture was added
218 to a Gene Pulser Cuvette with a 0.1 cm electrode gap (Bio-Rad). The cells were then
219 electroporated as performed previously (3). Electroporation was performed using a GenePulser
220 Xcell (Bio-Rad) at a voltage of 1,500 V, a capacitance of 25 µF, and a resistance of 400 Ω. After
221 electroporation, cells were resuspended with 1 ml of PYE then incubated shaking at 30°C for 3
222 hours. Cells were diluted 3-fold then spread on PYE + Kan plates (100 µl/plate). Plates were
223 incubated at 30°C for 5-6 days.
224
225 *Swarm assay*
226 Strains were grown overnight in PYE, diluted to an OD_{600} of 0.02, and allowed to grow
227 for two doublings (to OD_{600} of ~0.06 - 0.07). All strains were diluted to OD_{600} = 0.03 and 1 µl of
228 culture was injected into a 0.3% agar PYE plate. Isopropyl 1-thio-b-D-galactopyranoside (IPTG)
229 (final concentration 1.5 mM) and vanillate (final concentration 1 mM) was added to plate
230 mixture before pouring plates where applicable. Molten 0.3% agar in PYE (25 ml) was poured
231 in each plate. Plates were incubated at 30°C for 5 days. Plates were imaged using a BioRad
232 ChemiDoc MP Imaging System with Image Lab software. Swarm size was then quantified in
233 pixels using ImageJ software. All swarm sizes on a plate were normalized to the wild-type

swarm on that plate. Assays were performed in triplicate and average and standard deviation were calculated.

Short-term adhesion assay

Strains were grown overnight in PYE, diluted to an OD_{600} of 0.02, and allowed to grow for two doublings (to OD_{600} of ~0.06 - 0.07). All strains were diluted to OD_{600} = 0.05, at which time 0.5 ml of each strain was inoculated into a well of a 24-well dish and incubated at 30°C for 2 hours in triplicate. Cell culture was removed and wells were washed 3 times with 0.5 ml of fresh PYE. To each well was added 0.5 ml of 0.1% crystal violet and incubated at room temperature for 20 minutes. Crystal violet was removed from each well before the plate was washed by dunking in a tub of deionized water. Crystal violet bound to biomass was eluted with 0.5 ml acetic acid and the A_{589} was quantified using a Themo Nanodrop 2000 (Themo Scientific). Averages for each strain were calculated and then normalized to wild-type values inoculated into the same plate. These assays were performed three times for each strain and used to calculate average and standard deviation.

Lectin-binding assay and microscopy conditions

Holdfast staining was based on the protocol of (24). Strains of interest were grown overnight in PYE to an OD_{600} of 0.05 – 0.07. For each strain, 200 µl of culture were incubated in a centrifuge tube with 2 µl of Alexafluor 488 (Molecular Probes) for 20 minutes at room temperature. Cells were washed with 1 ml of sterile water then centrifuged 15,000 x g for 1 min at room temperature. The cell pellet was resuspended in 30 µl of sterile water. A 1% agarose pad (agarose in H_2O) was prepared for each strain on a glass slide to which 1 µl of culture was

added. Slides were then examined and photographed using an Olympus IX81 microscope by phase contrast and epifluorescence microscopy using a 100X Plan APO oil immersion objective The holdfast of GFP-labeled strains were stained with Alexafluor 594 conjugated to Wheat Germ Agglutinin and prepared for imaging as described above. Alexafluor 488 and GFP labeled strains were imaged with 470/20 nm excitation and 525/50 nm emission wavelengths. Alexafluor 594 labeled strains were imaged with 572/35 nm excitation and 635/60 nm emission.

Isolation of phage.

Surface water samples from freshwater bodies were collected from several sources in Lafayette County, Mississippi in 50 ml sterile centrifuge tubes and kept refrigerated. Samples were passed through 0.45 µm filters to remove debris and bacterial constituents. To isolate phage, 100 µl of filtered water was mixed with 200 µl mid-exponential *B. subvibrioides* cells and added to 2.5 ml PYE with molten 0.5% agar. The solution was poured onto PYE agar plates, allowed to harden, and then incubated at room temperature (~22^{o}C) for 2 days. Plaques were excised with a sterile laboratory spatula and placed into sterile 1.5 ml centrifuge tubes. 500 µl PYE was added and the sample was refrigerated overnight to extract phage particles from the agar. To build a more concentrated phage stock, the soft-agar plating was repeated with extracted particles. Instead of excising plaques, 5 ml of PYE was added to the top of the plate and refrigerated overnight. The PYE/phage solution was collected and stored in a foil-wrapped sterile glass vial, and 50 µl chloroform was added to kill residual bacterial cells. Phage solutions were stored at 4^{o}C.

Isolation of phage resistant mutants.

B. subvibrioides was mutagenized with EZ-Tn5 transposome as described above. After electroporation, cells were grown for 3 hr without selection, followed by 3 hr with kanamycin selection. Transformed cells (100 µl) were mixed with 100 µl phage stock (~1 x 10^{10} pfu/ml) and plated on PYE agar medium with kanamycin. Colonies arose after ~5 days and were transferred to fresh plates. Transformants had their genomic DNA extracted using the Bactozol kit (Molecular Research Center). Identification of the transposon insertion sites was performed using Touchdown PCR (25), with transposon specific primers provided in the EZ-Tn5 kit.

Phage sensitivity assays.

Two different phage sensitivity assays were used. First (hereafter referred to as the spotting assay) involved the mixing of cells and phage in liquid suspension and then spotting droplets on an agar surface. Each cell culture was normalized to OD_{600} = 0.03. The culture was then diluted 10^{-2}, 10^{-4} and 10^{-5} in PYE medium. For control assays, 5 µl of each cell suspension (including undiluted) was mixed with 5 µl PYE, then 5 µl of this mixture was spotted onto PYE plates, allowed to dry, then incubated at room temperature for 2 days. For the phage sensitivity assays, 5 µl of each cell suspension was mixed with 5 µl of phage stock (~1 x 10^{10} pfu/ml), 5 µl spotted onto PYE plates, allowed to dry, then incubated at room temperature for 2 days.

The second assay (hereafter referred to as the soft agar assay) involved creating a lawn of cells and spotting dilutions of phage on the lawn. Cell cultures were normalized to OD_{600} = 0.03 and 200 µl of cells were mixed with 4.5 ml PYE with molten 0.5% agar, mixed, poured onto a PYE agar plate, and allowed to harden. Phage stock (~1 x 10^{10} pfu/ml) was diluted in PYE media as individual 10X dilutions to a total of 10^{-7} dilution. 5 µl of each phage concentration

(10^{-1} to 10^{-7}, 7 concentrations total) were spotted on top of the soft agar surface and allowed to dry. Plates were incubated 2 days at room temperature.

Swarm suppressor screen

Individual colonies from a transposon mutagenesis were collected on the tip of a thin sterile stick and inoculated into a 0.3% agar PYE plate. Wild-type *B. subvibrioides* strains as well as *B. subvibrioides ΔdivK* were inoculated into each plate as controls. 32 colonies were inoculated into each plate including the 2 controls. Plates were incubated at 30°C for 5 days. Plates were then examined for strains that had expanded noticeably further than the parent *divK* strain from the inoculation point. Those strains of interest were then isolated for further testing.

Identification of swarm suppressor insertion sites.

Swarm suppressor insertion sites were identified by Inverse PCR (iPCR, (26)). Genomic DNA (gDNA) was purified using the DNeasy Blood & Tissue Kit (Qiagen). Digests were then prepared using 1 µg of gDNA and either AluI or HhaI incubated overnight at 37°C. Digests were heat inactivated for 20 minutes at 80°C then column cleaned using the DNA Clean and Concentrator kit (Zymo Research). Dilute ligations (100-500 ng DNA) were then prepared so that digested fragments would likely circularize. Ligations were incubated at 17°C overnight. Reactions were heat inactivated at 65°C for 20 minutes then column cleaned using the DNA Clean and Concentrator kit. The ligated DNA was used as the template in a PCR reaction with primers that anneal inside the transposon sequence. Primers used included AluIF (GCGTTGCCAATGATGTTACAGATGAG) and AluIR (GCCCGACATTATCGCGAGCCC) as well as HhaIF2 (TTACGCTGACTTGACGGGAC) and HhaIR2 (GGAGAAAACTCACCGAGGCA).

Given the large size of the resulting AluI fragment from the transposon sequence alone, another primer AluIFSeq (CGGTGAGTTTTCTCCTTCATTACAG) was designed specifically for sequencing after iPCR was complete. Primers were designed facing outward toward either end of the transposon such that the resulting PCR amplicon would be fragments that begin and end with transposon sequence with gDNA in between. PCR reactions were prepared using 10.75 µl H_20, 5 µl HF buffer (BioRad), 5 µl combinational enhancer solution (2.7 M betaine, 6.7 mM DTT, 6.7% DMSO, 55 µg/mL BSA), 1 µl of template DNA from each ligation, 1 µl each of their respective forward and reverse primers (primers based on what enzyme was used during digestion), 1 µl of 10 mM dNTP's (BioLine), and 0.25 µl iProof (BioRad). PCR conditions were as follows. Initial melt was set to 98°C for 30 seconds. Melting temperature was set to 98°C for 45 seconds, annealing temperature was set to 52°C for 20 seconds, extension temperature was set to 72°C for 2:30 seconds. and these three steps were cycled through 30 times. Final extension temperature was set to 72°C for 10 minutes. 5 µl from each reaction were run on a 1% agarose gel to check for fragments. Those reactions that tested positive for bands were drop dialyzed using 0.025 µm membrane filters (Milllipore) then prepared for sequencing with their respective primers. Samples were sent to Eurofins for sequencing.

Quantification of c-di-GMP.

Strains of interest were grown overnight in PYE to an OD_{600} of 0.05 – 0.07. Metabolites were then extracted from each sample and c-di-GMP was quantified using the protocol previously described in (27). Metabolites from each strain were extracted in triplicate. Remaining cellular material was dried at room temperature and resuspended in 800 µL 0.1M NaOH. Samples were incubated at 95°C for 15 minutes. Samples were then centrifuged for 10 min at 4°C,

20,800 x g. Protein levels were measured in triplicate for each sample using 10 µl from the pellet treatment and the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Intracellular concentrations measured by mass spectrometry were then normalized to protein levels.

Results

Deletion mutants in the *B. subvibrioides* DivJ-DivK-PleC system result in varied phenotypes compared to that of analogous *C. crescentus* mutations. In the previous study done in *Brevundimonas subvibrioides*, deletion mutants of the genes *divJ*, *divK*, and a *divJdivK* double mutant were made and partially characterized, uncovering some starkly different phenotypes compared to the homologous mutants in *C. crescentus*. However, characterization of this system was not complete as it did not extend to a key player in this system: PleC. As previously mentioned, *C. crescentus pleC* mutants display a lack of motility, pili, holdfast, and stalks (28). To begin examining the role of PleC in *B. subvibrioides*, an in-frame deletion of the *pleC* gene (Bresu_0892) was created. This strain, along with the previously published *divJ*, *divK*, and *divJdivK* strains, were used in a swarm assay to analyze motility. All mutant strains displayed reduced motility in swarm agar compared to the wild-type (Figure 1A, Supplemental Figure S1). This had been reported for the published strains (3). The mechanistic reasons for this are unclear. All were observed to produce flagella and were seen to swim when observed microscopically. The *divJ* strain has significantly filamentous cell shape which is known to inhibit motility through soft agar, but the *divK* and *divJdivK* strains actually have shorter than wild-type cells. The nature of the *pleC* motility defect is also unknown. The cell size of the *pleC* mutants was not noticeably different from that of wild-type cells (Figure 1B). The *C. crescentus pleC* mutant is known to have a paralyzed flagellum which leads to a null motility phenotype,

371 but *B. subvibrioides pleC* mutants were observed swimming under the microscope suggesting
372 that unlike *C. crescentus* their flagellum remains functional. While the mechanistic reason for
373 this discrepancy is unknown, it does provide another important difference in developmental
374 signaling mutants between the two organisms.

375 To further the phenotypic characterization, these strains were analyzed for the surface
376 adhesion properties using both a short-term adhesion assay as well as staining holdfast material
377 with a fluorescently-conjugated lectin. As previously reported, the *divK* and *divJdivK* strains had
378 minimal adhesion and no detectable holdfast material (Figure 1AB). It was previously reported
379 that the *divJ* strain had increased adhesion over wild-type, but in this study, it was found to have
380 slightly reduced adhesion compared to wild-type. It is not clear if this difference in results
381 between the two studies is significant. The *pleC* strain had reduced adhesion compared to wild-
382 type, but more adhesion compared to the *divK* or *divJdivK* strains. When analyzed by
383 microscopy, the *pleC* strain was found to still produce detectable holdfast (Figure 1B,
384 Supplemental Figure S2), which is a difference from the *C. crescentus pleC* strain where holdfast
385 was undetectable (28, 29).

386 An important component to the function of this signaling system is the subcellular
387 localization of DivJ and PleC to the stalked and flagellar poles respectively. As the localization
388 of these proteins had yet to be characterized in *B. subvibrioides*, GFP-tagged constructs were
389 generated such that the tagged versions were under native expression. Because *B. subvibrioides*
390 cells very rarely produce stalks under nutrient-replete conditions (30), holdfast material was
391 stained using a WGA lectin conjugated with a fluorophore that uses RFP imaging conditions.
392 When the *divJ-gfp* strain was analyzed (N = 403 cells), the majority of cells (82.9%) had no
393 detectable signal (Figure 1C). It is not clear if this means the cells are not producing DivJ or that

the level is below the detection limit. When cells had detectable fluorescence it was seen only as a unipolar focus (17.1%). When cells had detectable DivJ-GFP foci and labeled holdfast, the foci were found exclusively at the holdfast pole (N = 55 cells). When the *pleC-GFP* strain was imaged (N = 433 cells), most cells displayed a unipolar focus (68.8%). Only 28.9% of cells displayed no fluorescence. A small number of cells (2.3%) had seemingly bipolar PleC localization; these cells may be transitioning from the swarmer to stalked state. Of the cells that had detectable PleC-GFP foci and labeled holdfast (N = 38), all PleC-GFP foci were found at the pole opposite the holdfast (Figure 1C, Supplemental Figure S3). As it has been demonstrated that holdfast material is produced at the same pole as the stalk in *B. subvibrioides* (30), this result suggests that these proteins demonstrate the same localization patterns as their *C. crescentus* counterparts. DivK-GFP was seen to form different localization patterns in different cells (Figure 1C, Supplemental Figure S4). Of the 431 cells counted, the vast majority (85.8%) showed total cell fluorescence with no distinct localization. Bipolar localization was seen in 6.3% of cells, and unipolar localization was seen in 7.9% of cells. Of the cells that displayed unipolar localization and a detectable holdfast, DivK-GFP was localized almost exclusively to the stalked pole (96.3%, N = 27). These results contrast *C. crescentus* results in some ways. In *C. crescentus* DivK-GFP was found predominantly bipolarly localized (56%), with 12% displayed stalked pole localization and 32% displaying no detectable fluorescence (31), though this quantification was performed using stalked cells specifically. Diffuse total body fluorescence was not reported in that study, but it is known that DivK-GFP is diffuse in swarmer cells. It should be noted that the work in *C. crescentus* expressed *divK-gfp* from a low copy plasmid using the native promoter, while here a plasmid integration scheme was used to create a merodiploid; however, both strains had both tagged and untagged versions of *divK* each

417 expressed by native promoters, so it is not clear if strain construction is the cause of the
418 differences in localization pattern. Therefore, DivJ and PleC localizations match the *C.*
419 *crescentus* model, while DivK appears to spend most of the time delocalized (with some bipolar
420 and stalked pole localization), as opposed to *C. crescentus* where the protein appears to spend
421 most of the time bipolarly localized. This is another case where the histidine kinase results
422 somewhat match between organisms, while the response regulator results are quite different.

423 **Isolation of a bacteriophage capable of infecting *B. subvibrioides*.** Another important
424 developmental event in *C. crescentus* is the production of pili at the flagellar pole coincident
425 with cell division. Pili are very difficult to visualize, and in *C. crescentus* the production of pili
426 in strains of interest can be assessed with the use of the bacteriophage ΦCbK, which infects the
427 cell using the pilus. Resistance to the phage indicates the absence of pili. However,
428 bacteriophage that infect *C. crescentus* do not infect *B. subvibrioides* (data not shown) despite
429 their close relation. In an attempt to develop a similar tool for *B. subvibrioides*, a phage capable
430 of infecting this organism was isolated.

431 Despite the fact that *B. subvibrioides* was isolated from a freshwater pond in California
432 over 50 years ago, a phage capable of infecting the bacterium was isolated from a freshwater
433 pond in Lafayette County, Mississippi. This result is a testament to the ubiquitous nature of
434 *Caulobacter* and *Brevundimonas* species in freshwater environments all over the globe. This
435 phage has been named Delta, after the state's famous Mississippi Delta region. To determine the
436 host range for this phage, it was tested against multiple *Brevundimonas* species (Figure 2A).
437 Delta has a relatively narrow host range, causing the largest reduction of cell viability in *B.*
438 *subvibrioides* and *B. aveniformis*, with some reduction in *B. basaltis* and *B. halotolerans* as well.
439 None of the other 14 *Brevundimonas* species showed any significant reduction in cell viability.

Neither did Delta show any infectivity toward *C. crescentus* (data not shown). While *B. subvibrioides*, *B. aveniformis*, and *B. basaltis* all belong to the same sub-clade within the *Brevundimonas* genus (P. Caccamo, Y.V. Brun, personal communication), so do *B. kwangchunensis*, *B. alba* and *B. lenta*, all of which are more closely related to *B. subvibrioides* than *B. aveniformis* and all of which were resistant to the phage. Therefore, infectivity does not appear to fall along clear phylogenetic lines and may be determined by some other factor.

To begin identifying the infection mechanism of Delta, *B. subvibrioides* was randomly mutagenized with a Tn5 transposon and resulting transformants were mixed with Delta to select for transposon insertions conferring phage resistance as a way to identify the phage infection mechanism. Phage resistant mutants were readily obtained and maintained phage resistance when rescreened. A number of transposon insertion sites were sequenced and several were found in the pilus biogenesis cluster homologous to the *C. crescentus flp*-type pilus cluster. Insertions were found in the homologs for *cpaD*, *cpaE* and *cpaF*; it is known disruption of *cpaE* in *C. crescentus* abolishes pilus formation and leads to ΦCbK resistance (31-33). A targeted disruption was made in *cpaF* and tested for phage sensitivity by the soft agar assay (Figure 2B). The *cpaF* disruption caused complete resistance to the phage. The fact that multiple transposon insertions were found in the pilus cluster and that the *cpaF* disruption leads to phage resistance strongly suggest that Delta utilizes the *B. subvibrioides* pilus as part of its infection mechanism. The identification of another pili-tropic phage is not surprising as pili are major phage targets in multiple organisms.

Phage Delta was used to assess the potential pilus production in developmental signaling mutants using the soft agar assay (Figure 2C). The *divJ* mutant has similar susceptibility to Delta as the wild-type, suggesting this strain still produces pili. This result is consistent with the

463 *C. crescentus* result as the *C. crescentus divJ* mutant is ΦCbK susceptible (8). Conversely, the *B.*
464 *subvibrioides pleC* mutant shows a clear reduction in susceptibility to Delta, indicating that this
465 strain is deficient in pilus production. If so, this would also be consistent with the *C. crescentus*
466 *pleC* mutant which is resistant to ΦCbK (8, 28). With regards to the *divK* strain, if that mutant
467 was to follow the *C. crescentus* model it should demonstrate the same susceptibility as the *divJ*
468 strain. Alternatively, as the *divK* strain has often demonstrated opposite phenotypes to *divJ* in *B.*
469 *subvibrioides*, one might predict it to demonstrate resistance to Delta. As seen in Figure 2C, the
470 *divK* strain (and the *divJdivK* strain) shows the same level of resistance to phage Delta as the
471 *pleC* mutant. Therefore, in regards to phage sensitivity, the *divK* strain is once again opposite of
472 the prediction of the *C. crescentus* model. Interestingly, none of these developmental signaling
473 mutants demonstrate complete resistance to Delta as seen in the *cpaF* strain. This result suggests
474 that these mutations impact pilus synthesis, but do not abolish it completely.

475 **A suppressor screen identifies mutations related to c-di-GMP signaling.** As the *B.*
476 *subvibrioides divK* mutant displays the most unusual phenotypes with regard to the *C. crescentus*
477 model, this strain was selected for further analysis. Complementation of *divK* was attempted by
478 expressing wild-type DivK from an inducible promoter on a replicating plasmid, however
479 induction failed to complement any of the *divK* phenotypes (data not shown), indicating proper
480 complementation conditions have not yet been identified. Transposon mutagenesis was
481 performed on this strain and mutants were screened for those that restore motility. Two mutants
482 were found (Bresu_1276 and Bresu_2169) that restored motility to the *divK* strain, and
483 maintained this phenotype when recreated by plasmid insertional disruption. Both mutants were
484 involved in c-di-GMP signaling. The *C. crescentus* homolog of the Bresu_1276 gene, CC3100
485 (42% identical to Bresu_1276), was recently characterized in a subcluster of CheY-like response

regulators and renamed CleD (34). Function of CleD is, at least in part, initiated by binding c-di-GMP via an arginine-rich residue with high affinity and specificity for c-di-GMP. Upon binding, roughly 30% of CleD localizes to the flagellated pole of the swarmer cell. Nesper et. al suggests that CleD may bind directly to the flagellar motor switch protein, FliM. In *E. coli* and *Salmonella*, the flagellar brake protein YcgR interacts with FliM in a c-di-GMP dependent manner, biasing the motor in the smooth-running counter clockwise direction (35, 36), but YcgR is not a response regulator-type protein, and no obvious YcgR homologs are present in the *C. crescentus* or *B. subvibrioides* genomes (data not shown). Based upon the *C. crescentus* findings, it was hypothesized that increased c-di-GMP levels cause activation of CleD, which binds to the flagellar switch and inhibits flagellar function (34). In *C. crescentus*, *cleD* mutants are 150% more motile while their adhesion does not differ significantly from that of the wild-type. Unlike conventional response regulators, the phosphoryl-receiving aspartate is replaced with a glutamate in CleD. In other response regulators, replacement of the aspartate with a glutamate mimics the phosphorylated state and locks the protein in an active conformation. Alignment of CleD with orthologs from various *Caulobacter* and *Brevundimonas* species demonstrated that this was a conserved feature of CleD within this clade (Figure 3). Similar to *C. crescentus*, the swarm size of *B. subvibrioides cleD* mutant increased to 151% compared to wild-type. A knockout of *cleD* in the *divK* background led to a complete restoration of motility compared to that of wild-type, while adhesion did not appear to be affected (Figure 4A). These phenotypes correspond relatively well with the model given in Nesper et al. As CleD is thought to inhibit motor function, a cell lacking CleD would have less motor inhibition, leading to an increase in motility and a delay in surface attachment, though *cleD* disruption had no impact on

508 the phage sensitivity phenotypes of the wild-type or *divK*-derived strains (Supplemental Figure
509 S5).

510 Bresu_2169 is the homolog of the well-characterized *C. crescentus* diguanylate cyclase,
511 DgcB (61% identical amino acid sequence). In *C. crescentus*, DgcB is one of two major
512 diguanylate cyclases that work in conjunction to elevate c-di-GMP levels which in turn helps
513 regulate the cell cycle, specifically in regards to polar morphogenesis (37). It has been shown
514 that a *dgcB* mutant causes adhesion to drop to nearly 50% compared to wild-type while motility
515 was elevated to almost 150%. It was unsurprising to find very similar changes in phenotypes in
516 the *dgcB* mutant in wild-type *B. subvibrioides*. In the *dgcB* mutant, swarm expansion increased
517 by 124% while adhesion dropped to only 46% compared to wild-type (Figure 4A). Though the
518 *dgcB* mutant did not restore motility to wild-type levels in the *divK* background, the insertion did
519 cause the swarm to expand nearly twice as much as that of the *divK* parent. These phenotypes
520 are consistent with our current understanding of c-di-GMP's role in the *C. crescentus* cell cycle.
521 As c-di-GMP builds up in the cell, it begins to make the switch from its motile phase to its
522 sessile phase. Deleting a diguanylate cyclase therefore should prolong the swarmer cell stage,
523 thereby increasing motility and decreasing adhesion. Similar to *cleD*, *dgcB* disruption had no
524 impact on the phage sensitivity phenotypes of the wild-type or *divK*-derived strains
525 (Supplemental Figure S5).

526 **A *pleD* mutant lacks hypermotility in *divK* background.** Given the identification of
527 *dgcB* in the suppressor screen, it was of note that the screen did not identify the other well-
528 characterized diguanylate cycle involved in the *C. crescentus* cell cycle, PleD. PleD is an
529 atypical response regulator with two receiver domains in addition to the diguanylate cyclase
530 domain (38, 39). The *pleD* mutant in *C. crescentus* has been shown to suppresses the *pleC*

motility defect in *C. crescentus* which led to its initial discovery alongside *divK* (22, 38, 39). However, in a wild-type background, *pleD* disruption has actually been shown to reduce motility to about 60% compared to wild-type (22, 37). Additionally, a 70% reduction in adhesion is observed in *pleD* mutants which is thought to be a result of delayed holdfast production (22, 37, 40). Therefore, it was not clear whether a *pleD* disruption would lead to motility defect suppression in a *divK* background. To examine this, a *pleD* disruption was made in both the wild-type and *divK B. subvibrioides* strains (Figure 4A). The *divK* and *pleD* genes belong to the same two gene operon, where *divK* is the first gene. As previously published, deletion of *divK* was performed using an in-frame deletion and thus is not expected to impact *pleD* expression. As *pleD* is the latter of the two genes, plasmid insertion into *pleD* is not expected to impact *divK* expression.

In wild-type *B. subvibrioides*, *pleD* disruption resulted in little change to motility with swarms expanding to 105% of wild-type, while adhesion dropped to only 10% compared to wild-type. While this data supports the broader theory of c-di-GMP's role as the "switch" between the motile and sessile phase of the cell cycle, it does not align with those phenotypes seen in a *C. crescentus pleD* mutant. While adhesion is reduced in both organisms, the reduction in adhesion was much more drastic in *B. subvibrioides* than *C. crescentus*. Moreover, the motility phenotypes in homologous *pleD* mutants do not match. In *C. crescentus*, *pleD* mutants causes a decrease in motility by nearly 40% in the wild-type background (22, 37). In *B. subvibrioides*, motility is the same as wild-type (Figure 4A). Disruption of *pleD* also causes a reduction in phage sensitivity (Figure 7B, Supplemental Figure S5).

Another interesting detail discovered in performing these assays was the lack of change in phenotypes seen in the *pleD* disruption in a *divK* background. It is not surprising that

554 adhesion was not negatively impacted as it is already significantly lower in the *divK* strain
555 compared to wild-type. However, disrupting the *pleD* gene did not increase motility in the *divK*
556 mutant. In fact, motility was reduced to 89% compared to the *divK* control (Figure 4A).
557 Additionally, both *divK* and *pleD* mutants had the same reduction in phage sensitivity as the *divK*
558 *pleD* double mutant (Figure 7B, Supplemental Figure S5). It is not clear why disruption of the
559 diguanylate cyclase DgcB leads to increased motility in both the wild-type and *divK*
560 backgrounds, but disruption of another diguanylate cycle PleD does not increase motility in
561 either background. Interestingly, it was previously shown that DivJ and PleC do not act on DivK
562 alone, but in fact also have the same enzymatic functions on PleD phosphorylation as well (41).
563 It may be that PleD acts upon motility not through c-di-GMP signaling but instead by modulating
564 DivK activity, perhaps by interacting/interfering with the polar kinases. If so, then the absence
565 of DivK could block this effect.

566 **Suppressor mutants have altered c-di-GMP levels.** As these mutations are all involved
567 in c-di-GMP signaling, c-di-GMP levels in each strain were quantified to determine if the cellular
568 levels in each strain correspond to observed phenotypes. These metabolites were quantified from
569 whole cell lysates. In bacteria, high c-di-GMP levels typically induce adhesion while low c-di-
570 GMP levels induce motility. Therefore, it would be expected that hypermotile strains would
571 show decreased c-di-GMP levels. Instead, hypermotile strains of the wild-type background had
572 varying c-di-GMP levels (Figure 4B). The *pleD* knockout had reduced c-di-GMP levels as
573 predicted. While it may seem surprising that c-di-GMP levels are not affected in a *dgcB* mutant,
574 this in fact true of the *C. crescentus* mutant as well (37). This result suggests that the c-di-GMP
575 levels found in the *dgcB* strain do not appear to be the cause for the observed changes in motility

and adhesion. A comparison of c-di-GMP levels and phenotypic analyses between the organisms is presented in Table 1.

Perhaps the most interesting result is that the *cleD* mutant had the highest c-di-GMP levels of all strains tested. This is surprising as it is suggested by Nesper et. al. that CleD does not affect c-di-GMP levels at all, but rather is affected by them. CleD is a response regulator that contains neither a GGDEF nor an EAL domain characteristic of diguanylate cyclases and phosphodiesterases respectively. Instead it is thought CleD binds to c-di-GMP, which then stimulates it to interact with the flagellar motor. The data presented here suggests that there may be a feedback loop whereby increased motility in the swarm agar leads to increased c-di-GMP levels. One potential explanation is that this situation increases contact with surfaces. Yet the *cleD* mutant clearly shows decreased adhesion compared to wild-type despite the elevated c-di-GMP levels. Therefore, there must be a block between the high c-di-GMP levels and the execution of those levels into adhesion in this strain.

Very different results were obtained when c-di-GMP levels were measured in *divK* derived strains (Figure 4B). While a wide variety of motility phenotypes were observed in *cleD*, *dgcB*, and *pleD* disruptions in the *divK* background, their c-di-GMP levels are all nearly identical to that of the *divK* mutant. For the *dgcB divK* strain, once again the increase in motility occurs without a change in c-di-GMP levels. These results suggest that DgcB is not a significant contributor to c-di-GMP production in *B. subvibrioides*. While *pleD* disruption leads to decreased c-di-GMP levels in the wild-type background, no change is seen in the *divK* background. This means in the absence of PleD some other enzyme must be responsible for achieving these levels of c-di-GMP. Given the lack of impact DgcB seems to have on c-di-GMP signaling, it is tempting to speculate an as-yet characterized diguanylate cyclase is involved.

599 Lastly the elevated c-di-GMP levels seen in the *cleD* disruption are not seen when *cleD* is
600 disrupted in the *divK* background. This result suggests that whatever feedback mechanism leads
601 to elevated c-di-GMP levels is not functional in the *divK* mutant.

602 **Non-native diguanylate cyclases and phosphodiesterases cause shifts in c-di-GMP**
603 **levels but do not alter phenotypes in the *divK* strain.** As previously mentioned, c-di-GMP is
604 thought to assist in the coordination of certain developmental processes throughout the cell cycle.
605 The previous results found mutations in genes involved in c-di-GMP signaling could suppress
606 developmental defects, but the actual effect of the mutations appears uncoupled from effects on
607 c-di-GMP levels. In order to further investigate the connection between developmental defects
608 and c-di-GMP signaling, c-di-GMP levels were artificially manipulated. Plasmid constructs
609 expressing non-native c-di-GMP metabolizing enzymes previously used in similar experiments
610 in *C. crescentus* were obtained and expressed in *B. subvibrioides*. The diguanylate cyclase *ydeH*
611 from *Escherichia coli* was expressed from two different IPTG inducible plasmids; a medium
612 copy number pBBR-based plamid, pTB4, and a low copy number pRK2-based plasmid, pSA280
613 (22). The combination of the two different inducible copy number plasmids resulted in different
614 elevated levels of c-di-GMP (Figure 5B). A phosphodiesterase *pchP* from *Pseudomonas*
615 *aeruginosa* (42), as well as its active site mutant $pchP^{E328A}$ were expressed from pBV-MCS4, a
616 vanillate inducible medium copy number plasmid (23). The phosphodiesterase on a medium
617 copy plasmid was enough to decrease levels of c-di-GMP to either equivalent or lower levels as
618 is seen in the *divK* strain. The decrease was not observed when the active site mutant was
619 expressed, demonstrating that the reduction of c-di-GMP was the result of *pchP* expression.
620 Wild-type and *divK* strains were grown with IPTG and vanillate respectively to control for any
621 growth effects caused by the inducers.

622 Expression of the phosphodiesterase in the wild-type background caused a reduction in c-
623 di-GMP which would be predicted to increase motility and decrease adhesion. While this strain
624 had a large reduction of adhesion, it also had a small reduction in motility (Figure 5A).
625 However, these same results were obtained when this construct was expressed in wild-type *C.*
626 *crescentus* (22). It is interesting to note, though, that expression of the phosphodiesterase results
627 in similar c-di-GMP levels to that of the *divK* strain yet the phosphodiesterase strain
628 demonstrates much larger swarm sizes than the *divK* strain. The low copy diguanylate cyclase
629 plasmid did not appear to affect c-di-GMP levels (Figure 5B), and unsurprisingly did not appear
630 to affect either motility or adhesion. However, the medium copy diguanylate cyclase plasmid
631 increased c-di-GMP levels but had surprising phenotypic results. An increase in c-di-GMP
632 levels would be predicted to increase adhesion and decrease motility. When this construct was
633 expressed in wild-type *C. crescentus* there was a small decrease to adhesion but a very large
634 decrease in motility, almost to the point of non-motility (22); the motility results mirror the
635 prediction based on c-di-GMP levels. In *B. subvibrioides* the same construct produced a slight
636 decrease to adhesion, but motility actually increased instead of drastically decreasing. Here not
637 only do *B. subvibrioides* results differ from *C. crescentus* results, but the results contradict
638 predictions based on known c-di-GMP paradigms.
639 In the *divK* background strain, expression of either diguanylate cyclase increases c-di-
640 GMP levels, though the low copy diguanylate cyclase increase is not as dramatic as the medium
641 copy. However, neither expression level has a significant impact on motility or adhesion (Figure
642 5). Neither the phosphodiesterase nor its active site mutant cause a noticeable shift in the c-di-
643 GMP levels compared to the *divK* strain nor any noticeable impact on phenotype. In fact, though
644 the c-di-GMP levels differed dramatically between strains, the phenotypes of all six of these

strains are not impacted. T-tests performed between each strain and its respective control showed no significant difference. These results appear to be the antithesis of those found from the suppressor screen. While the suppressor mutants showed recovery in their motility defect compared to *divK*, their c-di-GMP levels did not significantly differ from each other or *divK*. Conversely, when c-di-GMP levels were artificially manipulated, alterations of c-di-GMP levels in the *divK* strain had no impact on phenotypes. These results suggest that DivK is somehow serving as a block or a buffer to c-di-GMP levels and their effects on phenotypes and calls into question the role c-di-GMP has in *B. subvibrioides* developmental progression.

Disruption of *pleD* in developmental signaling mutants does not alter *pleC* phenotypes, but does alter *divJ* phenotypes. As discussed above, disruption of *pleD* does not impact the adhesion or swarm expansion phenotypes of a *divK* mutant. To determine the epistatic relationship between *pleD* and other developmental signaling mutants of *B. subvibrioides*, the *pleD* disruption was placed in the *divJ*, *divJdivK*, and *pleC* strains and resultant mutants were analyzed for developmental defects (Figures 6 and 7). Disruption of *pleD* does not alter the phenotypes of the *divK* or *divJdivK* strains in swarm expansion, adhesion, holdfast formation, or phage sensitivity. The exception to this is the *divJdivK pleD::pCR* strain which had a small but statistically significant reduction in adhesion compared to the *divJdivK* parent ($p = 0.03$). These results are to be expected given previous results and the fact that the *divJdivK* strain has consistently phenocopied the *divK* strain. What was not expected was that disruption of *pleD* had no effect on *pleC* strain phenotypes. The *pleD* gene was originally discovered as a motility suppressor of the *C. crescentus pleC* mutant (13), but in *B. subvibrioides* *pleD* disruption does not alter any of the developmental phenotypes of the *pleC* strain. Instead, *pleD* disruption alters some, but not all, of the *divJ* mutant phenotypes. The *divJ pleD::pCR*

668 strain had wild-type levels of phage sensitivity just like the *divJ* parent, and microscopic
669 examination of the strain also revealed the strain had a filamentous cell morphology
670 characteristic of the *divJ* parent strain (Figure 7A). However, this strain had a significant
671 reduction in adhesion, holdfast was undetectable, and there was a small but statistically
672 significant increase in motility. Essentially, *pleD* disruption removes holdfast formation and
673 increases motility without affecting cell filamentation or pilus production, though swarm
674 expansion results are clouded by the filamentous cell morphology which impacts swarm
675 expansion independent of flagellum function or chemotaxis. These *divJ pleD::pCR* results stand
676 in stark contrast to the *divJdivK* results where all developmental phenotypes copy the *divK*
677 phenotypes. Perhaps this suggests that PleD may have a more specific role in the morphological
678 changes that occur during *B. subvibrioides* cell cycle progression.
679

680 **Discussion**

681 Across closely related bacterial species, high levels of gene conservation are commonly
682 observed. It has therefore been a long-standing assumption that information gathered from
683 studying a model organism can be extrapolated to other closely related organisms. Through this
684 study, by comparing and contrasting the developmental signaling systems of *C. crescentus* and *B.*
685 *subvibrioides*, it has been shown that these assumptions may not be as safe to make as previously
686 thought. Preliminary data raised a few questions by demonstrating major differences in the
687 phenotypes of *divK* mutants between species. Here the system was analyzed in greater depth in
688 *B. subvibrioides* by examining subcellular protein localization, developmental phenotypes of a
689 *pleC* mutant, and isolating a pilitropic bacteriophage to examine pilus production in multiple
690 developmental mutants. GFP tagging revealed that the subcellular localization patterns of DivJ

and PleC in *B. subvibrioides* are consistent with the *C. crescentus* proteins. DivJ was consistently detected at the holdfast (i.e. stalked) pole, while PleC was consistently detected at the non-holdfast (i.e. flagellar) pole. DivK was found in a variety of localization patterns. In *C. crescentus*, DivK is localized to the stalked pole in stalked cells, bi-polarly localized in predivisional cells, and delocalized in swarmer cells. In *B. subvibrioides*, stalked pole and bipolar localizations were observed, but the majority of cells displayed delocalized DivK-GFP. If *B. subvibrioides* adhered to the *C. crescentus* model, this would suggest that over 85% of the *B. subvibrioides* population in a growing culture is swarmer cells. Many of the cells with delocalized DivK were in rosettes and/or had detectable holdfast (Supplemental Figure S4, additional data not shown). Correlated with this is the fact that 82.9% of cells had no detectable DivJ-GFP foci, while only 28.9% lacked detectable PleC-GFP foci; the presence of PleC and the absence of DivJ would theoretically lead to complete dephosphorylation of DivK, which causes delocalization in *C. crescentus*, such as in swarmer cells (43). This suggests that many, if not most, *B. subvibrioides* stalked cells have delocalized DivK. One unusual facet of *B. subvibrioides* physiology is the fact that the doubling time of this organism in PYE is 6.5 hours (3), compared to the 1.5 hours of *C. crescentus* in the same media. It is not clear how the cell cycle is adjusted to account for this longer generation time. Perhaps the *B. subvibrioides* signaling system is held in a more swarmer cell like state even though the cells are morphologically more like a stalked cell. However, stalked pole localization is observed, so what induces the transition from delocalized to localized? More careful dissection of the *B. subvibrioides* cell cycle, with particular respect to signaling protein localization, may reveal the answer.

While the discovery that developmental protein localization in *B. subvibrioides* largely matches the localization in *C. crescentus* may not be surprising, this discovery makes the phenotypic results even more surprising (summarized in Table 2). Previously it was shown that the *B. subvibrioides divJ* mutant closely matches the phenotypes of the *C. crescentus divJ* mutant in regards to cell filamentation, holdfast production, adhesion and motility (3). Here pilus synthesis was added by the use of a novel bacteriophage, and here still *B. subvibrioides divJ* mirrored the *C. crescentus* results. In *C. crescentus divK* disruption/depletion leads to G1 cell stage arrest (15); i.e. in the stalked cell stage prior to entering the predivisional stage. As a consequence, the cells become extremely filamentous and do not produce flagella or pili. While adhesion of this strain was never tested, cells are seen the produce stalks that touch tip to tip in the rosette fashion, suggesting holdfast production. These phenotypes are similar to *divJ* phenotypes, which makes sense given both mutations ultimately lead to lower levels of DivK~P. Conversely, previously it was shown that *B. subvibrioides divK* deletion produced opposite phenotypic results in cell size, holdfast production and adhesion. Both mutants were compromised in motility, but the mechanistic reasons are unclear, especially given both strains produce apparently functional flagella, though the morphology defects of the *divJ* strain may have a bearing on these results. Here another difference was demonstrated between the strains as the *B. subvibrioides divK* strain had an intermediate phage resistance phenotype. While *B. subvibrioides divJ* largely matched *C. crescentus divJ*, and *B. subvibrioides* was largely opposite *C. crescentus divK*, *B. subvibrioides pleC* was an intermediate of *C. crescentus pleC*. In *C. crescentus pleC* mutants have normal cell size, but produce no pili, no holdfast (and thus are adhesion deficient), and are non-motile due to a paralyzed flagellum. Here it was found that *B. subvibrioides pleC* mutants have an intermediate phage resistance phenotype, suggesting

intermediate pilus production, intermediate adhesion with weak holdfast detection, and intermediate motility. Therefore, comparing signaling mutants between organisms results in the same, opposite, or intermediate results. These results are all the more confusing given the fact that localization patterns of the proteins are mostly conserved between the organisms. It seems unlikely that the phenotypic consequences are a result of altered localization patterns. This suggests that the phenotypic consequences are a product of altered downstream signaling. Even in *C. crescentus*, the exact connection between signaling protein disruption and phenotypic consequence are largely unknown. Careful mapping of the signaling systems between the mutation and the eventual phenotype is required in both organisms.

In an attempt to further map this system in *B. subvibrioides*, a suppressor screen was employed using the *divK* mutant as its phenotypes differed most dramatically from its *C. crescentus* homolog. Suppressor mutations were found in genes predicted to encode proteins that affected or were affected by c-di-GMP. This was not necessarily a surprising discovery. C-di-GMP is a second messenger signaling system conserved across many bacterial species used to coordinate the switch between motile and sessile lifestyles. Previous research in *C. crescentus* suggests that organism integrated c-di-GMP signaling into the swarmer-to-stalked cell transition. Mutations that modify c-di-GMP signaling would be predicted to impact the swarmer cell stage, perhaps lengthening the amount of time the cell stays in that stage and thus lead to an increase in swarm spreading in soft agar. However, further inquiry into c-di-GMP levels of *divK* suppressor mutants revealed discrepancies between c-di-GMP levels and their corresponding phenotypes. Firstly, CleD, a CheY-like response regulator that is thought to affect flagellar motor function, caused the strongest suppression of the *divK* mutant restoring motility levels to that of wild-type. Given that the reported function of CleD is to bind the FliM filament of the flagellar motor and

759 interfere with motor function to boost rapid surface attachment (34), it is expected that disruption
760 of *cleD* would result in increased motility and decreased adhesion which can be seen in both the
761 wild-type and *divK* background strains (Figure 4A). What was unexpected, however, was to find
762 that a lack of CleD led to one of the highest detected levels of c-di-GMP in this study, which was
763 surprising given that CleD has no predicted diguanylate cyclase or phosphodiesterase domains.
764 Yet when this same mutation was placed in the *divK* background, the c-di-GMP levels were
765 indistinguishable from the *divK* parent. Therefore, the same mutation leads to hypermotility in
766 two different backgrounds despite the fact that c-di-GMP levels are drastically different.
767 Consequently, the phenotypic results of the mutation do not match the c-di-GMP levels,
768 suggesting that c-di-GMP has little or no effect on the motility phenotype. A similar result was
769 seen with DgcB. Disruption of *dgcB* in either the wild-type or *divK* background resulted in
770 hypermotility, but c-di-GMP levels were not altered. Once again, the effect on motility occurred
771 independently of c-di-GMP levels. Disruption of *divK* seems to somehow stabilize c-di-GMP
772 levels. Even when non-native enzymes are expressed in the *divK* background the magnitude of
773 changes seen in the c-di-GMP pool is dampened compared to the magnitude of change seen
774 when the enzymes are expressed in the wild-type background. This may explain why *pleD* was
775 not found in the suppressor screen. While CleD and DgcB seem involved in c-di-GMP
776 signaling, their effect on the cell appears c-di-GMP-independent, while PleD appears to perform
777 its action by affecting the c-di-GMP pool. If that pool is stabilized in the *divK* strain, then
778 disruption of *pleD* will have no effect on either the c-di-GMP pool or on the motility phenotype.
779 However, it should be noted that c-di-GMP levels were measured from whole cell lysates and
780 does not reflect the possibility of spatial or temporal variations of c-di-GMP levels within the

781 cell. It is possible that CleD and DgcB have c-di-GMP dependent effects, but those effects are
782 limited to specific sub-cellular locations in the cell.

783 In *C. crescentus*, c-di-GMP is implicated in the morphological changes that occur during
784 swarmer cell differentiation, but c-di-GMP levels are also tied to the developmental network, and
785 more specifically CtrA activation, by two different mechanisms. First, through most of the cell
786 cycle the diguanylate cyclase DgcB is antagonized by the phosphodiesterase PdeA such that no
787 c-di-GMP is produced by DgcB (37). Upon swarmer cell differentiation PdeA is targeted for
788 proteolysis, leaving DgcB activity unchecked. This, combined with active PleD, increases c-di-
789 GMP levels in the cell. The elevated levels activate the protein PopA, which targets CtrA for
790 proteolysis, which is useful for swarmer cell differentiation as it will relieve the inhibition of
791 chromosome replication initiation performed by CtrA binding at the origin of replication. In the
792 second mechanism, it has been found that c-di-GMP is an allosteric regulator of CckA, the
793 hybrid histidine kinase responsible for phosphorylation of CtrA (44). As stated above, DivJ and
794 PleC have the same antagonistic phosphorylation activities on PleD as they do on DivK, and
795 phosphorylation induces PleD activity. During swarmer cell differentiation, isolated PleC is
796 replaced at the transitioning stalked pole by DivJ, and which leads to elevated PleD
797 phosphorylation and therefore higher enzymatic activity. This, combined with unchecked DgcB
798 activity, increases c-di-GMP levels which has been shown to inhibit kinase activity and stimulate
799 phosphatase activity of CckA, causing CtrA to be dephosphorylated and therefore deactivated.
800 Additionally, the same CckA phosphatase activity causes dephosphorylation of CpdR, which also
801 targets CtrA for proteolysis. It has also been shown that CpdR activity targets PdeA for
802 degradation, thereby permitting unchecked DgcB activity. Therefore, the elevation of c-di-GMP
803 levels during swarmer cell differentiation works redundantly to deactivate CtrA, which is

804 necessary for cell cycle progression, and serves to coordinate the morphological changes of
805 swarmer cell differentiation with the necessary changes in the signaling state of the
806 developmental network.

807 However, these models do not adequately explain some of the more unusual results
808 reported in this study. First, it is not clear why c-di-GMP levels appear stabilized in a *divK*
809 mutant in *B. subvibrioides*. In *C. crescentus* DivK has been shown to be an allosteric affector of
810 PleC, where non-phosphorylated DivK stimulates PleC to become a kinase and increase PleD~P
811 levels (45), though it is thought this activity only occurs during the onset of swarmer cell
812 differentiation. If one were to assume that DivK-driven PleC phosphorylation of PleD were the
813 sole mechanism of PleD phosphorylation, then theoretically the absence of DivK could mean
814 PleD is never phosphorylated and therefore c-di-GMP levels would not change above
815 background in a *divK* strain, but this would mean 1) observed DivJ kinase activity on PleD is
816 unimportant to PleD activity and it is only the phosphorylation of PleD by PleC (at the onset or
817 swarmer cell differentiation) that is important, and 2) PleD is the sole contributor to c-di-GMP
818 elevation and thus DgcB activity is not a meaningful contributor to measurable c-di-GMP
819 changes. Perhaps this second point is supported by our data here which shows a disconnect
820 between phenotype and c-di-GMP levels in *dgcB* mutants, and it was seen both here in *B.*
821 *subvibrioides* and in *C. crescentus* that overall c-di-GMP levels do not change much in *dgcB*
822 mutants (37). These models also do not explain why *pleD* disruption alters the adhesion
823 phenotypes of a *divJ* mutant. Assuming that DivJ is the PleD kinase, then PleD should be
824 inactive in a *divJ* strain and therefore disrupting *pleD* in a *divJ* background should have no effect.
825 In this study, it clearly does. Perhaps this result supports a hypothesis that only PleC
826 phosphorylation of PleD is biologically relevant. Additionally, assuming another PleD kinase is

827 active in a *divJ* strain, the results of *pleD* disruption should be redundant to *divJ* deletion, not
828 counter-productive. A *divJ* deletion should lead to decreased levels of DivK~P, which should
829 allow promiscuous interaction between DivL and CckA, ultimately leading to over-activation of
830 CtrA. Disruption of *pleD* should lead to decreased c-di-GMP levels (which was seen in this
831 study) which would not direct CckA into its phosphatase role, and also potentially lead to over-
832 activation of CtrA. Therefore, combining *divJ* and *pleD* mutations should be redundant, if not
833 additive. Yet in this study it was seen *pleD* disruption reversed the holdfast formation and
834 motility of the *divJ* strain. Even further, *pleD* disruption did not alter the cell filamentation or
835 pilus production of the *divJ* strain, which may suggest that PleD's role in cell cycle progression
836 is specific to holdfast and/or motility and argues against a role in CtrA regulation.

837 This research raises several questions. First, what is the exact role of c-di-GMP in cell
838 cycle progression of *B. subvibrioides*? Is this signal a major driver of the swarmer cell and
839 swarmer cell differentiation? Or have the various c-di-GMP signaling components found new
840 roles in the swarmer cell and the actual c-di-GMP is simply vestigial. What is the role of PleD in
841 cell cycle progression? Why are c-di-GMP levels so stable when DivK is removed? And lastly,
842 are the answers to these questions specific to *B. subvibrioides*, or can they be extrapolated back
843 to *C. crescentus*? Further investigation into c-di-GMP signaling in both organisms is required.
844

845 **Acknowledgements**

846 The authors wish to thank P. Caccamo and Y.V. Brun for providing most of the
847 *Brevundimonas* species, as well as the iPCR protocol that had been adapted for use in *C.*
848 *crescentus*, U. Jenal for providing several plasmids, Satish Adhikari and Kari Ferolito for their

849 help with cloning, and the Curtis lab for general support. This work was supported by the United
850 States National Science Foundation CAREER program award 1552647.
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991 **Table 1: Comparison of changes in c-di-GMP levels and phenotypes between *C. crescentus***
992 **and *B. subvibrioides* strains.**

Mutation	Organism[1]	Phenotype[2]			References
		c-di-GMP	Adhesion	Motility	
cleD	*C.c.*	N.D.	+/-[3]	++	(34)
	B.s.	++ (193%)	+/-	++	This study
dgcB	*C.c.*	+ (~105%)	+/-	++	(37)
	B.s.	+ (108%)	+/-	++	This study
pleD	*C.c.*	+/- (~70%)	+/-	+/-	(37)
	B.s.	- (27%)	+/-	+	This study

993 [1] *C.c.* = *C. crescentus*, *B.s.* = *B. subvibrioides*.

994 [2] ++ = above wild-type levels, + = wild-type or near wild-type levels, +/- below wild-type or
995 intermediate levels, - = null or near null levels, N.D. = not determined.

996 [3] Adhesion was measured of individual cells in microfluidic devices.

997

998 **Table 2: Comparison of developmental mutation phenotypes between *C. crescentus* and *B.***
999 ***subvibrioides*.**

Mutation	Organism[2]	Phenotype[1]					References
		Cell Size	Adhesion	Holdfast	Motility	Pili	
divJ	*C.c.*	Filament	++	++	-	+	(8, 46, 47)
	B.s.	Filament	+/++	++	-	+	(3), this study
divK	*C.c.*	Filament	N.D.[3]	N.D.[3]	-	-	(15, 48, 49)
	B.s.	Short	-	-	-	+/-	(3), this study
pleC	*C.c.*	Normal	-	-	-	-	(12, 13, 23, 28, 29)
	B.s.	Normal	+/-	+/-	+/-	+/-	This study
pleD	*C.c.*	Normal	-	+	+/-	+	(23, 37, 39, 40, 50)
	B.s.	Normal	-	-	+	+/-	This study
dgcB	*C.c.*	Normal	+/-	+	++	N.D.	(37)
	B.s.	Normal	+/-	+	++	+	This study

1000 [1] ++ = above wild-type levels, + = wild-type or near wild-type levels, +/- below wild-type or
1001 intermediate levels, - = null or near null levels, N.D. = not determined.

1002 [2] *C.c.* = *C. crescentus*, *B.s.* = *B. subvibrioides*.

1003 [3] Adhesion and holdfast production were not analyzed in this strain, but circumstantial evidence
1004 suggests holdfast material is produced.

1005

1006 **Figure Legends**

1007 **Figure 1. Deletions in *B. subvibrioides* developmental signaling genes results in varying**
1008 **physiological phenotypes.** A) Wild-type, *divJ*, *divK*, *divJdivK*, and *pleC B. subvibrioides* strains
1009 were analyzed for swarm expansion (dark bars) and adhesion (light bars) defects using a soft
1010 agar swarm assay and a short-term adhesion assay. Mutant strains were normalized to wild-type
1011 results for both assays. Deletion of *divJ* gives motility defects but minimal adhesion defects,
1012 similar to *C. crescentus divJ* results. *B. subvibrioides divK* and *divJdivK* strains give opposite
1013 results, with severe motility and adhesion defects. The *B. subvibrioides pleC* strain has reduced
1014 motility and moderately reduced adhesion, which is similar but not identical to the *C. crescentus*
1015 *pleC* strain. B) Lectin staining of holdfast material of wild-type, *divJ*, *divK*, *divJdivK*, and *pleC*
1016 strains. The *pleC* strain, despite having reduced adhesion in the short-term adhesion assay, still
1017 has detectable holdfast material C) GFP-tagged DivJ localizes to the holdfast producing pole,
1018 while PleC-GFP localizes to the pole opposite the holdfast. DivK-GFP displays bi-polar
1019 localization. These localization patterns are identical those of *C. crescentus* homologs.
1020
1021 **Figure 2. Bacteriophage Delta serves as a tool to investigate *B. subvibrioides* pilus**
1022 **production.** A) Phage Delta was tested for infection in 18 different *Brevundimonas* species.
1023 Control assays used PYE media instead of phage stock. Delta caused a significant reduction in
1024 *B. subvibrioides* and *B. aveniformis* viability, with some reduction in *B. basaltis* and *B.*
1025 *halotolerans* as well. B) Phage Delta was tested against wild-type and *cpaF*::pCR *B.*
1026 *subvibrioides* strains using a soft agar phage assay. Wild-type displayed zones of clearing with
1027 phage dilutions up to 10^{-7}, while the *cpaF* strain showed resistance to all phage dilutions. C) *B.*
1028 *subvibrioides* developmental signaling mutants were tested with phage Delta in soft agar phage

1029 assays. Wild-type shows clear susceptibility to Delta, as does the *divJ* strain suggesting that, like
1030 *C. crescentus divJ*, it produces pili. The *pleC* strain shows a 2-3 orders of magnitude reduced
1031 susceptibility to the phage, indicating reduced pilus production which is consistent with the *C.*
1032 *crescentus* phenotype. The *divK* and *divJdivK* strains display similar to resistance as the *pleC*
1033 strain. Here again, *divK* disruption causes the opposite phenotype to *divJ* disruption, unlike the
1034 *C. crescentus* results.
1035
1036 **Figure 3. CleD displays a conserved glutamate residue in place of an aspartate typical of**
1037 **response regulators.** CleD orthologs from various Caulobacter and Brevundimonas species
1038 were aligned by ClustalW, along with B. subvibrioides DivK. The shaded box indicates *B.*
1039 *subvibrioides* DivK D53, which is analogous to *C. crescentus* DivK D53 and is the known
1040 phosphoryl-accepting residue. This alignment demonstrates that CleD orthologs all contain a
1041 glutamate substitution at that site, which has been found to mimic the phosphorylated state and
1042 lock the protein in an active conformation in other response regulators.
1043
1044 **Figure 4. Phenotypes exhibited by *divK* suppressors do not coincide with intracellular c-di-**
1045 **GMP levels.** A) Swarm expansion (dark bars) and surface adhesion (light bars) of suppressor
1046 mutations tested in both the wild-type and *divK* background. Disruption of CleD, DgcB and
1047 PleD lead to increased motility in the wild-type background, but only CleD and DgcB lead to
1048 increased motility in the *divK* background. Disruptions in the wild-type background lead to
1049 varying levels of adhesion reduction, but the same disruptions had no effect on adhesion in the
1050 *divK* background. * indicates that motility is statistically insignificant from the *divK* parent,
1051 while ** indicates motility is statistically significant from the *divK* parent ($p < 0.05$). B) C-di-

GMP levels were measured using mass spectrometry then normalized to the amount of biomass from each sample. Despite disruptions causing increased motility in the wild-type background, those strains had different c-di-GMP levels. No disruption changed c-di-GMP levels in the *divK* background even though some strains suppressed the motility defect while others did not. These results show a discrepancy between phenotypic effects and intracellular c-di-GMP levels.

Figure 5. Artificial manipulation of c-di-GMP levels do not significantly affect phenotypes in the *divK* mutant. A) Swarm expansion (dark bars) and surface adhesion (light bars) of strains that have altered c-di-GMP levels caused by expression of non-native enzymes in the wild-type and *divK* background. Constructs including the *E. coli* diguanylate cyclase *ydeH* expressed from a medium copy plasmid (med DGC) and a low copy plasmid (low DGC), the *P. aeruginosa* phosphodiesterase *pchP* (PDE) as well as a catalytically inactive variant (inactive PDE). Bars below the x-axis outline inducer used for plasmids in each strain. In the wild-type background the medium copy DGC increased motility and decreased adhesion, which is opposite the expected outcome, while the PDE reduced motility and severely reduced adhesion. In the *divK* background, no expression construct significantly altered the phenotypes. * indicates both motility and adhesion were statistically insignificant from the control strain ($p > 0.05$). B) C-di-GMP levels were measured using mass spectrometry then normalized to the amount of biomass from each sample. In the wild-type background the medium copy DGC significantly increased c-di-GMP levels while the PDE reduced c-di-GMP levels. In the *divK* background, both DGC constructs increased c-di-GMP levels, though PDE expression has no effect, despite the fact that neither DGC construct has an effect on motility and adhesion phenotypes.

1075 **Figure 6. Disruption of *pleD* does not alter *pleC* or *divK* motility or adhesion, but does alter**
1076 ***divJ* motility and adhesion.**
1077 Wild-type, *pleD*, *divJ*, *divJpleD*, *divK*, *divKpleD*, *pleC*, and *pleCpleD B. subvibrioides* strains
1078 were analyzed for swarm expansion (A) and adhesion (B) defects using a soft agar swarm assay
1079 and a short-term adhesion assay respectively. Mutant strains were normalized to wild-type
1080 results for both assays. The *pleD* mutation has no effect on the adhesion or motility of the *pleC*
1081 or *divK* strains, but does reduce adhesion and increase motility of the *divJ* strain. In A), the bar
1082 with an asterisk indicates *divJ pleD::pCR* has statistically significant more swarm expansion than
1083 *divJ* ($p < 0.05$). In B), results with the same number of asterisks are not statistically significant
1084 from each other, but are statistically significant from results with a different number of asterisks
1085 ($p < 0.05$).

1086

1087 **Figure 7. Disruption of *pleD* does not alter *pleC* or *divK* holdfast production or phage**
1088 **sensitivity, but does alter *divJ* holdfast production.**

1089 A) Lectin staining of holdfast material of wild-type, *pleD*, *divJ*, *divJpleD*, *divK*, *divKpleD*, *pleC*,
1090 and *pleCpleD B. subvibrioides* strains. The wild-type and *divJ* strains have easily detectable
1091 holdfast material. The *pleC* and *pleC pleD* strains have greatly reduced but still detectable
1092 holdfast material, while all remaining strains have no detectable holdfast. This includes the *divJ*
1093 *pleD* strain, which still displays obvious cell filamentation despite no longer producing holdfast.
1094 B) *B. subvibrioides* wild-type, *pleD*, *divJ*, *divJpleD*, *divK*, *divKpleD*, *pleC*, and *pleCpleD* strains
1095 were tested with phage Delta in soft agar phage assays. While the *pleD* disruption alters the
1096 adhesion and holdfast phenotypes of the *divJ* strain, this mutation does not alter the phage

sensitivity of the parent, as both *divJ* and *divJpleD* strains have similar sensitivities to the phage as wild-type