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Global Gene Expression and Phenotypic Analysis of a *Vibrio cholerae* *rpoH* Deletion Mutant[▽]

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***Vibrio cholerae*, the cause of cholera, can grow in a variety of environments outside of human hosts. During infection, this pathogen must adapt to significant environmental alterations, including the elevated temperature of the human gastrointestinal tract. σ^{32}, an alternative sigma factor encoded by *rpoH*, activates transcription of genes involved in the heat shock response in several bacterial species. Here, we assessed the role of σ^{32} in *V. cholerae* physiology. In aggregate, our findings suggest that σ^{32} promotes *V. cholerae* growth at temperatures ranging at least from 15°C to 42°C. Growth of the *rpoH* mutant was severely attenuated within the suckling mouse intestine, suggesting that σ^{32}-regulated genes are critical for *V. cholerae* adaptation to conditions within the gastrointestinal tract. We defined the *V. cholerae* RpoH regulon by comparing the whole-genome transcription profiles of the wild-type and *rpoH* mutant strains after a temperature up-shift. Most of the *V. cholerae* genes expressed in an RpoH-dependent manner after heat shock encode proteins that influence protein fate, such as proteases and chaperones, or are of unknown function. Bioinformatic analyses of the microarray data were used to define a putative σ^{32} consensus binding sequence and subsequently to identify genes that are likely to be directly regulated by RpoH in the whole *V. cholerae* genome.**

Vibrio cholerae, the cause of the diarrheal disease cholera, is a facultative pathogen. This curved gram-negative rod grows well in a variety of aquatic ecosystems (37). In addition, when humans ingest food or water contaminated with *V. cholerae*, this microorganism can multiply in the small bowel. During the transition from aquatic habitats to the human gastrointestinal tract, and vice-versa, *V. cholerae* cells confront significant alterations in their environment. The molecular factors that enable *V. cholerae* to adapt to the abrupt changes in the environment, such as the elevated temperature and acidity that the organism encounters in the human host, are largely unknown. However, alterations in the patterns of total cellular transcription, mediated by a variety of alternative sigma factors and other transcriptional regulators, often constitute an important part of the cellular response to environmental stress.

Sigma factors interact with the core components of the RNA polymerase to generate a holoenzyme complex that is capable of initiating transcription at promoters. While σ^{70}, the major sigma factor, activates transcription of most genes in growing cells, alternative sigma factors enable the RNA polymerase to transcribe genes important for cellular adaptation to various stresses (14). In *Escherichia coli*, the alternative sigma factor σ^{32} activates transcription of genes involved in the heat shock response (52). This sigma factor, also referred to as RpoH, has also been implicated in the *E. coli* response to other stresses, including elevated hydrostatic pressure (1), hyperosmolar shock (3), and nutrient limitation (21).

A variety of mechanisms govern σ^{32} levels and activity in *E. coli*. Several promoters drive *rpoH* transcription (12, 31), and several regulatory proteins, including DnaA, CRP, and CytR, can influence *rpoH* transcription (22, 31, 48). Posttranscriptional and posttranslational mechanisms also control RpoH levels. Elevated temperatures induce changes in the *rpoH* mRNA secondary structure that promote its translation (23, 29, 30, 32, 41, 53). RpoH stability and activity are controlled by temperature (25), by sequestration by the DnaK-DnaJ-GrpE and GroES-GroEL chaperone complexes (13, 15, 42, 44), and by proteolysis by FtsH and HslU-HslV (20, 24, 45). The chaperones and proteases that govern RpoH stability and activity belong to the RpoH regulon; thus, regulation of cellular RpoH levels occurs through a complex negative-feedback loop.

σ^{32} is essential for *E. coli* growth at temperatures above 20°C (55). Recently, Zhao et al. used whole-genome arrays to characterize the *E. coli* RpoH regulon after induction of *rpoH* expression. This approach confirmed the list of genes previously known to be under σ^{32} control and identified many additional genes regulated either directly or indirectly by σ^{32} (54). More recently, Nonaka et al. also carried out a comprehensive analysis of the *E. coli* σ^{32} regulon (33).

While knowledge of *rpoH* regulation and the RpoH regulon in *V. cholerae* is limited, it is clear that σ^{32} from *E. coli* and *V. cholerae* are similar in sequence and function. The predicted amino acid sequences of σ^{32} of *V. cholerae* and *E. coli* are 70% identical, and overexpression of the *E. coli* σ^{32} in *V. cholerae* promoted transcription of the HtpG chaperone and production of other proteins regulated by σ^{32} in *E. coli* (34, 38). Chakrabarti et al. (6) reported that *dnaK* expression was induced by heat shock in *V. cholerae* and that the likely *dnaK* promoter possesses potential σ^{32} binding sites based on the *E. coli* consensus sequence. Even though the secondary structure of the *V. cholerae* *rpoH* mRNA is predicted to be slightly different from that in *E. coli*, Sahu et al. suggested that its translation is likely enhanced by

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elevated temperatures, as in *E. coli* (39). However, the kinetics of the heat shock response differ in these two γ-*Proteobacteria*. In *V. cholerae*, peak induction of heat shock proteins occurs 20 min after a temperature up-shift, whereas this peak occurs after only 5 min in *E. coli* (5, 38, 51). Some *V. cholerae* heat shock proteins have been identified (38), but very little is known about the *V. cholerae* RpoH regulon.

Here we constructed a *V. cholerae rpoH* deletion mutant containing a plasmid-borne inducible copy of *rpoH* to begin to explore the physiological role of σ^{32} in *V. cholerae*. Our findings suggest that, in rich medium, σ^{32} is beneficial for *V. cholerae* growth at temperatures from 42°C to 15°C or lower. Growth of the *rpoH* mutant was severely attenuated within the suckling mouse intestine, suggesting that σ^{32}-regulated genes are critical for *V. cholerae* to respond to conditions within the gastrointestinal tract. Microarray analyses were used to define the RpoH regulon. Most genes in the *V. cholerae* RpoH regulon identified here are either involved in protein fate, e.g., chaperones and proteases, or are of unknown function. Application of bioinformatic algorithms to the microarray data enabled us to propose a *V. cholerae* RpoH binding site consensus sequence. Using this sequence, we identified the subset of genes that are likely to be directly regulated by RpoH, as well as genes potentially regulated by RpoH that were not identified in our microarray analyses.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Vibrio cholerae* El Tor clinical isolate N16961 was used as the parental strain throughout this study. The annotated genome of this strain has been published (19). *E. coli* strain DH5α λ*pir* was used as the host strain for plasmid construction. *E. coli* SM10 λ*pir* was used as the donor in mating experiments. Cells were grown in Luria-Bertani (LB) medium and stored at −80°C in LB containing 20% glycerol.

The antibiotic concentrations used for bacterial selection were as follows for *V. cholerae*: 200 μg/ml streptomycin, 50 μg/ml kanamycin, 5 μg/ml chloramphenicol, and 80 μg/ml ampicillin. For *E. coli*, antibiotics were used at the following concentrations: 50 μg/ml kanamycin, 20 μg/ml chloramphenicol, and 100 μg/ml ampicillin. Arabinose and glucose were added at final concentrations of 0.1 and 0.2%, respectively. In the growth experiments described in Fig. 2, 10μl of serial 10-fold dilutions from freshly thawed frozen stocks were spotted on the indicated plates and incubated for 18 h (for 30°C, 37°C, and 42°C experiments) or for 48 h (for 15°C experiments). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was used at a final concentration of 40 μg/ml.

DNA and RNA manipulations. Plasmid DNA was extracted from *E. coli* with QIAGEN kits. Chromosomal DNA was extracted from N16961 with Genome DNA kits from Q-Biogene. RNeasy kits and RNase-free DNase kits (QIAGEN) were used to purify RNA from exponential-phase cells. RNA was purified from stationary-phase cells with TRIzol reagent (Invitrogen). Contaminating DNA was removed from RNA preparations with the DNA-free DNase I kit from Ambion. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs; SuperScript II reverse transcriptase was purchased from Invitrogen. PCR was performed with a PTC-200 thermal cycler from MJ Research, with *Pfx* Platinum polymerase (Invitrogen) or the HotStart *Taq* Mastermix from QIAGEN. The oligonucleotides for PCR amplification, 5′ rapid amplification of cDNA ends (RACE), and real-time reverse transcription-PCR (RT-PCR) were purchased from the Tufts University Core facility or from Operon (Huntsville, AL). Nucleotide sequencing was performed at the Tufts University Core facility.

Plasmid and strain construction. pRpoH contains the promoterless *rpoH* gene (*vc0150*) under the control of the arabinose-inducible P_{BAD} promoter. It was constructed by subcloning *rpoH* into pBAD18-Cm (17). The *rpoH* gene was amplified by PCR from N16961 chromosomal DNA using primers RSP3 and rpoH8 (Table 1). DNA sequencing confirmed that the subcloned *rpoH* sequence was identical to that of *vc0150*.

The allele exchange vector pCVD442Δ*rpoH*::Kn was constructed in the following steps. First, the 5′ and 3′ regions of the *rpoH* gene were amplified by PCR, using primer pairs rpoH3/rpoH4 and rpoH5/rpoH6 (Table 1). The 5′ end was purified as an XbaI/EcoRI fragment, and the 3′ end was purified as a BamHI/SphI fragment. The kanamycin resistance (Kn^r) cassette was amplified from plasmid pKD4 (8) with primers kan3 and kan5 (Table 1) and digested with EcoRI and BamHI. The three fragments were ligated with XbaI- and SphI-digested pCVD442 (10), a *pir*-dependent *sacB*-containing allele exchange vector. The resulting plasmid, pCVD442Δ*rpoH*::Kn, was then introduced by electroporation into the Mob^+ *E. coli* strain SM10 λ*pir*. The *rpoH* gene of strain N16961 was deleted by allele exchange with pCVD442Δ*rpoH*::Kn essentially as follows. In brief, the plasmid was initially transferred from SM10 λ*pir* by conjugation into N16961. One of the resulting Ampicillin-resistant (Ap^r) and Kn^r transconjugants was then electroporated with pRpoH. The transformants were grown at 15°C in the presence of kanamycin and chloramphenicol and then plated on sucrose-kanamycin-chloramphenicol plates (at 15°C) to select for cells that had lost the allele exchange vector after homologous recombination. The resulting strain, N16961 Δ*rpoH*::Kn (pRpoH), was designated LS1.

The structures of the strains and plasmids constructed for this study were all verified by PCR and DNA sequencing.

Plasmid loss assay. LS1 [N16961 Δ*rpoH* (pRpoH)] and LS2 [N16961 (pRpoH)] cells were grown overnight in LB supplemented with chloramphenicol and arabinose at 22°C. The cells were then diluted (1/1,000) at time zero into fresh medium supplemented with only arabinose and incubated either at 30, 37, or 42°C for 8 h or at 15°C for 32 h. Serial dilutions of the cultures were then plated on streptomycin- and arabinose-containing agar and incubated for 16 h at 30°C. Colonies were counted, and replicas were generated on plates supplemented with streptomycin, chloramphenicol, and arabinose. The plates were incubated at room temperature until colonies appeared, and they were subsequently com-

TABLE 1. Primers used in this study

Name	Sequence
0177Fd	TATTCGATTCCCTGAACTGGA
0177Rv	TTGACCAGAGAGCACCTTCA
0706Fd	GTGGCTTCTGCAATTCACG
0706Rv	TTCGCTCAGTTCCGTTTTAT
2675Fd	TGAAAGGCAATGCACGTAAA
2675Rv	CCTTGGTGCATCTGCAACTT
DSP	CGACGACCGATCAGACGCTTG
DSP1	CAATTTTGTAAGGCATGAT
DSP2	GATATCGCGCTGAACTTCTTCG
GroES2Fd	AAAATCAAACCGTGGCAAAG
GroES2Rv	TCCGTTTTCACACCGTAACC
HSP	CCTCGACATTCGCGACAATATG
HSP1	GGCATGATGATTACATTTAG
HSP2	GGATTACTAGCTTCACAATGCAAG
kan3	CGGAATTCCCCGCGCTGGAGGATCATC
kan5	CGGGATCCAGCTACTGGGCTATCTGGAC
KSP	GTTGGCTACCCGTGATATTGC
KSP1	GGAAAATGGCCGCTTTTC
KSP2	GGGTGTGGCGGACCGCTATC
lacZ1	CCCAAGCTTAACAATTTCACACAGGAAACAG
lacZ2	ATAGTTTAGCGGCCGCGAGAACAGAGAAATA GCGGC
PgrpE1	GAAGATCTTGAACGGCTTTTTCATAAGG
PgrpE2	CCCAAGCTTCACTTCATACTGGTCAGG
RpB2Fd	AACCTGTCTCAAGCCGGTTA
RpB2Rv	TTTCTACCAGTGCAGAGATGC
rpoH3	GCTCTAGATTGGTTTGCCCGTTTAGAT
rpoH4	CGGAATTCTCAATTCCTCATCATTCTCTGCTC
rpoH5	CGGGATCCATGCGTAAGCTGAAAGAAGC
rpoH6	ACATGCATGCCGGTATTCTATACCATCCC
rpoH8	TGCTCTAGACCTTTTCTTATCAAGCTG
rpoHH1	GCTCTAGAAATTCATGAATATGTGCTACG
rpoHH2	TCAGTGATGGTGATGGTGATGGAACTCGCCC ACCGCTTC
rpoHH3	CATCACCATCACCATCACTGATTTCACATTGC GTAACAGC
rpoHH4	TCATGCATGCCGGTATCTATACCATCCCTGAG
RSP	CTCTTGCACTAAGTCCGCCATT
RSP1	CTCAGGGTTAAAGCGTTT
RSP2	CCGCTTTCATCAGACCGATATT
RSP3	CCAGATCAGAGAATGATGAGGAA

pared to the master plates, allowing calculation of the ratio of streptomycin- and chloramphenicol-resistant (Sm^r Cm^r) cells to Sm^r cells. The same procedure was used to calculate this ratio for the cells at time zero.

Intestinal colonization assay. LS1 (Lac^+) and LS2 (Lac^-) cells were grown overnight at 22°C in LB in the presence of chloramphenicol and arabinose. The cultures were then diluted 1:1,000 into fresh LB supplemented with chloramphenicol and either arabinose or glucose and grown for an additional 1.5 h at 22°C. The experiment was carried out essentially as described previously (43). Briefly, one-to-one mixtures of LS1 and LS2 from the glucose and from the arabinose cultures were intragastrically inoculated into 5-day-old CD-1 suckling mice. In a parallel in vitro competition experiment, 30 μl of the same mixtures were diluted 100-fold in LB medium and grown for 20 h at 37°C. Mice were sacrificed 20 h after inoculation, and the small intestine was harvested and ground, and serial dilutions were plated on LB supplemented with X-gal, arabinose, and streptomycin.

Microarray analyses. To prepare RNA for microarray analyses, LS2 [N16961 (pRpoH)] and LS1 [N16961 Δ*rpoH* (pRpoH)] cells were grown as follows. First, the strains were grown at 15°C in LB supplemented with arabinose to an optical density at 600 nm (OD_{600}) of ~0.5. Then, the cells were harvested and resuspended in LB supplemented with glucose and incubated for 20 additional minutes at 15°C. Finally, the two strains were subjected to a heat shock at 37°C for 20 min, and then the cells were harvested and RNA was extracted. The subsequent steps of cDNA preparation and labeling, with microarray hybridization and data analysis, were carried out as previously described by Ding et al. (9). The experiment was repeated three times on independently isolated RNA samples, and dye swaps were performed on each RNA sample. Thus, in one experiment Cy3 (Amersham Biosciences) was used to label cDNA from LS1 and Cy5 (Amersham Biosciences) was used to label cDNA from LS2 and vice-versa, generating replicas. The results from the three experiments were averaged, and genes were counted as downregulated in LS1 compared to LS2 if the ratio of LS1/LS2 transcripts was ≤0.5 (see Table 2).

5′ RACE. To map *rpoH* promoters, 5′ RACE was performed on total RNA extracted from stationary-phase N16961 and from the isogenic *rpoE* mutant BD1581 (9). The 5′ RACE system kit (Invitrogen) was used according to the manufacturer's recommendations. The specific primer RSP1 (Table 1) was used to generate cDNA, and the nested primers RSP2 and RSP (Table 1) were used for the subsequent PCR steps.

To map the transcription start sites of *dnaK* and *vca0446*, 5′ RACE was performed on two of the LS2 RNA samples that were used for the microarray experiments. The specific primers DSP1 (for *dnaK*) and HSP1 (for *vca0446*) were used to generate cDNA. The nested primers DSP2 and DSP (for *dnaK*) or HSP2 and HSP (for *vca0446*) (Table 1) were used for the subsequent PCR steps.

The final products were purified from an agarose gel and sequenced.

Real-time RT-PCR analyses. The RNA samples used for the microarray experiments were also used for real-time RT-PCR analyses, as previously described (35). The specific oligonucleotide primer pairs GroES2Fd/GroES2Rv, 2675Fd/2675Rv, 0706Fd/0706Rv, and 0177Fd/0177Rv (Table 1) were used for PCR. The primers were designed with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and verified with Amplify software and by using the search for short, nearly exact match option of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The real-time RT-PCR assays were performed on two different sets of RNA. The reactions were performed and analyzed with an ABIPrism 7000 sequence detection system.

Bioinformatics. The *V. cholerae* RpoH binding site consensus sequence was generated with BioProspector (27). Bioprospector is designed to identify motifs conserved among groups of sequences. To utilize BioProspector, the user must initially specify the general structure of the motif being sought. The *E. coli* σ^{32} binding site consensus sequence motif is comprised of two conserved blocks separated by a gap of variable length (52). The blocks and gap widths used to identify the consensus sequence for the *V. cholerae* σ^{32} binding site were based on the *E. coli* σ^{32} binding site consensus sequence. Bioprospector was used to search for a consensus motif corresponding to this structure in the 200-bp sequence upstream of each of the RpoH-regulated genes presented in Table 2. The search parameters were adjusted until the motif identified corresponded to the published sequence of the *V. cholerae* σ^{32}-regulated *htpG* promoter (34). The final search parameters were as follows: motif, 2 blocks; width of the first motif block, 6; width of the second motif block, 8; minimum gap between the blocks, 13; maximum gap between the blocks, 16.

Identification of putative σ^{32} binding sites throughout the *V. cholerae* genome was then accomplished in two steps. First, the program PATSER (46) was used to search the upstream regions of all *V. cholerae* genes for sequences corresponding to the consensus motif of each block. Second, PromoterFinder, a C++ program, was developed and used to find those blocks separated by gaps of 13 to 16 bp in length. Each putative promoter was assigned a score corresponding to the average score of its associated blocks as determined by PATSER and was also assigned a rank among the scores of all the promoters.

Sequence comparisons were done with BLAST on the NCBI website (http://www.ncbi.nih.gov/BLAST/) and with DnaStrider software. Promoter predictions were performed by using Bprom on the Softberry website (http://www.softberry.com/berry.phtml).

Microarray accession number. The microarray data have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nih.gov/geo/) and assigned the following accession number: GSE6097.

RESULTS

Absence of RpoH induction impairs *V. cholerae* growth. Generating an *rpoH* deletion mutant to explore the role of the alternative sigma factor RpoH in *V. cholerae* physiology proved difficult. Despite several attempts, we were unable to replace the *rpoH* gene in the sequenced El Tor *V. cholerae* clinical isolate N16961 with a cassette conferring resistance to kanamycin, via allelic exchange, at temperatures ranging from 15°C to 37°C. However, the same strategy was successful when RpoH, provided by a plasmid that carries *rpoH* under the control of the arabinose-inducible P_{BAD} promoter, was introduced into N16961 prior to the allelic exchange (see Materials and Methods). The resulting N16961 Δ*rpoH* (pRpoH) strain was designated LS1.

To explore the growth phenotype of the *V. cholerae* Δ*rpoH* mutant, we assessed LS1 and LS2 [N16961 (pRpoH)] growth in LB broth supplemented with chloramphenicol and either arabinose, to induce *rpoH* expression, or glucose, to repress *rpoH* expression, at 22°C and 42°C. Transcriptome analyses presented below confirmed that these conditions altered *rpoH* mRNA levels as expected. We measured changes in OD_{600} and counted CFU on plates containing arabinose (Fig. 1). Growth rates were calculated as the change in OD_{600}/time. At both temperatures, the growth rates of wild-type strain LS2 were approximately the same in arabinose and glucose (Fig. 1A and B), suggesting that overexpression of *rpoH* does not alter *V. cholerae* growth. In contrast, the growth rate of the *rpoH* deletion mutant LS1 at 42°C was ~1.7 times greater in arabinose than in glucose. At 42°C, the number of LS1 CFU was approximately five times greater in arabinose- than in glucose-containing cultures (Fig. 1C). At 22°C, although the growth rates of LS1 were similar in arabinose and glucose (Fig. 1B), there was a somewhat higher (~2.0-fold) number of LS1 CFU in the arabinose-containing cultures (Fig. 1D). This discrepancy may, at least in part, result from the mild filamentation observed in LS1, but not LS2, at both 22° and 42°C in glucose-containing media (data not shown). Overall, these observations suggest that *rpoH* has a significant effect on *V. cholerae* growth at 42°C and a more subtle influence at 22°C, perhaps by influencing cell division.

***V. cholerae* σ^{32} may be essential at all temperatures.** To determine whether *V. cholerae* cells are able to survive in the absence of RpoH, we compared the frequency of pRpoH loss from the *rpoH* deletion mutant LS1 and the wild-type strain LS2 at temperatures ranging from 15°C to 42°C. In these experiments, overnight cultures of LS1 and LS2 were grown at 22°C in LB supplemented with arabinose and chloramphenicol, the marker carried by pRpoH. Then, the cultures were diluted in fresh LB supplemented with only arabinose and incubated at 30°C, 37°C, or 42°C for 8 h or at 15°C for 32 h. Regardless of the temperature, all of the Δ*rpoH* cells present at the end of the experiment carried pRpoH (Fig. 2, black bars).

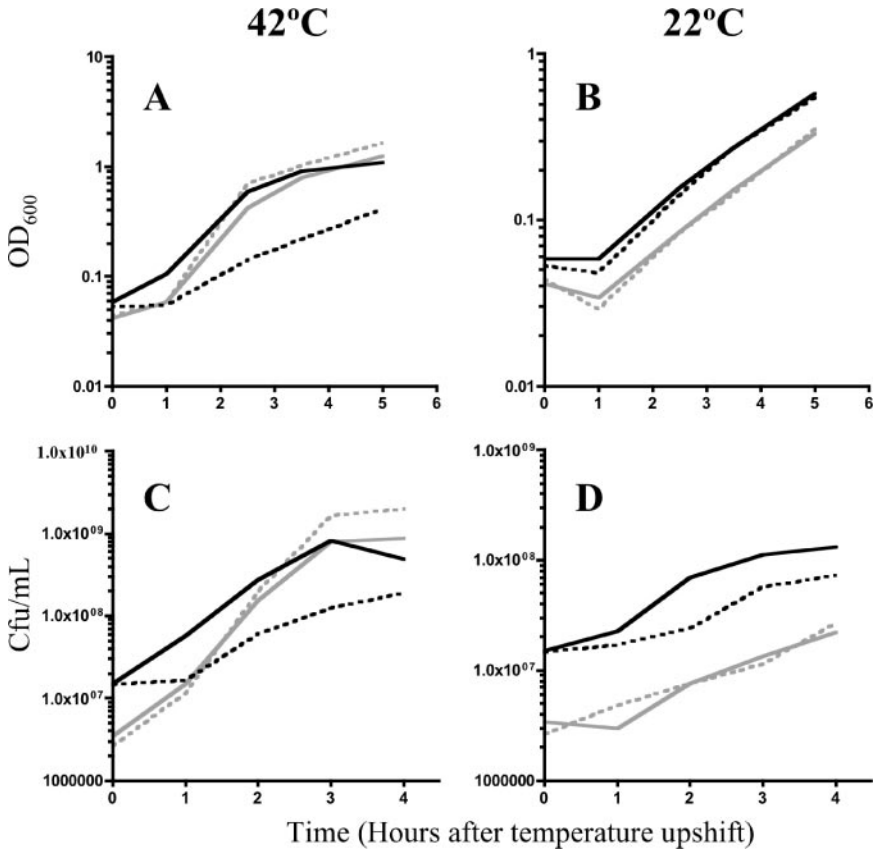


FIG. 1. Comparison of the growth and colony-forming ability of wild-type (LS2) and Δ*rpoH* (LS1) *V. cholerae* at 22°C and 42°C. LS1 (black lines) and LS2 (gray lines) cells were grown in LB with chloramphenicol and arabinose at 22°C to an OD_{600} of 0.03. The cells were then harvested, washed, and resuspended in fresh medium supplemented with chloramphenicol and either arabinose (solid lines) or glucose (dotted lines) and grown in a shaker incubator at 42°C (A and C) or 22°C (B and D). Each graph is representative of at least two independent experiments; the data in panels A and B and those in panels C and D come from two different experiments. Time zero indicates the time at which the cells were placed in the incubator at either 22°C or 42°C. Note that the *y* axis is a logarithmic scale.

In contrast, there was marked loss of pRpoH from the wild-type cells. After incubation at 30°C or 37°C, only 0.32% and 0.01% of LS2 cells, respectively, harbored pRpoH. The marked difference in the stability of pRpoH in LS1 and LS2 in the absence of selection indicates that regardless of temperature, provision of *rpoH* in *trans* is beneficial to the Δ*rpoH* mutant. Interestingly, a larger fraction of the wild-type cells still carried the plasmid after incubation at 42°C or 15°C than at 30°C or 37°C. Wild-type cells harboring the empty vector pBAD18-Cm showed a similar pattern of plasmid loss (Fig. 2). The similarity in the patterns of pRpoH and pBAD18-Cm loss from wild-type cells suggests that the excess of RpoH provided by pRpoH does not explain the differences in pRpoH loss observed at different temperatures; instead, temperature-dependent differences in plasmid segregation likely account for the marked difference in pRpoH loss from LS2 over the temperature range we tested.

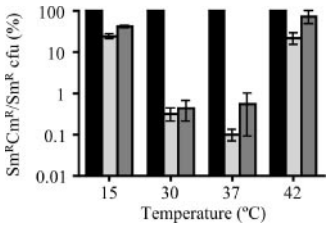


FIG. 2. Maintenance of a plasmid-borne copy of *rpoH* in wild-type versus Δ*rpoH V. cholerae* at various temperatures. Retention of the Cm^r plasmid pRpoH in LS1 (Δ*rpoH*) and LS2 (wild type) after 32 h at 15°C, or after 8 h at 30°C, 37°C and 42°C, is shown as the normalized ratio of Sm^r Cm^r to Sm^r CFU. The black bars represent LS1 cells, the light gray bars represent LS2 cells, and the dark gray bars represent wild-type cells carrying the empty vector pBAD18-Cm. Normalization was done by dividing the ratios of Sm^r Cm^r/Sm^r cells after incubation at the various temperatures by the ratio of Sm^r Cm^r/Sm^r cells at the time of inoculation ($t = 0$). Note that the *y* axis is a logarithmic scale. The results are the mean values of two experiments, and the error bars represent standard deviations.

RpoH is required for *V. cholerae* resistance to kanamycin at high temperatures. During the course of our experiments investigating the growth of the *V. cholerae rpoH* deletion mutant, we made the unexpected observation that *rpoH* influences *V. cholerae* resistance to the aminoglycoside antibiotic kanamycin in a temperature-dependent fashion. LS1 contains a Kn^r gene in the place of *rpoH*. When we plated LS1 on plates containing either arabinose or glucose, the results were remarkably different depending on whether we used kanamycin to select for LS1 CFU (Fig. 3A; compare the top and bottom halves of each

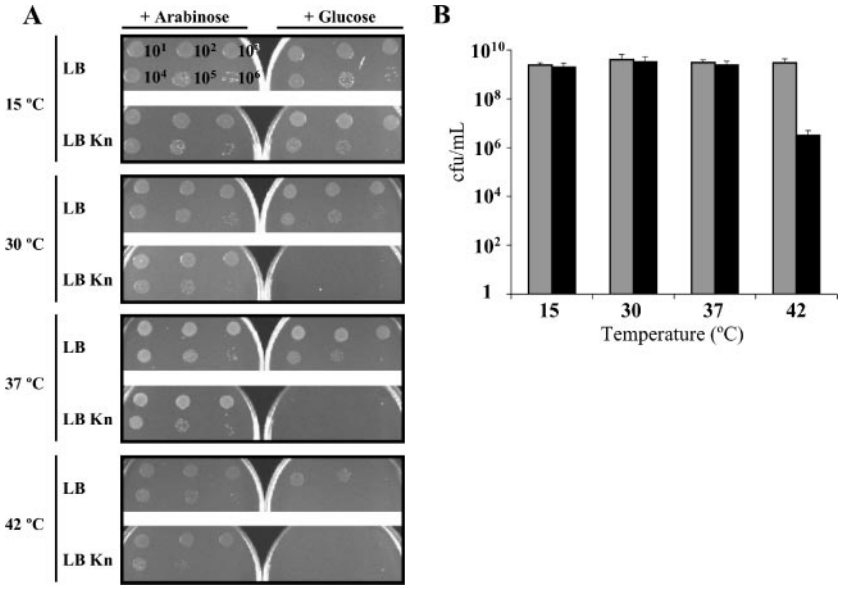


FIG. 3. Temperature influences the requirement of an *rpoH* deletion mutant for RpoH in the presence of kanamycin. In panel A, 10 μl of serial 10-fold dilutions from a freshly thawed frozen stock of LS1 were spotted on LB and LB-kanamycin (LB Kn) plates. The numbers in the upper panel indicate the dilution factor. Plates on the left contain arabinose (0.1%), and plates on the right are supplemented with glucose (0.2%). The CFU/ml of the freshly thawed frozen stocks plated on LB supplemented with chloramphenicol are shown in the bar graph (B). Gray bars indicate plates supplemented with arabinose, and black bars indicate plates supplemented with glucose. Note that the *y* axis is a logarithmic scale. The results are the mean values of at least two experiments, and the error bars represent standard deviations.

panel). We counted the number of LS1 [Δ*rpoH* (pRpoH)] CFU on LB plates containing either arabinose, to induce RpoH expression, or glucose, to repress RpoH expression, at four different temperatures, in the presence or absence of kanamycin. The results of a representative spot dilution experiment are shown in Fig. 3A. At 15°C, RpoH induction was dispensable for *V. cholerae* colony formation, independently of the presence of kanamycin. However, there was a dramatic reduction in the number of LS1 CFU on the medium containing glucose versus arabinose at 30°C and 37°C on plates containing the antibiotic. Finally, at 42°C, no LS1 CFU were detected on the plates supplemented with glucose and kanamycin (Fig. 3A, bottom half of the bottom panel). In contrast, LS1 colony formation was not impaired below 42°C in the presence of streptomycin (data not shown), another aminoglycoside antibiotic like kanamycin, or chloramphenicol, another antibiotic that, like kanamycin, inhibits protein synthesis (Fig. 3B). Thus, RpoH appears to be essential for *V. cholerae* resistance to kanamycin at high temperatures or for *V. cholerae* resistance to high temperatures in the presence of kanamycin.

RpoH is critical for *V. cholerae* growth in the intestine. We carried out competition experiments to compare the growth of LS1 and LS2 in the intestines of suckling mice to assess the importance of RpoH for *V. cholerae* growth in vivo. We designed these experiments to approximate the temperature shift that *V. cholerae* may undergo during infection of the human gastrointestinal tract. To prepare the inocula for these experiments, the two strains were initially grown overnight at 22°C in LB supplemented with chloramphenicol and arabinose, to ensure maintenance of pRpoH and expression of *rpoH*. Prior to mixing of the strains for in vivo inoculation, they were grown for an additional 1.5 h at 22°C in LB supplemented with chloramphenicol and either arabinose, to promote *rpoH* expression, or glucose, to repress *rpoH*. One-to-one mixtures of LS1 and LS2 grown in glucose or LS1 and LS2 grown in arabinose were intragastrically inoculated into 5-day-old suckling mice. After 20 h, the mice were sacrificed, their small intestine was harvested and ground, and serial dilutions of the mixture were plated on LB supplemented with X-gal and streptomycin. In a parallel in vitro competition experiment, the same inocula were diluted in LB medium and grown at 37°C for 20 h to compare the growth of these two strains in rich medium, at approximately the same temperature as the mice.

As shown in Fig. 4, LS1 was profoundly attenuated for

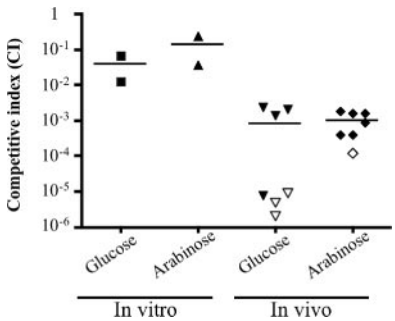


FIG. 4. Comparison of the growth of LS1 and LS2 in LB at 37°C and in suckling mice. Prior to inoculation, the strains were grown at 22°C in either glucose or arabinose. Then, the two strains were mixed 1:1 and inoculated into suckling mice or LB broth. The ratio of LS1 to LS2 CFU in intestinal homogenates or in the overnight LB broth cultures was divided by the ratio of these strains in the inocula to yield the competitive indices. Open triangles and diamonds represent mice that had no detectable LS1 CFU.

TABLE 2. *V. cholerae* RpoH-regulated genes

Gene category	Ratio[a]	*E. coli* regulon[b]			Designation[c]	Name	Product[c]
		υ	λ	τ			
Degradation of proteins, peptides, and glycopeptides	0.27	υ	λ	τ	***vc0188***	*prlC*	Oligopeptidase A
	0.05	υ	λ	τ	***vc0711***	*clpB-1*	ClpB
	0.45				*vc1343*		Peptidase, M20A family
	0.33	υ	λ	τ	***vc1920***	*lon*	ATP-dependent protease LA
	0.25	υ		τ	*vc2674*	*hslU*	Protease HslVU, ATPase subunit HslU
	0.25	υ	λ	τ	***vc2675****	*hslV*	Protease HslVU, subunit HslV
Protein folding and stabilization	0.33	υ	λ	τ	***vc0018***	*ibpA*	16-kDa heat shock protein A
	0.40	υ	λ	τ	***vc0854***	*grpE*	Heatshock protein GrpE
	0.05	υ	λ	τ	***vc0855***	*dnaK*	DnaK
	0.08	υ		τ	*vc0856*	*dnaJ*	DnaJ
	0.14	υ	λ	τ	***vc0985***	*htpG*	Heat shock protein HtpG
	0.06				*vc2664*	*groEL-1*	Chaperonin, 60-kDa subunit
	0.05				*vc2665*	*groES-1*	Chaperonin, 10-kDa subunit
	0.05	υ		τ	***vca0819****	*groES-2*	Chaperonin, 10-kDa subunit
	0.06	υ	λ	τ	*vca0820*†	*groEL-2*	Chaperonin, 60-kDa subunit
Regulatory functions	0.35		λ	τ	*vc0706**		σ^{54} modulation protein, putative
	0.26				*vc2485*		Transcriptional regulator, LysR family
Transcription	0.14				*vc0150*	*rpoH*	RNA polymerase σ^{32} factor
Transport and binding proteins	0.37				*vc0280*		Cadaverine/lysine antiporter CadB, putative
	0.47				*vca1028*	*ompS*	Maltoporin
	0.13				*vca0910*	*tonB1*	TonB1
	0.29				***vc0589***		ABC transporter, ATP-binding protein
	0.37				*vc0590*		Permease, putative
Biosynthesis of cofactors, prosthetic groups, and carriers	0.48				*vc0468*	*gshB*	Glutathione synthetase
	0.38				*vc1950*	*bizZ*	Biotin sulfoxide reductase
	0.42	υ			*vc2673*	*menA*	1,4-Dihydroxy-2-naphthoate octaprenyltransferase
Pathogenesis	0.36				***vca0446***		Hemagglutinin
	0.29				*vca0447*		Hemagglutinin-associated protein
Metabolism	0.49				*vc1589*	*aldC*	α-Acetolactate decarboxylase
	0.46				*vc1951*	*yecK*	Cytochrome *c*-type protein YecK
	0.11			τ	*vca0752*	*trxC*	Thioredoxin 2
Conserved hypothetical proteins	0.20				*vc0466*		
	0.41				*vc0467*		
	0.44				*vc0469*		
	0.40				*vc0490*		
	0.18		λ	τ	*vc0977*		
	0.48				***vc1055***		
	0.41				***vc1358***		
	0.45				*vc1871*		
	0.46	υ		τ	***vc2735***		
Hypothetical proteins	0.04				*vc0177**		
	0.44				*vc0189*		
	0.36				***vc0886***		
	0.49				*vc1080*		
	0.20				*vc2486*		
	0.17				*vc2663*		
	0.15				*vca0065*		
	0.46				***vca0396***		
	0.16				*vca0821*		

[a] To be included here, the ratio of mutant/wild-type transcript must be ≤0.5. Values are averages from three independent experiments.
[b] Genes that are also found in the *E. coli* regulon, defined as follows: τ, Zhao et al. (54); λ, Wade et al. (47); υ, Nonaka et al. (33).
[c] As annotated by The Institute for Genomic Research. The genes that possess a potential σ^{32} binding sequence in their promoter region are in boldface type. Symbols: *, gene chosen for real time RT-PCR; †, data not available for one of the three experiments for *vca0820* (*groEL-2*) (the ratio reported for this gene is the average from two of three experiments).

growth in vivo. Induction or repression of *rpoH* expression prior to inoculation did not rescue LS1 colonization. In both cases, the average LS1/LS2 competitive index (the ratio of LS1 to LS2 CFU recovered from individual mice divided by the ratio of LS1 to LS2 CFU in the inocula) for growth in vivo was ~0.001 (Fig. 4). These numbers represent an underestimate of the true degree of the in vivo growth impairment of the *rpoH* mutant, since several mice did not have any detectable LS1 in the small intestine. Thus, RpoH appears to be critical for *V. cholerae* growth in vivo. The maintenance of pRpoH in vivo and in vitro was similar (Fig. 1 and data not shown). All the LS1 CFU recovered from the intestinal homogenates harbored pRpoH whereas nearly all LS2 CFU lost the plasmid. The finding that induction or repression of RpoH production prior to inoculation did not appear to significantly influence LS1 colonization may suggest that RpoH is required throughout the duration of infection and not strictly for the shift in growth conditions that the organism undergoes at the beginning of the experiment. In LB, LS1 was also outcompeted by LS2 in the vitro competition experiment. Thus, provision of *rpoH* in *trans* without inducer does not appear to provide adequate RpoH to enable wild-type growth of the *rpoH* mutant in rich medium when competing with wild-type cells. However, the magnitude of the competitive defect of LS1 versus LS2 in vivo was more than 150 times lower (0.16 versus 0.001) than in vitro. The most likely explanation for this dramatic difference is that there is a greater necessity for RpoH and the genes that it controls for growth in the intestine than in rich medium.

The *V. cholerae* RpoH regulon. To begin to define the *V. cholerae* RpoH regulon, we compared the transcriptomes of LS1 [N16961 Δ*rpoH* (pRpoH)] and LS2 [N16961 (pRpoH)] following a temperature up-shift. The strains were initially grown at 15°C in medium containing arabinose, to induce RpoH expression, until the culture reached an OD_{600} of ~0.5. Then, the cells were shifted to medium containing glucose, to repress RpoH expression, and incubated at 15°C for an additional 20 min and subsequently shifted to 37°C for 20 min prior to extraction of RNA for cDNA synthesis. Thus, this comparison should yield knowledge of the RpoH-dependent genes that are induced by a heat shock similar to the change in temperature that occurs upon infection of the human gastrointestinal tract by *V. cholerae* from a natural water environment.

The transcripts of 49 genes were more abundant in wild-type cells than in Δ*rpoH* cells 20 min after the temperature up-shift (ratio of mutant/wild-type transcript, ≤0.5) (Table 2). As expected, the *rpoH* transcript was much less abundant (sevenfold) in the LS1 cells than in the LS2 cells. This confirmed that *rpoH* transcription was reduced by the shift to glucose in LS1 compared to LS2, which contains the native *rpoH* gene under the control of its own promoter. While 10 different categories of genes are found in the list of genes whose temperature up-shift induction depends on RpoH, more than 60% of the genes identified here are either involved in protein fate, like chaperones and proteases, or are of unknown function.

Most of the genes encoding products involved in protein fate have orthologs in the *E. coli* RpoH regulon, as recently defined by Zhao et al. (54), Nonaka et al. (33), and Wade et al. (47). Four other genes (*vc0706*, *vca0752* [*trxC*], *vc0977*, and *vc2735*) also appear to be common between the two regulons. Although

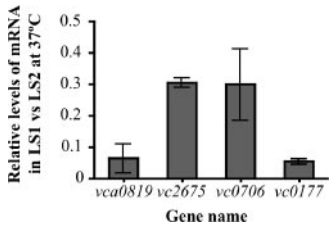


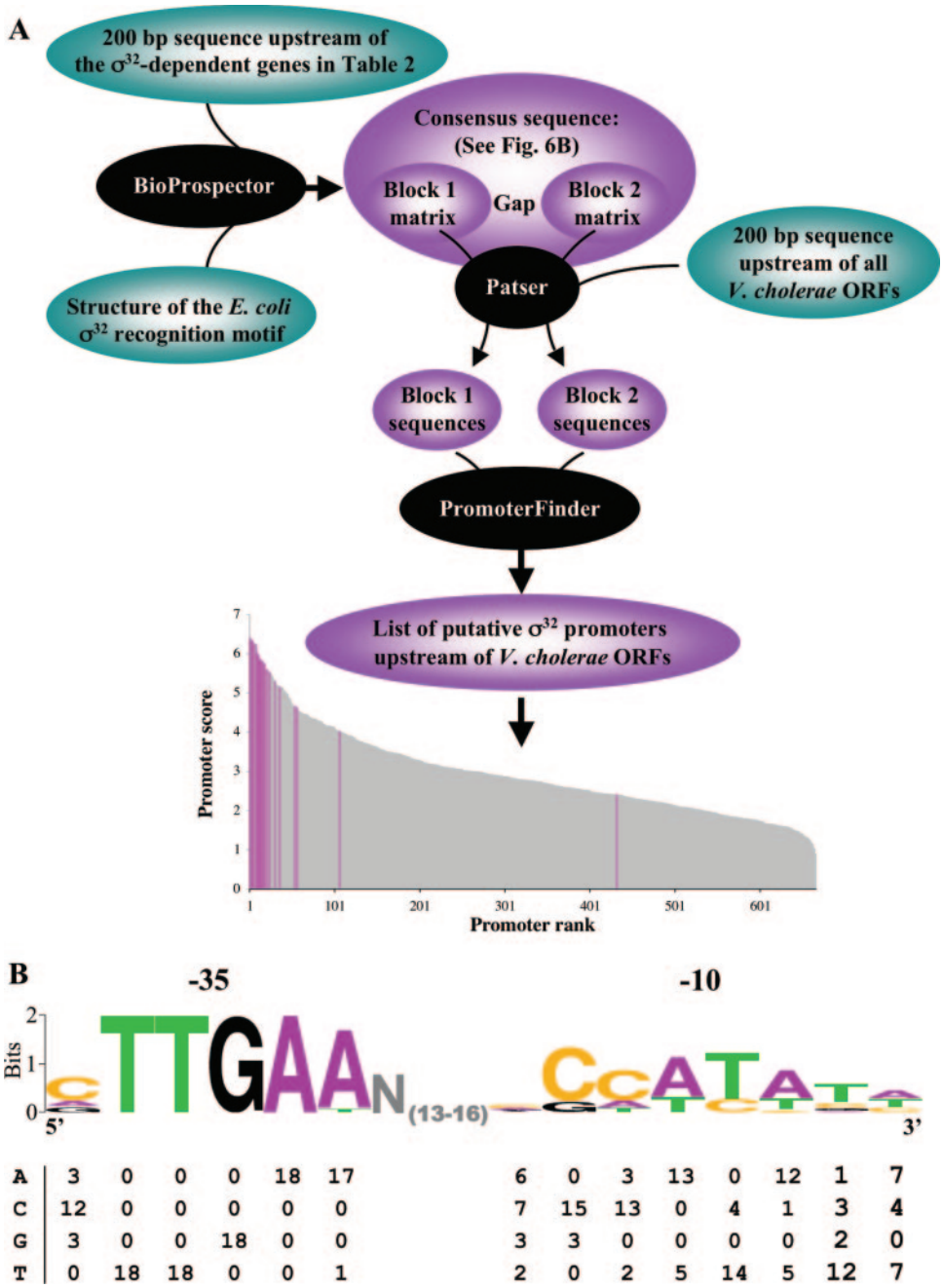
FIG. 5. Relative levels of transcripts of four RpoH-regulated genes in Δ*rpoH* versus wild-type *V. cholerae*. RNA was isolated from LS1 and LS2 20 min after a temperature up-shift from 15°C to 37°C. The names of the transcripts quantified by real-time RT-PCR are indicated below the *x* axis. The transcript levels were normalized to the amount of *rpoB* transcripts in the cells. The results presented are the mean values of two experiments. The error bars represent the standard deviations.

vc2735 was classified as encoding a "conserved hypothetical protein" by Heidelberg et al. (19), the product of this gene is homologous to *E. coli* Hsp15 (encoded by *yrfH*), a heat shock protein that possesses an S4-like RNA binding domain. *vc2735* is likely to be in an operon with *vc2736*, a gene also annotated as encoding a conserved hypothetical protein; *vc2736* bears similarity to the *E. coli* Hsp33 protein, a redox-regulated chaperone. Although *vc2736* did not meet the criteria to be included in Table 2, it is probably RpoH regulated, as its transcript level was reduced more than 1.5-fold in LS1 relative to LS2.

There are also several differences between the *V. cholerae* and *E. coli* RpoH regulons. Not all genes in the *V. cholerae* RpoH regulon that have *E. coli* homologs were found to be RpoH regulated in *E. coli* (e.g., *vc0466*, *vc0467*, *vc0468*, and *vc0469*). Twelve genes in the *V. cholerae* RpoH regulon do not have *E. coli* homologs; the products of these genes mainly belong to the "conserved hypothetical protein" category. Some of the RpoH-regulated genes that are involved in protein fate in *E. coli*, such as *ftsH*, were absent from our list. Although most of the genes whose products are involved in regulating the levels and activity of RpoH are present in our list (such as DnaK, DnaJ, and GroESL), the absence of *ftsH* suggests that RpoH regulation may differ in *V. cholerae* and in *E. coli*, as FtsH degrades RpoH in *E. coli* (20, 45).

Some of the RpoH-regulated genes listed in Table 2 may contribute to *V. cholerae* pathogenicity. Indeed, two genes, *vca0446* and *vca0447* (a hemagglutinin and a hemagglutinin-associated protein, respectively), in Table 2, are found in the "pathogenesis" category. Although *vca0065* is classified in the "conserved hypothetical protein" category, it appears to be a secreted Zn-metalloprotease and thus may play a role in *V. cholerae* virulence. Furthermore, RpoH-regulated genes that contribute to the cellular response to the various stresses encountered in vivo are also likely to contribute to pathogenicity. For example, the RpoH-regulated chaperone DnaK has been implicated in the virulence of *V. cholerae* (6) and other pathogens and is known to be a target of the human immune response to *V. cholerae* (38).

To verify the accuracy of the microarray data, we performed real time RT-PCR analyses on transcripts of four randomly chosen genes from Table 2. For these experiments we used two of the three RNA sample sets that were used for the microarray experiments. As seen in Fig. 5, the relative levels of



A	3	0	0	0	18	17		6	0	3	13	0	12	1	7
C	12	0	0	0	0	0		7	15	13	0	4	1	3	4
G	3	0	0	18	0	0		3	3	0	0	0	0	2	0
T	0	18	18	0	0	1		2	0	2	5	14	5	12	7

FIG. 6. Bioinformatics approach for determination of a putative *V. cholerae* σ^{32} binding site consensus sequence. (A) Protocol used to determine a consensus sequence for the *V. cholerae* RpoH binding site shown in panel B. Sequence databases are shown as blue ovals, computer programs are shown in black ovals, and outputs are shown as magenta ovals. The consensus sequence was used to search upstream of all *V. cholerae* ORFs to identify putative RpoH-dependent promoters. The graph at the bottom of panel A shows the score distribution of these putative RpoH-dependent promoters. The pink lines correspond to putative promoters identified upstream of RpoH-regulated genes in Table 2. (B) Consensus sequence of the RpoH binding site displayed with WebLogo (http://weblogo.berkeley.edu) (7, 40). The height of each column indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each nucleic acid at that position. The number of times each nucleotide is found at each position in the consensus is shown below the sequence. $N_{(13–16)}$ indicates the gap between the two blocks.

vca0819, *vc2675*, *vc0706*, and *vc0177* transcripts were all reduced in the Δ*rpoH* mutant relative to the wild-type strain. In fact there is a reasonably good concordance with the ratios of the transcripts ascertained with the RT-PCR and microarray techniques. This concordance lends considerable credence to the microarray data.

A putative RpoH binding site consensus sequence. To begin to assess which genes in Table 2 are under the direct control of σ^{32}, we used a bioinformatics-based approach (Fig. 6A; also see Materials and Methods) to define a putative σ^{32} binding site consensus sequence, cTTGAA($N_{13–16}$)(a/c)CCATat(a/t), where lowercase letters indicate less conservation among the

FIG. 7. *dnaK* and *vca0446* transcription start sites. (A) Two *dnaK* transcription start sites were identified by 5′ RACE. P1 and P2 are positioned 6 and 7 bp downstream from the −10 motif of the two predicted σ^{32} binding sites, respectively. (B) One transcription start site upstream from *vca0446* was identified by 5′ RACE. P1 is located 9 bp downstream from the −10 motifs of the predicted σ^{32} binding site. The translation start codon for each gene is boxed, and the likely Shine-Dalgarno (SD) sequence is indicated by a dashed line. The beginning of each ORF is shown with uppercase letters.

sequences aligned (shown in Fig. 6B). Higher sequence conservation was observed for the −35 region than for the −10 region. This consensus sequence is similar to the three reported *E. coli* σ^{32} binding consensus sequences (33, 47, 54). Eighteen potential σ^{32} binding sites were found upstream of 16 of the 49 genes in Table 2 (highlighted in pink in Fig. 6A). Thus, approximately one-third of the *V. cholerae* σ^{32} regulon appears to be under the direct control of this sigma factor. More than half of the genes found to be associated with a putative σ^{32}-regulated promoter are involved in protein fate.

To test the accuracy of our proposed σ^{32} promoter consensus sequence, we used 5′ RACE experiments to determine the transcription start sites of two genes predicted to be directly regulated by σ^{32}, *dnaK* and *vca0446*. Two potential σ^{32} binding sites are predicted upstream from *dnaK*. We identified two apparent transcription start sites for this gene. These sites, P1 and P2, are located 108 and 39 bp upstream from the putative *dnaK* translation start codon, respectively (Fig. 7A). P1 is located 6 bp downstream from the distal end of a predicted −10 motif, and P2 is 7 bp downstream from the second predicted −10 motif. Similarly, the apparent transcription start site for *vca0446*, which is located 27 bp upstream from the translation start codon, is 9 bp downstream from the distal end of the predicted −10 motif (Fig. 7B). The relative positions of the predicted σ^{32} binding sites upstream of *dnaK* and *vca0446*, with respect to the experimentally defined transcription start sites for these genes, are similar to the distances found between the −10 motifs and transcription start sites in bacterial promoters recognized by sigma factors from the σ^{70} family to which RpoH belongs (18). Furthermore, in the recent comprehensive analysis of the *E. coli* σ^{32} regulon, Nonaka et al. found that the distance between the −10 motif and the transcription start site for σ^{32}-regulated promoters varies from 6 to 9 bp (33). These observations provide experimental support for our proposed σ^{32} binding site consensus sequence.

A bioinformatics approach to identifying new RpoH-regulated genes. Using the consensus sequence we identified, we took a bioinformatics approach to complement our microarray data and potentially find additional genes that may be directly regulated by σ^{32}. First, we searched for the σ^{32} binding site consensus motif in the regions upstream of all *V. cholerae* open reading frames (ORFs) as a means of identifying possible σ^{32}-regulated promoters; 665 potential σ^{32} binding sites that likely correspond to σ^{32}-regulated promoters were found. Each of these was given a score corresponding to the average of the scores assigned by PATSER to its associated block 1 and block 2 sequences (Fig. 6A). In the PATSER scoring system, the closer a sequence is to the consensus matrix, the higher the score. Each of the putative promoters was then ranked according to its score, and promoter scores were plotted versus promoter ranks to create the graph shown at the bottom of Fig. 6A. Almost all of the 18 putative promoters upstream of genes in Table 2 are clustered among the highest-scoring promoters in Fig. 6A. The fact that promoters predicted upstream of RpoH-regulated genes ranked among the highest-scoring promoters supports the idea that the consensus motif identified by Bioprospector is specific to genes regulated by RpoH and may correspond to a potential RpoH binding site. However, this finding could also reflect the inherent circularity in our approach, i.e., the high scores of putative promoters found in regions upstream of RpoH-regulated genes might simply reflect the fact that these regions were among the sequences used by Bioprospector to identify the consensus motif. To address this possibility, we searched for a consensus motif upstream of 49 randomly selected *V. cholerae* genes by the approach used to determine the RpoH binding site consensus motif. We then used PATSER and PromoterFinder to search for sequences corresponding to this consensus motif upstream of all *V. cholerae* genes. Of 720 sequences identified, those identified in these 49 regions had an average rank of 241, which corresponds to the 66th percentile. In contrast, putative σ^{32}-dependent promoters predicted upstream of RpoH-regulated genes had an average rank of 41 out of 665 predicted promoters, corresponding to the 94th percentile. These findings suggest that the high-scoring putative promoters predicted upstream of the 16 RpoH-regulated genes correspond to actual RpoH binding sites.

Many of the 665 predicted σ^{32} binding sites are likely to be false positives because there is significant divergence among sequences recognized by sigma factors. However, this list also probably includes several σ^{32}-dependent promoters that were not identified in our microarray experiments. We examined whether any of the genes associated with the top 20% of putative σ^{32} binding sites (and not listed in Table 2) have homologs in the *E. coli* σ^{32} regulons as defined in three recent studies (33, 47, 54). We found three genes, *vc0636*, *vc0350*, and *vc0566*, homologous to *E. coli ftsJ*, *hflC*, and *b0161* (which encodes a putative protease), respectively, that met these criteria. On the other hand, several genes that were associated with high-scoring putative σ^{32} binding sites do not have homologs in *E. coli* but are also likely to be under the direct control of σ^{32} in *V. cholerae*. For example, the region upstream

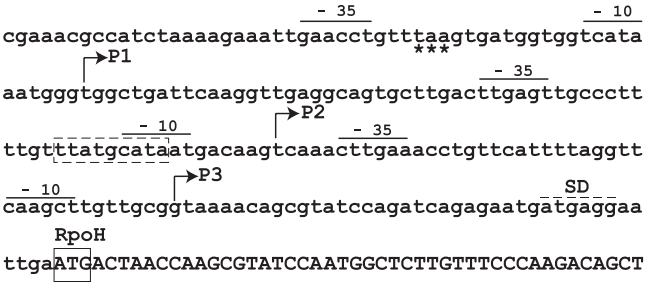


FIG. 8. Features of the *rpoH* promoter region. Three *rpoH* transcription start sites, P1, P2, and P3, were identified by 5′ RACE. The likely −10 and −35 elements for P2 and P3 suggest that these promoters depend on σ^{70}. Potential σ^{24} −10 and −35 sequences were identified upstream of the P1 transcription start site. The RpoH translation start site is boxed, and the likely Shine-Dalgarno (SD) sequence is indicated by a dashed line. The beginning of the *rpoH* ORF is shown with uppercase letters. The asterisks denote the *ftsX* translation stop codon. The dashed box indicates a putative DnaA binding sequence.

of *vc1379* has one of the highest-scoring potential σ^{32} binding sites. The ratio of LS1 to LS2 for *vc1379* transcripts was 0.6 and therefore did not meet our parameters to be included in the regulon; however it is probable that this gene is under the control of RpoH. Furthermore, BLAST analysis suggests that the product of this gene is distantly related to a DnaJ-like protein. Thus, even though this bioinformatic method yields many false predictions, our findings suggest that computational approaches can be a valuable complement to experimental studies.

***rpoH* transcription start site.** We also performed 5′ RACE experiments to explore the *V. cholerae rpoH* promoter region. *rpoH* transcription in *E. coli* utilizes four transcription start sites and several regulatory proteins (12, 22, 31, 48). Three of the four promoters are recognized by σ^{70}, whereas the fourth is σ^{24} dependent (49). Our previous work suggested that *rpoH* transcription in *V. cholerae* is also influenced by σ^{24} (RpoE), as *rpoH* mRNA levels were elevated in a *V. cholerae* strain with a deletion of *rseA* (9), the gene encoding the RpoE anti-sigma factor. We carried out 5′ RACE experiments on RNA extracted from N16961 and from an isogenic *rpoE* mutant strain (9). These experiments suggest that there are three *rpoH* promoters (designated P1, P2, and P3 in Fig. 8). P2- and P3-derived transcripts were detected in both wild-type and *rpoE* mutant cells. P2 and P3, the two promoters most proximal to the *rpoH* translation start site, are probably σ^{70} dependent, as there are reasonable σ^{70} −10 and −35 recognition elements upstream from these transcription start sites. These two promoters are also predicted to contain transcription start sites (scores of 4.22 and 3.32, respectively) by the Bprom program (available at www.softberry.com). On the other hand, P1 has features of a σ^{24}-dependent promoter, as described by Kovacikova and Skorupski (26), and, as expected, no putative σ^{24}-dependent *rpoH* transcript was amplified from the *rpoE* cells.

The *rpoH* promoter region in *E. coli* and other bacteria contains binding sites for regulators such as DnaA, CRP, and CytR (36). A putative DnaA box overlaps the −10 region of P2, but no other candidate regulatory binding sites were identified in the *V. cholerae rpoH* promoter region.

DISCUSSION

To begin to assess the role of σ^{32} in *V. cholerae* physiology, we constructed a *V. cholerae rpoH* deletion mutant containing an inducible plasmid-borne copy of *rpoH*. In aggregate, our findings suggest that this alternative sigma factor promotes *V. cholerae* colony formation at temperatures ranging from 42°C to 15°C or lower. σ^{32} has also been reported to promote *E. coli* growth over a similarly wide range of temperatures (55). However, in *E. coli*, σ^{32} is considered to be essential for growth at temperatures ≥20°C. We found that even at 42°C, the *V. cholerae rpoH* mutant could form colonies on plates that repress expression of *rpoH*. Even though glucose is a very potent repressor of the P_{BAD} promoter (17) that drives *rpoH* expression in the *rpoH* deletion mutant, it is possible that there is sufficient *rpoH* expression under these conditions to allow colony formation. Interestingly, early studies characterizing unsuppressed *E. coli rpoH* nonsense mutations led to the idea that *rpoH* is dispensable for growth at 30°C (50). After *E. coli rpoH* deletion and insertion mutants were constructed, this idea was found to be incorrect; the viability of the *E. coli rpoH* amber mutants at 30°C apparently depended on small amounts of σ^{32} in these cells (55). We also showed that, even in the absence of selection, no loss of an *rpoH*-bearing plasmid from the *rpoH* mutant was observed at temperatures ranging from 15°C to 42°C. In contrast, this plasmid was readily lost from the wild-type strain under the same assay conditions. These results suggest either that the necessity for RpoH influences plasmid segregation or that the cells that lose the RpoH-bearing plasmid do not survive under these conditions. The latter statement implies that RpoH is essential for *V. cholerae* survival at all temperatures.

Unexpectedly, we found that *rpoH* is essential for *V. cholerae* viability in the presence of kanamycin at 30°C or above. Similarly, the permissive growth temperature of *E. coli rpoH* mutants was also found to be reduced in the presence of kanamycin (55). The mechanistic basis for the reduction in viability of *V. cholerae* and *E. coli rpoH* mutants in the presence of this aminoglycoside is not known. Apparently, *rpoH* mutants cannot overcome the combined effects of absent or very small amounts of σ^{32} along with the toxicity of kanamycin. It is also possible that the activity of the neomycin phosphotransferase, the protein that mediates kanamycin resistance, depends on a σ^{32} -regulated gene.

As a facultative pathogen, *V. cholerae* can grow independently of human hosts in a variety of aquatic systems. While the temperature, pH, oxygen concentration, nutrient availability, and other environmental parameters in the intestinal niche occupied by *V. cholerae* are not known with precision, clearly these parameters do not replicate those found in rich media. In competition experiments between the *rpoH* mutant and the wild-type strain, the competitive advantage of the wild type over the *rpoH* deletion mutant was far more profound in the suckling mouse intestine than in LB. Thus, σ^{32}-regulated gene products appear to be more critical for *V. cholerae* growth within the intestine than in rich media. Since the inocula for the in vivo and in vitro competition experiments were both shifted from 22°C to 37°C, the requirement for σ^{32}-regulated genes in the heat shock response per se cannot explain the ~150-fold difference in the competitive indexes we found in the in vivo and in vitro competition experiments (0.001 versus

0.15, respectively). Instead, σ^{32}-regulated genes appear to be required for *V. cholerae* to adapt to non-temperature-related stresses encountered within the intestine.

Several σ^{32}-dependent processes could contribute to *V. cholerae* intestinal colonization. As in *Salmonella* (2), we found that σ^{32} promotes *V. cholerae* resistance to H_2O_2 (data not shown) and the intestine could provoke oxidative stress. Interestingly, we found that *gshB* (encoding glutathione synthetase), a gene that likely contributes to resistance to oxidative stress, is part of the σ^{32} regulon. In a screen for *V. cholerae* intestinal colonization factors, Merrell et al. reported that a transposon insertion in *gshB* profoundly reduced *V. cholerae* intestinal colonization (28). It has also been proposed that the intestine is relatively nutrient poor (28), and some of the σ^{32}-regulated genes listed in Table 2 may promote growth under nutrient-limiting conditions. Adherence of *V. cholerae* to the intestinal epithelium may promote σ^{32} activation, as has been observed following adherence of *Neisseria gonorrhoeae* to host epithelial cells (11). Adherence could stimulate changes in the cell membrane that activate σ^{E}, which appears to be a regulator of *rpoH* in *V. cholerae*. Recently, Nonaka et al. proposed that in *E. coli*, σ^{32}-regulated genes are important for maintaining membrane integrity (33). Perhaps one or more of the 18 genes of unknown function in the *V. cholerae* σ^{32} regulon contribute to membrane integrity in vivo.

Although we have not formally explored the mechanisms that control σ^{32} activity in *V. cholerae*, our observations suggest that this member of the γ-*Proteobacteria* has mechanisms for controlling the activity of this sigma factor that are similar, though not identical, to those in *E. coli*. In *V. cholerae*, as in *E. coli*, both σ^{70}- and σ^{24}-dependent promoters control *rpoH* transcription. In *E. coli*, regulatory proteins, including DnaA, CRP, and CytR (12, 22, 31, 48), are thought to influence *rpoH* transcription, but in *V. cholerae*, binding sites for the latter two proteins were not apparent in the region of the *rpoH* promoters, suggesting that different inducing stimuli may influence *rpoH* expression in *V. cholerae* and *E. coli*. In *E. coli*, σ^{32}-regulated genes control σ^{32} activity and stability. The *V. cholerae* σ^{32} regulon includes the chaperones that regulate σ^{32} activity in *E. coli* but does not include *ftsH*, the gene encoding the principal σ^{32}-degrading protease in *E. coli*. This is also the case in *N. gonorrhoeae*, where *ftsH* is not part of the σ^{32} regulon (16). Finally, in *V. cholerae* we found that the kinetics of RpoH production following heat shock are somewhat delayed compared to *E. coli* (data not shown).

We used microarrays to define the *V. cholerae* σ^{32} regulon by comparing the levels of transcripts of all annotated ORFs in the *rpoH* deletion mutant with those in the wild-type strain after a temperature up-shift. Recently, several studies used either genome-wide transcription profiling (33, 54) or chromatin immunoprecipitation coupled with microarrays (47) to define the *E. coli* σ^{32} regulon. In the former case, σ^{32}-regulated genes were identified as transcripts up-regulated after induction of σ^{32}, whereas Wade et al. (47) used heat shock to identify σ^{32} promoters. Even though different protocols were used to define the respective σ^{32} regulons, the *V. cholerae* and *E. coli* σ^{32} regulons are similar. Even the number of σ^{32}-regulated genes in the two species is similar; 20 min after the temperature up-shift we found that 49 genes were up-regulated in a σ^{32}-dependent fashion, while in *E. coli* 51 genes were up-regulated 15 min after induction of *rpoH* (54). We did not carry out a time course analysis of the *V. cholerae* σ^{32} regulon as was done with *E. coli*. Thus, it is possible that several genes, such as *clpX* and *ftsH*, that were not identified in the *V. cholerae* σ^{32} regulon but that are σ^{32}-regulated in *E. coli* would have been found if we had examined earlier time points. On the other hand, several *V. cholerae* σ^{32}-regulated genes, such as *gsbH*, are not found in the *E. coli* σ^{32} regulon. Finally, it will be most interesting to pursue the characterization of the genes coding for proteins of unknown function that belong to the RpoH regulon. We already identified VC2735 and VC2736 as proteins having the features of Hsp15 and Hsp33, respectively.

We used the list of σ^{32}-regulated genes derived from the microarray experiments and several bioinformatic algorithms to generate a putative *V. cholerae* σ^{32} binding site consensus sequence: cTTGAA(N_{13-16})(a/c)CCATat(a/t), where the uppercase letters indicate the most-conserved positions. Recently, several array-based approaches have been taken to define the *E. coli* σ^{32} regulon. All three of these studies use the array data to define potential σ^{32} consensus binding sites (33, 47, 54). These three consensus sequences are fairly similar to each other and to the putative *V. cholerae* σ^{32} consensus binding site identified here. This consensus sequence appears to be very close to that proposed by Wade et al. (47). The similarity of the *V. cholerae* and *E. coli* binding sites is not unexpected, since the *V. cholerae* and *E. coli* σ^{32} sequences are 70% identical. Furthermore, σ^{32} regions 2.4 and 4.2, which are involved in contacting the −35 and −10 motifs, respectively (4), are very conserved between the two sigma factors (100% and 77% identical, respectively). In *V. cholerae*, the −35 motif in the *V. cholerae* σ^{32} binding site appears to be more conserved than the −10 motif; this appears to be the case for the *E. coli* σ^{32} consensus sequence defined by chromatin immunoprecipitation coupled with microarrays, as used by Wade et al. (47). Given the overall similarity of the *V. cholerae* and *E. coli* σ^{32} binding sites, differences in the σ^{32} regulons between these related γ-*Proteobacteria* likely reflect gain or loss of σ^{32} binding sites in gene promoters during evolution.

While microarray analysis provides a powerful way to define the RpoH regulon, this approach can be complemented by a bioinformatics approach. To gather more information about the RpoH regulon, we used the consensus sequence we defined to search for potential RpoH promoters upstream of all *V. cholerae* genes and to identify RpoH-regulated genes that may have escaped detection because of the experimental conditions used in the microarray analysis.

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