Differences in Gene Expression between the Classical and El Tor Biotypes of *Vibrio cholerae* O1†

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Differences in whole-genome expression patterns between the classical and El Tor biotypes of *Vibrio cholerae* O1 were determined under conditions that induce virulence gene expression in the classical biotype. A total of 524 genes (13.5% of the genome) were found to be differentially expressed in the two biotypes. The expression of genes encoding proteins required for biofilm formation, chemotaxis, and transport of amino acids, peptides, and iron was higher in the El Tor biotype. These gene expression differences may contribute to the enhanced survival capacity of the El Tor biotype in environmental reservoirs. The expression of genes encoding virulence factors was higher in the classical than in the El Tor biotype. In addition, the *vieSAB* genes, which were originally identified as regulators of *ctxA* transcription, were expressed at a fivefold higher level in the classical biotype. We determined the VieA regulon in both biotypes by transcriptome comparison of wild-type and *vieA* deletion mutant strains. VieA predominantly regulates gene expression in the classical biotype; 401 genes (10.3% of the genome), including those encoding proteins required for virulence, exopolysaccharide biosynthesis, and flagellum production as well as those regulated by σ^E, are differentially expressed in the classical *vieA* deletion mutant. In contrast, only five genes were regulated by VieA in the El Tor biotype. A large fraction (20.8%) of the genes that are differentially expressed in the classical versus the El Tor biotype are controlled by VieA in the classical biotype. Thus, VieA is a major regulator of genes in the classical biotype under virulence gene-inducing conditions.

Vibrio cholerae is the causative agent of Asiatic cholera. It is estimated that the disease causes the deaths of 120,000 people worldwide every year and infects many more, mostly children between 1 and 5 years old (18, 27). The pathogen is endemic to coastlines and estuarine ecosystems around the world but can spread worldwide, causing pandemics (18, 35). *V. cholerae* strains are classified using the lipopolysaccharide O antigen, which identifies over 200 serogroups. Strains of *V. cholerae* that have epidemic and pandemic potential belong to serogroups O1 and O139. The O1 serogroup is further divided into classical and El Tor biotypes on the basis of their biochemical properties and phage susceptibilities (18, 27). *V. cholerae* has caused eight pandemics since 1817. The classical biotype was responsible for the first six pandemics, and the El Tor biotype is responsible for the seventh ongoing pandemic (18). The clinical manifestation of the disease caused by the classical biotype is more severe, while the El Tor biotype is considered to be more environmentally fit due to its capacity to eliminate the classical biotype during the last pandemic. These observations led to the postulation that the epidemic natures of the biotypes could be different (18, 27).

The major virulence factors cholera enterotoxin (CT) and the colonization factor toxin-coregulated pilus (TCP) are required for infection by both biotypes (27, 47). However, there are marked differences in the regulation of virulence factor production between the classical and El Tor biotypes (14). The production of the virulence factors CT and TCP is controlled by a complex transcriptional regulatory cascade (13). The transcription of the genes encoding CT and TCP is positively controlled by the regulatory proteins ToxRS and TcpPH, which control the expression of the most downstream regulator ToxT (15, 24, 32, 58). The expression of *tcpPH* is also under the control of two other regulatory proteins, AphA and AphB (30). In the classical biotype, the expression of *ctx*, *tcp*, and *toxT* in vitro is regulated by environmental signals, including pH, temperature, osmolarity, and amino acids; in contrast, the El Tor biotype requires a complex growth medium and specific growth parameters that include the incubation of cultures at 37°C under static conditions for 4 h, followed by overnight incubation with shaking at 37°C to induce virulence gene expression (26, 38). The molecular basis of differential regulation of virulence factor production in the two biotypes has been extensively studied (6, 14, 29, 30). These studies have shown that the variation in *tcpPH* production is due in part to DNA sequence differences between classical and El Tor *tcpPH* promoters and the resulting interaction of AphB with the *tcpPH* promoter (29, 30). It has also been shown that the timing of the transcription of *tcpPH* is different between the classical and El Tor biotypes (39).

Using the completely sequenced *V. cholerae* O1 El Tor genome and microarray technology, the genomic differences between different biotypes and serogroups of the pathogen have been determined on a genome-wide scale via DNA-to-DNA

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comparisons (17, 25). These studies have shown that there is a high degree of conservation in the gene content of different *V. cholerae* strains. In particular, as only 29 genes are missing in the classical biotype in comparison to El Tor, very small differences between the classical and seventh pandemic El Tor strains were detected (17). It is critical to note that genomic comparisons are unidirectional; genes that are present in the test strains but missing from the reference O1 El Tor strain N16961 cannot be detected. Moreover, short sequence differences in the genome are unlikely to be identified unless high-resolution microarrays and whole-genome sequencing approaches are utilized. If alterations to the genome are present in the coding sequences of master regulatory proteins or their upstream regulatory regions, the transcriptional profile of the organism may be dramatically altered. In such cases, the consequences of genome evolution can be detected at the transcriptome level. The transcriptome of an organism under a given growth condition as well as its capacity to modulate the transcriptome in response to extracellular and intracellular signals will greatly influence the overall fitness and epidemic and endemic potential of the organism.

In this study, we used transcriptional profiling to gain insight into variations in global gene expression between the classical and El Tor biotypes of *V. cholerae* O1 and determined that 13.5% of the total genes are differentially expressed between the two biotypes under conditions that induce virulence gene expression in the classical biotype. In particular, messages of the *vieS*, *vieA*, and *vieB* genes, which encode regulatory proteins that control *ctx* transcription through their modulation of intracellular cyclic diguanylate (c-di-GMP) concentration (51), were increased in the classical biotype. The *vieA* gene encodes a response regulator possessing hydrolase activity against c-di-GMP (43, 46) and performs the critical role of lowering c-di-GMP concentration to allow for virulence gene expression. To determine the contribution of VieA to the transcriptome under the classical biotype virulence gene-inducing condition, we compared the whole-genome expression profile of the classical wild type to that of a *vieA* deletion mutant. The results revealed that VieA is a major regulator of gene expression under classical virulence gene-inducing conditions regulating the expression of 10.3% of the genes. In contrast, VieA does not significantly contribute to the regulation of gene expression in the El Tor biotype.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* O1 classical strain O395 (hereafter referred to as the classical wild type [C-wt]) (5), O1 El Tor strain A1552 (E-wt) (57), and *vieA* in-frame deletion mutants generated in both biotypes C-Δ*vieA* (49) and E-Δ*vieA* (this work) were utilized. *V. cholerae* cultures were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) or M9 minimal medium supplemented with minimal essential medium vitamins (10 ml/liter; Gibco), trace metals (1 ml/liter of 5% MgSO$_4$, 0.5% MnCl$_2$ · 4H$_2$O, 0.5% FeCl$_3$, 0.4% trinitroloacetic acid), 0.75 mM KH$_2$PO$_4$, 0.5% glycerol, and 25 mM of the following L-amino acids: Asn, Arg, Glu, and Ser (43); the latter medium will be hereafter referred to as M9 + NRES. *V. cholerae* cultures were grown with aeration at 30°C unless otherwise noted.

Gene expression profiling. Whole-genome expression analysis was performed using RNA isolated from cultures grown in M9 + NRES at 30°C to an optical density at 600 nm (OD$_{600}$) of 0.65. A common reference RNA, which was a 1:1 ratio mixture of RNA isolated from C-wt and E-wt cells, was used for hybridization. Test RNA samples were isolated from C-wt and E-wt strains and the *vieA* mutants. Total RNA was isolated as described previously (56). RNA samples

from test and reference samples were used in a reverse transcription reaction as follows: 3 μg of RNA and 5 μg of random hexamers were denatured at 80°C for 8 min and then chilled on ice for 5 min. The reaction mixture, containing first-strand buffer, 0.01 M dithiothreitol, aminoallyl deoxynucleoside triphosphate labeling mix with amino acid-dUTP in a 3 (dTTP):2 (amino acid-dUTP) ratio, and 400 U SuperScript III (Invitrogen), was added to cooled samples and incubated at 42°C for 3 to 4 h. In order to hydrolyze the RNA in the cDNA-RNA mixture, samples were incubated at 65°C for 10 min in the presence of 100 mM NaOH and 10 mM EDTA. After hydrolysis, reactions were neutralized by the addition of 1 M HEPES, pH 7.0, at a final concentration of 0.5 M HEPES. cDNA was purified from the reactions using the QIAquick PCR purification kit (QIAGEN) using phosphate wash and phosphate elution buffers and dried. The samples were indirectly labeled by covalent coupling of the *N*-hydroxysuccinimide esters of the cyanine fluorophores to the aminoallyl-labeled cDNAs as follows. Cleaned and dried samples were resuspended in freshly prepared 50 mM Na-bicarbonate buffer, pH 9.0. One vial of Cy3/Cy5 fluor (Amersham CyDye postlabeling reactive dye pack) was mixed with each sample and incubated for 1 h in the dark at room temperature. Unincorporated dye molecules were removed using the QIAquick PCR purification kit (QIAGEN). Dye incorporation and cDNA concentrations were determined by measuring optical densities at 260, 550, and 650 nm. The amount of nucleotides in each sample was determined by using the following formula: amount of nucleotides (picomoles) = [OD$_{260}$ × volume (microliters) × 37 ng/μl × 1,000 pg/ng]/324.5 pg/pmol. The amount of incorporated dye molecules was determined by using the following formulas: picomololes Cy3 = [OD$_{550}$ × volume (microliters)]/0.15 and picomoles Cy5 = [OD$_{650}$ × volume (microliters)]/0.25. The nucleotide-to-dye ratio was then calculated by dividing picomoles of cDNA by picomoles of Cy dye. Samples with >100 pmol dye incorporation and <10 nucleotides per dye ratio were used in the hybridizations (this method is modified from The Institute for Genomic Research's [TIGR] microbial RNA aminoallyl labeling for microarrays protocol). For hybridizations, dye-coupled test samples were mixed with the corresponding reference sample and dried. Amine silane slides containing 70-mer oligonucleotides (which were purchased from Illumina), representing most of the open reading frames present in the *V. cholerae* N16961 genome, were UV cross-linked at 250 mJ and prehybridized in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 1% bovine serum albumin for at least 45 min at 42°C. The hybridization mix was prepared by resuspending dried samples in a solution containing 15 μg salmon sperm DNA, 15 μg tRNA, 3× SSC, and 0.1% sodium dodecyl sulfate. Concentrated hybridization mix was applied to the slides and incubated at 65°C for 12 to 20 h. After hybridization, slides were washed, dried, and scanned using an Axon scanner to determine the fluorescence in each open reading frame-specific spot. The raw data were obtained by using the software package GenePix 4.1 (Axon). Normalized signal ratios were obtained with LOWESS print-tip normalization using the Bioconductor packages (http://www.bioconductor.org) (19) in R environment. Differentially regulated genes were determined (with two biological and two technical replicates for each data point) with the significance analysis of microarrays (SAM) package (52) using twofold differences in gene expression and a 1% false discovery rate as a cutoff value.

Motility assays. LB soft agar plates (0.3% agar) were used to determine the motility of bacterial strains. Strains were first streaked for single colonies on LB agar and incubated overnight at 30°C. Single colonies were removed with a toothpick, stabbed into the LB soft agar plate, and incubated overnight at 30°C. The diameter of the zone of motility was measured after 20 h of incubation at 30°C.

RESULTS AND DISCUSSION

Transcriptome analysis of classical and El Tor biotype strains. With the exception of the differential regulation of virulence factor production in the two biotypes, which has been extensively characterized at the molecular level (6, 14, 29, 30), at present, relatively little is known about the transcriptome differences between the classical and El Tor biotypes. It is likely that other genes expressed in manners similar to those of virulence genes contribute to virulence gene expression either by acting as regulatory proteins or by influencing the physiological state of the cell, which may in turn influence virulence gene expression. To gain an understanding of the genes and processes that are differentially regulated in the two biotypes
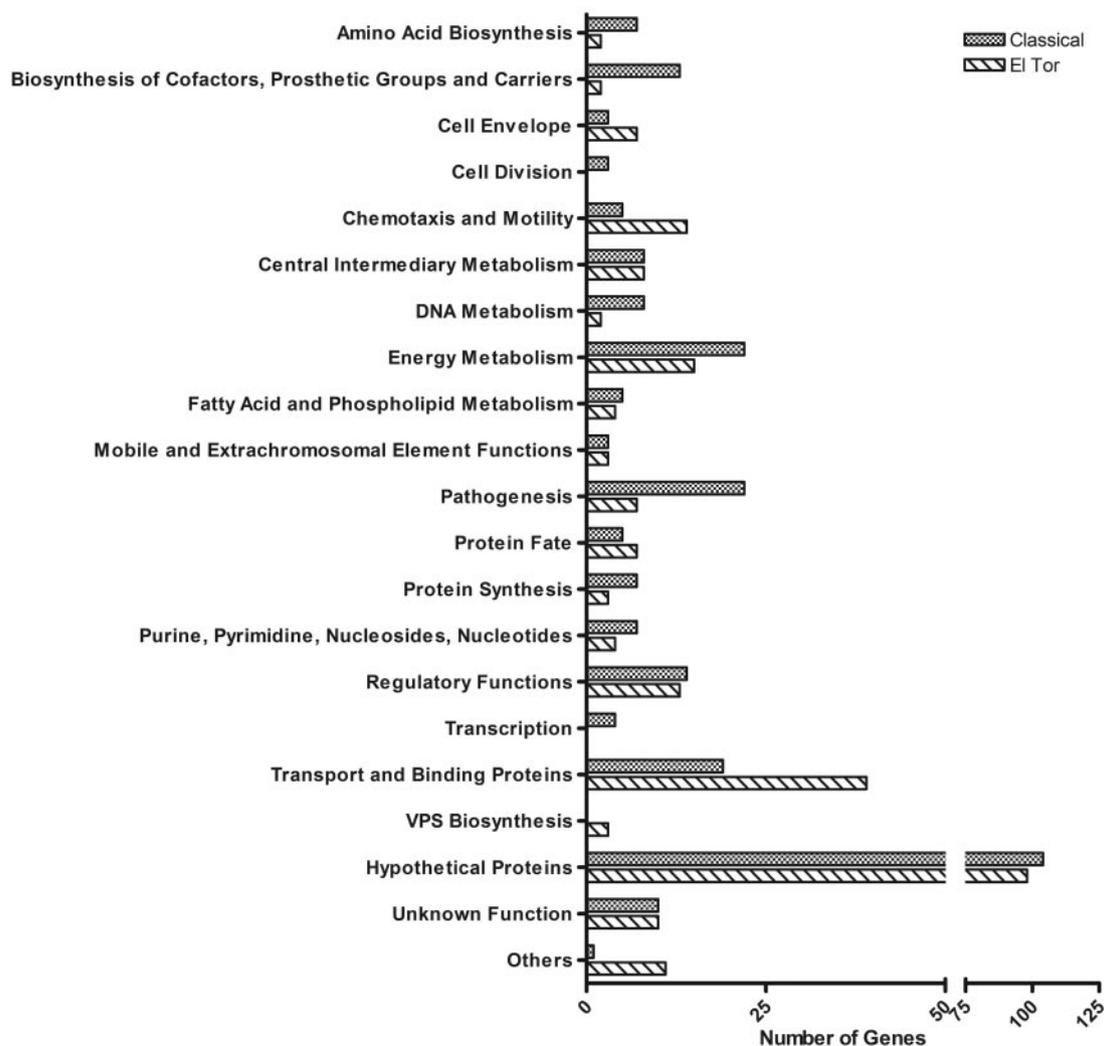
FIG. 1. Functional categories of the differentially expressed genes in *V. cholerae* biotypes. The number of genes whose expression is increased in classical or El Tor biotype is presented according to the functions assigned by TIGR genome database.

when grown under the conditions conducive to virulence factor production in the classical biotype (38), we compared transcriptomes of the two biotypes using RNA isolated from C-wt and E-wt. Both were grown in M9 + NRES at 30°C to an OD_{600} nm of 0.65. These conditions have been used by other investigators to induce virulence gene expression in C-wt (38, 50). RNA samples were used in indirect labeling reactions, and the labeled samples were hybridized onto a *V. cholerae* 70-mer oligonucleotide array. Differentially regulated genes were determined with SAM (52) by using a twofold difference in gene expression and a 1% false discovery rate as a cutoff value. We identified 271 genes with increased expression in C-wt and 253 genes with increased expression in E-wt. Differentially expressed genes classified according to their functional categories are shown in Fig. 1. For the complete list of differentially expressed genes, see Tables S1 and S2 in the supplemental material. Below we discuss a selected subset of these genes that was differentially regulated.

Expression profiling experiments were performed using RNA from cultures grown under conditions that activate vir-

ulence gene expression in the classical biotype (38). As expected, the expression of virulence genes was higher in C-wt. Specifically, *ctxA* and *ctxB* message abundance was higher by 3.7 and 3.6-fold, respectively (Fig. 2B). Similarly, the expression of the genes encoding the toxin coregulated pilus (*tcp*) were increased 11.5-fold on average (Fig. 2A). The expression of the genes encoding accessory colonization factors (*acfA*, *acfB*, and *acfC*) was 9.1-, 5.0-, and 4.1-fold higher, respectively. Message abundance for the genes encoding regulatory factors (*tcpPH* and *toxT*) controlling the transcription of *ctxAB* and *tcp* genes was increased 3.8-, 4.0-, and 7.3-fold, respectively. This finding is consistent with the previous reports that *tcpPH*, and in turn *toxT*, gene expression is higher in the classical biotype under classical inducing conditions (14, 39, 40). Similarly, as reported earlier, the transcription of *toxRS* as well as *aphAB* was not different between the two biotypes (31). It should be noted that the DNA sequence of the *tcpA* gene encoding the TCP pilin is different between classical and El Tor biotypes (25, 44). The microarrays used in this study contain unique 70-mer oligonucleotides for *tcpA* representing each biotype (El Tor, CATTT
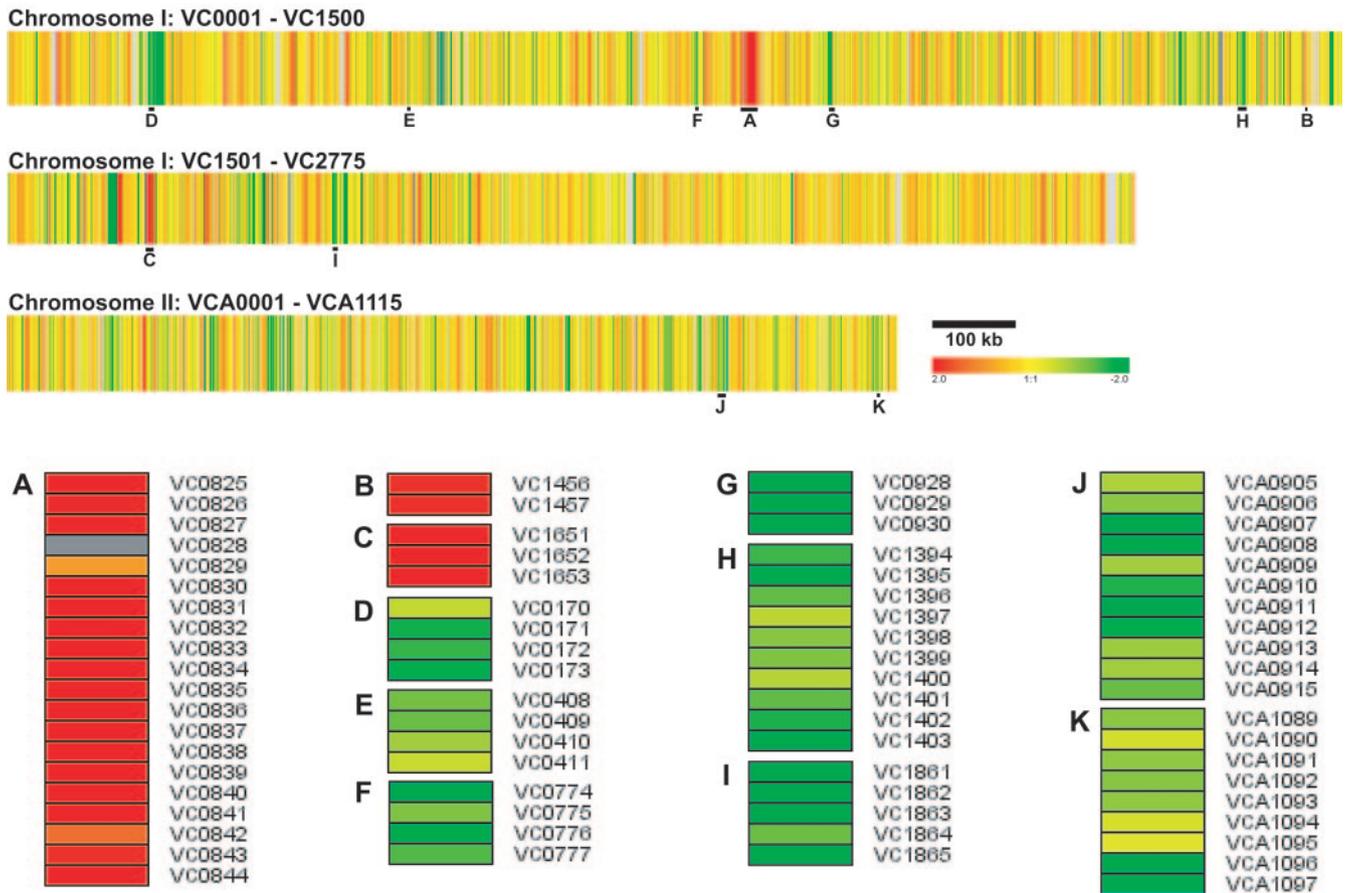
FIG. 2. Chromosomal mapping of the differentially expressed genes between the classical and El Tor biotypes. Differentially regulated genes are presented in genome order. The magnitude of expression is given with a color-coded scale (red, high in classical biotype; green, high in El Tor biotype; yellow, equally expressed in both biotypes). The expression profiles of selected gene clusters, (A) *tcp*, (B) *ctx*, (C) *vie*, (D) peptide ABC transporter, (E) *msh*, (F) vibriobactin biosynthesis, (G) *vps* region, (H) chemotaxis, (I) amino acid ABC transporter, (J) iron transport, and (K) chemotaxis, are shown.

CCACGAAACTCTGCAGCGAATAAAGCATTCGCAATTA CAGTCGGTGGCTTGACCCAAGCACAATG, and classical, GCGTAATGCAGCAGCTAATAAAGCATTTGCAATTTCA GTGGATGGTCTGACACAGGCTCAATGCAAGAC). Thus, as the 70-mers representing the *tcpA* gene are biotype specific, we were unable to compare *tcpA* message abundance differences between the two biotypes.

The *vieSAB* genes, which were originally identified as regulators of *ctxA* transcription during infection (33, 51), are differentially expressed in the two biotypes (Fig. 2C). The *vieSAB* gene products constitute a putative three-component signal transduction system in which *vieA* (VC1652) and *vieB* (VC1651) encode response regulators, and *vieS* (VC1653) encodes a sensor kinase. Recent studies have shown that the response regulator VieA also functions as a c-di-GMP phosphodiesterase and acts to maintain a low cellular concentration of c-di-GMP (46, 50, 51). Lower c-di-GMP levels in turn lead to an increase in *toxT*, *ctx*, and *tcp* gene transcription. In this study, for the first time, we show that *vieA*, *vieB*, and *vieS* genes are transcribed at higher levels of 5.0-, 6.7-, and 5.5-fold, respectively, in C-wt. These results suggest that Vie signal transduction also contributes to differential expression of virulence genes in classical and El Tor biotypes.

In addition to *vieSAB* genes, message levels for 11 genes predicted to encode regulatory proteins, VC0072, VC0278 (*cadC*), VC0290 (*fis*), VC1222 (*himA*), VC1286, VC1713, VC2485, VC2749 (*ntrC*), VCA0532, VCA0850, and VCA0952 (*vpsT*), were higher in the classical biotype. It will be of interest to determine whether any of these proteins contribute to biotype-specific virulence gene expression in *V. cholerae*.

Genes involved in purine and pyrimidine ribonucleotide biosynthesis showed higher levels of expression in C-wt. These include VC1004 (*purF*) amidophosphoribosyltransferase, VC0276 (*purH*) phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase, VC1491 (*pyrD*) dihydroorotate dehydrogenase, VC2510 (*pyrB*) aspartate carbamoyltransferase-catalytic subunit, VC2511 (*pyrI*) aspartate carbamoyltransferase-regulatory subunit, VC2389 (*carB*) carbamoyl phosphate synthase large subunit, and VC0767 (*guaB*) inosine-5′-monophosphate dehydrogenase. In addition, we observed 1.8-, 1.9-, and 2.0-fold higher expression of VC0051 (*purK*) phosphoribosylaminoimidazole carboxylase-ATPase subunit, VC2390 (*carA*) carbamoyl phosphate synthase small subunit, and VC1911 (*pyrF*) in C-wt, respectively, in comparison to E-wt. Intriguingly, the transcription of some genes involved in purine nucleotide and nucleoside interconver-

sion, including VC1916 (*cmk*) cytidylate kinase, VCA0197 (*guaC*) GMP reductase, VC0756 (*ndk*) nucleoside diphosphate kinase, and VC1129 (*gsk-1*) inosine-guanosine kinase, was lower in the classical biotype than in El Tor. As discussed above, a recently identified second messenger c-di-GMP regulates *V. cholerae* virulence gene expression. Under our experimental conditions, which induce virulence gene expression in C-wt, we also saw a difference in the expression of purine metabolism genes. It is possible that this difference may modulate cellular GTP and, in turn, c-di-GMP levels in the cell, leading to differential production of c-di-GMP in the classical and El Tor biotypes. We attempted to determine the c-di-GMP levels in classical and El Tor by using the two-dimensional thin-layer chromatography method that has been used previously (49). However, this method was not sensitive enough to reveal any possible differences in c-di-GMP levels under the conditions tested.

Vibrio species are capable of degrading chitin to *N*-acetyl-glucosamine, which can be used as a carbon and nitrogen source (34, 37). Of the five chitinase genes found in *V. cholerae*, three were differentially expressed in the two biotypes. The expression of VC1073, VCA0811, and VC1952 (*chiA-1*) was increased 2.0-, 9.2-, and 5.2-fold, respectively, in the classical biotype. Earlier studies have shown that the expression of VC1952 and VCA0811 (which function as chitinase and chitin binding proteins, respectively) is induced by chitin oligosaccharides and *N*-acetylglucosamine (37). In addition, it has been reported that VCA0811 is critical for the attachment of *V. cholerae* to epithelial cells and it is postulated that VCA0811 functions as a GlcNAc-specific lectin (28). In this study, we show that the putative cellular adhesion factor encoded by VCA0811 is differentially expressed in classical and El Tor biotypes.

The expression of a large set of genes was increased in E-wt relative to the classical biotype. One of the phenotypic differences between the two biotypes is the capacity of El Tor strains to produce a pilus termed mannose-sensitive hemagglutinin (MSHA) (23), even though the classical biotype has the necessary genes for making MSHA (17). Messages for the genes encoding MSHA pilin proteins, *mshA* (VC0409), *mshB* (VC0408), and *mshC* (VC0410) were 2.4-, 2.3-, and 1.8-fold higher in E-wt, respectively (Fig. 2E). The MSHA pilus is not critical for intestinal colonization (3, 45, 48); however, it facilitates adherence and subsequent biofilm formation by *V. cholerae* O1 El Tor to biotic and abiotic surfaces (8, 53, 54).

Another phenotypic characteristic that distinguishes the El Tor and classical biotypes is the hemolytic capacity of El Tor strains. In the *V. cholerae* genome, there are 13 genes predicted to encode hemolysins. According to our transcriptome analysis, two of the putative hemolysins (VCA0218 and VCA0646) were expressed at higher levels in the classical biotype and three of them (VC0930, VC1888, and VCA0219) showed higher expressions in E-wt. VCA0219 (*hlyA*) encodes the El Tor hemolysin (20, 21, 36), and here we show that this virulence factor is expressed at a higher level in E-wt.

V. cholerae produces an exopolysaccharide (VPS) that is required for biofilm formation. The VPS biosynthesis genes (*vps*) are localized in a 30-kb region in the large chromosome and organized into two clusters spanning VC0916 to VC0928 and VC0934 to VC0939. The expression of *vps* genes as well as the genes located between the two clusters is coregulated. It

was of interest that the expression of several genes located between *vpsI* and *vpsII* clusters, VC0928 (4.56-fold), VC0929 (3.70-fold), and VC0930 (8.33-fold), were increased in E-wt (Fig. 2G). Mutations in these genes alter biofilm development in *V. cholerae* (N. Fong and F. H. Yildiz, unpublished data). In addition, the expression of *vpsR*, encoding the positive transcriptional regulator of *vps* genes, was 2.17-fold higher in E-wt. In contrast, the expression of *vpsT*, which encodes the other positive transcriptional regulator of *vps* genes, was higher in C-wt. The significance of the differential expression of two positive regulators of *vps* genes has yet to be determined. As indicated above, the El Tor biotype is considered to be more environmentally fit; however, the physiological basis for this increased fitness has not been determined. Although our experiments were not performed under the conditions simulating natural habitats of *V. cholerae*, one can speculate that differences in *vps* gene expression and, in turn, increased capacity to produce VPS and form biofilms, could increase the environmental survival chances of the El Tor biotype.

A set of genes predicted to be involved in chemotaxis were differentially expressed in the El Tor and classical biotypes (Fig. 2H and K). *V. cholerae* has multiple homologs of *Escherichia coli* chemotaxis genes, and these are clustered into three different regions (25). Only one of the three *cheA* homologs (*cheA-2*) is responsible for chemotaxis in the classical and El Tor *V. cholerae* O1 biotypes when assayed in 0.3% LB soft agar motility plates (22). The message abundance of three of the *cheY* genes, VC1395 (*cheY-1*), VC1398 (*cheY-2*), and VCA1096 (*cheY-4*), which are response regulators, was 3.0-fold higher in E-wt, on average, in comparison to that in C-wt. In addition, the expression of VC1399 (*cheR-1*), VC1401 (*cheB-1*), and VC1402 (*cheW*) encoding for methyltransferase, methylesterase, and a putative purine-binding chemotaxis protein, respectively, was also higher in E-wt. Moreover, among 43 methyl-accepting chemotaxis proteins (MCPs), 7 of them (VC1248, VC1298, VC1394, VC1403, VC1643, VC1868, and VC1967) exhibited increased message abundance in E-wt. In contrast, the expression levels of three MCP genes (VC1289, VC1313, and VCA0068) were decreased in E-wt. Chemotaxis facilitates the adaptation of microorganisms to their environment by sensing and responding to physical and chemical changes. Differences in the expression of components of the chemotaxis signal transduction system, in particular, MCPs that sense environmental cues and transduce this information to modulate chemotaxis, may greatly influence environmental distribution and, in turn, the survival capacities of different biotypes.

Message abundance of VC1452 (*rstC*) and VC1455 (*rstR-1*) was increased in E-wt. These genes are located in RS1, a satellite phage of CTXφ, which harbors the genes encoding proteins involved in replication (RstA), integration (RstB), and transcriptional regulation (RstC and RstR). RstR functions as a repressor of both RS1 and CTXφ gene expression, and RstC functions as an antirepressor of RstR (10, 11). These observations suggest that CTXφ propagation may be different in the El Tor and classical strains and could greatly influence the evolution of new pathogenic *V. cholerae* variants.

The expressions of VC1583 (*sodC*) and VC2694 (*sodA*) that encode superoxide dismutase; VC0076, a universal stress protein A; VC0139, a DPS family protein; and VC1585 (*katB*), a catalase, were higher in E-wt, which may indicate that the

oxidative stress survival capacities of these two strains are different.

A large set of genes predicted to be involved in the transport of amino acids and peptides were expressed at higher levels in E-wt (Fig. 2D and I). These include VC0171 to VC0173 that encode peptide ABC transporter components; VC1861 to VC1864 that encode amino acid ABC transporter components; VCA1039, VC1362, and VCA0978 that each encode for a periplasmic amino acid binding protein; VCA0759 (*artI*), a periplasmic arginine binding protein; VC1168 (*gltP-1*) that encodes a proton/glutamate symporter; VC1658 (*sdaC-2*), a serine transporter; VC0784, a sodium alanine symporter; and VC1424 and VC0704 that both encode periplasmic spermidine/putrecine binding proteins. This observation suggests that there may be differences in preferred carbon, nitrogen, and energy sources between the classical and El Tor biotypes that could alter environmental survival and distribution.

Message abundance for a large set of genes involved in the acquisition and transport of iron was higher in E-wt. In particular, the genes involved in siderophore vibriobactin production and function are expressed at higher levels (Fig. 2F); these include VC2210 (*viuA*), VC2211 (*viuB*), VC0774 (*vibA*), VC0775, VC0776 (*fepB*), VC0777, and VCA0227 (*vct*). The expressions of additional genes encoding transport and binding proteins were also increased in E-wt (Fig. 2J). These include VCA0910 to VCA0911 that encode components of the TonB system transport protein, VCA0576 (*hutA*) and VCA0915 (*hutD*) that are required for heme and hemin transport, VC0608 (iron III-ABC transporer-periplasmic iron compound binding protein), VC0364 (bacterioferritin-associated ferredoxin), and VC1547 (biopolymer transport protein).

Genomic comparison of El Tor and classical biotypes revealed that 29 genes are missing in the classical biotype (9). We observed that 16 of these genes are expressed in E-wt under the experimental conditions used in this study.

Identification of VieA regulon via whole-genome expression analysis. VieA is a response regulator and has a c-di-GMP-specific phosphodiesterase activity (46, 51). The expression of *vieA* in classical strain O395 is elevated during growth in M9 + NRES (49). Under this growth condition, the loss of VieA phosphodiesterase activity results in a sixfold increase in the intracellular concentration of c-di-GMP (50). As *vieA*, *vieB*, and *vieS* genes are transcribed at higher levels of 5.0-, 6.7-, and 5.5-fold, respectively, in C-wt, we wanted to determine the contribution of the Vie signal transduction system and, in particular, that of VieA to the transcriptome of the classical biotype when grown under virulence gene-inducing conditions.

To identify VieA-regulated genes in the classical biotype, whole-genome expression patterns of the wild-type O395 (C-wt) and a derivative harboring an in-frame deletion of *vieA* (C-Δ*vieA*) were compared. The C-wt and C-Δ*vieA* strains were grown to mid-exponential phase in M9 + NRES. Gene expression data were analyzed by the SAM program (52) to identify genes whose expression levels were up to twofold different in the C-Δ*vieA* relative to the wild type. This analysis identified a total of 401 genes that were differentially expressed; of those, the expression of 241 was increased, while the expression of 160 was decreased in C-Δ*vieA* (Fig. 3 and see Table S3 in the supplemental material). Of the 401 differentially regulated genes, 109 genes were also differentially expressed between *V.*

cholerae classical and El Tor strains under the condition that induces classical virulence gene expression. Among the genes that overlap between the two data sets, the majority (75.5%) are regulated in the expected manner. For example, genes that exhibited increased expression in C-wt relative to E-wt were dependent on VieA for maximal expression. This category includes genes encoding the TCP colonization factor. Conversely, genes that showed increased expression in E-wt also exhibited increased expression in the C-Δ*vieA* strain relative to C-wt, suggesting that VieA is responsible for repressing the expression of these genes in the classical biotype. However, some genes that overlap between the two data sets (24.5%) do not fall into these categories but are regulated in the opposite manner. Additional regulatory factors may contribute to controlling the expression of these genes in the classical biotype. We also compared the transcriptomes of E-Δ*vieA* and E-wt. In contrast to the classical biotype, VieA regulates the expression of only a small set of genes in the El Tor biotype; the expression of one gene (VC1593) was increased, and the expression of four genes (VC0846, VC1098, VC2169, and VCA0509) was decreased in E-Δ*vieA* in comparison to E-wt. This finding highlights the importance of VieA's contribution to gene expression in the classical biotype under classical virulence gene-inducing conditions. Below we discuss sets of genes that are differentially expressed between C-wt and C-Δ*vieA*.

It was previously shown that C-Δ*vieA* exhibits increased expression of the *vps* genes and decreased expression of virulence genes (49, 50). Consistent with this, *vpsR* (VC0665) as well as most of the genes within and between the *vpsI* and *vpsII* clusters (VC0916 to VC0939) was overexpressed in C-Δ*vieA* (Fig. 4A). The deletion in *vieA* also resulted in increased expression of genes previously shown to be regulated by VpsR in transcriptome analysis (56). Specifically, most of the *eps* genes encoding a type II secretion system (Fig. 4B) and several of the most highly overexpressed genes (VC1888, VCA0849, VC1962, VCA0811, VC0157, and VCA0952) (see Table S3 in the supplemental material) are positively regulated by VpsR, suggesting that the regulation of these genes by VieA is indirect. Furthermore, since we have previously shown that VieA regulates *vpsR* transcription indirectly through controlling the concentration of c-di-GMP (49), we speculate that the overexpression of these genes in the C-Δ*vieA* is mediated by increased VpsR expression in response to elevated c-di-GMP concentration.

σ^E is an alternative sigma factor that is known to be activated in response to stresses that disrupt protein folding in the cell envelope (for reviews, see references 1, 2, and 16). *rpoE* itself, which encodes σ^E, and the downstream *rseABC* operon whose products control activity of σ^E, exhibited two- to four-fold increases in transcription in C-Δ*vieA* (Fig. 4F and see Table S3 in the supplemental material). The σ^E regulon in *V. cholerae* has recently been identified (12); using the data provided in this study, we determined the genes differentially expressed in C-Δ*vieA* which belong to the σ^E regulon (see Table S3 in the supplemental material). We observed that the second major class of genes, which had higher message abundance in C-Δ*vieA*, were those controlled by σ^E. In addition, homologs of the *E. coli* σ^E-regulated genes *rpoH*, *skp* (*ompH*), *fkpA*, *degP* (*htrA*), and *ecfE* all showed increased transcription. These results suggest that the loss of VieA causes the accu-
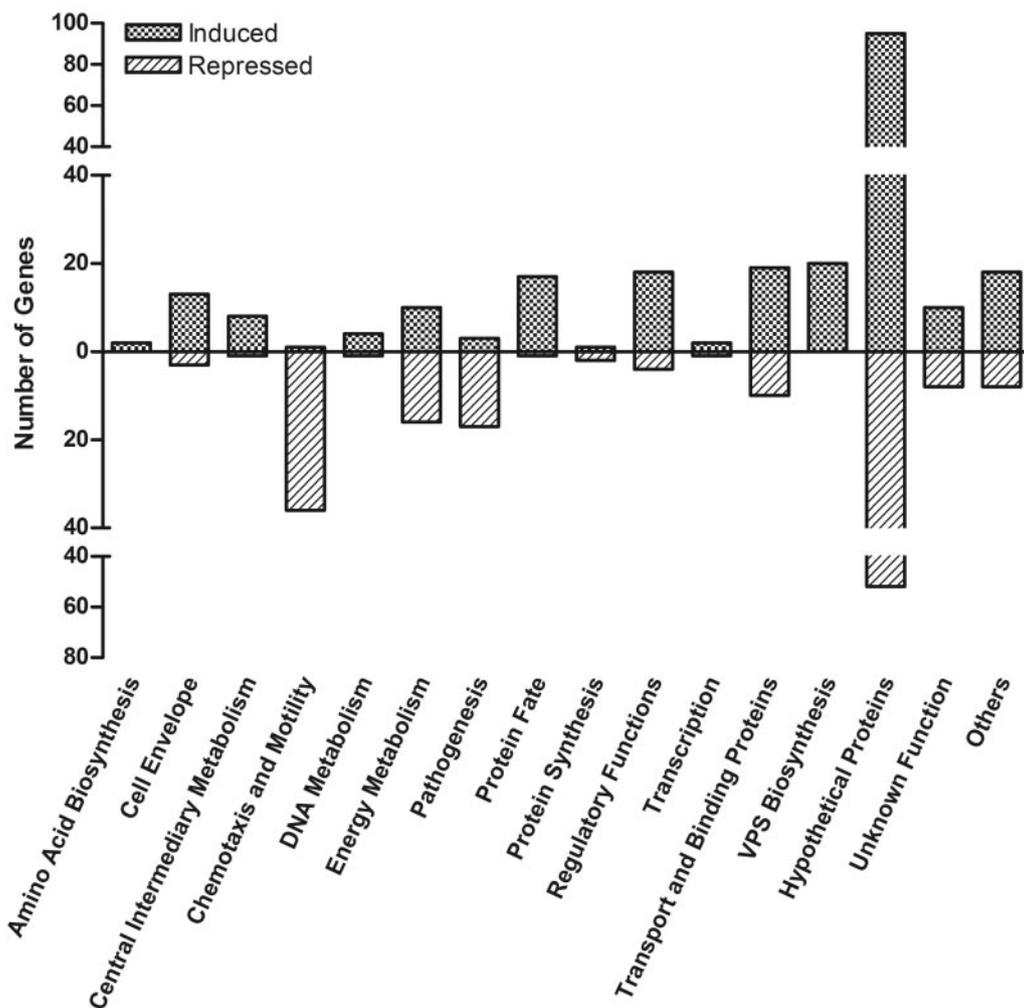
FIG. 3. Functional categories of the differentially expressed genes between C-wt and C-ΔvieA. The number of genes whose expression is increased or decreased in C-ΔvieA in comparison to the classical strain is presented according to the functions assigned by TIGR genome database.

mulation of misfolded proteins targeted to the outer membrane or periplasm. It is possible that these are proteins that are necessary for VPS biosynthesis or intermediates in the VPS pathway.

Among the genes that had lower message abundance in C-ΔvieA are *toxT* (VC0838) and virulence genes positively regulated by ToxT (Fig. 4C and see Table S3 in the supplemental material). These included genes in the *Vibrio p*athogenicity *is*land (VPI), which exhibited the largest decrease in transcription (Fig. 4C). Specifically, the expression of all of the genes in the *tcpA* operon was decreased. Since *tcpA* and other VPI genes are all positively regulated by ToxT (4, 7, 41, 55) and we have previously shown that VieA represses *toxT* transcription indirectly through control of c-di-GMP concentration (50), we propose that changes in the transcription of these genes in C-ΔvieA are due to elevated c-di-GMP concentration.

The membrane protein complexes TcpPH and ToxRS act in concert to activate *toxT* transcription (15, 24, 32). However, consistent with previous results, we observed no change in *toxRS* or *tcpPH* transcription in the C-ΔvieA strain (50). Thus, either c-di-GMP regulates *toxT* in a ToxRS-/TcpPH-independent manner or it regulates one or more of these regulatory proteins posttranscriptionally. The latter is unlikely with respect to ToxRS since the transcription of *ompU*, which is positively regulated by ToxRS (9), was not significantly changed in C-ΔvieA. The sum of these results suggests that c-di-GMP controls *toxT* transcription by affecting either the activity of TcpPH, the activity of ToxT itself, or the activity of an unidentified regulator.

The second major class of genes whose message abundance was decreased in the C-ΔvieA strain encodes proteins that function in flagellar motility and chemotaxis. Specifically, two operons of genes involved in the formation of the basal body-hook structure of the flagellum, all five flagellin subunit genes (*flaA* to *flaE*), and flagellar motor genes *motX* and *motY* exhibit decreased expression (Fig. 4D). In addition, several motility- and chemotaxis-related genes scattered throughout the *V. cholerae* genome are expressed at lower levels (see Table S3 in the supplemental material). Consistent with these transcriptional changes, C-ΔvieA exhibits reduced motility in semisolid agar (Fig. 5A and B), and this defect could be complemented
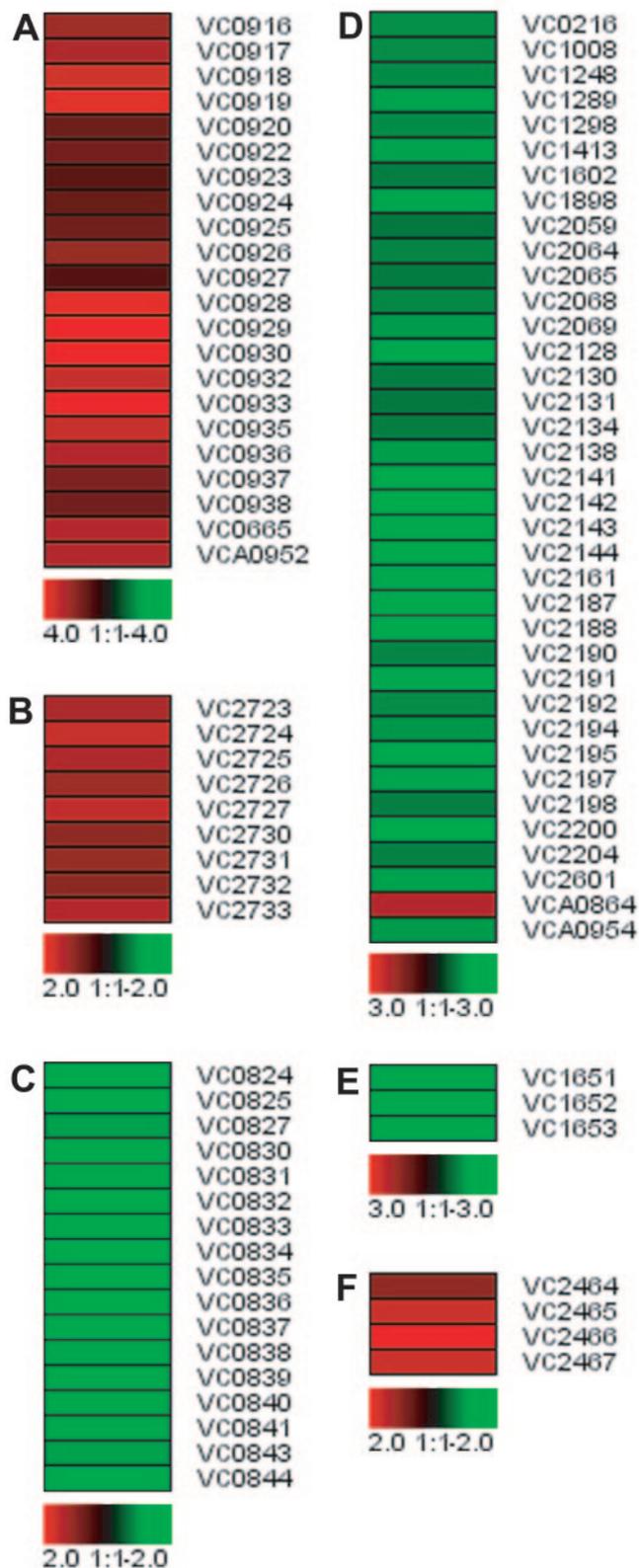
FIG. 4. Heat maps of differentially expressed genes between C-wt and C-ΔvieA. The magnitude of expression is given with a color-coded scale (red, induced; green, repressed). The expression profiles of selected gene clusters, (A) vps, (B) eps, (C) genes located in VPI, (D) genes involved in chemotaxis and motility, (E) vieSAB, and (F) rseABC and rpoE, are shown.
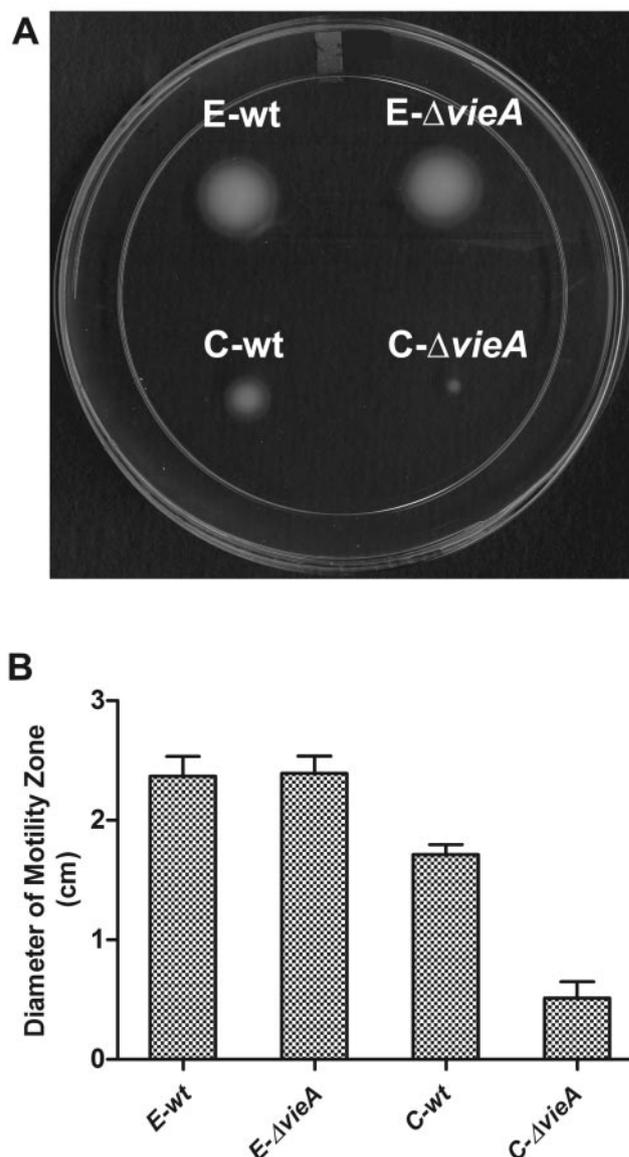

FIG. 5. (A) Motility phenotypes of E-wt, C-wt, E-ΔvieA, and C-ΔvieA on LB soft agar plates. (B) Bar graphs showing the diameters of the motility zones of E-wt, C-wt, E-ΔvieA, and C-ΔvieA after 20 h of incubation at 30°C.

in *trans* (data not shown). E-Δ*vieA*, on the other hand, remained motile (Fig. 5A and B).

To test whether the reduced motility phenotype of the C-Δ*vieA* strain is due to direct regulation by VieA or the increased c-di-GMP concentration in this strain, we overexpressed the VCA0956 diguanylate cyclase protein in the wild-type background. The induction of VCA0956 drastically inhibited motility in this assay (data not shown), which is consistent with the indirect regulation of genes encoding flagellar motility and chemotaxis components by c-di-GMP.

The *V. cholerae* motility and chemotaxis genes are regulated by a hierarchy of transcriptional regulators and have been divided into four classes (I to IV) based on the regulatory factors that are required for their expression (42). The genes

regulated by VieA fall primarily into class III and class IV. In particular, the *flgB-flgJ* operon, *flaG-fliS* operon, *flaA*, and *motX* are all class III genes and most of these exhibited a twofold or greater reduction in transcript level (Fig. 4D). In addition, the expression levels of *flaB*, *flaC*, *flaD*, *flaE*, and *motY*, all of which are class IV genes, were reduced as much as ninefold. These results suggest that the increased intracellular concentration of c-di-GMP in the C-$\Delta vieA$ strain interferes specifically with the activation of class III gene expression and that class IV genes are expressed at reduced levels because the basal body-hook structure is not properly assembled to allow the secretion of FlgM (anti-σ^{28}). Since the expression of *flrC*, which encodes the response regulator that activates the transcription of the class III genes, was not significantly affected by the $\Delta vieA$ mutation, the results suggest that c-di-GMP interferes with the activation of FlrC by phosphorylation.

Finally, many genes that exhibit increased or reduced expression in C-$\Delta vieA$ cannot be accounted for by changes in the expression of VpsR, σ^E, ToxT or the flagellar regulatory factors. There are, however, other transcriptional regulators that exhibit increased expression in C-$\Delta vieA$ that may regulate additional sets of genes. Alternatively, these genes may be regulated indirectly by the c-di-GMP second messenger. It should also be noted that the loss of *vieA* leads to a decrease in the expression of *vieB* and *vieS* (Fig. 4E), which are located on either side of *vieA*.

Through transcriptional profiling, we have identified VieA as a regulator of 401 genes in the classical biotype of *V. cholerae*. This analysis has confirmed the previously reported alterations in gene expression mediated by VpsR and ToxT and has defined additional gene families under the control of VieA. The expression of the σ^E regulon is inhibited by VieA. However, it is unclear whether σ^E expression is directly controlled by VieA or affected indirectly by the increased concentration of c-di-GMP. The expression of class III and IV genes required for motility and chemotaxis is positively regulated by VieA, and we demonstrated that this regulation occurs indirectly through the control of c-di-GMP concentration. Thus, when the concentration of c-di-GMP in the cell is high, motility is inhibited. Future studies will be directed at identifying how c-di-GMP alters the expression of these different classes of genes.

Genomes are dynamic as microorganisms are constantly evolving to increase their fitness and survival within dynamic and evolving ecosystems. The consequences of genome evolution can be distinguished by using comparative genomics or transcriptional profiling. In this study, we showed that the two biotypes of *V. cholerae* O1 exhibited markedly different transcriptional profiles when grown in a chemically defined growth medium. We also showed that the expressions of many of these genes are regulated by the transcriptional regulator VieA, whose message was produced at a higher level in the classical biotype. Our work suggests that to better understand population diversity and its contribution to the virulence and environmental survival of *V. cholerae*, variation in the transcriptome as well as genomic differences needs to be taken into consideration.

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