Indole acts as an Extracellular Cue Regulating Gene Expression in *Vibrio cholerae*

Ryan S. Mueller¹, Sinem Beyhan², Simran G. Saini¹, Fitnat H.Yildiz² & Douglas H. Bartlett¹*

¹Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92037, United States of America

²Department of Environmental Toxicology, University of California, Santa Cruz, Santa Cruz, CA 95064, USA.

* Corresponding author:

Marine Biology Research Division,

4305 Hubbs Hall,

8750 Biological Grade,

Scripps Institution of Oceanography, University of California, San Diego

La Jolla, CA 92037

United States of America.

Tel: 1-858-534-5233

Fax: 1-858-534-7313

Email: dbartlett@ucsd.edu
Indole has been proposed to act as an extracellular signal molecule influencing biofilm formation in a range of bacteria. For this study the role of indole in V. cholerae biofilm formation was examined. It was shown that indole activates genes involved in Vibrio polysaccharide (VPS) production, which is essential for V. cholerae biofilm formation. In addition to these genes, it was determined using microarrays that indole influences the expression of many other genes, including those involved in motility, protozoan grazing resistance, iron utilization and ion transport. A transposon mutagenesis screen revealed additional components of the indole - VPS regulatory circuitry. The indole signaling cascade includes the DksA protein along with known regulators of VPS production, VpsR and CdgA. A working model is presented in which global control of gene expression by indole is coordinated through σ54 and associated transcriptional regulators.
INTRODUCTION

Bacterial cells synthesize myriad small organic molecules to signal and adapt to environmental, physiological and population structure changes. These molecules include extracellular signals such as acyl homoserine lactones, butyrolactones, quinolones, a furanosyl borate diester, oligopeptides, 3-hydroxypalmitic acid methyl ester, and a hydroxyketone; and intracellular signals including cyclic nucleotides and ppGpp (25) (reviewed in 11). The phenotypic response to signaling compounds often involves traits that are beneficial under adverse conditions, such as biofilm formation, virulence, motility, bioluminescence, sporulation, competence and modification of carbon and energy utilization and macromolecule biosynthesis.

Indole is a relatively recent addition to the list of signaling molecules used by bacteria, and is produced as a by-product of the break down of tryptophan by the enzyme tryptophanase (TnaA, 18). Since the expression of the tnaA gene is controlled by catabolite repression, it is only transcribed during carbon limitation (57). As a result of this regulation, large quantities of indole are produced during the stationary phase of growth. Indole has long been known to act as a chemorepellent of E. coli (52), but only more recently indole has also been shown to control the expression of a wide assortment of genes and phenotypes unrelated to chemotaxis in many different bacteria. For example, in E. coli indole controls the expression of genes involved in amino acid metabolism (54), plasmid maintenance (13) and quorum sensing (32) among other functions (14). Additionally, indole may function as an inter-species signal contributing to biofilm formation in an assortment of different bacteria known to carry a copy of the tryptophanase gene (36). In E. coli specifically, the indole-driven transcriptional
response, which controls subsequent biofilm formation, is known to be controlled by the quorum sensing transcriptional regulator SdiA (32).

It was previously shown that control of biofilm formation by indole also extends to the etiological agent of the pandemic disease cholera, *Vibrio cholerae*. Transposon insertions in the tryptophanase gene contained within the genomes of two environmental strains of *V. cholerae* were shown to result in diminished biofilm formation by each mutant, and that supplementation of indole in the growth medium was able to complement the biofilm defect of these strains (38). Due to the reliance of these strains on the production of *Vibrio* polysaccharide (VPS) for biofilm formation, it was theorized that indole was influencing the regulation of VPS production.

The genes encoding the enzymes that catalyze VPS synthesis are contained within two operons within the *V. cholerae* genome (*vps*-I and *vps*-II predicted operons), and their regulation involve multiple transcriptional activators and repressors. The main activator of VPS production appears to be the $\sigma^{54}$-dependent transcriptional activator VpsR, which is essential for *vps* gene expression (58) and is a distant homolog of SdiA from *E. coli*. A secondary activator of *vps* genes is VpsT, which is not essential for *vps* transcription, but acts synergistically with VpsR to activate expression (6). Antagonizing these activities is the master transcriptional regulator of quorum sensing in *V. cholerae*, HapR, which is translated when autoinducer molecules accumulate in the extracellular environment. Since HapR is a repressor of *vps* expression, it is thought that quorum sensing acts to down-regulate VPS production and biofilm formation once the cell density increases above a given threshold (23). Superimposed on these regulatory mechanisms are proteins with GGDEF and/or EAL domains, which modulate
intracellular levels of the second messenger cyclic di-guanylic acid (c-di-GMP, 34, 51). C-di-GMP influences the regulators described above and ultimately many genes involved with motility, chemotaxis, virulence and biofilm formation (7). Thus, the regulation of processes such as biofilm formation is multi-faceted, depending on a variety of extracellular and intracellular signal molecules.

In this study it is shown that extracellular indole is also used as a signal in V. cholerae, and that it influences the expression of many different types of genes, including those involved in transport, virulence, biofilm formation and motility. Evidence is also provided that indole signaling proceeds through the RNA polymerase regulatory protein DksA, the VPS regulators VpsR and VpsT and the c-di-GMP second messenger system.

**MATERIALS AND METHODS**

**Strains, plasmids and growth conditions.** All strains and plasmids used in this study are listed in Table 1. Wild-type strains SIO and TP of V. cholerae were isolated from southern California coastal waters. All Escherichia coli and V. cholerae strains were grown in LB broth (37) supplemented with appropriate antibiotics at 37 °C, except when stated otherwise. Antibiotics used in this study were kanamycin (Km, 50 µg/mL for E. coli; 200 µg/mL for V. cholerae), chloramphenicol (Cm, 20 µg/mL for E. coli; 5 µg/mL for V. cholerae), gentamycin (Gm, 50 µg/mL), and ampicillin (Ap) and rifampicin (Rif) at a concentration of 100 µg/mL.

**V. cholerae mating.** E. coli strain S17-1λpir (46) was used as a donor for all conjugation experiments with strains of V. cholerae. All strains were grown overnight to stationary phase at 37°C. V. cholerae were subcultured at 1:100 dilution and grown in
LB medium at 22 °C until the mid-exponential phase of growth was reached (OD$_{600}$nm ~ 0.7). *E. coli* and *V. cholerae* (if necessary) were washed of antibiotics by centrifugation (2 minutes, 13,000 x g) and resuspended in equal amounts of fresh LB. One milliliter of washed *E. coli* was then added to 4 mL of *V. cholerae* and briefly vortexed. This mixture was then vacuum filtered onto a sterile membrane (0.45 µm pore size, 47mm diameter), which was placed atop of a LB agar plate and left overnight at 37 °C. Membranes were then transferred to tubes containing 10 mL of LB medium and cells were removed by vortexing. Dilutions were plated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS, 89 g/L, Difco) or LB agar, both supplemented with appropriate antibiotics, and grown at 37 °C overnight.

**DNA manipulations.** All PCR reactions were carried out with Expand High-Fidelity PCR kits (Roche) or Taq polymerase (Invitrogen). PCR purification was carried out using the MolBio PCR purification kit according to the manufacturer’s specifications and DNA sequencing was performed by SeqXcel, Inc. (San Diego, CA). Qiaprep Spin Miniprep kits (Qiagen) were used for plasmid purifications and restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA).

The method of Gene Splicing by Overlap Extension (SOEing) PCR as developed by Horton (26) was used to engineer in-frame deletions of specific genes within the *V. cholerae* genomes. PCR amplicons were designed and subcloned into pGPKm as previously described (38). After sequence verification, each plasmid was electroporated into *E. coli* S17-1λpir and conjugated into the desired *V. cholerae* strain (see above). Subsequent screening for plasmid integration events and in-frame deletion verification was then performed as explained in Mueller, *et al.* (38)
Transposon mutagenesis library generation, screening and mutant identification. Conjugations transferring plasmid pRL27 (30) into *V. cholerae* strain S9149 were carried out as described above. Transposon mutant libraries were made by arraying recovered ex-conjugants from the LB agar plates containing Rif 100 µg/mL and Km 200 µg/mL onto petri dishes with LB Km 200 µg/mL in 49-sample (7x7) grid format. After overnight incubation of these plates at 37 °C, each cell patch was replica-plated onto one LB agar plate supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 100 µg/mL) and one containing X-gal (100 µg/mL) and indole (500 µM). Each plate was grown at 37°C and the following day individual patches were qualitatively assessed for blue color production with and without indole. Putative mutants altered in *lacZ* expression were re-patched onto LB X-gal (100 µg/mL) plates with or without indole (500 µM). Mutants displaying phenotypes different from the parental S9149 strain were preserved at -80 °C in LB containing 15% glycerol (v/v).

The sequences of flanking DNA surrounding transposon insertions within these mutant strains were then retrieved using an arbitrary PCR technique first described by O’Toole, *et al.* (41) PCRs resulting in amplicons of approximately 500 base pairs were cleaned and sequenced using Sanger’s di-deoxy chain termination method (45). Recovered sequence data was analyzed by performing BLAST analysis (1) against the *Vibrio cholerae* strain N16961 genomic sequence (24).

Crystal violet quantification of biofilm formation. Strains were initially streaked onto LB agar and allowed to grow overnight at 37 °C. Biological replicates originating from three unique colonies for each strain were inoculated into 5 mL liquid cultures of LB medium and grown for ~16 hours. Five microliters of each culture was
then inoculated into 5 mL of fresh LB and grown overnight at 37 °C with moderate shaking. Biofilms formed by each strain were then quantified by a method presented by O’Toole (41). For each culture, 50 µL of crystal violet (0.1% w/v) was then added to allow for staining of the adherent cells. The medium was then washed from each tube and replaced with 5 mL of 95% ethanol (v/v) and the OD<sub>570nm</sub> of individual replicates were recorded. Pair-wise comparisons of the data of each sample were then analyzed for significance using a student’s t-test (α = .05).

**Auto-aggregation assay.** Strains were grown as above to obtain three biological replicates. After overnight growth in 5 mL of LB medium, each sample was allowed to settle for at least one hour. Once the flocculent particles within each culture had settled to the bottom of each tube, 200 µL was removed from the top portion of each culture and the OD of this sample was read at 600 nm using a Spectramax M2 microplate reader (Molecular Devices).

**Miller assays for β-galactosidase activity.** A protocol similar to that as described by Miller (37) was used to assay for alterations in lacZ expression. In brief, three biological replicates of each strain were grown overnight in LB broth and subcultured 1:1000 in fresh LB medium (+/- indole 500 µM from 1 M stock in methanol) and grown for an additional 20 hours, at which point the OD<sub>600nm</sub> was measured. One milliliter aliquots were then removed from each of these cultures and cells were centrifuged (2 minutes, 13,000 x g) and resuspended in 500 µL CPRG lysis buffer [250 mM Tris pH= 7.4, 2.5 mM EDTA, .25% Igepal (v/v)]. For samples where indole concentration was estimated, the supernatant of each was retained for later analysis. Cells were then lysed by freezing at -20 °C for 30 minutes and subsequently thawing at
room temperature. One hundred microliters of lysate was then added to 900 µL Z-buffer and β-galactosidase activity was assayed as outlined previously (37). Control experiments performed with cells grown in the presence of LB supplemented with methanol (0.05%, v/v), which is the indole solvent, demonstrated no appreciable change in vpsL::lacZ expression (data not shown).

For the co-culture experiment, three biological replicates of each strain were grown overnight in LB medium from single colonies and sub-cultured together in fresh LB at a 1:5000 dilution. Cultures were grown for 48 hours and ratios of each strain were monitored throughout using plating experiments. For all cell combinations, the Miller units calculated were normalized to the percentage of S9149 cells in the total OD, as represented by the percentage of S9149 colony counts obtained for each two-strain culture. In this manner, the calculated β-galactosidase activity was not reflective of all of the cells harvested, but only those producing LacZ.

For the conditioned media experiment, biological replicates of S9149 (indole’) and S9171 (indole”) were grown overnight in 11 mL of LB medium at 37 °C. One milliliter aliquots were removed from each tube for later use in inoculating new cultures. The remaining 10 mL of each culture were centrifuged to pellet the cells (10 minutes, 3,640 x g) and the resulting supernatants were filter sterilized through 0.22 µm membranes. To 3.8 mL of each of these conditioned supernatants, 200 µL of 20X YT (Yeast extract = 100 g/L; Tryptone = 200 g/L) was added. At this point, each biological replicate of S9149 was inoculated at a 1:1000 dilution into fresh LB medium, indole’ conditioned medium and indole” conditioned medium. After 24 hours growth, Miller assays were performed as described above.
**Indole concentration measurements.** The concentration of indole in the supernatants of cell cultures was measured by mixing 250 µL of supernatant with 250 µL trichloroacetic acid (20% w/v). After incubation on ice for 15 minutes each sample was centrifuged to remove precipitated proteins (10 minutes, 13,000 x g). This supernatant was then added to 500 µL Kovac’s reagent (Sigma-Aldrich Co.), vortexed and the OD₅₇₁nm was measured for 200 µL of the top layer. A standard curve of known indole concentrations was recorded and used to estimate the amount of indole in each sample.

**RNA isolation and transcription analysis using whole genome transcription profiling.** *V. cholerae* strains were initially grown on LB agar plates at 37 °C overnight. Individual colonies from each plate were inoculated into 5 mL of LB medium and grown overnight at 37 °C. Two colonies for each strain were picked and subcultured as biological replicates at a 1:1000 dilution in fresh LB (+/- indole 350 µM) and grown for 20 hours at 37 °C with moderate shaking. Two separate one milliliter samples from each culture were harvested as technical replicates, and RNA was isolated as described previously (59).

The microarrays used in this study representing the open reading frames present in *V. cholerae* genome were composed of 70-mer oligos and were printed at University of California, Santa Cruz. Whole genome expression analysis was performed using a common reference RNA, which was a 1:1 mixture of total RNA isolated from wild-type cells grown in LB and wild-type cells grown in LB supplemented with indole. cDNA synthesis, microarray hybridization and scanning were performed as described previously (7). Signal ratios were normalized with LOWESS print-tip normalization using the Bioconductor packages (21) in R environment. Significance Analysis of Microarrays
(SAM) program (53) was used to determine differentially regulated genes using ≥1.5 fold differences in gene expression and ≤1% false discovery rate as a cutoff value.

For meta-analysis of microarray experiments, a $\chi^2$-test was used to evaluate statistically significant differences between over- and under-expressed genes in previously published expression data sets and the results obtained in these current experiments. Data sets were downloaded from the supplemental tables of published reports (See Table 4) and genes were grouped together if they demonstrated a significant up-regulation or significant down-regulation under the treatment conditions. The resulting gene sets from each report were then individually compared to the sets of differentially regulated genes found in the study to determine the overlap between the two data sets. From this, a 2x2 $\chi^2$-test was performed and significance was assessed as having a p-value ≤ 0.01.

**GFP tagging of V. cholerae strains and confocal laser scanning microscopy (CLSM).** Tri-parental conjugations for inserting the green fluorescent protein (GFP) gene into the V. cholerae chromosome were performed as described previously (7). Biofilm formation by GFP-expressing strains within coverglass chambers (Nalge Nunc International) was assessed using CLSM. Biological replicates for each strain were inoculated from stationary phase cultures into chambers containing fresh LB medium (+/- indole, 500 µM) at a final concentration of $10^6$ cells/mL. Static growth was allowed to proceed for 6 hours at 37 °C, at which point all media was removed from the chambers and attached cells were washed twice with 100 mM Phosphate Buffered Saline (PBS, pH=7.0). After resuspension in 100 mM PBS, biofilms were visualized with a Nikon
RESULTS

Indole controls biofilm associated phenotypes in V. cholerae. A previous transposon mutagenesis screen for biofilm mutants in two environmental strains of V. cholerae, SIO and TP, identified the tryptophanase gene as being essential for proper biofilm formation (38). When the biofilms of these tnaA::Tn5 mutants were examined with crystal violet staining it was noted that the SIO mutant (S1101) formed ~2.3X less biofilm than its parental wild-type, and the TP mutant (T1101) formed ~6X less biofilm than the wild-type (Figure 1A). Since indole, a by-product of the tryptophanase reaction, had previously been identified as a factor controlling biofilm formation in other bacteria (36), the effect of exogenous indole addition on the biofilms of these tnaA mutants was tested. In this experiment, indole added exogenously at concentrations of 350 µM was able to fully complement the biofilm formation of these mutants to wild-type levels (Figure 1A).

The influence of indole on various properties of strain SIO was studied in more detail, due to its genetic tractability and the clear phenotypic differences between the smooth colonial phenotype of VPS defective mutants and the rugose colony structure of the wild-type strain. The biofilms formed by GFP-expressing SIO and S1101 (strains S4100 and S4101, respectively) were examined further using confocal laser scanning microscopy (CLSM) to investigate the morphological effects of indole on their biofilms. As seen in Figure 1B, the biofilms of the parental S4100 strain grown on glass coverslips
for 6 hours in LB broth under static conditions were distinctly different than the biofilms of S4101, the tryptophanase mutant. Without the addition of indole to the biofilm chamber, the S4100 strain began to form regular microcolonies covering the glass substratum with an average height of $25.8 \pm 5.0 \, \mu m$. In contrast, the microcolonies of S4101 biofilms were much smaller and exhibited sparse surface coverage. Additionally, these biofilms were thinner and only reached an average height of $15.5 \pm 2.6 \, \mu m$. When indole was added back to each strain, an up-shift in biofilm formation was discovered. While strain S4100 produced a slight increase in surface coverage when exogenous indole was added, a clear difference was seen for S4101 under these conditions. The biofilms of S4101 with exogenous indole nearly doubled in thickness to an average height of $28.0 \pm 4.4 \, \mu m$ and the surface coverage increased considerably and was not significantly different from that of the parental strain with indole.

Another notable phenotypic difference among the SIO-derived strains was that the tryptophanase mutant did not exhibit the auto-aggregative phenotype of its parental strain. Under stationary growth conditions wild-type cultures form multi-cellular clumps that settled to the bottom of the tube within liquid media. When liquid growth of the tryptophanase mutant was examined a lack of aggregation within the medium was evident. Figure 1C, which shows the OD$_{600nm}$ of the cultures of the SIO and S1101 strain with and without indole upon settling, demonstrates the aggregation effect. This figure also shows that when indole is added back to the tnaA mutant strain, the auto-aggregation phenotype is restored to near wild-type levels.

**Indole regulates VPS gene expression in V. cholerae.** In addition to affecting biofilm formation and auto-aggregation, indole induced the smooth colonies of S1101 to
revert to the rugose phenotype of the SIO parental wild-type when grown on LB agar plates (38). It has previously been shown that all three of these phenotypes are linked to over-production of exopolysaccharides (2, 55, 61). Therefore, it was hypothesized that indole is exerting its effects, at least in part, by regulating vps gene expression. Therefore, the β-galactosidase activity of modified SIO and S1101 parental strains carrying lacZ reporter fusions to the vpsL genes and deletions of the native lacZ genes, was monitored (S9171 and S9149, respectively). In these strains, β-galactosidase activity serves as a proxy for vps gene expression in the presence and absence of endogenously produced indole.

The initial experiment examined the simultaneous production of endogenous indole and β-galactosidase activity throughout the growth phases of S9171 grown in batch culture (Figure 2A). It was observed that indole production by this tnaA+ strain mirrored vpsL::lacZ expression. During early exponential-phase growth both indole and β-galactosidase levels are low. However, consistent with tnaA catabolite repression control (57), late exponential- and stationary-phase cultures produced increased amounts of indole, and concomitantly, increased amounts of β-galactosidase. While this experiment showed that extracellular levels of indole and vpsL induction correlate with one another, it did not address whether indole is responsible for vpsL induction.

To address this possibility, strain S9149 (∆tnaA) was used to monitor vpsL::lacZ expression under conditions of exogenous indole addition. Similar to the biofilm results reported above, S9149 vpsL::lacZ expression was approximately 10-fold less than its indole-producing parental strain, S9171 (Figure 2B). Full complementation of vpsL::lacZ induction occurred with concentrations of exogenous indole as low as 100 µM, which is
well below the observed concentration of endogenous indole produced by S9171. These data illustrate that indole supplied exogenously can regulate VPS expression when supplied at levels below physiologically relevant concentrations.

**Indole acts as an extracellular signal regulating VPS production.** To further address the hypothesis that endogenously produced indole acts as a signal controlling VPS in *V. cholerae*, experiments were performed to determine whether indole produced from an originating cell can be taken up by a different cell and elicit a response (i.e. *vpsL*:lacZ up-regulation). First, co-culture experiments were performed with the Lac⁻ indole -/+ donor strains (S2150-ΔlacZ, ΔtnaA and S2148-ΔlacZ, tnaA⁺, respectively) and an indole negative reporter strain (S9149-ΔtnaA, *vpsL*:lacZ). As shown in Figure 3A, the indole reporter strain produced very little β-galactosidase activity when grown in co-culture with the indole negative donor. However, when grown with the indole-positive donor the β-galactosidase activity increased ~8-fold. This increase is strikingly similar to the activity achieved when indole is added exogenously to the co-culture conditions with the indole negative donor and reporter strains, demonstrating that exogenously and endogenously produced indole have the same VPS regulatory effect.

An experiment was also performed in which strain S9149 was grown by itself in conditioned or unconditioned medium (Figure 3B). S9149 grown in fresh LB medium produced very little *vpsL*:lacZ reporter activity, which was similar to the result when S9149 was grown in a supernatant from an indole-negative strain (“SI-” in Fig. 3B; derived from strain S2150). However, when S9149 was grown in conditioned medium derived from an indole producing strain (“SI+” in Fig. 3B; derived from strain S2148), the level of β-galactosidase production rose significantly (>3-fold). These results
reinforce the conclusion that the VPS regulatory response is produced specifically by indole and not another component of the supernatant.

It was also investigated whether indole influences biofilm formation and VPS production in clinical strains of *V. cholerae* in addition to the non-O1/O139 environmental strains tested. It was found that addition of indole to cells of *V. cholerae* O1 El Tor strain N16961 carrying an in-frame deletion of the *tnaA* gene (smooth variant 24) caused significant increases in biofilm formation and *vpsL* transcription compared to cells grown without indole (data not shown). Thus, indole regulates biofilm formation and *vps* transcription in both clinical and environmental strains of *V. cholerae*.

**Transcriptional regulation by TnaA activity and indole production extends beyond VPS genes.** To explore whether indole regulation of gene expression extends beyond genes involved in VPS biosynthesis, whole-genome expression profiles from the wild-type SIO strain and strain S1101 (*tnaA::Tn5*) grown to stationary phase in LB medium either in the presence or absence of exogenously added indole were obtained and compared. Significantly regulated genes were defined using SAM analysis (53) as having ≤1% false-positive discovery rates and ≥1.5-fold transcript abundance differences between each sample. Four sets of pair-wise comparisons were made from the resulting transcriptome profiles in order to assess the influence of indole and/or tryptophanase activity on gene expression. Table S2 of the supplementary information provides the complete list of differentially regulated genes within each data set. The first comparison made was between the transcriptomes of SIO and S1101 to understand the effects of a tryptophanase mutation on gene expression (SIO/S1101). The second comparison examined the effects of indole alone by matching the expression data of the
tryptophanase mutant, S1101, grown with or without exogenous indole
[S1101(+I)/S1101]. The next comparison evaluated the expression changes of the indole-
producing S10 strain grown in the presence of additional exogenous indole to S10 grown
solely in LB medium, [S10(+I)/S10]. Here, the consequences of artificially high indole
concentrations on gene expression were evaluated. Finally, the expression data from the
S10 strain and S1101 grown with indole were compared to determine whether indole
alone could complement the expression changes resulting from a tnaA mutation
[S10/S1101(+I)]. Table 2 displays the summarized results of these comparisons noting
the number of differentially regulated genes in each pair.

The dataset comparing the wild-type S10 strain to S1101 contained the most
genes demonstrating significant induced/reduced expression, suggesting that both indole
production and tryptophanase activity can alter gene expression. Figure 4A displays
Venn diagrams illustrating the overlap between each additional expression profile
comparison and the S10/S1101 data set. When the S10(+I)/S10 expression profile was
compared to S10/S1101 only 34% of genes differentially expressed were shared between
data sets, possibly due to the artificially high indole conditions present in the former. In
contrast, many of the genes from the indole complementation expression profiles
[S10/S1101(+I) and S1101(+I)/S1101] are shared with the S10/S1101 data set (74% and
75%, respectively). The complementation effect of indole can be seen in the heatmap of
Figure 4B, where the 327 of the 507 genes significantly reduced/induced in expression in
the S10/S1101 data set show no significant difference in expression in the S10/S1101(+I)
data set (i.e. genes colored blue or yellow in the S10/S1101 column and colored black in
the S10/S1101(+I) column).
Differentially expressed genes were grouped according to their annotated functional roles in order to determine which categories may be influenced by indole production. Not surprisingly, genes involved in cell envelope maintenance and production were strongly up-regulated by indole, which is clearly demonstrated by the heatmap of the VPS genes shown in Figure 5A. In total, 16 of the 19 genes contained within the two vps operons were up-regulated by indole when all gene profile comparisons were considered. Additionally, some of these demonstrate the strongest differential expression between the SIO and S1101 expression profiles, with six demonstrating at least ~10-fold activation in the SIO profile.

Another notable functional category that was differentially regulated by indole, is the group containing genes responsible for general cellular processes (Figure 5B). Included within this functional class are many genes that have a role in chemotaxis and flagellar biosynthesis. These include many of the annotated chemotaxis (Che) proteins and methyl-accepting chemotaxis proteins (MCPs) of the *V. cholerae* genome (24). The vast majority of these genes demonstrate significant down regulation in the presence of exo- or endogenous indole, coinciding with microarray studies performed with *E. coli* showing a similar down-regulation chemotaxis gene transcription in the presence of indole (27).

The transport and binding protein functional category also demonstrated overall down-regulation in response to indole. This group includes genes involved in amino acid transport, iron uptake and carbohydrate transport. Figure 5C shows genes from the largest sub-group of genes within this category that function as iron transport systems and in siderophore production and transport. Most of these genes were repressed only under
artificially high indole conditions where gene expression of wild-type SIO grown in LB supplemented with exogenous indole was compared to SIO grown solely in LB medium.

Figure 5C also shows a set of genes that demonstrate a relative increase in expression and are annotated to fall within a group of genes with previously unknown functions. However, recent work has shown that the *hcp* (VC1415 and VCA0017), *vgrG* (VCA0018 and VCA0123) and *vasK* (VCA0120) genes of this group are involved in virulence-associated secretion (VAS), which has a role in infection and virulence towards eukaryotic cells (for reviews see 9, 19). For example, it has been shown that the *vas* genes and their homologues within other species function in mediating: (i) effective infection and nitrogen fixation by *Rhizobium leguminosarum* within nodules of the pea plant (10), (ii) disease in fish by the piscine pathogen *Edwardsiella tarda* (47), (iii) death of the phagocytic eukaryote *Dictyostelium discoideum* by *V. cholerae* (43), (iv) and pellicle and biofilm formation in *Vibrio parahaemolyticus* and *Actinobacillus actinomycetemcomitans*, respectively (17, 27).

In addition to the above five genes, the array results indicate that all but one of the surrounding genes of the VAS operon (VCA0107-VCA0123; Supplementary Information -Table S2) are indole-induced (Figure 5C). Seven of these genes demonstrated moderate to strong significant activation by indole (greater than ~2.5-fold). Although the *vas* genes did not show a significant increase in transcript abundance in the pair-wise comparison between S1101 grown on LB supplemented with indole versus growth on LB medium alone, semi-quantitative RT-PCR measuring the abundance of VCA0108 transcript demonstrated a 1.7-fold increase in S1101 cells grown in the presence of indole than without. Therefore, it appears that in the S1101 background the VAS operon can be up-
regulated by indole; although, its detection may be below the limits of the microarray experiments performed.

**Tryptophanase and indole have a role in grazing resistance.** Since indole induces genes involved in VAS secretion and toxin production, it was hypothesized that the tryptophanase gene may be important for grazing resistance. For these experiments using the *Dictyostelium discoideum* grazing model (43), strain SIO could not be used due to its high sensitivity to grazing by this amoeba. Therefore, *V. cholerae* strain TP was used since it was discovered to be resistant to *D. discoideum* grazing. Standard plaque assays were performed on nutrient agar supplemented with indole at levels that were not toxigenic to the *D. discoideum* (data not shown).

Table 3 shows that when strain T1101 (*tnaA::Tn5*) was examined for its resistance, a dramatic decrease in its ability to survive under grazing pressure was observed relative to the wild-type TP strain. However, when indole was supplied exogenously partial grazing resistance was restored in T1101. In addition, it was observed that a strain (T1144) carrying a mutation in the *vas* operon was also susceptible to grazing whether grown with or without indole. These results suggest that indole may stimulate VAS production leading to increased grazing resistance.

It was also found that exogenous indole could slightly increase grazing resistance in *V. cholerae* strain N16961 and *Klebsiella aerogenes*, which both carry full copies of the *vas* operon within their genomes, yet are grazing susceptible when grown without indole. It should be noted, though, that a VAS-connection to the phenotypes of *V. cholerae* N16961 and *K. aerogenes* has not been conclusively established.
**X² analysis suggests patterns of regulation involved in the indole response.**

Various activators and repressors that are known to influence exopolysaccharide production were differentially regulated in the transcriptome comparisons. Included within these are genes involved in: (i) direct transcriptional activation of vps (vpsR and vpsT), (ii) quorum sensing (cqsA, luxQ, fis and hapR), (iii) c-di-GMP biosynthesis and degradation (mbaA and cdgA), and (iv) membrane stress sensing and response (cpxPAR, rpoE and rseA). To determine whether the gene sets regulated by indole and tryptophanase are significantly similar to the regulons of some of these other known transcriptional regulators of *V. cholerae* and to predict possible key players of the indole response a X² analysis was performed.

The results of this analysis are shown in Table 4. Several patterns emerge from these analyses that suggest roles for specific transcriptional regulators in the indole response. The data sets with the most significant correlation to SIO/S1101 expression profile are from experiments performed under conditions of altered VPS production. These include transcriptome analyses performed with mutant strains of the vpsT and vpsR transcriptional activators, and with GGDEF protein mutants (mbaA and cdgA) or cells grown with artificially high levels of c-di-GMP. Even when the 18 vps genes were excluded from these analyses there was still significant overlap between the data sets, indicating that the similarities extend beyond VPS production (data not shown).

Other regulons, which appear to be similar to the indole/tnaA-regulated gene sets, are regulons controlled by the nucleic acid binding protein Hfq and the alternative sigma factor σ⁵⁴ (RpoN). Hfq has been shown previously to be involved in quorum sensing in *V. cholerae* (33), and σ⁵⁴ regulates motility and virulence and other global regulators
including HapR and $\sigma^{38}$ (RpoS) (43, 59), many of which are also regulated in the presence of indole.

Other regulators are not significantly correlated with the indole/tnaA regulon, however. These include: HapR, the master regulator of quorum sensing; ToxR, a master regulator of pathogenesis in virulent strains of \textit{V. cholerae}; and RpoE, a regulator of the membrane stress response. Whole genome hybridization microarray experiments indicate that strain SIO does not contain many of the genes (e.g. VPI-1 and CTX elements) found within the traditionally defined ToxR regulon of \textit{V. cholerae} (M. Miller, personal communication, 8), which may account for some of these results.

Since lack of significant correlation between the microarray experiments could be due to various reasons (i.e. experimental design or strain variations, etc.), the roles of the quorum sensing and membrane stress response systems in contributing to indole-driven gene expression were tested. In-frame deletions of key genes for each system were constructed (quorum sensing mutants - \textit{fis} and \textit{luxO}; membrane stress - \textit{rpoE} and \textit{cpxA}), and the ability of these mutant strains to respond to indole was examined using the \textit{vpsL::lacZ} reporter gene system. When grown under conditions with or without exogenous indole, none of the strains were impaired in the up-regulation of the \textit{vpsL::lacZ} fusion in the presence of indole (data not shown).

To also ensure that indole, due to its hydrophobic nature, is not triggering a membrane stress response as seen in other bacteria when provided at artificially high concentrations (40), we investigated the ability of indole to depolarize the membrane of \textit{V. cholerae} cells. Here it was found that physiologically relevant concentrations of indole (0.5 mM) did not have a measurable effect on membrane polarity (data not shown).
shown). Taken together, the above results support the view that neither the membrane stress nor quorum sensing systems play a role in the indole signaling cascade.

**Indole regulation of VPS proceeds through DksA, VpsR and GGDEF domain-containing proteins.** In order to further develop the details of the indole-signaling circuit, a transposon mutagenesis screen was performed to look for mutants that display an indole non-responsive phenotype. Of the approximately 11,000 transposon mutants screened, four unique genes with transposon insertions were recovered which demonstrated a consistent low-level of vpsL::lacZ expression that did not change significantly upon the addition of exogenous indole (Table 5). Interestingly, three of these four strains carried mutations in sequences coding for GGDEF-domain containing proteins (VCA0074 and VC1376) or in a hypothetical protein immediately upstream of one of these genes (VCA0075). Furthermore, each of these genes was recovered multiple times from independent transposition events (VCA0075 - 5 independent mutations, VCA0074 and VC1376 - 2 independent mutations each), demonstrating the importance of these genes in controlling the indole-induced VPS response and suggests a near-saturation of the genome by the transposon mutagenesis screen.

VCA0074, which has been named cdgA, has diguanylate cyclase activity and increases intracellular c-di-GMP levels, which leads to the production of VPS and the formation of biofilms in *V. cholerae* (34). On the *V. cholerae* genome, the cdgA gene resides within a two gene operon and is located immediately downstream of VCA0075. A transposon mutation in VCA0075 has previously been shown to also affect the biofilm formation of *V. cholerae* (38); although, it is not clear whether this is a direct
consequence of VCA0075 mutation by the transposon or an indirect result of polar effects on downstream cdgA expression.

Previous transcriptome experiments were reviewed to understand what genes potentially control the expression of the cdgA operon (see Table 4 for references). These data show that the cdgA operon is only differentially regulated in response to mutations in known regulators of VPS expression (ΔhapR, ΔvpsR, ΔvpsT, ΔrpoN and ΔvpvC) or in cells grown under conditions of artificially high intracellular c-di-GMP concentration. The cdgA operon is always up-regulated by activators of VPS production and down-regulated by repressors. It did not exhibit any differential regulation in microarray experiments comparing rpoE, hfq, toxR, and rhyB mutants that also do not alter the overall regulation of the VPS operons. Considering that there are 41 genes encoding GGDEF-domain containing proteins in the V. cholerae genome it is striking that cdgA is consistently transcribed under conditions of VPS gene activation.

Only one mutant was recovered that does not respond to indole and contains an insertion in a gene or operon whose product(s) are not known to affect the production of GGDEF proteins. This mutant contains an insertion in the dnaK suppressor protein gene, dksA, which is the third of eight genes in an operon and codes for a protein thought to interact directly with the RNA polymerase holoenzyme (E). Through this physical interaction, DksA promotes the dissociation of E from the housekeeping sigma factor σ70 allowing the RNA polymerase molecule to interact with alternative sigma factors such as σ54 (RpoN), (reviewed in 22). Interestingly, rpoN was not differentially regulated in any of the whole genome profile comparisons made, suggesting that indole’s effect is post-transcriptional, such as at the level of DksA activity. Similar to previous failed attempts
to complement transposon mutations in strain SIO (38), we were not able to restore indolet responsiveness when the wild-type *dksA* gene was supplied *in-trans*. However, construction of an in-frame deletion of the *dksA* gene resulted in an indole non-responsive phenotype (data not shown), suggesting that polar effects within the operon where *dksA* is found are not responsible for this observed phenotype.

A second class of mutants was found to produce no measurable β-galactosidase activity. Included in this class are two hypothetical proteins, one of which is predicted to be membrane localized, and two transporter genes. None of these genes have a known role in VPS production. A fifth mutant contained a transposon insertion in the *hmpA* gene, which encodes a soluble flavohemoglobin known to counteract nitrosative stress in *Salmonella enterica* (3). As VPS production has previously been shown to counteract oxidative stress (61), HmpA may play a role as a sensor, which can regulate VPS production in response to oxidative stress. The last mutant in this group contained a disruption in the *vpsR* gene, which is a key regulator of VPS production (58), and is known to combine with other regulators including VpsT and GGDEF-domain proteins to direct *vps* transcription (6). Therefore, it was not surprising that a *vpsR* mutant demonstrated no measurable β-galactosidase activity. These results indicate that the indole-induction of VPS requires VpsR, c-di-GMP, DksA and additional factors.

**DISCUSSION**

**Indole signaling controls physiologically important functions in *V. cholerae*.**

Recently, it has been proposed that the molecule indole, which is a natural product of the breakdown of tryptophan by the enzyme tryptophanase, can act as a stationary phase
signal molecule inducing biofilm formation (36, 54). Previous work showed that a mutation in the tryptophanase gene of \textit{V. cholerae} strains influences biofilm formation and the ability to produce rugose colonies, and it was speculated that this was a result of reduced VPS production (38). In this current study, it has been shown that indole secreted by one cell can act as an extracellular signal that is sensed by others within a population, and that the perception of this indole signal leads to the coordinated up-regulation of \textit{vps} gene expression, and the associated phenotypes of biofilm formation and rugose colonial morphology.

These results correspond with those of Martino, \textit{et al.} (36), who demonstrated that indole enhances the biofilm formation of many different microorganisms carrying the tryptophanase gene. However, they are in contrast to a report indicating that indole down-regulates biofilm formation in \textit{E. coli} (32). Similarly, we have found that biofilm formation by the rugose variant of \textit{V. cholerae} strain 92A1552 is not affected by deletion of the \textit{tnaA} gene (unpublished results), suggesting that indole’s effects may be strain specific. While the underlying reason for this difference is unknown, comparison of transcriptome expression patterns of the \textit{tnaA} mutant of \textit{V. cholerae} strain we used and the \textit{tnaA} mutant of \textit{E. coli} K-12 (32) provides a possible partial explanation. Both \textit{V. cholerae} SIO and \textit{E. coli} K-12 appear to down-regulate genes involved in cell motility and chemotaxis (27 genes in \textit{V. cholerae} and seven in \textit{E. coli}). While motility enhances biofilm formation in \textit{E. coli} (56), it is not required for biofilm development on glass or plastic by \textit{V. cholerae} strain SIO (38), which may account for the observed differences in biofilm formation in response to indole by these species.
Another set of genes that were differentially regulated by indole in the transcriptome experiments localizes to the VAS operon, which is important for resistance of grazing by protozoa (43). These genes are often carried by plant- or gut-associated bacteria (15), which are exposed to high levels of indolic compounds within these environments (28, 49). Thus, the evidence presented here linking VAS operon regulation to indole is compelling and deserves further investigation for other bacteria in different ecological settings (e.g. VAS-mediated root or pathogenic infection model systems).

From the microarray experiments it was noted that the expression of genes involved in flagellar biosynthesis, the vps-I and -II operons, and genes of the VAS operon was changed by indole. In each case, the regulation of these genes is known to depend on specific $\sigma^{54}$-dependent transcriptional activators: FlrC for flagellar biosynthesis (42), VpsR, for vps transcription (58, 59) and VasH, a regulator of the VAS operon (43). Since indole does not appear to influence the expression levels of rpoN gene (which encodes the $\sigma^{54}$ protein), it appears that this effect may be in part due to indole-induced alterations in $\sigma^{54}$-dependent transcriptional regulator gene expression. Indole appears to down-regulate flrC (~1.7-fold in SIO/S1101 comparison) and up-regulate vasH (~2.3-fold in SIO/S1101 comparison), consistent with the patterns of motility and VAS gene expression found in response to indole. In contrast, vpsR does not show a significant expression change in response to indole, and indole-controlled VPS regulation may result from post-transcriptional effects on VpsR.

**Indole signaling involves known regulators of VPS synthesis and the dnaK suppressor protein, DksA.** It is apparent that indole regulon is composed of many of the same genes that are governed by known regulators of VPS synthesis, such as VpsR,
VpsT and multiple GGDEF-domain containing proteins (e.g. CdgA and MbaA).

However, the expression profile of genes controlled by HapR, another known VPS regulator, did not correlate with the microarray results in any of the comparisons performed. Although HapR is the major quorum sensing transcriptional regulator in some strains of *V. cholerae* (62) and is known to affect the transcription of numerous regulators of VPS such as *vpsT*, *vpsR* and *cdgA*, these regulators can also function independently of HapR to control VPS production (6). In fact, many strains of *V. cholerae* often carry mutations in the *hapR* gene rendering the gene non-functional and presumably inactivating the quorum sensing mechanisms of these strains (24, 29). This information combined with our results showing that quorum sensing mutations had no effect on the up-regulation of VPS by indole lead us to conclude that indole regulation is dependent on regulators such as VpsR, VpsT and GGDEF proteins rather than quorum sensing regulators.

Previous studies also have shown that VpsR and CdgA are involved in a regulatory cascade controlling VPS production, with VpsR acting to up-regulate *cdgA* expression, and CdgA increasing the intracellular concentrations of the second messenger c-di-GMP through its guanylate cyclase activity (6). Rising c-di-GMP concentrations then appear to signal increased transcription of genes involved in VPS production and repression of genes responsible for motility (7). Synthesis of these results with ours implicates indole as an inducer of this c-di-GMP regulatory cascade.

A third gene that is thought to be a component of this signal cascade is *vpsT*, another known transcriptional activator of *vps* (12). *vpsT* shows consistent up-regulation by indole in the transcriptome comparisons (~2.8-fold in SIO/S1101 comparison);
therefore, it could also have a role in indole induction of VPS. To test its role, multiple
attempts to make mutations within the \( vpsT \) gene were performed, but the mutant was
never recovered. Although \( \Delta vpsT \) derivatives of clinical strains of \( V. \) cholerae have
previously been made, VpsT may have an essential role in the genetic background of the
strains used in this study. A role for VpsT in the indole response cannot be ruled out.

Another significant finding is that the gene \( dksA \) appears to be involved in the
indole response. Recently, it has been shown that the protein DksA along with the
intracellular alarmone ppGpp can bind directly to RNA polymerase (RNAP) and
indirectly modulate its associations with different sigma factors (reviewed in 22). This
binding increases the available intracellular pool of RNAP able to interact with \( \sigma^{54} \). As
more RNAP holoenzyme-containing \( \sigma^{54} \) (E\( \sigma^{54} \)) is produced, increased interactions with
 \( \sigma^{54} \)-dependent transcriptional activators takes place and transcription from \( \sigma^{54} \) promoters
proceeds at higher levels. It has been proposed that DksA can act in concert with ppGpp
to passively promote transcription from \( \sigma^{54} \) promoters (5). No previous findings have
demonstrated that indole can influence ppGpp production or DksA activity. However, it
has been shown that various indole derivatives, such as indole-based antimicrobial
compounds and indole-3-acetic acid, can influence RelA-based production of ppGpp
(48, 50). The role of \( dksA \) in the indole response and the fact that \( \sigma^{54} \)-dependent
transcriptional regulators control indole-controlled genes provide circumstantial evidence
for a role for \( \sigma^{54} \) in the indole response.

A proposed model of the indole signaling cascade in \( V. \) cholerae. Based on the
data presented in this report, a model to guide future investigation of indole regulation is
proposed in Figure 6. In this scenario catabolite-repressed tryptphanase activity
increases as carbon and energy supplies begin to dwindle, leading to a concomitant increase in indole production and excretion by individual cells. Once in the extracellular environment it is unclear how the cells receive the indole signal. It has been suggested the Mtr transporter can actively transport indole (57), or, due to its hydrophobic nature, indole might directly diffuse through the membrane (20). Once the signal is received, though, we propose that this indole signal may promote DksA-mediated interactions between RNAP and $\sigma^{54}$, possibly in a ppGpp-dependent manner. Another possibility is that indole can interact directly with DksA to promote differences in RNAP availability, although both hypotheses deserve future evaluation. As the association between RNAP and $\sigma^{54}$ enhances, many genes under the direct control of $\sigma^{54}$ are then activated including the VAS and VPS operons. Down-regulation of the $rpoS$ (which encodes the $\sigma^{38}$ protein) gene also is observed, which could result from the ability of $\sigma^{54}$ to negatively regulate the transcription of $rpoS$ (59). Further, since the $\sigma^{38}$ regulon is known to include genes involved in siderophore production and iron acquisition in other strains of Vibrio cholerae (39, 59) and in Vibrio vulnificus (31), it is possible that the down-regulation of the iron acquisition machinery observed is due to decreased $rpoS$ expression (Figure 6).

The indole regulation of genes involved in motility and VPS production is known to also result from downstream effects on the c-di-GMP signal system of V. cholerae (7, 51). The results of the transposon mutagenesis screen support this model, since the majority of indole non-responsive mutations are in genes coding for GGDEF-domain containing proteins. It is proposed that once VpsR is activated, the protein can interact with Eo$\sigma^{54}$ to enhance the expression of cdgA and vpsT. Both genes appear to have VpsR promoter binding sites (59) and are up-regulated in the expression profiles. The increase
in CdgA leads to an increase in c-di-GMP levels within the cell, which in turn stimulates
vps expression and represses motility gene expression, leading to the overall
enhancement of biofilm formation in *V. cholerae* strain SIO. Thus, it appears that
ultimate regulation of many of indole-responsive genes is dependent on the c-di-GMP
intracellular signaling system.

Given the multiple roles of biofilms and VAS in stress protection, predator-prey
interactions and virulence towards eukaryotic hosts, the production of the indole
signaling molecule by the tryptophanase enzyme during carbon and energy limitation
must have an important role in environmental survival.
ACKNOWLEDGEMENTS

We’d like to thank Ilham Naili for help in the preparation of tryptophanase mutant strain, in addition to thank Federico Lauro, Fiona Tomas and Xavier Mayali for aiding with statistical analysis.

This work was supported by NIH grant AI46600-02 and funding from grant CEQI0047 provided by the UC Marine Council Coastal Environmental Quality Initiative.
REFERENCES


Lactobacillus delbrueckii subsp. bulgaricus isolated from handmade yogurt. J. Food Prot. 70:223-
227.

Maintenance of nitric oxide and redox homeostasis by the Salmonella flavohemoglobin Hmp. J.

single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. Gene 109:
167-168.

2006. The guanosine tetraphosphate (ppGpp) alarmone, DksA and promoter affinity for RNA polymerase in

rugosity and biofilm formation in Vibrio cholerae: comparison of VpsT and VpsR regulons and

responses of Vibrio cholerae to increased cyclic di-GMP level. J. Bacteriol. 188:
3600-3613.


Curr. Opin. Microbiol. 11:3-8.

Rhizobium leguminosarum strain that are involved in temperature-dependent protein secretion.
Mol. Plant Microbe Interact. 16:53-64.

311:1113-1116.

rugose colonial morphology of Vibrio cholerae O1 El Tor. J. Bacteriol. 186:
1574-1578.


Protein expression in Escherichia coli S17-1 biofilms: impact of indole. Antonie van
Leeuwenhoek 91:71-85.

proteins) cluster in Vibrio cholerae and other proteobacteria through in silico analysis. In Silico
Biol. 3:287-300.


determinants of biofilm development of opaque and translucent Vibrio parahaemolyticus. Mol.

18. Evans, W. C., W. Richard, C. Handley, and F. C. Hapgood. 1941 The tryptophanase-indole
reaction: Some observations on the production of tryptophanase by Esch. coli; in particular the
effect of the presence of glucose and amino acids on the formation of tryptophanase. Biochem. J.
35:207-212.


Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M.
Genome Biol. 5:R80.
Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P.
Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L.
Bassler. 2003 Indole can act as an autoinducer and its role in virulence factor production.
pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon
SdiA. BMC Microbiol. 7:42.
2004. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in Vibrio
32. Lim, B., S. Beyhan, and F. H. Yildiz. 2007. Regulation of vibrio polysaccharide synthesis and
virulence factor production by CdgC, a GGDEF-EAL domain protein, in Vibrio cholerae. J.
extracellular signal to regulate biofilm formation of Escherichia coli and other indole-producing
Harbor, NY.
35. Mueller, R. S., D. McDougal, D. Cusumano, N. Sodhi, S. Kjelleberg, F. Azam, and D. H.
Bartlett. 2007. Vibrio cholerae strains possess multiple strategies for abiotic and biotic surface


# TABLES

## Table 1

Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant Genotype or Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-12π</td>
<td>recA, pro, hsdR, RP4-2-Tc::Mu::Tn7</td>
<td>(46)</td>
</tr>
<tr>
<td><strong>V. cholerae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>92A1552R</td>
<td>Wild-type 92A1552-Rugose variant</td>
<td>(60)</td>
</tr>
<tr>
<td>AR2101</td>
<td>92A1552R ΔtnaA</td>
<td>This study</td>
</tr>
<tr>
<td>92A1552S</td>
<td>Wild-type 92A1552-Smooth variant</td>
<td>(60)</td>
</tr>
<tr>
<td>AS2101</td>
<td>92A1552S ΔtnaA</td>
<td>This study</td>
</tr>
<tr>
<td>N16961</td>
<td>Wild-type N16961</td>
<td>(24)</td>
</tr>
<tr>
<td>N2101</td>
<td>N16961 ΔtnaA</td>
<td>This study</td>
</tr>
<tr>
<td>SIO</td>
<td>Wild-type SIO</td>
<td>(44)</td>
</tr>
<tr>
<td>S1101</td>
<td>SIO tnaA::Tn5, Km^R</td>
<td>(38)</td>
</tr>
<tr>
<td>S2101</td>
<td>SIO ΔtnaA</td>
<td>(38)</td>
</tr>
<tr>
<td>S2148</td>
<td>SIO ΔlacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S2150</td>
<td>SIO ΔlacZ, ΔtnaA</td>
<td>This study</td>
</tr>
<tr>
<td>S4100</td>
<td>SIO, rRNA::gfp, Gm^R</td>
<td>This study</td>
</tr>
<tr>
<td>S4101</td>
<td>SIO tnaA::Tn5, rRNA::gfp Km^R, Gm^R</td>
<td>This study</td>
</tr>
<tr>
<td>S9149</td>
<td>SIO ΔlacZ, ΔtnaA, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9170</td>
<td>SIO ΔlacZ, ΔtnaA, ΔvpsR, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9171</td>
<td>SIO ΔlacZ, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9185</td>
<td>SIO ΔlacZ, ΔtnaA, ΔrpoE, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9190</td>
<td>SIO ΔlacZ, ΔtnaA, ΔcpxA, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9209</td>
<td>SIO ΔlacZ, ΔtnaA, ΔcpxA, ΔrpoE, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9211</td>
<td>SIO ΔlacZ, ΔtnaA, Δfis, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9213</td>
<td>SIO ΔlacZ, ΔtnaA, ΔluxO, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9216</td>
<td>SIO ΔlacZ, ΔtnaA, VC1376::Tn5, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9218</td>
<td>SIO ΔlacZ, ΔtnaA, VC1673::Tn5, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9219</td>
<td>SIO ΔlacZ, ΔtnaA, cdgA::Tn5, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>S9224</td>
<td>SIO ΔlacZ, ΔtnaA, dksA::Tn5, ΔvpsL::lacZ</td>
<td>This study</td>
</tr>
</tbody>
</table>
S9225  SIO  ΔlacZ,  ΔtnaA,  VCA0075::Tn5,  ΔvpsL::lacZ  This study
S9226  SIO  ΔlacZ,  ΔtnaA,  VC0338::Tn5,  ΔvpsL::lacZ  This study
S9227  SIO  ΔlacZ,  ΔtnaA,  hmpA::Tn5,  ΔvpsL::lacZ  This study
S9228  SIO  ΔlacZ,  ΔtnaA,  VC0143::Tn5,  ΔvpsL::lacZ  This study
S9229  SIO  ΔlacZ,  ΔtnaA,  VC1731::Tn5,  ΔvpsL::lacZ  This study
S9230  SIO  ΔlacZ,  ΔtnaA,  ΔdksA,  ΔvpsL::lacZ  This study

TP   Wild-type TP (44)
T1101 TP  tnaA::Tn5,  KmR (38)
T1137 TP  vgrG-1::Tn5,  KmR (38)
T1144 TP  VCA0109::Tn5,  KmR (38)

Plasmids
pCC12   pRS415 vpsL promoter,  Ap\(^\prime\) (12)
        pFLcm   pFL122L containing  cat  gene from pBSL181 (38)
pFLcdgA pFLcm,  cdgA operon,  Cm\(^\prime\) This study
pGPKm   pGP704 sac28 containing  Km\(^\prime\) gene (38)
pGPcpxA pΔcpxA in pGPKm This study
pGFis    pΔfis in pGPKm This study
pGPLacZ pΔlacZ in pGPKm This study
pGPLuxO pΔluxO in pGPKm This study
pGPrpoE pΔrpoE in pGPKm This study
pGPtrnaA pΔtnaA in pGPKm This study
pGPypsL::lacZ ΔvpsL::lacZ in pGPKm This study
pGPvpsR pΔvpsR in pGPKm This study
pRL27   Tn5-R127  oriR6K  Km\(^\prime\) (30)
pMC11   pGP704::miTn7-GFP,  Gm\(^\prime\)  Ap\(^\prime\) (7)
pUX-BF13 oriR6K helper plasmid,  mob/oriT,  (4) provides the  Tn7  transposition function in  trans;  Ap\(^\prime\)
Table 2 Whole genome expression profiles of SIO and S1101.

<table>
<thead>
<tr>
<th>Pairwise Comparison</th>
<th>Total Genes</th>
<th>Regulated</th>
<th>Up-regulated</th>
<th>Down-regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIO/S1101</td>
<td>507</td>
<td>255</td>
<td>252</td>
<td></td>
</tr>
<tr>
<td>S1101(+I)&quot;/S1101</td>
<td>64</td>
<td>45</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>SIO(+I)&quot;/SIO</td>
<td>218</td>
<td>83</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>SIO/S1101(+I)&quot;</td>
<td>242</td>
<td>128</td>
<td>114</td>
<td></td>
</tr>
</tbody>
</table>

" (+I) – cells grown in the presence of 350 µm exogenous indole
Table 3  Qualitative assessment of the plaque assay demonstrating the resistance and/or susceptibility of multiple strains of bacteria to grazing by the phagocytic eukaryotic predator *D. discoideum* in the presence and absence of indole (500 µM).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Grazing Resistance Phenotype (a, b, c)</th>
<th>No Indole</th>
<th>Indole Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em> strain TP</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>T1101 (<em>tnaA::Tn5</em>)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>T1144 (<em>VCA0109::Tn5</em>)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>V. cholerae</em> strain N16961</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>K. aerogenes</em></td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* ++ no significant plaque formation covering the majority of the plate

*b* + small circular zones of clearing covering less than half of the plate

*c* - no plaques observed
Table 4 $\chi^2$ analysis of the tryptophanase/indole expression profiles compared to published data sets.

<table>
<thead>
<tr>
<th>Expression profile</th>
<th>S1101</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-di-GMP</td>
<td>102.31</td>
<td>(7)</td>
</tr>
<tr>
<td>$\Delta vpsR$</td>
<td>73.58</td>
<td>(6)</td>
</tr>
<tr>
<td>$\Delta vpsT$</td>
<td>50.95</td>
<td>(6)</td>
</tr>
<tr>
<td>$\Delta cdgA$</td>
<td>48.38</td>
<td>(6)</td>
</tr>
<tr>
<td>$\Delta hfq$</td>
<td>13.43</td>
<td>(16)</td>
</tr>
<tr>
<td>$\Delta mbaA$</td>
<td>21.82</td>
<td>(35)</td>
</tr>
<tr>
<td>$\Delta rpoN$</td>
<td>44.12</td>
<td>(59)</td>
</tr>
<tr>
<td>$\Delta rseA$</td>
<td>17.73</td>
<td>(16)</td>
</tr>
<tr>
<td>$\Delta hapR$</td>
<td>8.04</td>
<td>(6)</td>
</tr>
<tr>
<td>$\Delta rpoE$</td>
<td>3.19</td>
<td>(16)</td>
</tr>
<tr>
<td>$\Delta toxR$</td>
<td>1.01</td>
<td>(8)</td>
</tr>
</tbody>
</table>

* Critical values $\geq 11.35$ represent $p$-value $\leq .01$ with three degrees of freedom and are shown in bold, italic font
Table 5 Transposon mutants altered in \(vpsL::lacZ\) production.

<table>
<thead>
<tr>
<th>TIGR #</th>
<th>Strain</th>
<th>Altered Gene Name/Conserved Domains</th>
<th>Predicted Function</th>
<th>(\beta)-Galactosidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>LB</td>
<td>LB + Indole</td>
</tr>
<tr>
<td>Parental</td>
<td>S9149</td>
<td>(\Delta)maA, (\Delta)vpsL::lacZ</td>
<td></td>
<td>16 ± 3.1 31 ± 4.9</td>
</tr>
<tr>
<td>Indole Non-responsive mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC0596</td>
<td>S9224</td>
<td>(dksA) - (dnaK) suppressor protein</td>
<td>Regulatory functions</td>
<td>10.3 ± 2.3 14 ± 3.3</td>
</tr>
<tr>
<td>VC1376</td>
<td>S9216</td>
<td>GGDEF family protein</td>
<td>Cell Signaling</td>
<td>16 ± 2.8 21 ± 1.8</td>
</tr>
<tr>
<td>VCA0074</td>
<td>S9218</td>
<td>(cdgA)- GGDEF family protein</td>
<td>Cell Signaling</td>
<td>4.7 ± 0.7 5.2 ± 2.2</td>
</tr>
<tr>
<td>VPS(-) mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC0338</td>
<td>S9226</td>
<td>Sodium symporter</td>
<td>Ion transport</td>
<td>0 0</td>
</tr>
<tr>
<td>VC1673</td>
<td>S9219</td>
<td>AcrB-D-F Transporter</td>
<td>Ion transport</td>
<td>0 0</td>
</tr>
<tr>
<td>VCA0183</td>
<td>S9227</td>
<td>(hmpA)- Flavohemoglobin</td>
<td>Electron transport</td>
<td>0 0</td>
</tr>
<tr>
<td>VC0665</td>
<td>S9170</td>
<td>(vpsR)- (\sigma^{54}) dependent transcriptional regulator</td>
<td>Regulatory functions</td>
<td>0 0</td>
</tr>
<tr>
<td>VC0143</td>
<td>S9228</td>
<td>Hypothetical protein</td>
<td>Unknown</td>
<td>0 0</td>
</tr>
<tr>
<td>VC1731</td>
<td>S9229</td>
<td>Conserved hypothetical protein</td>
<td>Unknown</td>
<td>0 0</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Phenotypes of *V. cholerae* tryptophanase mutants. A. Biofilm accumulation of wild-type (WT) SIO and TP strains and their corresponding *tnaA::Tn5* mutants S1101 and T1101 grown in LB (grey bars) and LB supplemented with indole (350 µM, white bars). B. Confocal micrographs of the biofilms of S4100 and S4101 grown in LB (+/- Indole 500 µM) for six hours under static conditions. Top picture is XY-axis and bottom picture is Z-axis. C. Measure of the auto-aggregation phenotype of SIO and S1101 grown in LB with Indole 500 µM (white bars) or without (grey bars). Error bars represent one standard deviation from the mean of three biological replicates for each strain.

Figure 2. Indole production correlates to increased *vpsL::lacZ* transcription. A. Measured endogenous indole (white boxes) and β-galactosidase (black circles) production by strain S9171 (ΔlacZ, *vpsL::lacZ*) over a 24-hour period. B. Comparison of the β-galactosidase production in response to endogenous indole production strain S9171 and exogenous addition of indole to strain S9149 (ΔlacZ, ΔtnaA *vpsL::lacZ*). Error bars represent one standard deviation from the mean of three biological replicates for each strain.

Figure 3. Endogenously produced indole acts as a signal to stimulate the expression of *vpsL* in recipient cells. A. In the co-culture experiment, an indole-negative donor strain (S2150 - “I(-) Donor”) does not up-regulate *vpsL::lacZ* expression in the reporter strain (S9149 - “Reporter”; grey bar), while the indole-positive donor strain (S2148 - “I(+) Donor”) can complement *vpsL::lacZ* expression to similar levels as when 0.5 mM indole
(“I”) is added exogenously (white bars). B. The same up-regulation by indole is seen when the S9149 reporter strain is grown in conditioned media grown from indole-positive supernatant from S2148 (“SI+”; white bar) and indole-negative supernatant from S2150 (“SI-”; dark grey bar) conditions. Growth of S9149 in LB alone also did not stimulate vpsL::lacZ up-regulation (“LB”; light grey bar). For the co-culture experiment Miller Units are normalized to the percentage of S9149 cells from the total OD (see materials and methods). Error bars represent one standard deviation from the mean of three biological replicates for each strain.

**Figure 4.** Overlap of genes found to be significantly altered in expression between the SIO/S1101 expression profile and remaining pair-wise comparisons. A. Venn diagrams show the shared and unique genes found between the differentially expressed genes of SIO/S1101 grown in LB compared with: SIO grown with indole versus SIO grown without indole [SIO(+I)/SIO]; SIO grown without indole and S1101 grown with indole [SIO/S1101(+I)]; and S1101 grown with indole versus S1101 grown without indole [S1101(+I)/S1101]. B. Heatmap depicting differences in expression between all genes within the union of the SIO/S1101 and SIO/S1101(+I) expression profiles (569 genes; see middle Venn diagram of Fig. 4A). Compact views of genes are presented using the log$_2$ based color scale shown at the bottom of the panels (yellow = induced, blue = repressed, black = no significant change).

**Figure 5.** Heatmaps of selected functionally-relevant genes that are significantly altered in transcriptional levels from each expression profile examined. A. Selected genes of the
V. cholerae genome believed to be responsible for VPS biosynthesis. B. Selected genes functionally categorized as having a role in cellular processes, including many genes thought to be involved in flagellar biosynthesis, motility and chemotaxis. C. Genes involved in iron transport and Virulence-Associated Secretion (VAS). Compact views of genes are presented using the log_2 based color scale shown at the top right (yellow = induced, blue = repressed, black = no significant change).

Figure 6. Model of indole-regulated gene expression. Indole affects DksA activity and downstream associations between RNA polymerase holoenzyme and σ^{54}. This transcriptional complex can: (i) repress rpoS transcription and its activation of iron acquisition mechanisms, (ii) couple with VasH to enhance VAS operon transcription, and (iii) combine with VpsR to autoregulate VpsR expression in addition to upregulation of CdgA and VpsT expression. Up-regulation of CdgA leads to increases in c-di-GMP levels and subsequent repression of motility and chemotaxis genes and activation of vps genes. An asterisk follows the corresponding protein products of genes that are non-indole responsive and were recovered in the transposon mutagenesis. No arrowhead proceeds from RpoN to VpsR, due to the lack of experimental evidence establishing this link.