Introduction to revised application

This is the resubmission of application F31AI106288-01, "Regulation of *Vibrio* Biofilm Formation by H-NS Repression and Anti-repression". As a minority Ph.D. student I am grateful for the reviewer's effort and suggestions for improving my research training plan at UAB. This resubmission has undergone an extensive revision and changes, both in response to the reviewers' comments and based on new preliminary data.

Responses to reviewer 1

Major concerns were identified as "*lack of a stepwise approach, heavy reliance on ChIP and the study of c-di-GMP makes the application quite broad*". In the revised research plan we propose a more direct set of hypothesis-driven experiments to test the novel hypothesis that c-di-GMP enhances biofilm formation in *V. cholerae* by diminishing H-NS repression at *vps* promoters in a stepwise manner.

Responses to weaknesses in the research training plan:

1. "*Aim 1 is quite large and involves testing multiple genes in multiple mutants*". We have already identified VpsT as a candidate c-di-GMP-dependent H-NS anti-repressor at the *vps* operons I and II. In aim 1, we will directly test the hypothesis that VpsT functions as an anti-repressor and determine the molecular mechanism of this regulation.

2. *"The use of ChIP assays with mutants will not determine whether a gene is directly affecting H-NS binding*". Although this is correct, we have already provided solid evidences that VpsT directly antagonizes H-NS repression at the *vpsA* and *vpsL* promoters. To determine the mechanism of H-NS anti-repression at the *vpsT* promoter, I believe it is justified to test the transcription factors already demonstrated to directly interact with this promoter prior to conduct a screening for new factors (which we propose as an alternative approach). The alternative approach features the required sensitivity to identify additional regulatory proteins acting at the *vpsT* promoter (see letter of support).

3. *"A negative ChIP assay result led to a claim that there is indirect control of vpsR by H-NS"*. We are not going to work with the *vpsR* promoter and references to this matter were deleted from the document.

4. "*An anti-repressor will be purified using an IMPACT-CN system. It is not clear how this works and why this direct approach was not the primary one*". The IMPACT-CN system (NEB) works by placing a gene of interest down or upstream of a self-cleavable splicing element termed intein tag, which allows affinity purification on a chitin column. We have already purified H-NS and VpsT proteins using this system yielding proteins with optimal biological activities for the *in vitro* assays being proposed in aim 1.

5. *"Cyclic-di-GMP increases slightly in an H-NS mutant but it is not clear if this is meaningful?"* The section of the "*H-NS effect on the intracellular c-di-GMP content"* has been discarded in the new research plan.

6. "*A 60-fold increase in c-di-GMP is questionable as this could change a lot of indirect effectors*". We agree with the reviewer and propose to increase c-di-GMP in a dose-response manner to cover changes in c-di-GMP within physiological ranges.

Responses to reviewer-2 *addressing the Weaknesses in the Research Training Plan:*

1. "*Screening approach for anti-repressors is based on MS identification, which may or may not be limited by the quantity of the proteins*". See point 2 of responses to reviewer 1.

2. "*Focus on the details of promoter regulation instead of exploiting the major findings for studying pathogenicity*". We appreciate the reviewer's suggestion. Cells in a biofilm have been reported to exhibit a lower infective dose. Thus, in the revised application we address the relevance of H-NS regulation of biofilm development on biofilm infectivity using the standard suckling mouse colonization cholera model.

Responses to Weaknesses in the Training Potential

"The candidate has been working in the sponsor's laboratory for 2 years, limiting the training potential. All aims depend of a very limited set of techniques". The training potential of the new research plan has been strengthened by including a broader set of techniques which include *in vitro* studies of transcription regulation, confocal microscopy and animal models of cholera. In addition, I have identified new research advisors (see letters of support) and I will be co-sponsored by Dr. Turnbough (UAB), which will provide his expertise in transcription regulation.

Responses to reviewer-3 *addressing the Weaknesses in the Research Training Plan:*

1. "*The possibility that an anti-repressor binds directly to H-NS has not been considered*". This possibility is not discarded in the new research plan approaches (see in aim 1 the section *"Reconstruction of the vps operon transcription regulation in vitro"*)

2. *"Is a PCR fragment of 250 bp going to be long enough for footprinting?"* In aim 1 we are going to use promoter deletions and *lacZ* fusions to sound out where the DNA sequence requirements for H-NS and VpsT regulation lay out on the promoters. With that we will use DNA fragments spanning 250 to 350 bp to determine the exact binding sites by using a novel Dnase I footprint assay as outlined.

A. SPECIFIC AIMS

Cholera is an acute, waterborne diarrheal disease caused by the Gram-negative bacterium, *Vibrio cholerae* of serogroups O1 and O139. Cholera continues to be a major public health concern in endemic areas of Africa and Asia, where it occurs following a predictable seasonal pattern. Further, recent outbreaks in non-endemic regions exhibit a trend toward increased severity and duration [1]. The prevalence of this illness has been estimated to be 5 million cases of disease and 130,000 deaths per year. The major virulence factors expressed by *V. cholerae* are cholera toxin (CT), which is responsible for the profuse, rice-watery diarrhea, and the toxin coregulated pilus (TCP), a type IV bundle-forming pilus required for intestinal colonization [2]. *V. cholerae* can be found in brackish and estuarine waters as free-swimming planktonic cells or in the form of multi-cellular communities, known as biofilms, attached to plankton or in suspension [3, 4]. The development of mature biofilms involves a complex genetic program that includes the expression of motility for the initial attachment [5, 6]; expression of three types of pili, including TCP [7-10] and the biosynthesis of a polysaccharide matrix encoded by the *vps* genes [11-13]. *V. cholerae* within biofilms are more resistant to environmental stresses [14-16]. In addition, biofilm formation is involved in cholera transmission since *Vibrios* in the biofilm are more resistant to acid inactivation during passage through the stomach [16]. *V. cholerae in vivo*-formed biofilms present in fresh cholera stool are in a stage of transient hyperinfectivity that enhances their dissemination through the fecal-oral route [17-19]. In *V. cholerae,* the expression of *vps* genes is controlled by a complex network of transcriptional regulators, which include quorum-sensing and cyclic diguanylate (c-di-GMP) signaling [20-25]. We have demonstrated that the histone-like nucleoid structuring protein (H-NS) represses biofilm formation by silencing the transcription of *vps* genes [26]. H-NS is a DNA-binding protein involved in structurally organizing the nucleoid of prokaryotic cells and in transcriptional repression [27]. Promoters repressed by H-NS can be activated by transcription factors that disrupt the interaction between H-NS and the promoter and act as anti-repressors [28-30]. We have found that H-NS occupation at one *vps* promoter is antagonized by the c-di-GMP receptor protein VpsT and that artificially increasing the c-di-GMP content significantly failed to induce biofilm formation in a *hns* mutant compared to a wild type strain. Our goal is to test the novel hypothesis that c-di-GMP enhances biofilm formation in *V. cholerae* by diminishing H-NS repression at *vps* promoters and characterize the molecular interactions responsible for this regulation. To this end, we propose the following specific aims:

Aim 1. To determine the mechanism of H-NS repression and anti-repression regulating V. cholerae vps operons I and II. In this aim, we will characterize the interaction between H-NS and VpsT at the *vpsA and vpsL* promoters and determine the mechanism by which H-NS repression is antagonized. We will construct promoter deletions and *lacZ* transcriptional fusions to determine the DNA sequence requirements for H-NS and VpsT regulation. Then, we will use electrophoresis mobility shift assays (EMSA), DNase I footprinting and *in vitro* transcription to determine how these transcription factors interact to regulate transcription initiation at the *vpsA and vpsL* promoters.

Aim 2. To determine the relationship between c-di-GMP induction of vps genes and H-NS repression. In this aim we will test the hypothesis that c-di-GMP positively enhances biofilm formation by inducing an anti-repression cascade starting at the *vpsT* promoter. To this end, we will identify the transcription factor that counteracts H-NS repression at the *vpsT* promoter and use chromatin immunoprecipitation to demonstrate that increasing the intracellular c-di-GMP content diminishes H-NS occupancy at *vpsT* promoter.

Aim 3. Molecular architecture and properties of hns biofilms. In this aim we will address the role of H-NS in the development of mature biofilms. To this end will study the kinetics of biofilm development, tridimensional architecture, stability and the presence of eDNA in *hns* mutant biofilms, compared to wild type using laser scanning confocal microscopy. In addition, we will conduct mouse competitive colonization assays to compare the infectivity of wild type and *hns* biofilms.

B. RESEARCH STRATEGY

B.1 SIGNIFICANCE

A major obstacle to the eradication of cholera is the persistence of *V. cholerae* in aquatic environments, which is facilitated by their capacity to form biofilms [14, 31]. *V. cholerae* cells in biofilms are more resistant to environmental stresses, including biocides, classical antibiotics, and disinfectants [32-35], as well as to protozoan grazing [14-16]. *Vibrios* in the biofilm are also more resistant to acid inactivation during passage through the stomach [16], and mutations that block biofilm matrix exopolysaccharide biosynthesis impair colonization of the suckling mouse small intestine [36]. *Vibrios* can also form biofilms during infection [18], and these biofilms, formed *in vivo*, are in a stage of transient hyperinfectivity [19]. The lower oral infective dose of *V. cholerae* biofilms enhances rapid dissemination of the disease during outbreaks [17]. Biofilms formed *in vivo* can also adopt a quiescent physiological state in aquatic environments [37]. These dormant cells, called conditionally viable environmental cells, are infective and can survive for longer periods in fresh and estuarine waters [18]. A better understanding of the factors that regulate biofilm development is necessary to control the epidemic spread of pathogenic *V. cholerae*. We recently reported that the histone-like nucleoid structuring protein (H-NS) is a repressor of biofilm development [26]. In the proposed research effort, we will (i) elucidate the mechanism of biofilm repression by H-NS, (ii) determine how this global regulator interacts with other transcription factors that enhance the biosynthesis of the biofilm exopolysaccharide matrix, and (iii) characterize the biofilm formed in *hns* mutants.

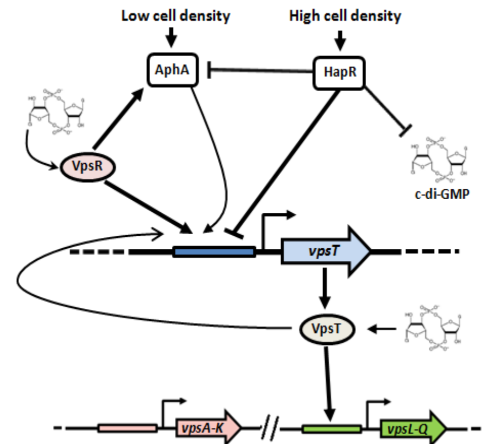
B.2. INNOVATION

The transition between planktonic and biofilm lifestyles is regulated by quorum sensing (QS) [23, 25, 38, 39] and the second messenger cyclic diguanylate (c-di-GMP) [40-43], which activates the expression of *Vibrio* polysaccharide (VPS), a major component of biofilms [13]. The precise mechanism of this regulation remains poorly understood. We propose to test the novel hypothesis that c-di-GMP enhances biofilm formation in *V. cholerae* by diminishing H-NS repression at *vps* promoters. Previous approaches to study the regulation of *vps* expression have relied on transcriptional profiling, the use of transcriptional reporters, and *in vitro* protein-DNA binding assays. These approaches, however, may not fully reveal the complexity of the protein-protein and protein-DNA interactions that occur in cells. For instance, electrophoresis mobility shift assays (EMSA) are not expected to reveal the stage of the DNA in the cell or the presence of other interacting proteins. An innovative aspect of our proposal is the use of chromatin immunoprecipitation (ChIP), a method to determine the physical interaction between a transcription factor and its target promoter in cells. ChIP is particularly suitable for regulators, like H-NS, that exhibit a broader DNA binding specificity. We recently developed a ChIP protocol to elucidate the interaction between H-NS and *V. cholerae* promoters that regulate motility and *vps* gene expression [26, 44]. With this protocol, we showed that H-NS binds to the promoters of three *vps* genes required for biofilm formation to repress their expression [26]. In this study, we propose to combine ChiP, EMSA, DNAse protection assays, *in vitro* transcription, regulatory mutants and confocal microscopy to determine the role of H-NS in biofilm development.

B.3. APPROACH

Overview of the transcriptional network controlling vps expression. The genes responsible for VPS biosynthesis are clustered in two operons, in which *vpsA* (UDP-N-acetylglucosamine 2-epimerase) and *vpsL* (glycosyltransferase) are the first genes of operons I and II, respectively [13]. The expression of these operons is regulated by QS and c-di-GMP signaling (reviewed in [20, 45]). QS is a cellular communication process involving the production, secretion, and detection of signaling molecules, known as autoinducers [46]. QS allows bacteria to sense their population density and synchronize their behavior to function socially [46]. At low cell density, the concentration of autoinducers is low, and *V. cholerae* expresses the QS regulator AphA [25], which activates the promoter of the *vps* gene activator, VpsT [23, 47, 48]. Expression of VpsT is also activated by a second transcription factor, VpsR [21, 47]. The second messenger c-di-GMP binds to VpsT and VpsR to enhance their activity [21, 49, 50]. At low cell density, the intracellular levels of c-di-GMP are relatively high [22], enhancing the activity of VpsR and VpsT [21, 50]. VpsT then activates VPS biosynthesis by binding to the *vpsL* promoter [49]. At high cell density, *Vibrios* express the QS regulator HapR [38, 51, 52]. Expression of HapR negatively influences VPS expression by lowering c-di-GMP levels [22, 53] and by directly repressing the *vpsT* and *aphA* promoters [21, 22]. An overview of the regulation of *vps* genes is shown in ***Fig.1***. In a previous study, we demonstrated that H-NS negatively affects biofilm formation [26]. H-NS is a global regulator and DNA-binding protein involved in structurally organizing the nucleoid of prokaryotic cells [54]. It consists of an N-terminal oligomerization domain connected by a flexible linker to a nucleic acid binding domain [54, 55]. Both oligomerization and DNA binding are required for the biological activities of H-NS [27]. In regulating transcription, H-NS negatively affects gene expression by binding to promoters exhibiting AT-rich, highly curved DNA regions [56].

Fig. 1. Regulatory network controlling vps gene expression. At low cell density, expression of the VPS matrix is positively enhanced by the QS regulator AphA and by c-di-GMP receptors VpsR and VpsT. At high cell density the QS regulator HapR diminishes c-di-GMP and represses the expression of *vps* genes. *Symbols:↓, positive regulation;⊥, negative regulation.*



B.3.1. Aim 1: To determine the mechanism of H-NS repression and anti-repression regulating V. cholerae vps operons I and II.

Preliminary data. We have demonstrated that H-NS represses the expression of *vpsA*-, *vpsL*- and *vpsT*-*lacZ* promoter fusions [26]. We purified *V. cholerae* H-NS and developed an anti-H-NS polyclonal antibody [44]. We found that H-NS expression can be detected at low cell densities concurrently with *vps* genes [44]. Further, EMSA demonstrated that H-NS binds to the *vpsA*, *vpsL*, and *vpsT* promoters *in vitro*. We used a *V. cholerae* strain expressing H-NS tagged with the FLAG epitope to conduct ChIP assays and thereby to determine the interaction between H-NS and these promoters in cells. As shown in ***Fig. 2***, H-NS occupied the *vpsA*, *vpsL*, and *vpsT* promoters. In these experiments, we used the *tcpA* promoter as a positive control, based on previous studies showing that this gene is transcriptionally silenced by H-NS [28]. As negative controls we used the promoter of VC1922, which is not regulated by H-NS, as determined in a qRT-PCR assay, and a DNA sequence located within the *rpsM* ORF, to which H-NS is not expected to bind as a repressor.

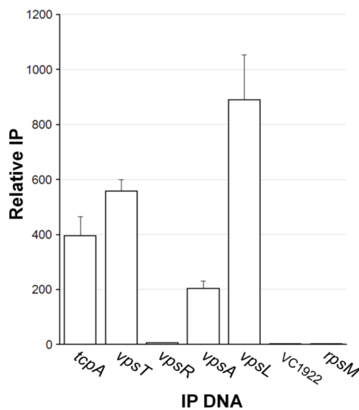


Fig. 2. ChIP analysis of H-NS occupancy of vps promoters. *V. cholerae C7258 expressing H-NS-FLAG was grown in LB medium at 37°C to OD_{600} 0.5. Quantification of H-NS occupancy at selected promoters was determined by qPCR using promoter-specific primers. H-NS occupancy is expressed as relative immunoprecipitation (IP), the amount of promoter DNA immunoprecipitated with anti-FLAG monoclonal antibody (mAb) standardized by the amount immunoprecipitated with the unrelated anti-Xpress mAb. Each value represents the mean of three experiments, and error bars indicate the standard deviations [26].*

H-NS repression is commonly counteracted by transcription factors that can disrupt its interaction with promoters and function as anti-repressors [28, 30, 44, 57-64]. As shown in ***Fig. 1***, AphA, VpsR, and VpsT positively affect *vps* transcription [21, 23, 49], making it likely that one or more of these regulators could function as an anti-repressor. In ***Fig. 3*** we show that (i) H-NS occupancy at the *vpsA* promoter is higher in a Δ*vpsT* mutant compared to wild type (Wt) (***3A***); (ii) Wt H-NS occupancy at the *vpsA* promoter is restored by genetic complementation with plasmid pTTQ18-*vpsT* upon induction with IPTG (***3A***); (iii) VpsT binds *in vitro* to the *vpsA* and *vpsL* promoters in a c-di-GMP-dependent manner (***3B***); and (iv) VpsT protein tagged with the FLAG epitope interacts with the *vpsA* and *vpsL* promoters in the cell when the c-di-GMP pool is artificially increased by ectopic expression of diguanylate cyclase (DGC) VCA0956 from the arabinose promoter in plasmid pAT1662 [43] (***3C***).

Hypothesis: based on these results we propose that VpsT functions as an anti-repressor of H-NS at the *vpsA* and *vpsL* promoters. Anti-repression could result from VpsT displacing H-NS from its DNA binding site, preventing H-NS oligomerization or a direct interaction between the VpsT and H-NS proteins. To test our hypothesis and elucidate the molecular mechanism of this regulation we propose to conduct the experiments described below.

Determine the sequence requirements for H-NS and VpsT regulation of vpsA and vpsL transcription First, we will determine the minimal DNA sequence required for a regulated transcription initiation at the *vpsA* and *vpsL* promoters. To this end, we will use PCR to amplify a series of 50-100-bp incremental deletions starting from the 5' end of the *vpsA* and *vpsL promoters*. The amplified fragments will be ligated to a promoterless *lacZ* gene as described in [44] and introduced in *V. cholerae* C7258Δ*lacZ*, C7258Δ*lacZ*Δ*hns*, C7258Δ*lacZ*Δ*vpsT and* C7258Δ*lacZ*Δ*hns*Δ*vpsT*. Then, we will measure β-galactosidase activity [65] as an indicator of promoter activity. This investigation will determine the minimum sequence required for full promoter activity and the minimum sequence for H-NS repression and for VpsT activation. In parallel, we will determine the transcription start site of *vpsA* and *vpsL* by primer extension mapping as described in [66]. This and other experiments proposed in this aim will be carried out by me in Dr. Charles Turnbough's lab at UAB.

Possible outcomes: H-NS could contain multiple sites of nucleation [56] along the promoters, and, by shortening them from the 5' end, the β-galactosidase activity could increase (reaching the derepressed levels observed in Δ*hns* mutant); until a point is reached at which some essential promoter element is removed. Alternatively, H-NS nucleation sites could be closely clustered so that most of the deletions that retain full promoter activity will exhibit similar levels of H-NS repression. With regard to VpsT binding determinants, β-galactosidase activity in the Δ*vpsT* background should be lower compared to Wt for fragments exhibiting H-NS

repression and retaining the VpsT binding site. Similarly, fragments exhibiting H-NS repression and lacking the VpsT binding site should express similar and low activities. Finally, if VpsT acts as an anti-repressor, promoter activities should be similar in Δ*hns* and Δ*hns*Δ*vpsT* mutants.

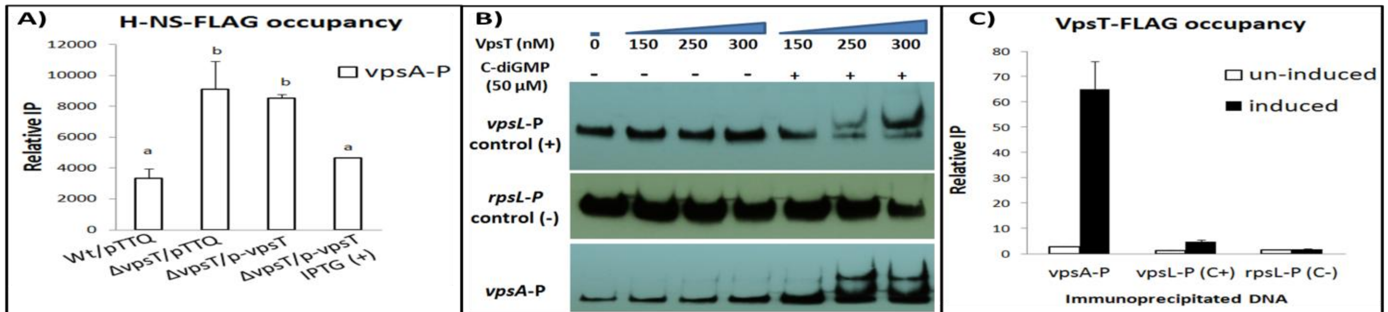


***Fig. 3**. **Regulation of the vpsA promoter by H-NS** **and** **VpsT. A. ChIP analysis of H-NS occupancy at the vpsA promoter**. From left to right: Strain C7258 expressing H-NS-FLAG with empty vector pTTQ18 [67] (Wt/pTTQ); isogenic vpsT deletion mutant with empty vector (ΔvpsT/pTTQ); vpsT mutant with pTTQ18-vpsT (ΔvpsT/p-vpsT); vpsT mutant with pTTQ18-vpsT induced by addition of 1 mM IPTG (ΔvpsT/p-vpsT IPTG +). Letters represent different statistical populations at P<0.05. **B. Binding of VpsT to DNA promoters.** DIG-labeled DNA fragments containing the vpsL- (positive control), rpsL- (negative control) and vpsA-promoters were incubated with increasing concentrations of purified VpsT and with or without 50 μM c-di-GMP. **C. ChIP analysis of VpsT occupancy at vps promoters**. Strain C7258 expressing VpsT-FLAG was transformed with plasmid pAT1662 encoding DGC VCA0956 expressed from the arabinose promoter. The c-di-GMP pool was artificially increased by addition of arabinose (0.2%). Immunoprecipitated DNA was quantified by qPCR using primers specific to the vpsA, vpsL and rpsL promoters.*

Determine the H-NS and VpsT binding sites at the *vpsA and vpsL promoters*

In this study we will determine the H-NS and VpsT binding sites at the *vpsA* and *vpsL* promoters by DNAse I footprinting [68]. Briefly, PCR fragments of ~300 bp will be generated with 6FAM-labeled primers as described in [69]. Binding reactions will consist of fluorescently-end-labeled DNA, purified H-NS or VpsT, c-di-GMP and the appropriate binding buffer [26, 49]. Control reactions will be incubated with BSA. After incubation, protein-DNA complexes will be digested with DNase I (several digestion optimization experiments will be tested using 2-10 ng/μL of DNase I). The reactions will be stopped by heat inactivation and DNA fragments purified with the PCR Purification kit (Qiagen). Digested DNA reactions will be mixed with GeneScan-500 LIZ size standards and analyzed in an automated fluorescent capillary electrophoresis instrument. In parallel, the promoters will be sequenced with the Thermo Sequenase Dye Primer Manual Cycle Sequencing kit (USB) and the FAM-labeled primer, and the entire sequencing reaction will be loaded onto the capillary electrophoresis instrument. Then, the Thermo Sequenase sequenced promoter and the DNase I digestion products will be aligned, through the use of the GeneMapper 3.7 and the size standards, to assign correct nucleotides to each peak from the DNA footprint.

Possible outcomes: Mapping of the H-NS binding sites will provide an insight into its repression mechanism. For instance, H-NS could (i) bind close to -35 and/or -10 promoter elements to block promoter recognition by RNA polymerase (RNAP) [70]; (ii) interact with a region sufficiently close to the sequence protected by RNAP, suggesting a protein-protein interaction that stalls the RNAP [71-75]; (iii) bind to a site shared with a specific activator of *vps* operon transcription [76], or (iv) trap RNAP within a hairpin by a DNA looping mechanism [77-81]. An overlap between VpsT and H-NS DNAse I protected sites would suggest that VpsT acts by hindering H-NS access to the promoter. Alternatively, the binding sites may not overlap each other, suggesting that VpsT could induce a conformational change upon binding to the DNA that weakens H-NS interaction or prevent its oligomerization along the promoter. Both mechanisms of H-NS anti-repression have been reported [28, 59, 60, 82-85].

Reconstruction of *vps* operon transcription regulation *in vitro*

In this section we propose to conduct a competitive EMSA between H-NS and VpsT at the *vpsA and vpsL* promoters. We have purified VpsT protein from *E. coli* using the IMPACT-CN system (NEB). The assay will be performed by adding either protein to pre-bound DNA complexes of the other. Briefly, DNA will be pre-incubated with the lowest concentrations of H-NS (66 nM) or VpsT (350 nM) that gives a complete DNA band shift. After 15 min at 30°C, increasing amounts of H-NS or VpsT will be added and incubation continued for an additional 15 min. The reaction mixture will be resolved by polyacrylamide gel electrophoresis and DIG-labeled DNA bands detected by enhanced chemoluminescence. This experiment will determine if one protein inhibits binding of the other, provided that the mobility of each protein-DNA complex is different. If the electrophoresis mobilities of the DNA complexes are indistinguishable, we will identify the H-NS-DNA complexes using an anti-H-NS polyclonal antibody to determine if H-NS is displaced from the DNA.

Finally, we will characterize the transcriptional regulation of *vps* operon promoters by H-NS and VpsT by *in vitro* transcription [86] using purified components with reconstituted RNA polymerase carrying σ^{70} (RNAP-σ^{70}).

DNA templates and purified RNAP-σ[70] will be prepared in the Turnbough's lab [87]. The kinetics of transcript biosynthesis will be followed in the presence-absence of each regulator (H-NS and VpsT). The effect of preventing the binding of VpsT to the promoter on the transcription will be examined by excluding c-di-GMP from the reaction mixture.

Possible outcomes: *In vitro* reconstruction of the interplay between H-NS and VpsT in the regulation of *vps* operon transcription will reveal the anti-repression mechanism. For instance, the EMSA studies will determine if VpsT diminishes H-NS access to an overlapping binding site on the DNA, or if it inhibits H-NS oligomerization from a different site leading to the formation of VpsT-DNA-H-NS complexes with intermediate mobilities. The functional significance of these interactions will be corroborated with *in vitro* transcription. Importantly, this assay will confirm whether VpsT functions as anti-repressor or if it posses additional enhancer functions. In addition, testing transcription in the absence of c-di-GMP should confirm that VpsT binding to the DNA is critical to H-NS anti-repression, since c-di-GMP-mediated oligomerization interface is critical for DNA binding and VpsT function [49].

Anticipated problems and solutions. Methods used in this aim will be carried out at Turnbough's lab as an additional training. Assays like primer extension mapping, *in vitro* transcription and the purification of RNAP-σ[70] from *E. coli* strains are done routinely at the Turnbough's lab providing confidence for additional troubleshooting. We are going to use a novel DNA footprint analysis that uses fluorescently labeled primers and sequencing on an automated capillary electrophoresis instrument [69]. The sequencing and alignment steps of the protocol will be provided as a service for fee at the UAB Heflin Center for Genomic Sciences Genomic Core. Should we have technical issues with the new protocol we will use the standard assay using radioactively labeled nucleotides and polyacrylamide slab gel electrophoresis [68].

B.3.2. Aim 2: To determine the relationship between c-di-GMP induction of vps genes and H-NS repression.

Preliminary data: Transcription of *vpsT* is up-regulated in response to an increase in c-di-GMP content [40]. Induction of the *vpsT* promoter by elevated c-di-GMP results from binding of the second messenger to VpsR, which in turn acts at *vpsT* and *aphA* promoters to enhance their transcription [21]. We have shown that H-NS binds to the *vpsT* promoter *in vitro* and *in vivo* to repress its transcription [26]. In ***Fig. 4*** we show that artificially increasing the c-di-GMP pool results in a 22-fold enhancement of biofilm formation in Wt *V. cholerae* but only a 3.6-fold induction in an isogenic Δ*hns* mutant. This result suggests that c-di-GMP enhances biofilm formation by inducing an anti-repression cascade starting at the *vpsT* promoter (see *Fig.1*).

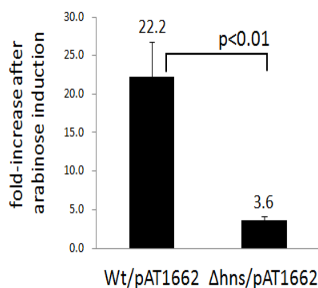


Fig. 4. H-NS and c-di-GMP reciprocally control biofilm formation. *Cyclic-di-GMP enhancement of biofilm was determined in Wt V. cholerae and Δhns* mutant *strains carrying pAT1662 [43] using the crystal violet assay* [88, 89] *. The c-di-GMP pool is increased upon addition of arabinose 0.2%. Bars represent the mean of six independent cultures, and error bars the standard error of the mean.*

Hypothesis: based on our preliminary data we hypothesize that c-di-GMP enhances biofilm development by releasing the *vpsT* promoter from H-NS repression. VpsT then acts to release the *vpsA* and *vpsL* promoters from H-NS repression (Aim 1). To test our hypothesis we will conduct the experiments described below.

H-NS occupancy at the vpsT promoter in regulatory mutants. In this section we will examine if any of the transcription factors known to directly interact with the *vpsT* promoter diminishes the H-NS promoter occupancy. To this end, we have constructed Δ*aphA*, Δ*vpsR*, and Δ*vpsT* mutants from *V. cholerae* C7258 by allelic exchange using the suicide vector pCVD442 and sucrose selection [90, 91]. The deletion mutants were confirmed by DNA sequencing and were used to integrate the *hns-flag* allele into the *hns* locus by homologous recombination to express H-NS-FLAG from native transcription/translation signals as described in [44]. The mutants expressed levels of H-NS-FLAG similar to the Wt precursor (data not shown). For ChIP, Wt and mutants strains expressing H-NS-FLAG will be grown in LB to mid-exponential phase, and protein-DNA interactions will be captured by cross-linking cells with formaldehyde, followed by cell lysis and fragmenting the chromosomal DNA to a 300-400 bp average size. Protein-DNA complexes will be immunoprecipitated (IPed) by incubation with anti-FLAG M2 mAb (Sigma) or the unrelated anti-Xpress mAb (Invitrogen) for a mock ChIP and pulled down with Protein A/G agarose beads (Imgenex). The beads will be collected, washed, and IPed complexes will be eluted by incubation in TE buffer containing 1% SDS (65°C, 30 min). IPed DNAs will be purified and qualitatively detected by PCR and agarose gel electrophoresis using primers specific to the promoter being tested. Real-time quantitative PCR (qPCR) will be used to quantitate promoter occupancy by H-NS-FLAG. The quantity of IPed DNA is calculated as the percentage of promoter DNA present in an input sample (prior to IP), using the formula: $IP = 2^{(Ct_{input} - Ct_{IP})}$, where Ct is the fractional threshold cycles of the input and IP samples. The relative IP is calculated by standardizing the IP of each sample by the IP of the corresponding mock ChIP.

Possible outcomes: If deletion of *aphA*, *vpsR,* or *vpsT* enhances H-NS occupancy at the *vpsT* promoter, we will conclude that the deleted regulatory gene encodes a putative anti-repressor since they are known to bind

to and directly activate the *vpsT* promoter. For genetic complementation we will use PCR to clone *aphA*, *vpsR* or *vpsT* and express these regulators under the control of the inducible Lac promoter in pTTQ18 [67] as described above for complementation of the Δ*vpsT* mutant (**Aim 1, *Fig. 3***). Genetic complementation of a regulatory mutant with its deleted allele encoding the anti-repressor should restore Wt H-NS occupancy levels at the *vpsT* promoter. If deleting known activators of *vpsT* do not result in increased H-NS occupancy at its promoter, we will follow a different approach outlined in the next section.

Alternative approach to identify transcription factors that antagonize H-NS at the vpsT promoter. We will use the Dynabeads® Streptavidin Trial Kit (Invitrogen) for isolating sequence-specific DNA- and RNA-binding proteins. Briefly, we will amplify the *vpsT* promoter region by PCR and label the DNA fragments with biotin-dUTP using terminal transferase (Roche). The labeled DNA target will be immobilized onto the streptavidin-coupled Dynabeads and incubated with a lysate of strain C7258Δ*hns*. Regulatory proteins bound to the promoter DNA will be washed and isolated using magnetic separation. Then, bound proteins will be eluted and characterized by polyacrylamide gel electrophoresis and identified by mass spectrometry (MS) at the UAB Targeted Metabolomics and Proteomics Laboratory (see letter of support). A similar experiment will be conducted using a lysate supplemented with 50 and 500 µg/mL of purified H-NS protein. Hits will be determined using a MS relative abundance (ratio) assay and will be evaluated by constructing deletion mutants and measuring the effect of deleting the corresponding promoter-binding protein on *vpsT-lacZ* expression and biofilm formation in wild-type and Δ*hns V. cholerae*.

Possible outcomes: In a hit representing an anti-repressor, the presence of H-NS in the lysate should diminish its interaction with the labeled promoter in a dose-dependent manner. In this case, we opted for a MS relative abundance assay, using an AB SCIEX TripleTOF® 5600 System, which can detect proteins in the attomole range (10^{-18} moles). This analysis will allow us to identify proteins that are noticeably more abundant in one treatment versus another (Δ*hns vs.* H-NS-supplemented lysates) with appropriate sensitivity to postulate possible interactions that will be further analyzed as outlined in aim 1.

Effect of c-di-GMP on the H-NS occupancy at vps promoters. The objective of these experiments is to directly prove that c-di-GMP enhances biofilm by releasing *vps* promoters from H-NS repression. In this section we will determine H-NS occupancies at *vpsA, vpsL* and *vpsT* promoters upon artificially increasing the c-di-GMP pool**.** The c-di-GMP pool will be increased by inducing the expression of the soluble DGC, VCA0956, from the arabinose promoter [43] with an inducing range of arabinose (*i.e.* 0.002-0.2%). This strategy has been shown to increase the intracellular c-di-GMP concentrations in a dose-dependent manner [21]. *V. cholerae* C7258/*hns::hns-flag* will be transformed with plasmid pAT1662 encoding VCA0956. The transformants will be grown in LB to OD_{600} 0.3 with L-arabinose to increase c-di-GMP (doses range: 0.002 to 0.2% and an un-induced control). H-NS promoter occupancy will be determined by ChIP, as described above (***Fig. 2***). If we find that increasing c-di-GMP diminishes H-NS promoter occupancies in a dose-dependent manner, we will conclude that the second messenger enhances biofilm formation by inducing the expression and/or activity of an anti-repressor. The same analysis will be done in strains lacking the c-di-GMP-receptor proteins VpsR and VpsT [21, 49]. To this end, we will transform pAT1662 into C7258/*hns::hns-flag* containing deletions of *vpsT* and/or *vpsR* and repeat the ChIP as above. We will further test our hypothesis by increasing the c-di-GMP pool in Wt and *hns* mutants containing chromosomally integrated *vpsA*-, *vpsL*- and *vpsT-lacZ* promoter fusions. The strains will be grown in LB at 37°C to mid-exponential phase, the c-di-GMP pool will be enhanced (arabinose range 0.002 to 0.2% and an un-induced control) and β-galactosidase activity will be measured as an indicator of promoter activity. If our hypothesis is correct, increasing the c-di-GMP pool should have a significantly smaller effect on the activity of these promoters in the Δ*hns* genetic background compared to the Wt strain.

Possible outcomes: Based on our results, we expect to find a direct antagonistic relationship between increasing c-di-GMP and H-NS occupancy and repression at the *vps* promoters studied. Similarly, we expect that c-di-GMP will not affect H-NS promoter occupancy in the receptor mutant strains. In this case we will conclude that VpsR and/or VpsT, when induced and activated by c-di-GMP, act as H-NS anti-repressors. We acknowledge the possibility of an unknown factor functioning as anti-repressor at the *vpsT* promoter (which we proposed above to identify by MS). This hypothetical factor might not be a c-di-GMP binding protein. In this case we would conclude that c-di-GMP acts by inducing VpsT to release the downstream *vpsA* and *vpsL* promoters from H-NS repression.

Anticipated problems and solutions. In this aim we predict that c-di-GMP enhances biofilm formation by inducing an anti-repression cascade starting at the *vpsT* promoter. We propose to study the H-NS occupancy by ChIP at the *vpsT* promoter in key regulatory mutants for c-di-GMP signaling. I have constructed the mutant strains and determined that they express similar levels of H-NS-FLAG. An alternative approach is considered in case none of the known positive direct regulators of *vpsT* expression functions as H-NS anti-repressor. In this case we will use a MS assay, which features the required sensitivity to identify protein abundance in the different conditions to be tested. We will define the H-NS-anti-repression mechanism at the *vpsT* promoter as outlined in *aim 1*. We have used DGC VCA0956 and liquid chromatography coupled to mass-spectrometry (LC/MS) to enhance and measure c-di-GMP content, respectively. Since we do not have an active site mutant

of VCA0956 to use as a control, results will be confirmed by increasing the c-di-GMP content by over-expression of the DGC, QrgB, which is alien to *V. cholerae* [22]. Further, we will use a dose-response approach when increasing intracellular c-di-GMP to determine if H-NS occupancy of *vps* promoters is decreased in a dose-dependent manner within physiological c-di-GMP concentrations. The exact c-di-GMP content will be determined by LC/MS as we reported before in [53].

B.3.3. Aim 3: Molecular architecture and properties of hns biofilms.

Preliminary data: The spatial organization and properties of *Vibrio* biofilms are determined by the polysaccharide and protein constituents of the extracellular matrix [92]. In a recent RNA-Seq analysis conducted in the Benitez laboratory it was found that H-NS represses the expression of two major matrix proteins: RbmA (Δ*hns*/Wt ratio: ~10, p=1.5E-05) and RbmC (Δ*hns*/Wt ratio: ~12, p=3.3E-06). Extracellular DNA (eDNA) is another important component of the biofilm matrix [93] and biofilms of *E. coli hns* mutants lack this component [94]. In *V. cholerae*, eDNA plays an important role in biofilm architecture, detachment and the fitness of biofilm clumps in the host [93]. Based on these evidences, we hypothesizes that *V. cholerae hns* biofilms could differ substantially from wild type in molecular architecture, eDNA content, stability and infective dose. To test this hypothesis we will conduct the experiments described below.

Molecular architecture and composition of hns biofilms. *V. cholerae* C7258 and derivative Δ*hns* cells will be allowed to form static biofilm in LB from 4 to 24 h, and the biofilm will be stained with Syto-9 and examined by laser scanning confocal microscopy (LSCM), as described in [26]. A quantitative analysis of the structural parameters defining mature tridimensional biofilms [5, 6, 39] will be performed by analyzing image stacks with the aid of the software COMSTAT [95]. With COMSTAT we will analyze the biofilm biomass, maximum thickness, roughness coefficient and the average diffusion distance. These parameters provide measures of quantity, biofilm heterogeneity [95] and the distance over which fluids diffuse [96]. Extracellular DNA will be quantified as described previously [97] and visualized on agarose gels (0.8%) and in LSCM analysis by staining with BOBO-3 (Invitrogen), which is membrane impermeable and specifically stains eDNA [98]. VPS will be visualized by staining with Cy3-labeled wheat germ agglutinin (NANOCS), which recognizes N-acetylglucosamine residues in the VPS [99].

Stability of hns biofilms. The stability of Wt and *hns* biofilms will be studied using a detachment assay [88]. Briefly, static biofilms will be allowed to form over 40 h and the planktonic cells-containing supernatants will be replaced by phosphate buffered saline pH7.4 (PBS). The percent of cells detached from the biofilm will be determined at different time periods (1-4 h) by plate counting. As a control, biofilms will be mechanically dispersed with glass beads and detachment expressed as a percentage of cells from fully dispersed biofilms.

Comparison of the colonization fitness of Wt and hns biofilms in vivo. We will perform suckling mice competitive colonization assays to compare the magnitude of biofilm-induced hyperinfectivity in Wt and Δ*hns* *V. cholerae*. These experiments will be performed at SRI (IACUC approval # 12-07-038B) as described in [100]. Briefly, 4 to 5-day-old CD-1 mice will be starved for 6 h and inoculated by oral gavage with 50 µL of inoculum. To prepare the inocula, biofilm-derived cells (*lacZ*$^-$) and planktonic cells (*lacZ*$^+$) will be mixed at 1:1 ratios in LB broth to a final titer of 10^5 colony forming units (CFU). It is well established that *lacZ* mutation had no intrinsic effect on the infectivity of *V. cholerae* [19, 93, 101]. The exact input ratio will be determined by differential counting on LB agar containing X-Gal. Inoculated mice will be maintained for 16 h in a styrofoam box at 30°C, then euthanized and the small intestine will be dissected and homogenized in 5 mL of PBS. The homogenates will be appropriately diluted and plated on LB agar containing X-Gal. The competitive index (CI) is calculated as the white/blue (biofilm/planktonic) output ratio normalized to the white/blue input ratio. To obtain biofilm-derived cells, biofilms will be allowed to form under static conditions in LB and in borosilicate glass tubes for 48 h at 30°C. Then, the biofilms will be rinsed with LB, removed mechanically from the glass tubes and adjusted in fresh LB to the desired concentration. For planktonic cells, the respective strain will be grown 16 h in LB and then adjusted to the desired concentration.

Possible outcomes: We expect that Δ*hns* biofilms will mature faster than Wt due to enhanced secretion of VPS and matrix proteins that dictate the architecture of mature tridimensional biofilms. Similarly, we expect that the overall architectural parameters and detachment from the biofilm will be significantly affected in Δ*hns* strains, since *hns* mutations are pleiotropic [102] and H-NS could be required for eDNA production as described in *E. coli* [94]. The CI biofilm/planktonic should be $>$ 1 for both, Wt and Δ*hns* mutant, consistent with a report demonstrating that growth in biofilms induces a hyperinfectious phenotype [19]. However, the hyperinfectivity of Wt and Δ*hns* biofilms, compared to their corresponding planktonic counterparts, could differ significantly depending on their respective molecular architecture, composition and stability.

Anticipated problems and solutions. The use of LSCM in this section surpasses the crystal violet assay to address biofilm architecture and will serve as an additional training. Dr Rexford Asare at SRI, an expert in confocal microscopy and imaging, has agreed to provide technical and analytical assistance.

SUMMARY STATEMENT
(Privileged Communication)

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Release Date: **08/06/2013**

Application Number: **1 F31 AI106288-01A1**

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Review Group: **ZRG1 F13-C (20)**
Center for Scientific Review Special Emphasis Panel
Fellowships: Infectious Diseases and Microbiology

Meeting Date: **07/18/2013**
Council: **OCT 2013**
Requested Start:

PCC: **X79A B**
Dual PCC: **M000SD**
Dual IC(s): **GM**

Project Title: **Regulation of Vibrio Biofilm Formation by H-NS Repression and Anti-repression.**

Requested: **4 years**

Sponsor: **Turnbough, Charles L**
Department: **Dept. of Microbiology**
Organization: **UNIVERSITY OF ALABAMA AT BIRMINGHAM**
City, State: **BIRMINGHAM ALABAMA**

SRG Action: **Impact Score: 20**
Next Steps: **Visit http://grants.nih.gov/grants/next_steps.htm**
Human Subjects: **10-No human subjects involved**
Animal Subjects: **30-Vertebrate animals involved - no SRG concerns noted**

1F31AI106288-01A1 Ayala-Figueredo, Julio

RESUME AND SUMMARY OF DISCUSSION: Revisions have improved this fellowship application from an excellent applicant seeking training in bacterial pathogenesis with a project that explores the regulation of biofilm formation in *Vibrio cholera*. Enthusiasm for the applicant is based on a strong academic record, good productivity, excellent letters and significant research experience. The addition of the co-sponsor, in this revision, is a strength and the sponsors and collaborators are considered excellent with strengths in research and training. The project is important and is supported by preliminary data. Improvement in the experimental plan, from revision, has raised enthusiasm for the application. Minor concerns are expressed over the third aim, however, based on a perception that the goals are somewhat superficial and unrelated to the focus of the project. The environment is excellent and there is confidence that the applicant will learn a variety of valuable skills.

DESCRIPTION (provided by applicant): Cholera, a waterborne disease caused by Vibrio cholerae of serogroups O1 and O139, is characterized by the passage of voluminous, rice-watery stools. In endemic areas in Asia and Africa, this disease continues to be a public health concern, with a prevalence of 5 million cases and 130,000 deaths per year. A major obstacle to the eradication of cholera is the persistence of V. cholerae in aquatic environments, which is facilitated by its capacity to form biofilms. Vibrios in biofilms are more resistant to environmental stresses, including biocides and disinfectants. Further, Vibrios in a biofilm exhibit a hyperinfective phenotype that facilitates the rapid dissemination of the disease in outbreaks. Thus, a better understanding of the factors that regulate biofilm formation is important for the prevention and control of cholera. The genes responsible for the biosynthesis of the Vibrio polysaccharide (VPS) extracellular matrix, a major component of biofilms, are located in two operons, in which vpsA and vpsL are the first genes of operons I and II, respectively. These genes are regulated by quorum sensing, the second messenger cyclic diguanylate (c-di-GMP), and by the transcription factors AphA, VpsR and VpsT. We recently showed that the histone-like nucleoid structuring protein (H-NS) directly represses the expression of genes involved in the biosynthesis of the VPS matrix. A common theme in H-NS transcriptional silencing is the presence of anti-repressors that disrupt its interaction with DNA promoters. The objective of this application is to test the novel hypothesis that c-di-GMP enhances biofilm formation in V. cholerae by activating the expression/activity of H-NS anti-repressors acting at vps promoters. To this end, we will characterize the interaction between H-NS and the c-di-GMP-receptor protein VpsT in the regulation of the vpsA and vpsL promoters using a combination of genetic and molecular biology approaches (Aim 1). In Aim 2, we will identify the transcription factor(s) capable of antagonizing H-NS repression at the vpsT promoter and use chromatin immunoprecipitation (ChIP) to demonstrate that elevated c-di-GMP intracellular content initiates an anti-repression cascade at the vpsT promoter that is subsequently transmitted to the downstream vpsA and vpsL promoters. Finally, in Aim 3, we will use confocal microscopy and a mouse competitive colonization assay to characterize the molecular architecture, composition and properties of the biofilm expressed in V. cholerae hns mutants.

PUBLIC HEALTH RELEVANCE: Biofilm formation is involved in cholera transmission and in survival of pathogenic Vibrio cholerae in aquatic environments. With this research effort, we will determine the mechanism by which a bacterial regulator, known as the histone-like nucleoid structuring protein, modulates this process.

CRITIQUE 1:

Fellowship Applicant: 2
Sponsors, Collaborators, and Consultants: 2
Research Training Plan: 3
Training Potential: 3
Institutional Environment & Commitment to Training: 2

Overall Impact/Merit: This is a revised submission from a second year applicant at the University of Alabama at Birmingham that proposes to test the hypothesis that c-di-GMP enhances biofilm formation in *V. cholerae* by diminishing H-NS repression at *vps* promoters. The applicant was responsive to previous reviewers, has been very productive, and has submitted an improved research plan. The environment is outstanding, as are the sponsor and collaborators. The only part of the application that dampened enthusiasm was the third aim of the research training proposal. It wasn't clear why the applicant shifted focus from VPS to e-DNA in the third aim. Another issue with the third aim is that the interpretation of results from the virulence studies may be difficult due to the pleitropy of the *hns* mutant. The proposed virulence studies are only the first step and it isn't clear what new information will be obtained from doing them. There are many missed opportunities in the proposed virulence studies.

1. Fellowship Applicant:

Strengths

- The applicant has contributed to three publications in two years, that lay the groundwork for the proposal indicating that he is already productive in this laboratory.
- The applicant has a strong academic background with a few Cs but mostly As and Bs.
- The applicant has a broad research background, for a second year graduate student, with publications and Abstracts in *Streptomyces* and endocrinology as well as *Vibrio.*

Weaknesses

- None

2. Sponsors, Collaborators, and Consultants:

Strengths

- The sponsor is well supported by the Southern Research Institute and the NIH on separate projects.
- The sponsor has mentored 26 predoctoral and postdoctoral scientists.
- Dr. Turnbough has mentored 35 predoctoral and postdoctoral scientists and is a major collaborator on this project.
- Dr. Turnbough brings expertise in the area of transcriptional regulation to the project that was missing from the previous submission.
- The applicant has enlisted Dr. Ascare as a consultant for the LCSM for biofilm imaging.
- The applicant has also enlisted Dr. Barnes for help from the Targeted metabolomics and Proteomics Laboratory.

Weaknesses

- Dr. Turnbough has no experience with *Vibrio*, per se, but this is a really minor weakness due to the similarities between *Vibrio* and *Escherichia*.

3. Research Training Plan:

Strengths

- There are three recent publications supporting the feasibility of the proposed research training plan.

- The applicant has provided additional preliminary data supporting the training plan.
- The applicant has included alternative experiments in the second aim to bolster the proposed CHiP experiments.
- The third aim will provide additional training opportunities for the applicant in examining biofilms.
- The third aim will address the question of relevance for HNS in an infection model.

Weaknesses

- Despite the inclusion of additional experiments in the second aim, there is still a very heavy reliance on CHiP for the proposed research training plan. The proposed alternative experiments will likely more directly test the hypothesis proposed n the second aim.
- The third aim proposes experiments on eDNA wheras the first and second aims are focused on vps expression. It isn’t clear why there is this shift in focus.
- The pleitropy of the *hns* mutant will make interpretation of the virulence data proposed in the third aim challenging.

4. Training Potential:

Strengths

- The applicant will learn how to analyze biofilm structure.
- The applicant will also determine how to assess virulence for *Vibrio* with the proposed research plan.

Weaknesses

- The proposal is almost CHiP-centric and the applicant appears to have already mastered this technique.

5. Institutional Environment & Commitment to Training:

Strengths

- The institution’s resources and commitment appear to be outstanding.

Weaknesses

- None

Protections for Human Subjects:

Not Applicable (No Human Subjects)

Vertebrate Animals:

Acceptable

- All issues are addressed.

Biohazards:

Acceptable

- The PI's lab has been working with Vibrio cholerae for many years. The laboratory is equipped for this BSL2 organism.

Resubmission:

- The applicant answered previous queries with more data and responded well to the weaknesses raised.

Training in the Responsible Conduct of Research:

Acceptable

Select Agents:

Not Applicable (No Select Agents)

Budget and Period of Support:

Recommend as Requested

CRITIQUE 2:

Fellowship Applicant: 1
Sponsors, Collaborators, and Consultants: 2
Research Training Plan: 3
Training Potential: 4
Institutional Environment & Commitment to Training: 1

Overall Impact/Merit: This application is by a promising young scientist with a history of research experience. The letters attest that the applicant is of high quality and serious about his research. This resubmission is improved in many ways. Adding Dr. Turnbough as a co-sponsor to help conduct and interpret experiments in aims 1 and 2 strengthened the proposal. The main hypothesis being tested in aims 1 and 2 seems reasonable and the experiments, for the most part, doable. Addition of the biofilm experiments in aim 3 largely seem confirmatory. The sponsor is well suited to advise the applicant on this research. The training potential is moderate. It seems that much of the methodologies being employed in this proposal are already mastered by the applicant. More detail from the sponsor on how he would groom the applicant to be an independent scientist would have been nice, although this was a minor concern.

1. Fellowship Applicant:

Strengths

- The letters are uniformly excellent, with hard-working and imaginative/creative consistently used to describe him.
- The applicant has significant research experience that has resulted in a number of publications which is excellent for someone at this stage of his career.
- The applicant has received two fellowships to study abroad in Belgium.

Weaknesses

- Grades were a little weak as an undergraduate, however they have steadily improved.

2. Sponsors, Collaborators, and Consultants:

Strengths

- Enlisting Dr. Turnbough, an experienced prokaryotic molecular biologist, as a co-sponsor is a positive.
- The primary sponsor, Dr. Benitez, has a solid publication record pertaining to V. cholerae and Hns.
- The sponsor appears to have funding through 2016.

Weaknesses

- None noted.

3. Research Training Plan:

Strengths

- The preliminary data provides a strong rationale for the hypothesis to be tested in aim 1.
- Biofilm formation by V. cholerae is a key aspect of its pathogenesis, thus understanding regulation of the vps polysaccharide genes is important.
- The applicant has had some good success showing that he is probably capable of carrying out much of the work described in aims 1 and 2.

Weaknesses

- The applicant does not address the possibility that VpsT can act both as an anti-repressor of hns and independently as an c-di-GMP dependent transcriptional activator.
- The proposal is not well written, with it being difficult to follow the logic behind what's being tested sometimes.
- Some preliminary data showing the approach using dynabeads to identify potential factors that antagonize Hns would be helpful.
- Aim 3 seems to provide limited information. Vps production is known to influence in vitro biofilm formation, so an effect would be expected. Focusing on eDNA seems to be peripheral to the main thrust of the research.

4. Training Potential:

Strengths

- The applicant will have an opportunity to do significant work at two different research institutions, which will broaden his research experience.
- The applicant will be introduced to some mammalian animal model work.
- The applicant will have access to seminars at both institutions.

Weaknesses

- The skill set to be gained by the applicants training seems to be somewhat limited since most of the experimentation described in aims 1 and 2 are techniques the applicant has already mastered.
- The training plan written by the sponsor is a bit light. What opportunities to be provided to the applicant (e.g. teaching/mentoring) are not clear.

5. Institutional Environment & Commitment to Training:

Strengths

- The addition of Turnbough at UAB is a positive, making most of the proposed research feasible.
- The letter from Dr. Asare indicates a willingness to help conduct the biofilm confocal microscopy experiments in aim 3.

Weaknesses

- None noted.

Protections for Human Subjects:

Not Applicable (No Human Subjects)

Vertebrate Animals:

Acceptable

- The five points are thoroughly addressed.

Biohazards:

Acceptable

Resubmission:

- The applicant did a nice job addressing most of the referees concerns.

Training in the Responsible Conduct of Research:

Comments on Format (Required):

- Has taken courses and will take courses at UAB.

Comments on Subject Matter (Required):

- Appropriate.

Comments on Faculty Participation (Required):

- unclear

Comments on Duration (Required):

- unclear

Comments on Frequency (Required):

- unclear

Select Agents:

Not Applicable (No Select Agents)

Budget and Period of Support:

Recommend as Requested

CRITIQUE 3:

Fellowship Applicant: 3
Sponsors, Collaborators, and Consultants: 2
Research Training Plan: 3
Training Potential: 2
Institutional Environment & Commitment to Training: 2

Overall Impact/Merit: An extremely well-written, hypothesis-driven research plan to determine the mechanisms of transcriptional regulation at promoters critical for expression of exopolysaccharide (VPS) in the important human pathogen *Vibrio cholerae*. A motivated applicant with several years of bench experience prior to graduate school and a training plan that includes additional course work. Addition of a senior co-sponsor with expertise in transcriptional regulation and plans for experiments in Aim 1 to be done in the co-sponsors lab increases the training potential.

1. Fellowship Applicant:

Strengths

- After BS in Biochemistry 2005 from Univ. Havana, has worked in several scientific research positions with biotechnology focus until starting graduate school is 2011. As a result, is a co-author on a number of research publication including two second-author papers most recently
- Strong scholastic record.
- Appears very motivated to pursue training in basic research with strong interest in translation. Has clearly laid out career goals.

Weaknesses

- None noted

2. Sponsors, Collaborators, and Consultants:

Strengths

- A primary mentor with very solid publication record in the field of the applicant's proposal and excellent record of mentorship.
- Well-respected and accomplished senior co-mentor with expertise in transcriptional regulation.
- Primary mentor funding primarily through internal institutional grant through early 2014 and an NIH contract for a specific project and a shared SC1 grant through mid-2016.

Weaknesses

- Primary mentor has applied for additional funding but unclear if funded.

3. Research Training Plan:

Strengths

- Aim 1 is hypothesis driven with sufficient preliminary data to support feasibility. The experimental approach is a well presented and comprehensive approach to elucidating transcriptional regulation by VpsT and H-NS at the vps I and II operon promoters. There is consideration of different possible results and of limitations and alternate approaches.

- Aims 2 and 3 are similarly well constructed with discussion of alternate approaches.

Weaknesses

- The proposal is not strong on innovation, although it is a clearly important transcriptional analysis of VPS expression.
- No mention of the statistical test used to assess data presented in figures.
- In Aim 3, resuspension of biofilm cells in fresh LB is very likely to alter their physiology. Why not resuspend in cell-free biofilm culture supernatant after removal of planktonic cells?

4. Training Potential:

Strengths

- Applicant has created a nicely detailed training plan for a combination of course-work, seminars, meetings and very appropriate conferences over the duration of the project.
- Sponsor's lab appears to consist of several postdoc-level and staff-level members, so the applicant will have experienced colleagues at hand for advice.
- Sponsor plans for applicant to attend national meetings.
- Many of the experiments proposed in Aim I will be done in the co-sponsor's lab. This will provide training in additional techniques that are commonly used in that lab and also increase the pool of scientists and trainees who are available for practical, day-to-day advice on experiments.
- The high quality of grant construction in the Research Plan likely reflects strong mentorship in this critical skill area.

Weaknesses

- Concern that coursework will consume a greater % effort than expected.
- Concern that the time needed for manuscript preparation for any first-author manuscript is very underestimated.
- Sponsor does not comment specifically on the training plan as laid out by the applicant, so it is unclear if sponsor is supportive of the course work proposed.

5. Institutional Environment & Commitment to Training:

Strengths

- Very strong academic institution.

Weaknesses

- None noted

Protections for Human Subjects:

Not Applicable (No Human Subjects)

Vertebrate Animals:

Acceptable

- VA section covers all elements of NIH VAS checklist.

Biohazards:

Acceptable

- Has received BSL-2 training.

Resubmission:

- Has addressed reviewer critiques well.

Training in the Responsible Conduct of Research:

Acceptable

Comments on Format (Required):

- 8/2012 completed a formal course at UAB.

Comments on Subject Matter (Required):

- Course covers the topics pertinent to NIH guidelines

Comments on Faculty Participation (Required):

- Formal course offered by University but does not specifically comment on faculty. Does comment on role of faculty and sponsor in informal discussion regarding RCR.

Comments on Duration (Required):

- 48 hours of instruction (3 credit hours)

Comments on Frequency (Required):

- course completed

Select Agents:

Not Applicable (No Select Agents)

Resource Sharing Plans:

Acceptable

- Any new bacterial mutants generated will be made shared after publication.

Budget and Period of Support:

Recommend as Requested

THE FOLLOWING RESUME SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE ON THE FOLLOWING ISSUES:

VERTEBRATE ANIMAL (Resume): ACCEPTABLE

SCIENTIFIC REVIEW OFFICER'S NOTES: Training in the responsible conduct of research was adequately described.

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

NIH has modified its policy regarding the receipt of resubmissions (amended applications). See Guide Notice NOT-OD-10-080 at http://grants.nih.gov/grants/guide/notice-files/NOT-OD-10-080.html.
The impact/priority score is calculated after discussion of an application by averaging the overall scores (1-9) given by all voting reviewers on the committee and multiplying by 10. The criterion scores are submitted prior to the meeting by the individual reviewers assigned to an application, and are not discussed specifically at the review meeting or calculated into the overall impact score. Some applications also receive a percentile ranking. For details on the review process, see http://grants.nih.gov/grants/peer_review_process.htm#scoring.

MEETING ROSTER

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CENTER FOR SCIENTIFIC REVIEW
Fellowships: Infectious Diseases and Microbiology
ZRG1 F13-C (20) L
July 18, 2013 - July 19, 2013

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Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.