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A Systems Biology Approach To Modeling *Vibrio cholerae* Gene Expression under Virulence-Inducing Conditions▽

Sanjat Kanjilal,[1] Robert Citorik,[2] Regina C. LaRocque,[2] Marco F. Ramoni,[3] and Stephen B. Calderwood[2]*

Harvard-MIT Division of Health Sciences and Technology, Harvard Medical School, Boston, Massachusetts 02115[1]; Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts 02114[2]; and Children's Hospital Informatics Program, Harvard-MIT Division of Health Sciences and Technology, Harvard Medical School, Boston, Massachusetts 02115[3]

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***Vibrio cholerae* is a Gram-negative bacillus that is the causative agent of cholera. Pathogenesis *in vivo* occurs through a series of spatiotemporally controlled events under the control of a gene cascade termed the ToxR regulon. Major genes in the ToxR regulon include the master regulators *toxRS* and *tcpPH*, the downstream regulator *toxT*, and virulence factors, the *ctxAB* and *tcpA* operons. Our current understanding of the dynamics of virulence gene expression is limited to microarray analyses of expression at selected time points. To better understand this process, we utilized a systems biology approach to examine the temporal regulation of gene expression in El Tor *V. cholerae* grown under virulence-inducing conditions *in vitro* (AKI medium), using high-resolution time series genomic profiling. Results showed that overall gene expression in AKI medium mimics that of *in vivo* studies but with less clear temporal separation between upstream regulators and downstream targets. Expression of *toxRS* was unaffected by growth under virulence-inducing conditions, but expression of *toxT* was activated shortly after switching from stationary to aerating conditions. The *tcpA* operon was also activated early during mid-exponential-phase growth, while the *ctxAB* operon was turned on later, after the rise in *toxT* expression. Expression of *ctxAB* continued to rise despite an eventual decrease in *toxT*. Cluster analysis of gene expression highlighted 15 hypothetical genes and six genes related to environmental information processing that represent potential new members of the ToxR regulon. This study applies systems biology tools to analysis of gene expression of *V. cholerae in vitro* and provides an important comparator for future studies done *in vivo*.**

Vibrio cholerae is a Gram-negative, motile bacillus that is the pathogenic agent of the diarrheal disease cholera. The WHO estimates that there are 5 million cases of cholera worldwide a year, with more than 100,000 deaths (22). Infection occurs when bacteria pass from the external environment into humans through fecal-oral transmission (19). Once inside the body, *V. cholerae* causes a severe, secretory diarrhea that can lead to death through dehydration. There are over 200 serogroups of *V. cholerae* that are distinguished by the O antigen of the lipopolysaccharide. The only serogroups that are known to cause epidemic or pandemic cholera are serogroups O1 and O139. The O1 serogroup can be further broken down into the classical and El Tor biotypes, with the latter representing the dominant strain of the ongoing global pandemic. Both biotypes can be further subdivided into the Ogawa and Inaba serotypes.

Organisms that survive passage through the acidic stomach enter the small intestine, where they produce cholera toxin (CT), a prototypical A_1B_5 subunit ADP-ribosylating enterotoxin. The B pentamer of the exotoxin binds to the ganglioside GM_1 on the surfaces of absorptive intestinal epithelial cells and translocates the A subunit into the host cytoplasm. The enzymatically active A subunit activates adenylate cyclase through ADP-ribosylation of its G protein, resulting in an increase in cyclic AMP levels and subsequent efflux of chloride ions into the intestinal lumen. The resulting osmotic shift in the lumen results in poor water absorption and copious, watery diarrhea (14). The other essential virulence factor of *V. cholerae* is the toxin-coregulated pilus (TCP), a type IV pilus that (i) facilitates colonization of the gastrointestinal tract by mediating bacterial-bacterial interactions to protect them from the shear stresses of the intestine (20, 28) and (ii) serves as the receptor for the bacteriophage encoding the cholera toxin genes (41).

The genes for cholera toxin are encoded in the filamentous bacteriophage CTXΦ, which enters bacterial cells by binding to TCP and then integrating into the bacterial chromosome and converting a nontoxigenic strain into a toxigenic strain (41). TCP is assembled by a set of genes located within the *tcpA* operon on a 40-kb pathogenicity island that also contains other virulence genes that are jointly regulated (23). Both the *ctxAB* and *tcpA* operons are activated primarily by the ToxT transcription factor, a member of the AraC protein family (16). ToxT also positively regulates expression of accessory colonization factor (*acf*) genes, the gene for aldehyde dehydrogenase (*aldA*), and ToxR-activated gene A (*tagA*) (29). Expression of *toxT* is itself positively regulated by two sets of dimeric transcription factors, ToxRS and TcpPH. ToxR is a transmembrane protein with a cytoplasmic DNA binding domain (35) whose activity is greatly enhanced by the presence of another transmembrane protein, ToxS. TcpP is a homologue of ToxR and is similarly localized to the membrane, with a DNA binding cytoplasmic domain; a second protein, TcpH, enhances the stability of TcpP (3). Both ToxR and TcpP bind the *toxT*

* Corresponding author. Mailing address: Gray-Jackson 504, 55 Fruit Street, Boston, MA 02114. Phone: (617) 726-3811. Fax: (617) 726-7416. E-mail: scalderwood@partners.org.
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promoter; however, it appears that TcpP is more directly responsible for the transcription of *toxT*, with ToxR playing a more indirect role (25). ToxR is known to regulate at least 17 genes besides *toxT*, including the outer membrane porin genes *ompU* and *ompT* (8). ToxR has also been shown to directly stimulate *ctxAB* expression, independently of ToxT (7). These ToxR-regulated virulence genes are collectively called the ToxR regulon, after the first discovered master regulator (35).

The sequencing of the genome of the *V. cholerae* O1 El Tor strain N16961 in 2000 opened the door to using high-throughput technologies to analyze this bacterium (15). The major advantage of these approaches is that they give researchers the opportunity to examine the effects of genetic and/or environmental perturbations on the expression of thousands of genes or proteins simultaneously without observation bias. DNA microarrays have been used to identify members of the *rpoH* regulon required for the *V. cholerae* heat shock response (39). Other work has elucidated the changes in the transcriptional profile of *V. cholerae* from early to late infection directly in humans (27). Two recent studies used analysis of the *in vivo* transcriptome of *V. cholerae* to highlight the existence of an "escape" response to help the bacteria detach from the intestinal epithelial surface and express a "preenvironmental" response to help prepare the organism for exit from the host into the marine environment (34, 38).

Studies using animal and human models have highlighted the temporal nature of virulence gene expression in *V. cholerae* (28); however, studies of global gene expression *in vivo* are impractical due to the technical difficulties of creating and resolving reporter gene constructs for all 3,890 open reading frames (ORFs) of the *V. cholerae* genome. Furthermore, genome profiling studies of humans are able to capture the *V. cholerae* transcriptome in early and late infection, using vomit and stool samples of infected patients for these phases (27), but miss gene expression in the small intestine, the site of active colonization.

To overcome these difficulties, we utilized a systems biology approach to assay gene expression at multiple time points during the growth of *V. cholerae* under virulence-inducing conditions to allow for high temporal resolution of the entire genome in parallel as virulence gene expression occurs over time. Previous studies have shown that El Tor *V. cholerae* strains grown in bicarbonate (1, 17) or in bicarbonate-containing AKI medium (18) activate virulence gene expression *in vitro*; we utilized growth in AKI medium in this study for analysis of gene expression at multiple time points. We assessed when the major ToxR regulon genes were expressed in relation to one another and then used the expression profiles of known virulence genes to identify additional genes regulated in parallel that might play a yet-unidentified role in pathogenesis. These potential virulence genes may enhance our understanding of the infectious process.

MATERIALS AND METHODS

Media and bacterial growth. *V. cholerae* strain N16961 was streaked on LB agar containing streptomycin (100 μg/ml). After overnight incubation, a single colony was inoculated into 25 ml of LB broth with streptomycin (100 μg/ml) and grown overnight. A 10-ml inoculum of the overnight culture was then transferred to 1 liter of either AKI medium (1.5% Bacto peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% $NaHCO_3$) to induce cholera virulence gene expression or LB medium, both of which were preheated to 37°C to minimize the cold shock transcriptional response. Cultures in AKI medium were grown initially under stationary microaerophilic conditions at 37°C with a flask-to-medium volume ratio of 1:1. Once cultures reached mid-log phase (optical density at 600 nm [OD_{600}] of ~0.300), the culture was switched to aerobic growth conditions, with a flask-to-medium volume ratio of 2:1 and shaking at 220 rpm (18). LB medium cultures were incubated aerobically with a flask-to-medium volume ratio of 2:1 and shaking at 220 rpm at 37°C for the entire growth curve. OD_{600} measurements of growth in AKI medium were done in triplicate, while the LB medium growth curve was done once.

Measurement of cholera toxin production. Detection of cholera toxin was done to confirm activation of the virulence cascade as previously described (5). One-milliliter samples of culture supernatants were taken from each time point and centrifuged at 3,000 × *g* for 5 min to remove bacterial cells and debris. Purified supernatants were then serially diluted 2-fold on enzyme-linked immunosorbent assay (ELISA) plates coated with GM_1-ganglioside. Goat anti-cholera toxin subunit B (List Biological Laboratories) was used as the primary antibody, with rabbit anti-goat conjugated to horseradish peroxidase used as the secondary antibody and hydrogen peroxide as the detecting reagent. The cholera toxin B subunit at 1 mg/ml was used to generate the standard curve.

Isolation of bacterial RNA for transcriptional analysis. Thirteen time points were taken every 25 min starting at 1 h and continuing until 6 h after the beginning of growth in either LB or AKI medium. A solution of 50% ethanol–50% acetone (chilled to −20°C) plus rifampin (200 μg/ml; Sigma-Aldrich) was added 1:1 to an aliquot of the growth medium at each time point to halt transcription and preserve RNA (37). Samples were immediately stored at −80°C overnight. The volume of sample used for transcriptional analysis varied inversely with the OD_{600} of the culture aliquot, with lower optical densities requiring larger volumes of sample to generate enough signal for subsequent microarray hybridization (OD_{600} of 0.000 to 0.060, 100-ml aliquot; OD_{600} of 0.061 to 0.100, 50-ml aliquot; OD_{600} of 0.101 to 0.250, 25-ml aliquot; OD_{600} of >0.250, 10-ml aliquot). Culture aliquots were thawed at room temperature and spun at 3,000 rpm at 4°C for 10 min to pellet cells. The supernatant was removed, and the cells were resuspended in 1 ml of lysis buffer. The RNeasy minicolumn kit was used for mRNA purification and on-the-column DNase treatment according to the manufacturer's specifications (Qiagen). The concentration of RNA in samples was quantitated using spectrophotometry, and the RNA was visualized on a 1% agarose gel to confirm RNA integrity.

Microarray hybridization. The *V. cholerae* microarray (J. Craig Venter Institute, http://www.jcvi.org) contains all 3,890 annotated ORFs of *V. cholerae* El Tor strain N16961. Seventy-base-pair oligomers from the ORF of each gene are spotted on each slide in triplicate. mRNA from time points following growth in AKI and LB media was purified, labeled, hybridized, and quantitated as previously described (4, 10). Genomic DNA from *V. cholerae* O1 El Tor strain N16961 was isolated using the Easy-DNA kit (Invitrogen) according to the manufacturer's specifications and was used as a universal internal control to normalize the quality of the array and to allow for comparison between time points and conditions.

Microarray data analysis. Microarray slides were scanned and spot intensities quantitated using the ScanArray Express (PerkinElmer; version 4.0) software. All spots were checked manually for artifacts. Data were analyzed using the software package Limma, within the "R" computing environment (40). Limma is part of the Bioconductor project (http://bioconductor.org [13]) and has been validated across a range of microarray data sets. Data normalization within and between arrays was performed using standard techniques (42), and log fold changes in expression of each gene were estimated using linear modeling. Fold changes were first calculated for LB medium and AKI medium separately. The final expression profile represents the expression of each gene in AKI medium at each time point after subtracting expression of that gene at that time point in LB medium, with expression given relative to that at the 1-h time point. We did the expression profiling in AKI medium in triplicate at each time point and then averaged those results before subtracting the results for growth in LB medium. For genes that had little change in expression in LB medium, this had little effect on the profile other than to average the fold changes among the AKI medium replicates.

Clustering analysis of gene expression. For the clustering analysis, we used the cluster analysis of gene expression dynamics (CAGED) algorithm, a Bayesian clustering method that is ideally suited for time series microarray data (36). The algorithm models the time series profile of each gene using a polynomial or autoregressive model and then iteratively merges the time series into clusters of genes with similar expression patterns based on a user-defined similarity measure and the overall posterior probability of the model. The algorithm stops when remaining mergers no longer increase the model's posterior probability. Theo-

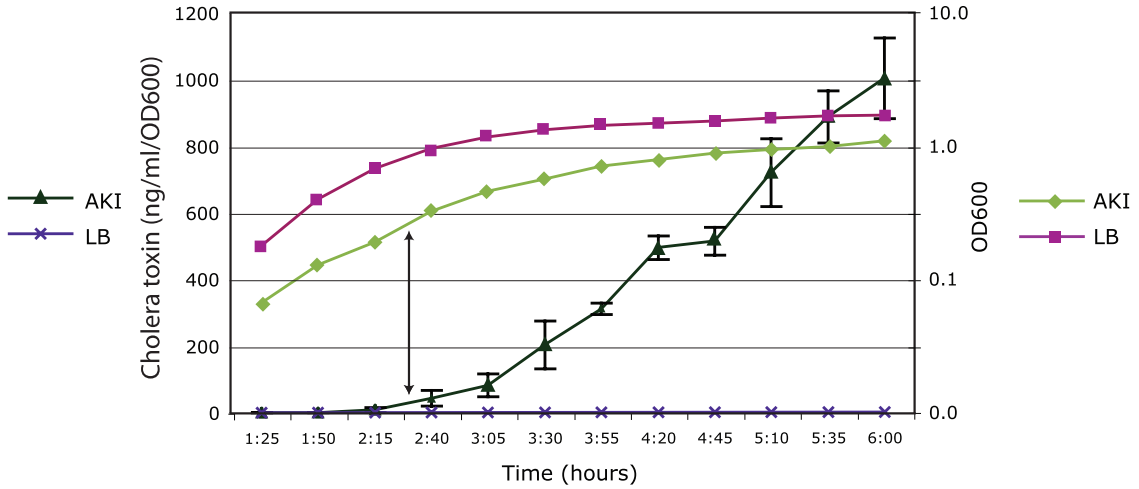

FIG. 1. *V. cholerae* O1 El Tor N16961 growth curves and toxin production in LB and AKI media. Average AKI medium and LB medium growth curves and toxin production are shown. Toxin concentrations in ng/ml/OD_{600} appear on the left *y* axis. The OD_{600} log scale for growth curves appears on the right *y* axis. The arrow represents the time at which AKI medium cultures were switched to shaking (aerobic) growth conditions. LB medium cultures were grown aerobically throughout the growth curve.

retically, each cluster represents a separate biologic process, generating a unique time series profile. For this analysis, we used fourth-order polynomial equations and Euclidean distance as our model parameter and similarity measure, respectively. Both the prior precision (the sample size of imaginary observations that builds the prior distribution) and the Bayes factor threshold (the minimum value that must be met for two series to be merged) were set to the default value of 1.

RESULTS

***V. cholerae* produces significant amounts of cholera toxin under inducing conditions.** El Tor *V. cholerae* strains grown *in vitro* produce toxin under AKI medium conditions (18). We utilized this property, as well as the inability of *V. cholerae* to produce toxin in standard LB medium, to evaluate the impact of toxin-inducing and noninducing conditions on gene expression. Figure 1 compares the growth curves of *V. cholerae* El Tor strain N16961 grown in AKI medium and LB medium, superimposed with levels of toxin production over time. *V. cholerae* grew more rapidly and reached a higher steady-state cell density in LB medium than in AKI medium. Expression of cholera toxin was seen specifically under AKI medium conditions, as expected. Bacteria were already in the exponential phase of growth by 1 h postinoculation in both media and reached stationary phase by 3 h in LB and 4 h in AKI medium. The delayed growth in AKI medium likely reflects the more limited nutrients than in LB medium and the growth initially under microaerophilic conditions. Cholera toxin was detectable in the supernatants of cultures grown in AKI medium shortly after the cultures were switched to aerating conditions at ca. 2.5 hours and increased linearly through the 6-h time point.

***V. cholerae* virulence genes are upregulated in AKI medium versus in LB medium.** In order to investigate the effect of growth under AKI medium conditions on *V. cholerae* global gene expression, we compared the dynamic transcriptomes of *V. cholerae* grown in LB and AKI media at multiple time points during the exponential and stationary phases of growth. c*txA* had the highest peak fold change of all genes and also exhibited large fluctuations in expression over a range of 7 $\log_2$ at the various time points. There was the suggestion of periodicity in *ctxA* expression, but the three AKI medium replicates had differing amplitudes and frequencies of these periodic swings (Fig. 2). The time series expression profile of *ctxB* followed the general trend of *ctxA*'s but lacked the periodicity and large fluctuations. Expression of both genes in LB medium remained near baseline over the entire growth curve. The full data sets of expression of all genes are available as supplementary material via the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/).

Major virulence genes and regulators display differing levels of gene expression over time. Of the genes known to be involved in regulation of virulence gene expression in *V. cholerae* (*toxRS*, *tcpPH*, and *toxT*), there was a brief spike in *tcpP* expression at 1 h 50 min, followed by significant upregulation of *toxT* during growth under AKI medium conditions (Fig. 3), suggesting that growth under AKI medium conditions upregulates *toxT* independently of expression of ToxRS. All of these genes (*toxRS*, *tcpPH*, and *toxT*) remained at baseline during growth in LB medium throughout the time course (data not shown).

The genes of the *tcpA* operon were also expressed at significantly higher levels in AKI medium. In contrast to what occurred with *ctxAB*, whose expression did not increase until after the expression of *toxT* spiked at 2 h 40 min, the time course profiles of the *tcpA* operon increased simultaneously with the increase in *toxT*. Nearly all *tcpA* operon members increased in expression from 1 h 25 min until 2 h 40 min and then transiently decreased shortly after the point at which the cultures were switched to aerating conditions; they increased again at 3 h 30 min (Fig. 3). There was considerable variation in expression between the operon members within a replicate, with less interreplicate variation. *tcpA* showed a negative fold change earlier in the time series in all AKI medium replicates, but then expression tended upward during the stationary phase of growth. Expression of the *tcpA* operon in LB medium was generally close to baseline throughout, except with *tcpE* and *tcpB*, which were expressed at levels 0.5- to 1.7-$\log_2$-fold above

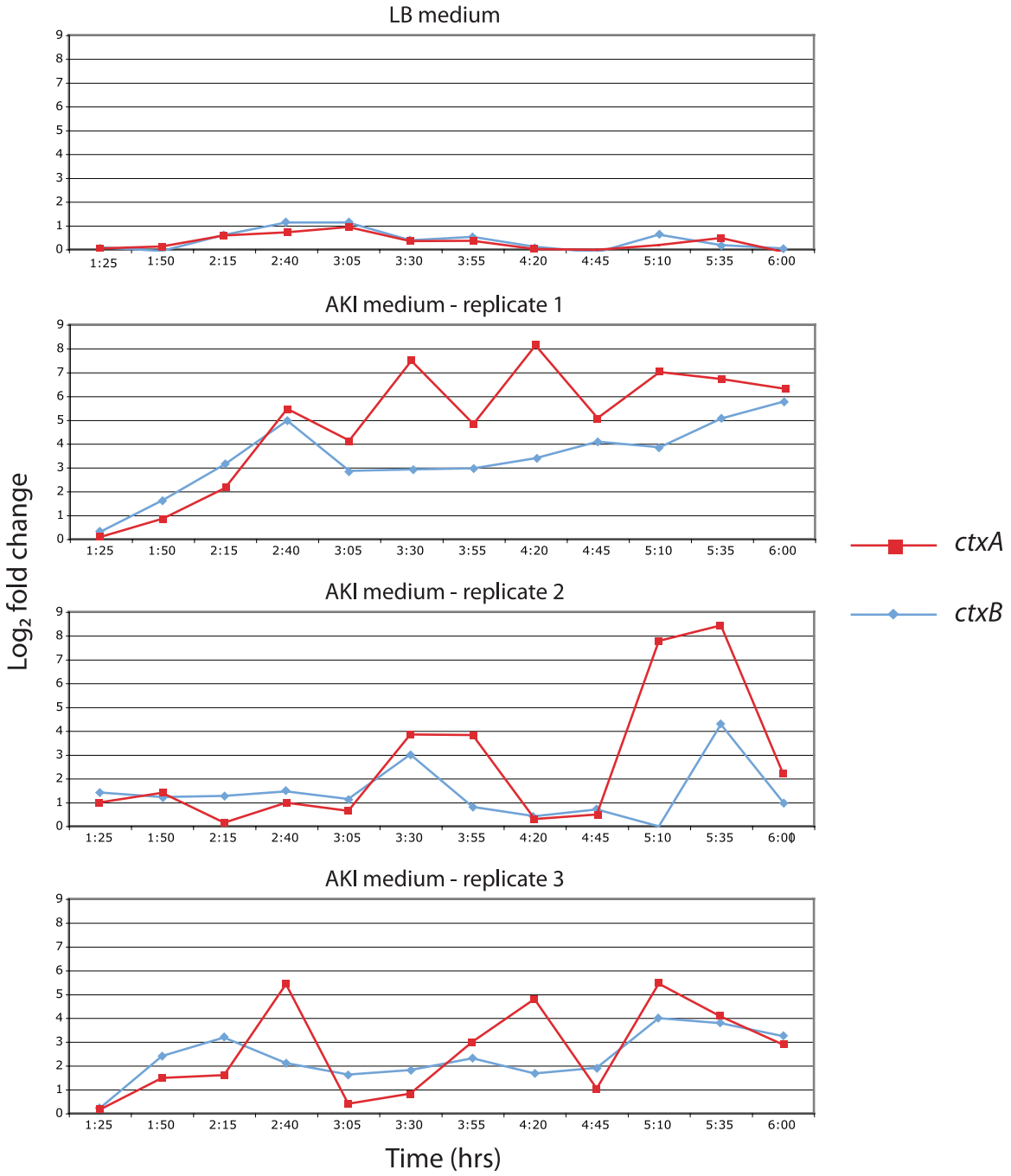

FIG. 2. Comparison of levels of cholera toxin gene expression in LB medium and AKI medium replicates. Each data point represents the $\log_2$ fold change from the expression level at 1 h. Cultures were switched to aerating conditions at 2 h 30 min.

baseline throughout, and *tcpR*, whose expression remained below −1.2 $\log_2$ after 3 h 30 min (data not shown).

We examined genes for similarities in their time series profiles during growth under toxin-inducing conditions. The overall shape of the *toxT* profile was quite similar to the shapes of profiles of its downstream targets *ctxA*, *tcpB*, *tcpC*, *tcpE*, *tcpQ*, *tcpR*, *tcpS*, and *tcpT*, at least to 4 h 20 min. The time expression profile of *toxT* was less similar to those of other downstream targets, *ctxB*, *tcpA*, *tcpD*, *tcpF*, and *tcpJ*, although the reason for the difference from other members of the same *ctxAB* and *tcpA* operons is unknown (data not shown).

Expression profiles of the accessory colonization factors (*acfABCD*) increased early in the time course, peaked at 3 h 30 min, and then gradually decreased as bacteria entered stationary phase. Expression of the gene for the Ccm2-related protein (VCA1042), as well as of *acfD* and *tagD*, oscillated in phase with *toxT* between 2 h 40 min and 4 h 20 min, before expression decreased to baseline by 6 h. Expression of *tagD* was negatively

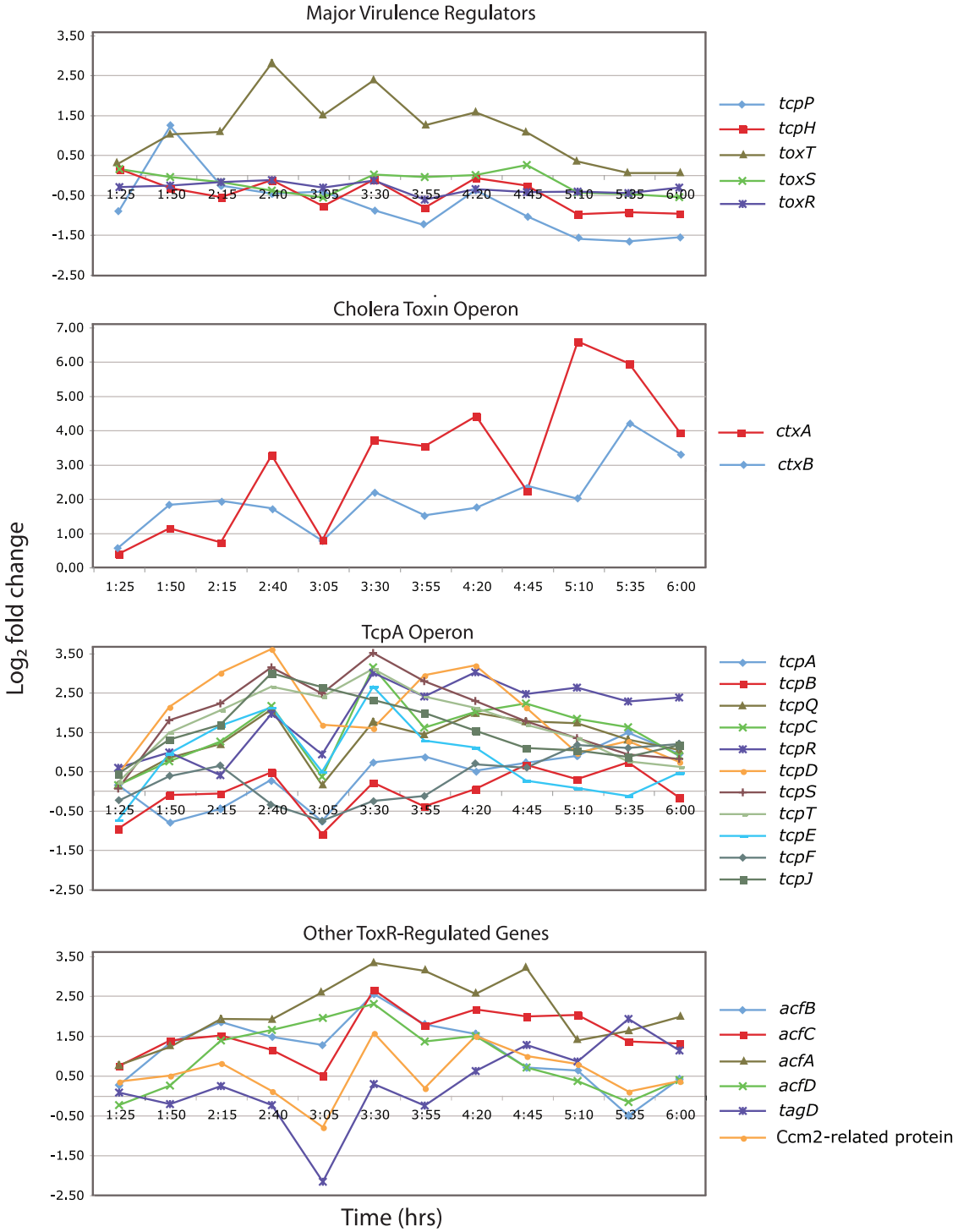

FIG. 3. Time series expression profiles of major virulence genes and gene regulators. Each data point represents the $\log_2$ fold change averaged from three AKI medium replicates with respect to expression in LB medium at 1 h. Cultures were switched to aerating conditions at 2 h 30 min.

regulated initially, reaching a minimum of -2.2 $\log_2$ below baseline at 3 h 5 min before rebounding and remaining near 1.0 $\log_2$ above baseline by the end of growth (Fig. 3). None of these genes were upregulated in LB medium (data not shown).

Potential new members of the *V. cholerae* virulence regulon identified through cluster analysis. We used the CAGED algorithm on our time series data as an unsupervised method of identifying potential new members of the virulence regulon. The analysis was limited to the 127 genes that exhibited more than ± 2-$\log_2$-fold changes in AKI medium at any point in the time series and that also did not have a similar fold change during growth in LB medium. Presumably, these are the genes

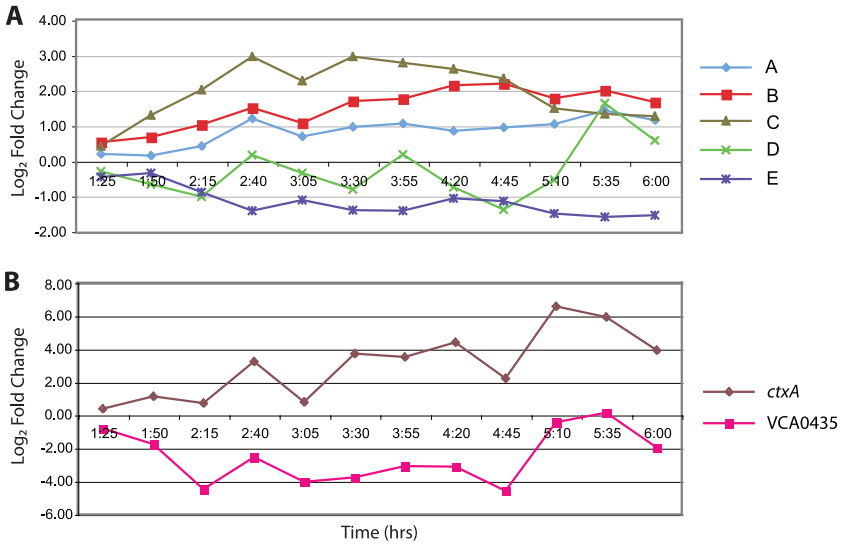

FIG. 4. (A) Cluster time series expression profiles. Each data point represents the average expression of all genes within the cluster, normalized to expression in LB medium and to time at 1 h. Cluster A contains 41 genes, cluster B 32 genes, cluster C 6 genes, cluster D 5 genes, and cluster E 41 genes. (B) Time series expression profiles of *ctxA* and VCA0435. These two genes did not cluster with any genes due to the sizes of their positive and negative fold changes, respectively.

that are most closely associated with toxin production and other virulence attributes. This assumption is supported by the fact that this subset contains 10 of the 13 genes identified through transcriptome analysis to be downregulated in a *toxT* mutant (2) and also contains the cholera toxin operon, *toxT* itself, and the *acf* operon.

Using the model parameters described in Materials and Methods, the CAGED analysis generated five distinct clusters, labeled A to E, suggesting five different biologic processes associated with pathogenesis. Cluster A contains 41 genes, cluster B 32 genes, cluster C 6 genes, cluster D 5 genes, and cluster E 41 genes. All known virulence genes except for *ctxA* grouped into clusters A to C. The series profile shown in Fig. 4A represents the average levels of expression of the genes over time and provides a visual representation of the expression pattern of each cluster. The series profile for clusters A and B have similar patterns, peaking late in the growth curve, with cluster B genes expressed at a higher average value. Cluster C expression peaked earlier and genes were expressed to a greater degree than with cluster A and B genes, reaching 3.0 $\log_2$ above baseline between 2 h 40 min and 3 h 30 min. Cluster D was negatively regulated, oscillating between -1.4 and 0 $\log_2$ until 5 h 10 min, when expression rose ~2-$\log_2$-fold in 25 min to 1.6 $\log_2$. The expression of cluster E tended downward and reached a plateau around -1.4 $\log_2$ below baseline from 2 h 40 min until 6 h. *ctxA* and locus VCA0435 did not cluster with any other genes due to their very large positive and negative fold changes, respectively (Fig. 4B).

Gene clusters were further divided into functional categories: virulence genes, transcription/translation genes, metabolism genes, miscellaneous genes, and unknown genes (Table 1). Cluster A, which contained *toxT*, *tcpE*, *acfB*, *acfD*, *tagD*, and *tagE*, also included 11 hypothetical genes of unknown function. Cluster B contained *tcpC*, *tcpQ*, *tcpR*, *ctxB*, and *acfC*, along with four hypothetical genes, and cluster C contained *tcpD*, *tcpJ*, *tcpS*, *tcpT*, and *acfA*. Other genes of interest in clusters A to C included three ATP-binding cassette active-transport genes (VC1092, VC1882, VCA0759), an ion transporter (VCA1093), and two signal transduction genes (VC0200, VCA0232). A subunit of SecY (VC2576), a member of the protein complex involved in cholera toxin secretion, was included in cluster A. A large number of housekeeping genes associated with transcription/translation and cellular metabolism also showed a time course of expression identical to that of genes specific to virulence.

The negatively regulated clusters D and E contained 2 and 17 hypothetical genes, respectively. Among the other genes belonging to these groups were the oxygen sensor *fexA* (VC2368), an anaerobic C_4-dicarboxylate transporter (VCA0205), the phage protein RstB1 (VC1453), a factor-for-inversion stimulation protein (VC0290), a heat shock gene (VC1663), and the flagellar assembly protein MinD (VC2067).

DISCUSSION

Previous studies of *V. cholerae* virulence gene regulation have focused on a limited number of individual genes or time points, comparison of expression levels between two conditions or strains, or expression in two different samples from humans (4, 27, 28). This work represents the first attempt to utilize systems biology tools to study expression at the whole-genome level, with high temporal resolution, during growth of the organism *in vitro* under virulence-inducing conditions. We show that virulence genes of El Tor *V. cholerae* O1 are expressed at much higher levels under toxin-inducing conditions than in standard medium. We found that *tcpP* and *toxT* were expressed early during mid-log-phase growth in AKI medium despite no increase in *toxRS* expression at that point. The fact that we saw only a brief spike in *tcpP* expression may reflect the very short half-life of the *tcpPH* transcript (ca. 2 min), shown previously

TABLE 1. Results of cluster analysis

Cluster	Category	Common name or characteristic	Locus
A	Virulence	TagD protein	VC0824
		Toxin-coregulated pilus biosynthesis protein E	VC0836
		TCP pilus virulence regulatory protein	VC0838
		Accessory colonization factor AcfB	VC0840
		TagE protein	VC0843
		Accessory colonization factor AcfD	VC0845
	Unknown	Hypothetical protein	VC0712
		Hypothetical protein	VC0821
		Conserved hypothetical protein	VC0842
		Hypothetical protein	VC1221
		Conserved hypothetical protein	VC2278
		Conserved hypothetical protein	VCA0006
		Hypothetical protein	VCA0393
		Hypothetical protein	VCA0394
		Hypothetical protein	VCA0480
		Hypothetical protein	VCA0817
		Hypothetical protein	VCA0932
	Transcription/translation	DNA polymerase III, beta chain	VC0013
		Ribosomal protein L11	VC0324
		Ribosomal protein L10	VC0326
		Elongation factor G	VC0361
		Ribosomal protein L13	VC0570
		Elongation factor G	VC2342
		Ribosomal protein L36	VC2575
		Ribosomal protein L24	VC2585
		Ribosomal protein S3	VC2590
		Ribosomal protein L3	VC2596
	Metabolism	Citrate lyase, gamma subunit	VC0797
		Cytochrome *d* ubiquinol oxidase, subunit II	VC1843
		NADH:ubiquinone oxidoreductase, Na translocating, alpha subunit	VC2295
		UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	VC2542
		Aspartate ammonia-lyase	VC2698
		C_4-dicarboxylate transporter, anaerobic	VC2699
		ATP synthase F_o, B subunit	VC2768
		ATP synthase F_o, A subunit	VC2770
		3,4-Dihydroxy-2-butanone 4-phosphate synthase	VCA1060
	Other	LysE/YggA family protein	VC0481
		Oligopeptide ABC transporter, permease protein	VC1092
		Lipoprotein-releasing system permease protein	VC1882
		Preprotein translocase, SecY subunit	VC2576
		Na^+/H^+ antiporter, putative	VCA0193
B	Virulence	Toxin-coregulated pilus biosynthesis protein Q	VC0830
		Toxin-coregulated pilus biosynthesis outer membrane protein C	VC0831
		Toxin-coregulated pilus biosynthesis protein R	VC0832
		Accessory colonization factor AcfC	VC0841
		Cholera enterotoxin, B subunit	VC1456
	Unknown	Hypothetical protein	VC2098
		Hypothetical protein	VCA0408
		Hypothetical protein	VCA0619
		Conserved hypothetical protein	VCA0689
	Transcription/translation	Transcription antitermination protein NusG	VC0323
		DNA-directed RNA polymerase, beta subunit	VC0328
		Ribosomal protein S7	VC0360
		Ribosomal protein L21	VC0435
		Cell division protein FtsH	VC0637
		Ribosomal protein L25	VC1640
		Ribosomal protein S11	VC2573
		Ribosomal protein L14	VC2586
		Ribosomal protein S17	VC2587
		Ribosomal protein S19	VC2592
		Ribosomal protein L31	VC2679
	Metabolism	Aldehyde dehydrogenase	VC0819
		Long-chain-fatty acid transport protein	VC1043
		Phosphoserine aminotransferase	VC1159
		Glyceraldehyde 3-phosphate dehydrogenase	VC2000
		Enolase	VC2447

Continued on following page

TABLE 1—*Continued*

Cluster	Category	Common name or characteristic	Locus
	Other	Iron(III) compound receptor	VC0200
		NifR3/Smm1 family protein	VC0291
		Antioxidant, AhpC/Tsa family	VC0731
		Enterobactin synthetase component F-related protein	VC1579
		Enterobactin receptor VctA, authentic frameshift	VCA0232
		Arginine ABC transporter, periplasmic arginine-binding protein	VCA0759
		DnaJ-related protein	VCA0788
C	Virulence	Toxin-coregulated pilus biosynthesis protein D	VC0833
		Toxin-coregulated pilus biosynthesis protein S	VC0834
		Toxin-coregulated pilus biosynthesis protein T	VC0835
		Leader peptidase TcpJ	VC0839
		Accessory colonization factor AcfA	VC0844
	Transcription/translation	Ribosomal protein L19	VC0564
D	Unknown	Hypothetical protein	VC0507
		Hypothetical protein	VCA0306
	Other	RstB1 protein	VC1453
		Chaperonin, 60-kDa subunit	VC2664
		C_4-dicarboxylate transporter, anaerobic	VCA0205
E	Unknown	Conserved hypothetical protein	VC0340
		Hypothetical protein	VC0546
		Hypothetical protein	VC0569
		Conserved hypothetical protein	VC0758
		Conserved hypothetical protein	VC0770
		Hypothetical protein	VC0967
		Conserved hypothetical protein	VC1153
		Conserved hypothetical protein	VC1354
		Hypothetical protein	VC1794
		Hypothetical protein	VC1924
		Hypothetical protein	VC1999
		Hypothetical protein	VC2149
		Conserved hypothetical protein	VC2216
		Hypothetical protein	VC2306
		Hypothetical protein	VC2351
		Conserved hypothetical protein	VC2523
		Conserved hypothetical protein	VCA0835
	Transcription/translation	RNase P protein component	VC0006
		Ribosomal protein L9	VC0369
		Alanyl-tRNA synthetase	VC0545
		Peptidyl-tRNA hydrolase	VC2184
		Elongation factor Ts	VC2259
		Site-specific recombinase IntI4	VCA0291
	Metabolic	Pyrroline-5-carboxylate reductase	VC0460
		Glutathione synthetase	VC0468
		Hypoxanthine phosphoribosyltransferase	VC0585
		Inositol monophosphate family protein	VC0745
		Cytidine deaminase	VC1231
		3-Hydroxydecanoyl-(acyl carrier protein) dehydratase	VC1483
		Mannose-6-phosphate isomerase	VC1827
		L-Asparaginase I	VC1995
		3-Oxoacyl-(acyl carrier protein) reductase	VC2021
		Long-chain fatty acid–coenzyme A ligase, putative	VC2484
		Periplasmic nitrate reductase	VCA0678
	Other	ATP-dependent DNA helicase RecQ	VC0196
		Factor for inversion stimulation protein	VC0290
		Cellobiose/cellodextrin-phosphorylase, putative	VC0612
		Heat shock protein HslJ	VC1663
		Heme exporter protein C	VC2055
		MinD-related protein	VC2067
		Aerobic respiration control protein FexA	VC2368
None (unclustered)	Virulence	Cholera enterotoxin, A subunit	VC1457
	Unknown	Hypothetical protein	VCA0435

(33). Members of the *tcpA* operon were also upregulated early, while *ctxAB* was expressed after the increase in expression of *toxT* and shortly after the switch to shaking conditions. Once initiated, however, *ctxAB* expression continued to increase independently of *toxT* levels. Using our time series clustering method, we distilled gene expression profiles into five distinct clusters containing known virulence genes and identified 15 hypothetical genes positively associated with virulence gene expression that are potential new members of the ToxR regulon and represent future targets of study.

The temporal nature of virulence gene expression in *V. cholerae* is of critical importance in understanding disease pathogenesis. Lee et al. showed in a murine model that expression of *tcpA* increased early as the bacteria began microcolonization of the upper intestine, while expression of *ctxAB* occurred later, after bacteria had attached to intestinal epithelial cells. They hypothesized that *tcpA* is induced before *ctxAB* to ensure that only bacteria that are adherent to cells secrete toxin, thereby increasing the efficiency of intoxication (28). Merrell et al. and Schild et al. described the activation of hyperinfectious and preenvironmental genetic programs late in infection, as the bacteria prepare to exit the intestine and reenter the external environment (31, 38). These results point to a highly evolved sequence of adaptive responses in *V. cholerae* as the organism colonizes the intestine, causes infection, and then escapes back into the environment to repeat the process again. Although growth in AKI medium cannot mimic the full complexity of the spatiotemporal interactions between host and pathogen, we present evidence that the major ToxR regulon members show temporal variations during *in vitro* growth in AKI medium similar to those that occur during intestinal colonization. Activation of the *tcpA* operon occurred during the exponential growth phase, while expression of cholera toxin genes increased later (Fig. 3). This suggests that although ToxT is responsible for *tcpA* and *ctxAB* operon expression, its ability to act on their respective promoters *in vitro* is dependent on different factors. The *tcpA* operon appears to be more responsive to cell density signals, perhaps relayed via quorum-sensing molecules, whereas the *ctxAB* operon may be triggered by different environmental conditions. The temporally distinct expression of the *tcpA* operon early and *ctxAB* later is analogous to the *in vivo* hypothesis put forth by Lee et al. (28) but in response to different signals.

Growth in AKI medium did not appear to influence *toxRS* expression, and there was only a brief spike of *tcpP* expression seen at 1 h 50 min. The low levels of *tcpPH* expression contrast with prior work by Murley et al. and Kovacikova and Skorupski, who showed upregulation of *tcpPH* in El Tor strains in AKI medium at the end of static phase and after overnight growth, respectively (24, 33). If we reexamine our data without normalizing to LB medium or to time at 1 h, we can reconcile these results, since *tcpPH* transcript levels were found to tend upward at the end of 6 h of growth (data not shown). Also, the *tcpPH* message has a very short half-life (33), and results seen previously with promoter fusions may give results quite different from gene microarray results, as demonstrated here to detect the presence of specific mRNA at individual time points. The constitutive level of *toxR* expression in both media is consistent with prior research by Medrano et al., who demonstrated that ToxR appears early during static growth in AKI medium and is present constitutively thereafter (30). Our results suggest that higher levels of *toxT* expression in AKI medium occur at a time when the proximal regulators, ToxRS, are not upregulated. Activation of *toxT* expression at this point could be the result of an unknown positive regulator or of specific environmental signals during growth in AKI medium.

The cholera toxin operon responds in phase to *toxT* expression starting at 2 h 40 min but, interestingly, continues to increase in expression even as *toxT* expression declines. This behavior suggests that ToxT is required for the initiation of cholera toxin gene expression but perhaps not for its continued expression *in vitro*. These results are consistent with the work of Medrano et al., who demonstrated that cholera toxin mRNA continues to increase linearly through 10 h of growth despite the disappearance of *toxT* after hour 5 (30). Those authors proposed that ToxR is present constitutively but that it is able to bind to the promoter region of *toxT* only during the early stationary phase of growth, when it receives the proper signals to switch to the "on" conformation. There are at least three possible explanations for continued *ctxAB* transcription despite decreasing levels of *toxT* expression. The first is that ToxR directly binds to the *ctxAB* promoter, bypassing *toxT*. Direct activation of *ctxAB* by ToxR has been shown before (7), but Dirita et al. showed that ToxT was the primary activator of *ctxAB in vitro* (9). Another possibility is that *toxT* is present at later time points mainly as part of a larger transcript of the *tcpA* operon, in which *toxT* resides, but that the oligonucleotide array did not detect this multigene transcript as efficiently. This possibility is supported by footprinting studies that have shown the *toxT* transcript attached to a *tcpA*-dependent message (6). A third possibility is that ToxT activates a feed-forward loop that stimulates *ctxAB* expression, which after initiation no longer requires ToxT for further stimulation. Possible mediators of this feed-forward loop remain to be identified.

The overall dynamics of the gene expression profiles under AKI medium conditions showed oscillation of expression across multiple virulence operons beginning at 2 h 15 min and dampening by 4 h 20 min. Genes exhibiting this behavior include *toxT*, the *ctxAB* operon, most (but not all) members of the *tcpA* operon, *acfC*, *acfD*, *tagD*, and the Ccm2-related protein gene. The oscillatory behavior of nodes in a complex system has been well documented in eukaryotic and prokaryotic genetic networks (11, 26) and reflects the dynamic balance achieved between positive- and negative-feedback loops on downstream elements after perturbation (2). In agreement with previous work on biologic networks, the timing of these oscillations is more precise than the amplitude (26, 32). It has been posited that variations in amplitude are secondary to noise generated during protein production, a property inherent in all biochemical circuits (2). Notably, the *ctxA* transcript showed variation in both the amplitudes and frequencies of its oscillations in AKI medium among three replicates (Fig. 2). This behavior has been associated with negative-feedback loops containing multiple regulators, termed repressilators, which have been shown to be noisy with regard to both the timing and amplitude of oscillations (12). Potential candidates for regulators are suggested through cluster analysis and are the targets of future investigation.

The CAGED algorithm clusters time series profiles together on the assumption that similarly shaped profiles are generated

by genes involved in the same biological process. Though our results suggest five unique gene transcription profiles during growth in AKI medium, it is more likely that there is only one positive and one negative module. The existence of three different clusters all containing genes from the *tcpA* operon is likely the result of the same fundamental process being split up due to differing transcriptional dynamics and the message stability of the genes. We were unable to conclusively generate sets of genes that correspond to "early" and "late" regulators. This may be because our time intervals were too broad to capture the necessary changes in transcription at precise time intervals, or it may be that the sequence of gene activation seen in animal/human models of cholera is not fully replicated *in vitro*.

The primary significance of the cluster analysis is the inclusion, based on a systems biology approach to expression profiling, of potential new genes in the ToxR regulon which were not previously suggested to be related to the expression of virulence genes. The majority of these candidates, particularly the metabolic and transcription-related genes, may have similar expression profiles but not be related to virulence. An example is aldehyde dehydrogenase (*aldA*, VC0819), a recognized downstream target of ToxT that was included in cluster B. Although its presence in a cluster with other ToxT-regulated genes helps to validate our results, its broad role in metabolism may make unlikely the possibility that it also plays a major role in pathogenesis. Among the remaining genes, the presence of several ABC transporters, an ion transporter, and two signal transducers suggests an important role for processing of information about the environment; these represent better candidates for genes important in the pathogenic process. Their interactions with known members of the ToxR regulon, along with functional characterization of the 11 hypothetical genes in cluster A and four hypothetical genes in cluster B, are the subjects of future research.

This study is limited by the fact that it was performed *in vitro*. Work by Lee and colleagues has shown that the gene-regulatory relationships seen in the laboratory are not the same as those seen in natural infection, likely due to the absence of essential environmental signals from the gut (28). Although our data show clear temporal activation of the ToxR regulon genes in AKI medium, the complex sequence of gene expression described *in vivo* was not as clearly present, for example, in the separation in time between expression of key regulators and expression of downstream effectors. There could be two reasons for this. The first is that a temporal resolution of 25 min between time points is not sufficiently fine to detect the changes. The second explanation is that the milieu of factors during growth in AKI medium does not fully capture the intestinal environment; this is more likely the case. Despite this, *in vitro* study of *V. cholerae* gene expression clearly still has relevance to clinical disease, as many experiments cannot be performed in animal models or directly in humans and thus require an alternative, albeit imperfect, surrogate.

A systems biology approach to identifying potential pathogenesis genes using time series microarrays is based on the principle that genes that show similar dynamic expression profiles as known virulence genes are likely to be related to the pathogenetic process. The ultimate goal is to build an accurate topology of the genetic network responsible for pathogenesis, thus allowing rational therapeutic and vaccine design. One of the disadvantages of this current approach, however, is illustrated by the fact that not all genes known to be parts of the same operon were upregulated to the same extent in the time series profiles. One possible explanation for this is that the oligonucleotides on the array specific to each gene in a multigenic operon may have differing affinities for binding the multigenic transcript and therefore give differing levels of up- or downregulated expression of genes in the same operon. For example, expression of *ctxB* was clearly not as significantly upregulated as *ctxA*, and some of the genes in the *tcpA* operon were less upregulated than others, including *tcpA* itself. In interpreting a time series profile of gene expression as in this paper, it may be more prudent to look at the overall expression of genes in an operon, rather than the expression of specific individual genes within each operon.

In this study, we analyzed the global gene expression profile of *V. cholerae in vitro*. By analyzing time-dependent profiles, we have gained insight into the mechanisms of information processing that are common to many different types of genetic networks of prokaryotes and eukaryotes. Our data show appropriate temporal expression of major virulence genes, suggesting that growth in AKI medium, coupled with a high-resolution time series design, allows for the preliminary characterization of the dynamic interactions between known and newly identified genes that are associated with virulence. Further studies of these interactions in appropriate *in vivo* situations will contribute to our understanding of pathogenesis.

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REFERENCES

1. **Abuaita, B. H., and J. H. Withey.** 2009. Bicarbonate induces *Vibrio cholerae* virulence gene expression by enhancing ToxT activity. Infect. Immun. **77:** 4111–4120.
2. **Alon, U.** 2007. Network motifs in developmental, signal transduction, and neuronal networks, p. 97–124. *In* U. Alon (ed.), An introduction to systems biology: design principles of biological circuits. Chapman & Hall/CRC, Boca Raton, FL.
3. **Beck, N. A., E. S. Krukonis, and V. J. DiRita.** 2004. TcpH influences virulence gene expression in *Vibrio cholerae* by inhibiting degradation of the transcription activator TcpP. J. Bacteriol. **186:**8309–8316.
4. **Bina, J., J. Zhu, M. Dziejman, S. Faruque, S. Calderwood, and J. J. Mekalanos.** 2003. ToxR regulon of Vibrio cholerae and its expression in vibrios shed by cholera patients. Proc. Natl. Acad. Sci. U. S. A. **100:**2801–2806.
5. **Bina, J. E., D. Provenzano, C. Wang, X. R. Bina, and J. J. Mekalanos.** 2006. Characterization of the Vibrio cholerae vexAB and vexCD efflux systems. Arch. Microbiol. **186:**171–181.
6. **Brown, R. C., and R. K. Taylor.** 1995. Organization of tcp, acf, and toxT genes within a ToxT-dependent operon. Mol. Microbiol. **16:**425–439.
7. **Champion, G. A., M. N. Neely, M. A. Brennan, and V. J. DiRita.** 1997. A branch in the ToxR regulatory cascade of Vibrio cholerae revealed by characterization of toxT mutant strains. Mol. Microbiol. **23:**323–331.
8. **DiRita, V. J., C. Parsot, G. Jander, and J. J. Mekalanos.** 1991. Regulatory cascade controls virulence in Vibrio cholerae. Proc. Natl. Acad. Sci. U. S. A. **88:**5403–5407.
9. **DiRita, V. J., M. Neely, R. K. Taylor, and P. M. Bruss.** 1996. Differential expression of the ToxR regulon in classical and El Tor biotypes of Vibrio cholerae is due to biotype-specific control over toxT expression. Proc. Natl. Acad. Sci. U. S. A. **93:**7991–7995.
10. **Dziejman, M., E. Balon, D. Boyd, C. M. Fraser, J. F. Heidelberg, and J. J. Mekalanos.** 2002. Comparative genomic analysis of Vibrio cholerae: genes

that correlate with cholera endemic and pandemic disease. Proc. Natl. Acad. Sci. U. S. A. **93:**1556–1561.
11. **Eichenberger, P., M. Fujita, S. T. Jensen, E. M. Conlon, D. Z. Rudner, S. T. Wang, C. Ferguson, K. Haga, T. Sato, J. S. Liu, and R. Losick.** 2004. The program of gene transcription for a single differentiating cell type during sporulation in Bacillus subtilis. PLoS Biol. **2:**e328.
12. **Elowitz, M. B., and S. Leibler.** 2000. A synthetic oscillatory network of transcriptional regulators. Nature **403:**335–338.
13. **Gentleman, R. C., V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. H. Yang, and J. Zhang.** 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. **5:**R80.
14. **Gill, D. M., and C. A. King.** 1975. The mechanism of action of cholera toxin in pigeon erythrocyte lysates. J. Biol. Chem. **250:**6424–6432.
15. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. 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. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.
16. **Higgins, D. E., E. Nazareno, and V. J. DiRita.** 1992. The virulence gene activator ToxT from *Vibrio cholerae* is a member of the AraC family of transcriptional activators. J. Bacteriol. **174:**6974–6980.
17. **Iwanaga, M., and K. Yamamoto.** 1985. New medium for the production of cholera toxin by *Vibrio cholerae* O1 biotype El Tor. J. Clin. Microbiol. **22:**405–408.
18. **Iwanaga, M., K. Yamamoto, N. Higa, Y. Ichinose, N. Nakasone, and M. Tanabe.** 1986. Culture conditions for stimulating cholera toxin production by Vibrio cholerae O1 El Tor. Microbiol. Immunol. **30:**1075–1083.
19. **Kaper, J. B., J. G. Morris, and M. M. Levine.** 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.
20. **Kirn, T. J., M. J. Lafferty, C. M. Sando, and R. K. Taylor.** 2000. Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by Vibrio cholerae. Mol. Microbiol. **35:**896–910.
21. Reference deleted.
22. **Kosek, M., C. Bern, and R. L. Guerrant.** 2003. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull. World Health Organ. **81:**197–204.
23. **Kovach, M. E., M. D. Shaffer, and K. M. Peterson.** 1996. A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in Vibrio cholerae. Microbiology (Reading) **142:**2165–2174.
24. **Kovacikova, G., and K. Skorupski.** 1999. A *Vibrio cholerae* LysR homolog, AphB, cooperates with AphA at the *tcpPH* promoter to activate expression of the ToxR virulence cascade. J. Bacteriol. **181:**4250–4256.
25. **Krukonis, E. S., R. R. Yu, and V. J. DiRita.** 2000. The Vibrio cholerae ToxR/TcpP/ToxT virulence cascade: distinct roles for two membrane-localized transcriptional activators on a single promoter. Mol. Microbiol. **38:**67–84.
26. **Lahav, G., N. Rosenfeld, A. Sigal, N. Geva-Zatorsky, A. J. Levine, M. B. Elowitz, and U. Alon.** 2004. Dynamics of the p53-Mdm2 feedback loop in individual cells. Nat. Genet. **36:**147–150.
27. **Larocque, R. C., J. B. Harris, M. Dziejman, X. Li, A. I. Khan, A. S. G. Faruque, S. M. Faruque, G. B. Nair, E. T. Ryan, F. Qadri, J. J. Mekalanos, and S. B. Calderwood.** 2005. Transcriptional profiling of *Vibrio cholerae* recovered directly from patient specimens during early and late stages of human infection. Infect. Immun. **73:**4488–4493.
28. **Lee, S. H., D. L. Hava, M. K. Waldor, and A. Camilli.** 1999. Regulation and temporal expression patterns of Vibrio cholerae virulence genes during infection. Cell **99:**625–634.
29. **Matson, J. S., J. H. Withey, and V. J. DiRita.** 2007. Regulatory networks controlling *Vibrio cholerae* virulence gene expression. Infect. Immun. **75:**5542–5549.
30. **Medrano, A. I., V. J. DiRita, G. Castillo, and J. Sanchez.** 1999. Transient transcriptional activation of the *Vibrio cholerae* El Tor virulence regulator *toxT* in response to culture conditions. Infect. Immun. **67:**2178–2183.
31. **Merrell, D. S., S. M. Butler, F. Qadri, N. A. Dolganov, A. Alam, M. B. Cohen, S. B. Calderwood, G. K. Schoolnik, and A. Camilli.** 2002. Host-induced epidemic spread of the cholera bacterium. Nature **417:**642–645.
32. **Mihalcescu, I., W. Hsing, and S. Leibler.** 2004. Resilient circadian oscillator revealed in individual cyanobacteria. Nature **430:**81–85.
33. **Murley, Y. M., J. Behari, R. Griffin, and S. B. Calderwood.** 2000. Classical and El Tor biotypes of *Vibrio cholerae* differ in timing of transcription of *tcpPH* during growth in inducing conditions. Infect. Immun. **68:**3010–3014.
34. **Nielsen, A. T., N. A. Dolganov, G. Otto, M. C. Miller, C. Y. Wu, and G. K. Schoolnik.** 2006. RpoS controls the Vibrio cholerae mucosal escape response. PLoS Pathog. **2:**e109.
35. **Peterson, K. M., and J. J. Mekalanos.** 1988. Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect. Immun. **56:**2822–2829.
36. **Ramoni, M. F., P. Sebastiani, and I. S. Kohane.** 2002. Cluster analysis of gene expression dynamics. Proc. Natl. Acad. Sci. U. S. A. **99:**9121–9126.
37. **Roberts, C., K. L. Anderson, E. Murphy, S. J. Projan, W. Mounts, B. Hurlburt, M. Smeltzer, R. Overbeek, T. Disz, and P. M. Dunman.** 2006. Characterizing the effect of the *Staphylococcus aureus* virulence factor regulator, SarA, on log-phase mRNA half-lives. J. Bacteriol. **188:**2593–2603.
38. **Schild, S., R. Tamayo, E. Nelson, F. Qadri, S. B. Calderwood, and A. Camilli.** 2007. Genes induced late in infection increase fitness of Vibrio cholerae after release into the environment. Cell Host Microbe **2:**264–277.
39. **Slamti, L., J. Livny, and M. K. Waldor.** 2007. Global gene expression and phenotypic analysis of a *Vibrio cholerae rpoH* deletion mutant. J. Bacteriol. **189:**351–362.
40. **Smyth, G. K., Y. H. Yang, and T. Speed.** 2003. Statistical issues in cDNA microarray data analysis. Methods Mol. Biol. **224:**111–136.
41. **Waldor, M. K., and J. J. Mekalanos.** 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science **272:**1910–1914.
42. **Yang, Y. H., S. Dudoit, P. Luu, D. M. Lin, V. Peng, J. Ngai, and T. P. Speed.** 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. **30:**e15.