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The Type II Secretion Pathway in *Vibrio cholerae* Is Characterized by Growth Phase-Dependent Expression of Exoprotein Genes and Is Positively Regulated by σ^E

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Vibrio cholerae, an etiological agent of cholera, circulates between aquatic reservoirs and the human gastrointestinal tract. The type II secretion (T2S) system plays a pivotal role in both stages of the lifestyle by exporting multiple proteins, including cholera toxin. Here, we studied the kinetics of expression of genes encoding the T2S system and its cargo proteins. We have found that under laboratory growth conditions, the T2S complex was continuously expressed throughout *V. cholerae* growth, whereas there was growth phase-dependent transcriptional activity of genes encoding different cargo proteins. Moreover, exposure of *V. cholerae* to different environmental cues encountered by the bacterium in its life cycle induced transcriptional expression of T2S. Subsequent screening of a *V. cholerae* genomic library suggested that σ^E stress response, phosphate metabolism, and the second messenger 3′,5′-cyclic diguanylic acid (c-di-GMP) are involved in regulating transcriptional expression of T2S. Focusing on σ^E, we discovered that the upstream region of the T2S operon possesses both the consensus σ^E and σ^70 signatures, and deletion of the σ^E binding sequence prevented transcriptional activation of T2S by RpoE. Ectopic overexpression of σ^E stimulated transcription of T2S in wild-type and isogenic ΔrpoE strains of *V. cholerae*, providing additional support for the idea that the T2S complex belongs to the σ^E regulon. Together, our results suggest that the T2S pathway is characterized by the growth phase-dependent expression of genes encoding cargo proteins and requires a multifactorial regulatory network to ensure appropriate kinetics of the secretory traffic and the fitness of *V. cholerae* in different ecological niches.

The type II secretion (T2S) system is a sophisticated machine utilized by many Gram-negative bacteria to transport proteins that contribute to their virulence and/or survival in environmental habitats (1–4). Depending on the bacterial species, the T2S complex consists of 12 to 15 different proteins that, via multiple protein-protein interactions, assemble a secretion apparatus that bridges the cytoplasmic membrane with the outer membrane (5). This secretory pathway is responsible for the passage of folded and/or oligomeric cargo proteins (exoproteins and T2S substrates) from the periplasm across the outer membrane. After translocation, the T2S substrates are either released to the extracellular milieu or remain anchored to the bacterial cell surface (6). The array of T2S exoproteins is comprised of toxins, enzymes capable of breaking down a variety of biological materials, and lipoproteins participating in respiration and/or biofilm formation, as well as proteins facilitating bacterial attachment to biotic and abiotic surfaces (2–4, 6).

The organization and functions of the individual constituents of the T2S complex have been studied in several model organisms, including *Vibrio cholerae*, the etiological agent of the epidemic diarrheal disease cholera. In *V. cholerae*, the components of the T2S system are encoded by two divergent operons, *epsCDEFGHIJKLMN* and *epsAB* (where *eps* stands for extracellular protein secretion), as well as the *vcpD* (*pilD*) gene (7). The model of the T2S machine includes an outer membrane protein, the secretin EpsD; inner membrane platform proteins (EpsC, -F, -L, and -M); a major pseudopilin, EpsG; the minor pseudopilins EpsH through EpsK; a cytoplasmic T2S ATPase, EpsE; and a prepilin peptidase, PilD, which is shared with the type IV secretion system (5). The secretion of the *V. cholerae* principal virulence factor, cholera toxin, is dependent upon a functional T2S system (8). Cholera toxin is produced and secreted by toxigenic strains of *V. cholerae* after successful colonization of the human small intestine and is largely responsible for the voluminous diarrhea, the hallmark symptom of cholera (9). The genes encoding cholera toxin, *ctxAB*, are not an integral component of the *V. cholerae* genome but are carried by the lysogenic filamentous bacteriophage CTXphi, which specifically infects *V. cholerae* (10). Hence, the environmental nonpathogenic strains of *V. cholerae* can be converted into toxigenic strains (11). Intriguingly, the exit of CTXphi from its host depends upon functional EpsD, making the T2S system an important contributor to the horizontal transfer of the key virulence gene (12, 13). The importance of T2S in the life cycle of *V. cholerae* extends beyond the transport of cholera toxin and release of CTXphi, because at least 20 cargo proteins are translocated to the extracellular milieu via this secretory pathway (2, 14). The functions of only a small subset of these additional T2S exopro-

TABLE 1 Strains and plasmids used in this study

Strain/plasmid	Description/relevant genotype[a]	Reference/source
V. cholerae strains		
O395	Wild-type classical O1 biotype; Sm[r]	Laboratory collection
O395Δ*rpoE*	O395 Δ*rpoE* Sm[r]	37
N16961	Wild-type El Tor O1 biotype; Sm[r]	Laboratory collection
C6706	Wild-type El Tor O1 biotype; Sm[r]	Laboratory collection
C6706Δ*hapR*	C6706 Δ*hapR* Kan[r]	This study
E. coli strains		
MC1061	F⁻ *araD139* Δ(*ara-leu*)7697 Δ(*lac*)X74 *rpsL hsdR2 mcrA mcrB1*	49
MM294(pRK2013)	Donor of transfer function for triparental conjugation	50
NEB5α	*fhuA2* Δ(*argF-lacZ*)U169 *phoA glnV44* Φ80 Δ(*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
Plasmids		
pMMB67-EH	Expression vector; P_tac promoter; Amp[r]	51
pBBR*lux*	*ori p*BBR1; promoterless *luxCDABE*; Cm[r]	20
pACYC177	*ori p15A* Amp[r] Kan[r]	52
pAES106	*rpoEP2* cloned into pBBR*lux*; Cm[r]	17
pKD4	*ori* R6Kγ; Amp[r] FRT Kan[r] FRT	22
pNEB193	Cloning vector pUC19 derivative	NEB
pPCR-Script Amp SK(+)	Cloning vector; Amp[r]	Stratagene
pCVD442	*ori* R6k *mob* RP4 *sacB* Amp[r]	23
p*eps-lux*	*epsP1-P2* cloned into pBBR*lux*; Cm[r]	This study
p*eps70-lux*	*epsP1* cloned into pBBR*lux*; Cm[r]	This study
p*vesA-lux*	*vesAP* cloned into pBBR*lux*; Cm[r]	This study
p*vesB-lux*	*vesBP* cloned into pBBR*lux*; Cm[r]	This study
p*vesC-lux*	*vesCP* cloned into pBBR*lux*; Cm[r]	This study
p*tarp-lux*	*tarpP* cloned into pBBR*lux*; Cm[r]	This study
pRpoE	pMMB67-EH carrying P_tac-*rpoE*^N16961	This study
pHapR	pMMB67-EH carrying P_tac-*hapR*^C6706	This study
pNEBΔ*hapR*	pNEB193 carrying 2,636-bp DNA fragment containing Kan[r] cassette flanked by 553 bp upstream and downstream of *hapR*	This study
pCVDΔ*hapR*	pCVD442 containing upstream region of *hapR*, Kan[r], and downstream of *hapR*	This study

[a] Sm[r], streptomycin resistant; Amp[r], ampicillin resistant; Cm[r], chloramphenicol resistant.

teins have been addressed, including sialidase (neuraminidase), hemagglutinin protease (HapA), chitinases, and a chitin-binding protein (GbpA). These proteins contribute to disease pathogenesis and/or to the persistence of *V. cholerae* in natural aquatic habitats during interepidemic periods (2).

Cholera remains one of the top global infectious disease threats (15), and therefore, understanding the basic mechanisms of *V. cholerae* virulence and environmental survival is a prerequisite for the development of new therapeutic treatments or preventive measures. Previous studies demonstrated that genetic manipulations leading to removal of either the entire *epsC-epsN* (*epsC-N*) operon or inactivation of the individual *eps* genes or *pilD* led to dramatic consequences for *V. cholerae* pathophysiology (16–18). In addition to loss of cargo protein secretion and virulence, the T2S mutants displayed growth defects, perturbed cell envelope integrity, altered iron homeostasis, and induction of the extracytoplasmic RpoE stress response. These pleiotropic effects further underscore the vital role of T2S in *V. cholerae* biology, suggesting the T2S pathway as a molecular target for therapeutic and preventive interventions against cholera. While many studies have focused on understanding the architecture and functions of the T2S components, significantly less is known about the regulation of biogenesis of the T2S complex, the recognition of exoproteins, and the secretory traffic. Until they have been defined, opportunities to target T2S therapeutically are likely to remain limited.

Thus, in this study, we began to characterize the transcriptional regulation of T2S and the patterns of cargo gene expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *V. cholerae* strains were derived from either El Tor (N16961 and C6706) or the classical O395 biotype. Bacteria were streaked from −80°C on Luria-Bertani (LB) agar supplemented with appropriate antibiotics. Antibiotics were used in the solid and liquid media in the following concentrations: chloramphenicol (30 μg/ml or 4 μg/ml for *Escherichia coli* or *V. cholerae*, respectively), carbenicillin (50 μg/ml), and kanamycin (50 μg/ml). All *E. coli* strains were cultured in LB medium at 37°C. *V. cholerae* was propagated in LB medium at 37°C or 25°C, as well as the conditions used for either maximal induction or repression of virulence gene expression (19) as specified in the text. All media utilized in this study were purchased from Difco.

DNA manipulations. To construct transcriptional fusion reporters, DNA fragments of 173, 440, 446, 440, and 499 bp containing promoter regions of the *epsC-N* operon, *vesA*, *vesB*, *vesC*, and VCA0148 encoding a *TagA-r*elated *p*rotein (Tarp), respectively, were amplified using the primers listed in Table 2. The truncated version of the *eps* promoter (*eps70-lux*), which lacked the σ^E signature sequence, was constructed by utilization of the primer pair eps70-F and eps70-R (Table 2). The *V. cholerae* N16961 genome sequence was used as a template to design primers, and a purified chromosomal DNA (Promega Wizard Genomic DNA Purification Kit) from either *V. cholerae* N16961 or C6706, as indicated in the text, was used in PCRs. All reactions were performed using Q5 High-Fidelity

TABLE 2 Oligonucleotide primers designed and used in this study

Purpose	Name	Sequence (5′ to 3′)[a]
Promoter fusion construction	eps-F	<u>GGATCC</u>CATCGCAATATAACACAAGC
	eps-R	<u>ACTAGT</u>CAGTCTCACCTCTTCTGCGC
	eps70-F	GATATC<u>ACTAGT</u>AAGCTTATTACATTTGACTATTATTCA
	eps70-R	GATATC<u>GGATCC</u>GGCATCGCAATATAACACAAGC
	vesA-F	CTCGAG<u>ACTAGT</u>ATTCGCCAGAAAGAAGTGGG
	vesA-R	CTCGAG<u>GGATCC</u>GCGTCACCTCATTGGTTGA
	vesB-F	CTCGAG<u>ACTAGT</u>TGTATCTCGGCTTACTGCT
	vesB-R	CTCGAG<u>GGATCC</u>AACTCAATCCTTTCATTAATCA
	vesC-F	CTCGAG<u>ACTAGT</u>AGATACCTCTGATAACTCCC
	vesC-R	CTCGAG<u>GGATCC</u>GGCTGTAAATCTTGTATCTC
	tarp-F	CTCGAG<u>ACTAGT</u>AATGCCGTACAAGGTTTT
	tarp-R	CTCGAG<u>GGATCC</u>CAGCAAATAGTTACACAATAATCA
Cloning wild-type alleles	rpoE-F	ATGCAT<u>CCCGGG</u>AGGGGAATAACAATAGGAGTGA
	rpoE-R	ATGCAT<u>TCTAGA</u>TCATTACGGAATTTGCGTTA
	hapR-F	ATGCAT<u>GAATTC</u>CAACTCAATTGGCAAGGATA
	hapR-R	ATGCAT<u>TCTAGA</u>GCTGCCCAAGAAACTAGTTC
Creating ΔhapR knockout	pNEB193-F	CTCTAGACTTAATTAAGGATCC
	pNEB193-R	GACTCTAGACTTAATTAAGGATCC
	hapR-U-F	CCTTAATTAAGTCTAGAGTCTTCCACCAGCAATACATC
	hapR-U-R	CAGCCTACACTTTGCTCTAATGATTATTTTGTTATTTG
	kan-F	TTAGAGCAAAGTGTAGGCTGGAGCTGCT
	kan-R	GCCCAAGAAATGGGAATTAGCCATGGTCC
	hapR-D-F	TAATTCCCATTTTCTTGGGCAGCACAAG
	hapR-D-R	GCCTGCAGGTTTAAACAGTCGTCACTGCAAGATATGTTAGATG
	hapR-F-Sal	CGAA<u>GTCGAC</u>TTCCACCAGCAATACATCTTTAC
	hapR-R-Sca	CGAA<u>GAGCTC</u>GTCACTGCAAGATATGTTAGATG
Sequencing genomic library	pACYC-F	CCGAAGAACGTTTTCCAATG
	pACYC-R	GAGTACTCAACCAAGTCATTCTGA

[a] F, forward; R, reverse; U, upstream; D, downstream. The underlined nucleotides refer to restriction enzyme sites.

DNA Polymerase (New England BioLabs), with oligonucleotides synthesized by Integrated DNA Technologies. The PCR products obtained were first cloned into the pPCR-Script Amp SK(+) cloning vector (Stratagene). Subsequently, the individual DNA fragments containing promoters of the respective genes were inserted into SpeI-BamHI-digested pBBR-*lux* in front of a promoterless *luxCDABE* reporter (20).

Construction of plasmids carrying either the *rpoE* or *hapR* gene placed under the control of the P_{TAC} promoter was accomplished as follows. First, *rpoE* and *hapR* coding sequences were amplified using chromosomal DNA isolated from *V. cholerae* N16961 and C6706, respectively, as templates, and oligonucleotides listed in Table 2. The resulting PCR products of *rpoE* and *hapR* were cloned into the corresponding XmaI-XbaI and EcoRI-XbaI sites within a broad-host-range expression vector, pMMB67-EH (Table 2). The expression of both *rpoE* and *hapR* was induced with 100 μM isopropyl-β-D-1-thiogalactopyranoside (IPTG).

To create an in-frame deletion of the *hapR* gene in *V. cholerae* C6706, first, the pNEBΔ*hapR* plasmid was generated using the assembly method of Gibson et al. (21). The 500-bp regions upstream and downstream of the *hapR* gene were amplified by PCR using the *V. cholerae* C6706 genome as a template and the oligonucleotide pairs hapR-U-F/hapR-U-R and hapR-D-F/hapR-D-R, respectively (Table 2). The kanamycin resistance gene was amplified from plasmid pKD4 (22) using primers kan-F and kan-R. Plasmid pNEB193 (NEB) was linearized in a PCR using primers pNEB193-F and pNEB193-R. Subsequently, 100 ng of linearized vector was mixed with a 3-fold excess of inserts and 10 μl of Gibson Assembly Master Mix (NEB), followed by incubation at 55°C for 1 h. Chemically competent *E. coli* NEB5α (Table 1) cells were transformed with 5 μl of the assembled product. The construct obtained was amplified using primers hapR-F-Sal and hapR-R-Sca, digested with restriction enzymes SalI and

ScaI, and cloned into a similarly cleaved suicide vector, pCVD442 (23). The resulting plasmid, pCVDΔ*hapR*, was introduced into *V. cholerae* C6706 by conjugation, and in-frame deletion of the *hapR* gene was achieved by allelic-exchange procedures according to the methods described by Sikora et al. (17).

All the constructs obtained were sequenced at the Center for Genome Research and Biocomputing at Oregon State University. Plasmids were introduced into *V. cholerae* by triparental conjugation using as a donor *E. coli* MC1061 carrying a particular plasmid and a helper strain, MM294, as previously described (17). After conjugation, *V. cholerae* strains were selected by plating bacterial mixtures on thiosulfate-citrate-bile salts-sucrose agar supplemented with appropriate antibiotics.

Assessment of transcriptional expression using promoter-lux reporter fusions. To examine the kinetics of the transcriptional activity of promoter-*luxCDABE* (*lux*) fusions, bacterial strains at the stationary phase of growth (optical density at 600 nm [OD_{600}], about 4) were diluted 1:100 in appropriate fresh culture media, as specified in the text, and delivered in technical triplicates into 96-well, white, clear-bottom microtiter plates (Greiner-BioOne). Technical triplicates of the respective culture media (controls for both OD_{600} and contamination) and the culture of *V. cholerae* carrying the pBBR-*lux* vector (background luminescence control) were included. The total volume in each well on the microtiter plate was 200 μl. The individual microtiter plates were incubated in a Synergy HT Multi-Mode Microplate reader (BioTek) with moderate shaking for 16 h. The turbidity of bacterial cultures (OD_{600}) and the relative luminescence units (RLU) were recorded every 20 min, and the data are presented as means with calculated standard errors of the mean (SEM) obtained from experiments performed in at least biological triplicates.

The relative fold change was used to compare the expression of indi-

vidual promoter-*lux* fusions in classical *V. cholerae* during growth under conditions that result in either maximal induction (LB, pH 6.5; 30°C) or repression (LB, pH 8.5; 37°C) of virulence gene expression (19). The highest activity of a respective promoter-*lux* fusion (maximum RLU) detected during bacterial growth under each condition was normalized by the optical density of the culture (17). Subsequently, the relative fold change was calculated by dividing the normalized promoter-*lux* activity under virulence gene-inducing conditions by its corresponding activity under virulence gene-inhibiting conditions. All experiments were performed independently at least four times, and means with calculated SEM are reported.

To examine the effects of various exogenous factors on transcriptional expression of either *eps-lux* or *rpoEP2-lux*, assays were performed as previously described (17, 18). Briefly, *V. cholerae* strains carrying particular transcriptional fusions were cultured with aeration in LB medium at 37°C until the mid-logarithmic phase of growth ($OD_{600} = 0.5$). Then, equal amounts of the cultures were divided into individual test tubes containing the examined compound, including polymyxin B sulfate (100 or 50 U per ml for N16961 or O395, respectively), the iron chelator 2-(pyridin-2-yl)pyridine (2,2′-dipyridyl at 100 μM final concentration), dimethyl sulfoxide (DMSO) (vehicle), or bile salts (0.1% final concentration). The RLU and turbidities of untreated, treated, and mock-treated bacterial cultures were examined at 30 min postexposure. The reported values of promoter-*lux* activities are expressed as RLU normalized by the turbidity of the culture per 1.0 ml (17). Experiments were conducted on five independent occasions, and mean values with associated SEM are reported.

The effect of overproduction of RpoE on the transcriptional activity of *eps* was assessed in *V. cholerae* strains carrying either pRpoE and p*eps-lux* or pRpoE and p*eps70-lux*. The control strains harbored empty pMMB67-EH vector and either p*eps-lux* or p*eps70-lux* (Table 1). In all experiments, overnight cultures of *V. cholerae* carrying appropriate plasmids, as specified in the text, were inoculated in a 1:100 ratio into fresh medium supplemented with antibiotics. The expression of *rpoE* was induced with 100 μM IPTG when the cultures reached the mid-logarithmic phase of growth ($OD_{600} = 0.5$). To correct for any potential effect of IPTG on transcriptional activity, the inducer was also added to control strains of *V. cholerae*. Both bioluminescence and bacterial growth were assessed at 30 min from induction as described above. Likewise, the effect of overproduction of HapR on p*eps-lux* activity was examined in both N16991 and O395. In addition, the function of HapR in regulation of *eps* expression was assessed in C6706 wild-type and isogenic ΔhapR knockout strains using the experimental design outlined above, as well as by monitoring the kinetics of *eps-lux* activity throughout 16 h of bacterial growth in LB medium at 37°C in a microtiter plate format, as explained previously. All data were collected during at least five independent experiments, and the reported values represent the means with accompanying SEM.

Design of a genetic screen and screening for *eps* regulators. The *V. cholerae* O395 chromosomal DNA was purified using the Wizard DNA kit (Promega). Chromosomal DNA (25 μg) was partially digested with HincII and separated on a 1% agarose gel, and 2- to 7-kb DNA fragments were excised from the gel, purified, and cloned into HincII-digested pACYC177 (Table 1). The *E. coli* MC1061 transformants carrying the library plasmids and p*eps-lux* were spread on LB agar supplemented with kanamycin and chloramphenicol. Subsequently, all the colonies were screened for loss of resistance to a β-lactam antibiotic, carbenicillin, as successful cloning into the HincII site resulted in disruption of the *bla* gene carried within pACYC177. Individual bacterial colonies that failed to grow on LB agar plates containing carbenicillin were inoculated into each well on 96-well, white, clear-bottom plates (Greiner BioOne) containing 200 μl of LB medium supplemented with kanamycin and chloramphenicol. The *eps-lux* activities (in RLU) and turbidities (OD_{600}) of the cultures were measured after 16 h of static incubation at 37°C using a microplate reader as described above. The bioluminescence was normalized by bacterial growth (RLU/OD_{600}). Clones showing significant changes in biolu-

minescence in comparison to the control strain, *E. coli* carrying both p*eps-lux* and pACYC177, were reassayed. Plasmid DNA was isolated from bacterial cultures that displayed 6-fold or higher induction in the measured luminescence compared to the control strain. The inserted fragments of *V. cholerae* chromosomal DNA were sequenced using primers listed in Table 2. The sequences obtained were searched against the *V. cholerae* O395 genome in the KEGG database using BLASTn.

SDS-PAGE and immunoblotting. The *V. cholerae* strains were grown as described in the text, and samples were withdrawn at the indicated time points of the experiments and centrifuged at $4,000 \times g$ for 5 min to pellet bacterial cells. Subsequently, the cells were suspended in 1× LDS loading buffer (Invitrogen) supplemented with dithiothreitol (50 mM). Samples of whole-cell lysates were matched by equivalent OD_{600} units and resolved in 4 to 20% Tris-glycine gradient gels (Bio-Rad). Proteins were transferred onto 0.2 μM polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using Trans-blot Turbo (Bio-Rad). The membranes were blocked in 5% milk in phosphate-buffered saline (PBS) (pH 7.0; Li-Core) supplemented with 0.1% Tween 20 and probed with polyclonal anti-EpsE antiserum (1:10,000 dilution) followed by incubation with Immun-Star goat anti-rabbit (GAR)-horseradish peroxidase (HRP) conjugate (Bio-Rad; 1:10,000 dilution). The immunoblots were developed using Clarity Western ECL Substrate (Bio-Rad) on a Chemi-Doc MP System (Bio-Rad).

Statistical analyses. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software) and R version 3.0.2 base distribution. An unpaired Student's *t* test was used to analyze the data. Detrended (residual) correlations between the mean *eps-lux* activity levels for each of the experiments described above and shown in Fig. 1 were computed by first obtaining the overall mean *eps-lux* activity time series, averaging over all replicates across all experiments. Then, the detrended mean *eps-lux* time series were obtained by subtracting the overall mean time series from the mean time series for each experiment. Finally, Pearson's product-moment correlation coefficient was used to assess the similarity in the residual time series between different experiments. A correlation coefficient near 1 indicates that the two residual time series exhibit almost exactly the same shape, whereas negative correlation coefficients result when one residual series tends to be high when the other is low, indicating different activity profiles.

RESULTS

Kinetics of expression of the T2S genes. In *V. cholerae*, replacement of the 173 bp located upstream of the first gene in the *epsC-N* operon with the arabinose-inducible promoter P_{BAD} allows the controlled biogenesis of the T2S complex, indicating that this chromosomal fragment contains the region driving expression of T2S (17, 24). To examine the expression of the T2S genes, we generated a transcriptional fusion, *eps-lux*, by placing this fragment of DNA in front of the promoterless *luxCDABE* operon located on a low-copy-number plasmid, pBBR322 (20). We chose this transcriptional reporter system because it permits uninterrupted monitoring of gene expression throughout bacterial growth if assays are executed in microtiter plates incubated in a plate reader. We reasoned that transcriptional expression of the T2S could be uniquely regulated in classical and El Tor biotypes of *V. cholerae* due to differences in the regulation of virulence factor production and transcriptome profiles (25, 26). Therefore, the plasmid carrying *eps-lux* was introduced by conjugation into classical (O395) and El Tor (N16961 and C6706) strains of *V. cholerae*. Subsequently, the kinetics of *eps-lux* expression (in RLU) and the turbidity of the cultures (OD_{600}) were monitored continually every 20 min during 16 h of culture in LB medium at the temperatures encountered by the bacteria in the human host (37°C) and in the environment (25°C).

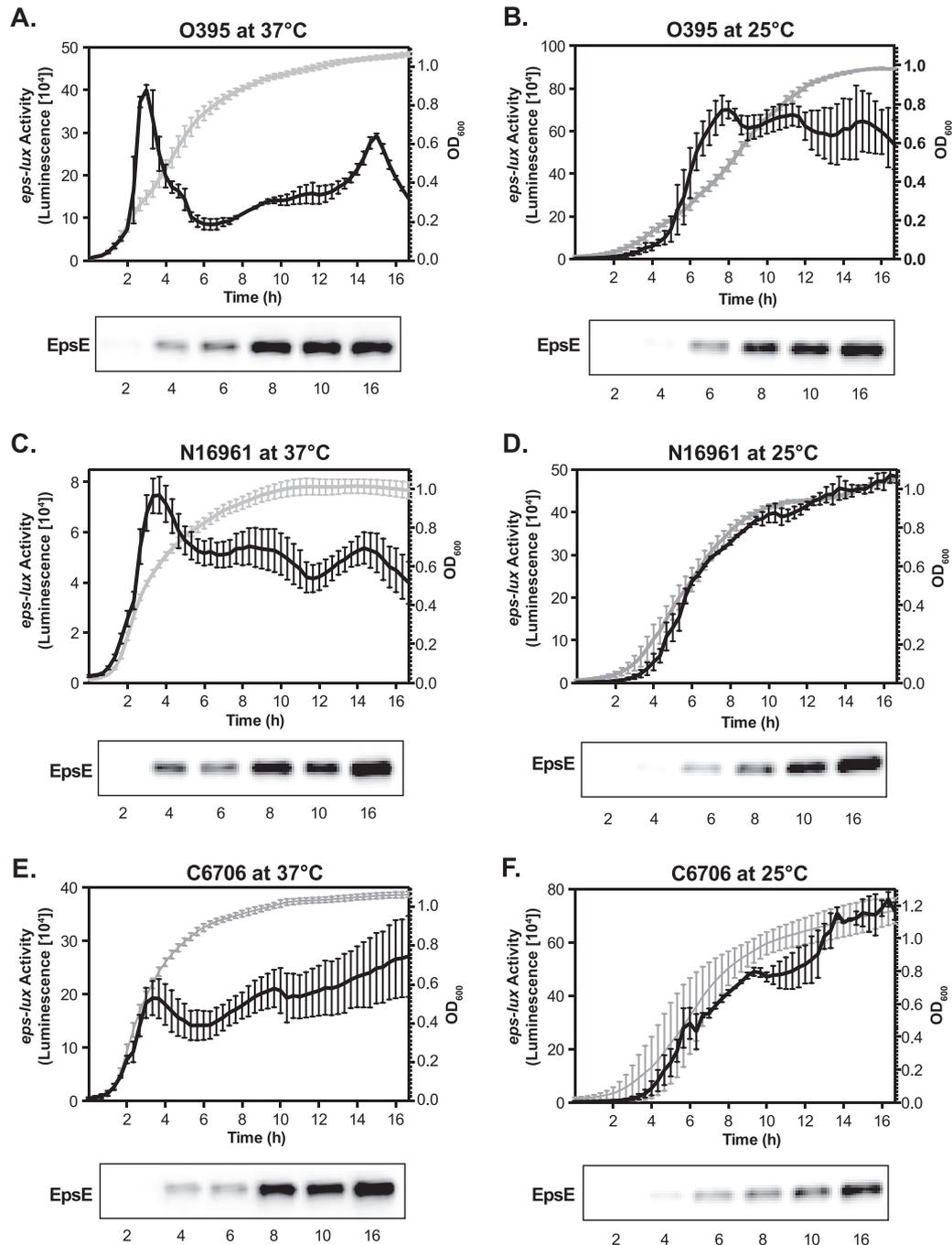In all *V. cholerae* strains grown at 37°C, the *eps-lux* activity

FIG 1 Kinetics of *eps* expression in *V. cholerae* classical (O395) and El Tor (N16961 and C6706) strains. Expression of the *eps-lux* transcriptional fusion was monitored in *V. cholerae* O395 (A and B), N16961 (C and D), and C6706 (E and F). The bacterial strains were cultured in LB medium at either 37°C (A, C, and E) or 25°C (B, D, and F) in 96-well, white, clear-bottom microtiter plates. The turbidity of the cultures at OD_{600} (gray) and *eps-lux* activity (luminescence) (black) were assessed in technical triplicates and recorded every 20 min for 16 h. Each datum point represents the mean and SEM obtained from at least three independent experiments. The levels of the T2S component EpsE were detected in whole-cell extracts of *V. cholerae* strains by immunoblotting analyses with antisera against EpsE. Bacterial cells were withdrawn at 2, 4, 6, 8, 10, and 16 h of growth, as indicated. Samples were matched by equivalent OD_{600} units.

peaked in the mid-logarithmic phase of growth (at an OD_{600} of 0.5) and remained continuously expressed (Fig. 1A, C, and E). The constitutive transcriptional expression of T2S was also observed during growth under conditions either inducing or repressing virulence gene expression and in media triggering catabolite repression (LB broth supplemented with 0.4% glucose, maltose, or su-

crose) (data not shown). Interestingly, during the cultivation of *V. cholerae* at the environmental temperature in either LB medium or marine broth, the transcriptional activity of *eps-lux* seemed to follow bacterial growth, which was particularly apparent in both El Tor strains (Fig. 1B, D, and F and data not shown). As expected, the proliferation rates of all bacterial strains were lower at 25°C

than at 37°C, and mid-exponential growth stage was reached after about 6 and 8 h from back-dilution for the El Tor and classical isolates, respectively (Fig. 1). In addition, the lower temperature had a stimulatory effect on *eps-lux* expression, and the maximal values of detected transcriptional activity in O395, N16961, and C6706 were 1.75-, 6.88-, and 2.77-fold higher, respectively, than those measured at 37°C (Fig. 1). Immunoblotting analyses using antisera against the T2S ATPase EpsE showed that the overall transcriptional expression pattern corresponded to the expression of T2S at the protein level, although the detected amounts of EpsE were not increased at 25°C in comparison to those detected at 37°C. This discrepancy could be caused by the posttranscriptional regulation of gene expression or could be due to different protein stabilities.

Next, we applied a series of statistical analyses to thoroughly compare the obtained *eps-lux* expression profiles shown in Fig. 1. The relationship between the *eps-lux* activity and bacterial growth was evaluated for each series using the Pearson correlation coefficient. Corroborating our earlier conclusions, the highest correlation was observed during the cultivation of *V. cholerae* at 25°C (see Fig. S1A in the supplemental material). This was especially clear in both El Tor strains, where the correlation values were 0.996 and 0.984 for N16961 and C6706, respectively. The lowest correlation between the *eps-lux* expression kinetics and bacterial growth (0.303) was observed in the classical strain at 37°C (see Fig. S1A in the supplemental material). To directly compare *eps-lux* expression from one *V. cholerae* strain-temperature combination to that from another, a correlation matrix for the six different mean *eps-lux* time series was computed, and a hierarchical-clustering dendrogram was constructed (see Fig. S1B and C, respectively, in the supplemental material). These analyses showed that in all *V. cholerae* isolates, *eps-lux* activities at 37°C grouped together, and the activities measured at 25°C clustered together (see Fig. S1C in the supplemental material). Subsequently, the overall trend in *eps-lux* activity was computed as the overall mean time series and then detrended by subtracting the estimated trend series (Fig. 2 A). To assess the similarity in the residual time series of *eps-lux* expression in all *V. cholerae* strains and at all temperatures, Pearson's product-moment correlation coefficients were calculated. The detrended correlations between the time series in the different experiments provided strong evidence of similar patterns of *eps-lux* expression between classical and El Tor *V. cholerae* biotypes under common temperature conditions (Fig. 2B). Finally, we considered how the timing of the peak *eps-lux* luminescence differs between the strain-temperature experiments (see Fig. S2 in the supplemental material). The time series of mean *eps-lux* luminescence was plotted in black, when there was a statistically significant difference (at an α level of 0.05) between the mean *eps-lux* luminescence at that time point and the peak mean, and it was plotted in red when there was no statistically significant difference between the mean *eps-lux* luminescence and the peak mean. This analysis showed that at 37°C, the peak *eps-lux* luminescence occurs around minute 200 in all *V. cholerae* strains (see Fig. S2A, C, and E in the supplemental material). There were, however, some differences among the three panels in that there was a sharp decline in the O395 strain at 37°C, whereas in the El Tor strains, the time series effectively plateau. In addition, the location of the peak was clearly influenced by the two different experimental temperatures (see Fig. S2 in the supplemental material).

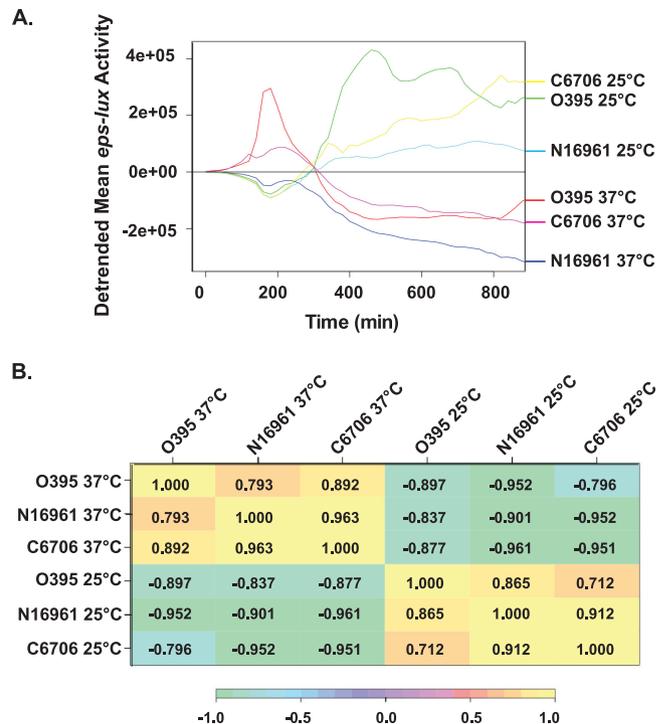In summary, these additional statistical explorations indicated

FIG 2 Detrended correlation of *eps* expression in *V. cholerae*. (A) The detrended mean time series of *eps-lux* activity in all examined *V. cholerae* strains cultured at 37°C and 25°C (as indicated) was computed by first estimating the overall mean time series and then subtracting the overall estimated mean trend series. (B) Correlation between the detrended mean time series and a heat map illustrating the correlation matrix. The correlation value legend is shown below.

common patterns between Fig. 1A, C, and E (*eps-lux* at 37°C) and similarly between panels B, D, and F (*eps-lux* activity at 25°C), which further supported the idea that in *V. cholerae* the transcriptional expression of T2S is temperature regulated. Despite the strong correlation in the detrended time series (Fig. 2B), a clear difference was still observed between the classical strain O395 and the El Tor strains in terms of the *eps-lux* activity profile, which seemed to peak more sharply in the classical strain than in the El Tor strains at a given temperature (see Fig. S2 in the supplemental material).

HapR stimulates expression of T2S. Quorum sensing has been shown to positively regulate the expression of T2S in several Gram-negative bacteria (27, 28). Among the *V. cholerae* strains utilized in our studies, only C6706 possesses a functional master regulator of quorum sensing, HapR (29, 30). We noted that although all the strains displayed similar *eps-lux* expression profiles at 37°C, there was a 2.8-fold difference in the peak of transcriptional activity (at 200 min) between the El Tor strains C6706 and N16961 (Fig. 1E and C; see Fig. S2 in the supplemental material). Two lines of investigation were undertaken to determine whether HapR stimulates expression of T2S. The *hapR* knockout strain was constructed in *V. cholerae* C6706 using homologous recombination, and the *hapR* gene from strain C6706 was cloned under the control of the IPTG-inducible promoter P$_{TAC}$, located on a broad-host-range plasmid, pMMB67-EH (Table 1). Subsequently, either p-HapR or the empty vector pMMB67-EH (p) was introduced by conjugation into N16961 and O395 carrying p*eps-lux*. The bacte-
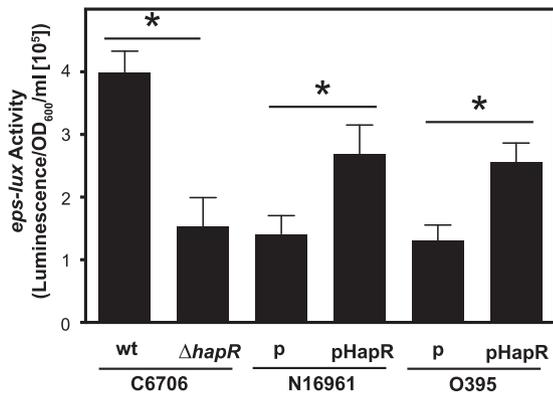
FIG 3 HapR stimulates expression of *eps*. The *V. cholerae* strains N16961 and O395 carrying either pMMB67-EH (p) or pHapR were grown with aeration in LB medium at 37°C until the mid-logarithmic phase of growth ($OD_{600} = 0.5$). Then, all the cultures were treated with IPTG at a final concentration of 100 μM. The activity of *eps-lux* was determined at 30 min postexposure and normalized by the density of the cultures. Means and SEM were obtained from five independent experiments (*, $P < 0.05$). wt, wild type.

rial strains were cultured in LB medium with aeration, and IPTG was added to all cultures in the mid-logarithmic phase of growth ($OD_{600} = 0.5$). The expression of *eps-lux* was determined 30 min posttreatment and normalized by bacterial growth (Fig. 3). The genetic inactivation of *hapR* resulted in a 2.6-fold decrease in the *eps* transcriptional activity. This difference was even more dramatic when the wild-type and isogenic *hapR* knockout strains of *V. cholerae* C6706 were cultured in a 96-well microtiter plate format (see Fig. S3 in the supplemental material). Corroborating these findings, the ectopic overproduction of HapR resulted in statistically significant 1.9- and 1.96-fold increases in *eps-lux* expression in N16961 and O395, respectively, in comparison to the control strains carrying the empty plasmid (Fig. 3). Thus, we concluded, that HapR might positively regulate the expression of genes encoding the T2S system in El Tor and classical strains of *V. cholerae*.

Growth phase-dependent expression patterns of genes encoding T2S-dependent cargo proteins. The pattern of expression of the T2S system could be dictated by the necessity to support the secretion of different cargo proteins at distinct phases of bacterial growth. It has been shown, for example, that GbpA is produced and secreted to the extracellular milieu in the early exponential phase, while secretion of HapA occurred in the stationary phase (31, 32). Given that T2S is responsible for the transport of at least 20 proteins (2, 14), we reasoned that other T2S-dependent proteins might display similar temporal expression patterns. To begin to address this hypothesis, transcriptional reporter fusions were constructed for four recently identified T2S-dependent exoproteins: the serine proteases VesA, VesB, and VesC, as well as a putative metalloprotease, TagA-related protein (Tarp) (14, 33). The individual plasmids p*vesA-lux*, p*vesB-lux*, p*vesC-lux*, and p*tarp-lux* were introduced into N16961 (as an El Tor representative) and the classical strain O395. Subsequently, the kinetics of expression of the respective cargo genes were assessed at both 37°C and 25°C in a 96-well microtiter plate format as described above. Although there were differences in the magnitudes of activities of individual transcriptional fusions between the analyzed strains, the overall patterns were strikingly similar during growth at 37°C. The tran-

scription of *vesA-lux* reached maximum expression in the late stationary phase, while the activities of *vesB-lux* and *vesC-lux* peaked in the mid-exponential phase (Fig. 4A and C). At the environmental temperature, similarly to the *eps-lux* activity, the transcriptional expression of the examined T2S substrates was significantly increased in comparison to that at 37°C (Fig. 4). There were 2-, 10-, and 8-fold-higher bioluminescences measured for *vesA-*, *vesB-*, and *vesC-lux* fusions in O395, respectively, while in N16961, there were on average 7-, 4-, and 4-fold augmentations in expression of the respective cargo genes (Fig. 4B and D). Further, in the O395 strain, the expression curves of *vesA-* and *vesC-lux* at 25°C were similar to those observed at 37°C, while expression of *vesB* reached the maximum in the mid-logarithmic phase and was continuously expressed at similar levels through the end of the experiment (Fig. 4A and B). In the El Tor *V. cholerae*, expression of *vesB* followed bacterial growth, with a peak of activity shifted toward entry into the stationary phase (Fig. 4D). Further, expression of *vesC* did not show a distinct peak of activity, which was clearly noticeable in O395; instead, after reaching its maximum at the mid-exponential phase, it plateaued and gradually decreased after the bacterial cultures entered the stationary phase of growth (Fig. 4D). Interestingly, the activity of *tarp-lux* was diminished to the background level (RLU detected in bacteria carrying pBBR*lux*) in the examined strains under the tested growth conditions, with the exception of strain O395 cultured at 25°C, suggesting that expression of this T2S substrate is specifically regulated (Fig. 5; see Fig. S4A in the supplemental material).

Next, we asked whether the virulence-inducing and virulence-repressing conditions affect the transcriptional activities of *eps* and genes encoding selected exoproteins. In these experiments, we utilized a classical strain, O395, because it is well established that the production of cholera toxin and other crucial virulence factors in the classical biotype of *V. cholerae* can be maximized or minimized in the laboratory setting by culturing the bacteria in LB medium at initial pH 6.5 and 30°C or pH 8.5 and 37°C, respectively (19, 34). Under both conditions, the expression patterns for all tested promoter fusions were similar to those tested in LB at 37°C, with the exception of *tarp-lux*, which was active only under virulence-inducing conditions, with a peak of activity in the mid-logarithmic phase of *V. cholerae* growth (see Fig. S4B in the supplemental material). The overall activities of *eps*, *vesB*, *vesC*, and *tarp* were 8-, 30-, 6-, and 100-fold upregulated, respectively, under virulence-inducing conditions, in comparison to those under virulence-inhibiting conditions (Fig. 5). In contrast, the expression of *vesA* was diminished by about 3-fold.

Together, these studies revealed the growth phase-dependent expression of four cargo genes and suggested a relationship between the pattern of transcriptional expression of T2S and its exoproteins. Future proteomic analysis will be required to address the secretion dynamics of the T2S-dependent secretory traffic.

Exogenous factors stimulating expression of *eps*. We subsequently examined whether other physiologically relevant stimuli besides temperature have an impact on *eps* transcriptional activity. During passage through the gastrointestinal tract, ingested *V. cholerae* faces several challenges, including nutrient competition (e.g., iron availability) and exposure to a number of different potentially toxic compounds, such as bile salts and antimicrobial peptides. In these experiments, *V. cholerae* carrying p*eps-lux* was initially cultured under permissive conditions in LB medium at 37°C. Subsequently, when the turbidity reached an OD_{600} of about
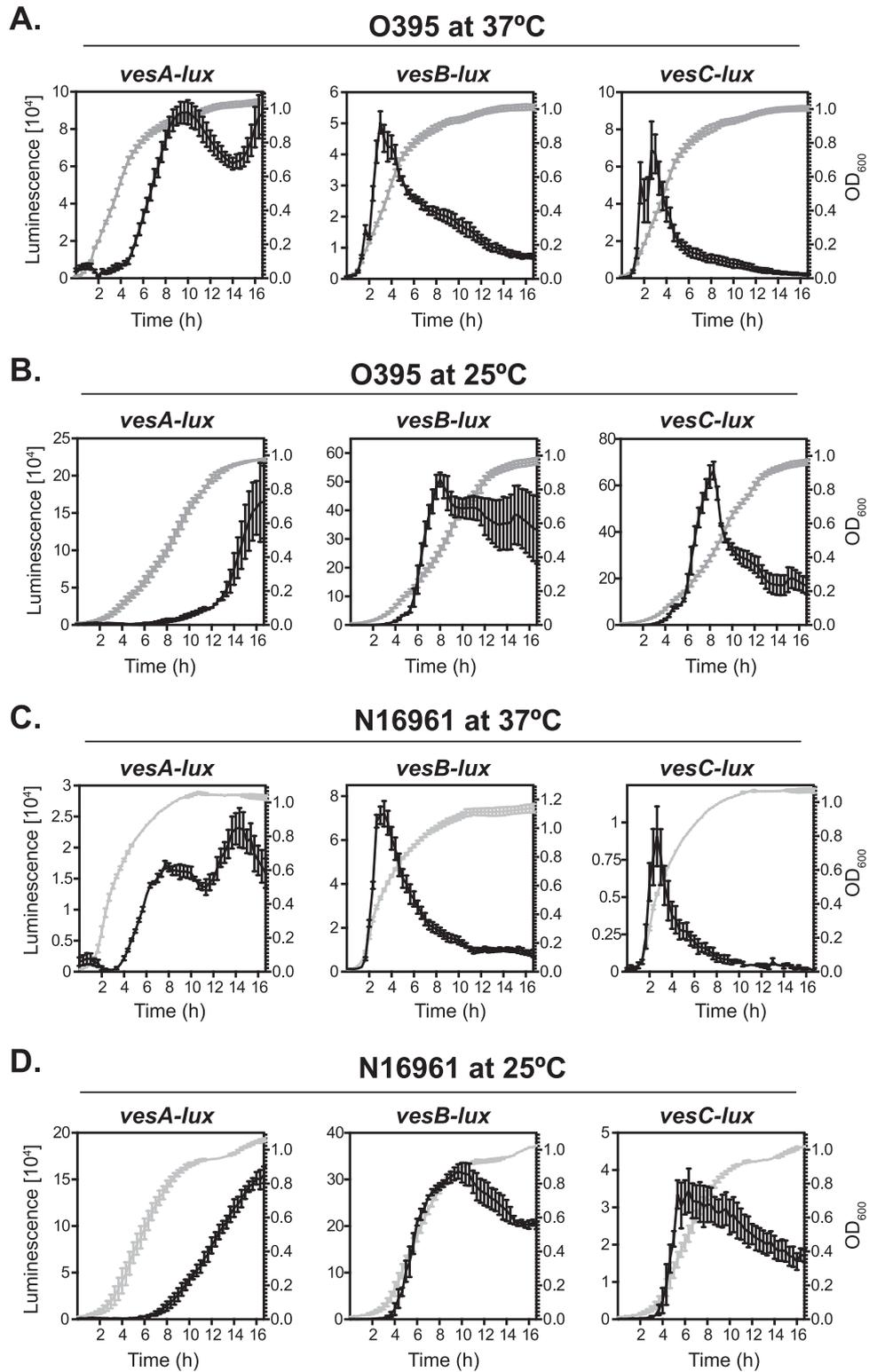
FIG 4 Growth phase-dependent expression of *vesA*, *vesB*, and *vesC* genes encoding T2S cargo proteins. Shown are the activities of the *vesA-lux*, *vesB-lux*, and *vesC-lux* transcriptional fusions in either O395 (A and B) or N16961 (C and D) cultured at either 37°C (A and C) or 25°C (B and D) in LB medium in 96-well microtiter plates. The turbidity of the cultures at OD$_{600}$ (gray) and the RLU (black) were assessed in technical triplicates and recorded every 20 min for 16 h. Each datum point represents the mean and SEM ($n = 4$).
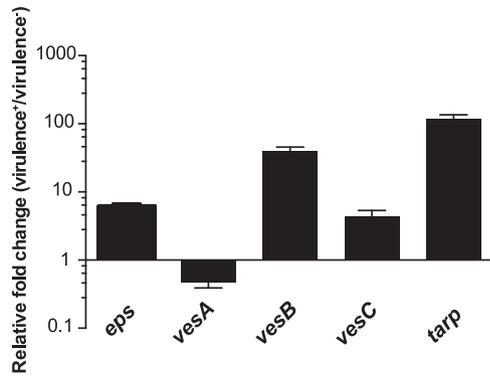
FIG 5 Transcriptional activities of T2S and its cargo proteins are differentially regulated under conditions inducing and repressing virulence gene expression. The expression of *eps-lux*, *vesA-lux*, *vesB-lux*, *vesC-lux*, and *tarp-lux* was assessed in classical *V. cholerae* O395 under conditions either inducing (LB, pH 6.5; 30°C) or inhibiting (LB, pH 8.5; 37°C) virulence gene expression. The highest promoter activity recorded for a particular growth condition was normalized by the OD_{600} of the bacterial culture. The relative fold change in gene expression was calculated by dividing the normalized promoter-*lux* activity under virulence gene-inducing conditions by its corresponding activity under virulence gene-inhibiting conditions. Each bar represents the mean and SEM obtained from at least four independent experiments.

0.5, equal volumes of cultures were split and either treated or not treated with bile salts (0.2%), iron chelator (2,2′-dipyridyl; 100 μM), and antimicrobial peptide (polymyxin B sulfate at 100 U or 50 U/ml for N16961 and O395, respectively). The *eps-lux* activity and bacterial growth were examined at 30 min postexposure (Fig. 6A and data not shown). All the tested compounds did not alter bacterial growth, while the *eps-lux* activity increased 1.5-, 2-, and 4-fold in comparison to the untreated or vehicle-treated (dimethyl sulfoxide) cultures of El Tor *V. cholerae* (Fig. 6A). The transcriptional activity of *eps* in the classical strain increased 1.4- and 2-fold after treatment with 2,2′-dipyridyl and polymyxin B sulfate, respectively, but was unaffected in the presence of bile salts (data not shown). Thus, our studies suggested that, in addition to temperature, other exogenous signals encountered by *V. cholerae* in the human host could also impact the expression of the genes encoding the T2S system. However, the secretion of cargo proteins, as examined by measurements of the extracellular serine- and metalloprotease activities, which are associated with a functional T2S pathway (2, 14), was not increased in the presence of these exogenous cues (see Fig. S5 in the supplemental material). Expression of these cargo proteins likely remains under the control of different regulatory factors. At this point in the investigations, however, the possibility that the production and secretion of other exoproteins were not affected cannot be precluded.

Genetic screen to identify endogenous factors activating expression of *eps*. We took a genetic-screening approach to gain insights into the endogenous factors of *V. cholerae* that regulate the transcriptional activity of the T2S complex. The chromosomal library of *V. cholerae* O395 was constructed by cloning 4- to 7-kb fragments within the *bla* gene located on a cloning vector, pACYC177. The library was introduced into the *E. coli* strain carrying p*eps-lux*. After transformation, bacteria were spread onto LB agar plates supplemented with kanamycin and chloramphenicol to select transformants carrying pACYC177 and p*eps-lux*, respectively. All the bacterial colonies obtained were patched on LB agar with chloramphenicol and, additionally, with either kanamy-
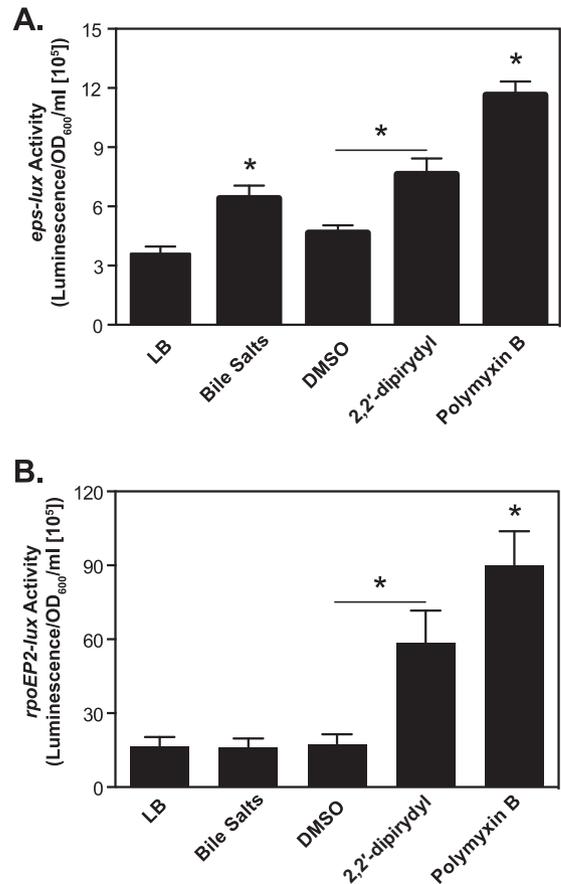

FIG 6 Expression of *eps* and *rpoEP2* in *V. cholerae* N16961 in response to physiologically relevant environmental cues. *V. cholerae* N16961 harboring either p*eps-lux* (A) or p*rpoEP2-lux* (B) was cultured in LB medium at 37°C until the mid-logarithmic phase of growth (OD_{600}, about 0.5). Subsequently, equal volumes of cultures were split into separate test tubes and either treated or not treated with bile salts (0.1% final concentration), iron chelator (2,2′-dipyridyl; 100 μM final concentration), DMSO (vehicle control), or antimicrobial peptide (polymyxin B sulfate; 100 U/ml). The expression of individual reporter fusions was determined at 30 min postexposure and normalized by the optical density of the cultures. Means and SEM were obtained from at least four independent experiments (*, $P < 0.05$).

cin or carbenicillin. The *E. coli* cells that contained a cloned fragment of *V. cholerae* genomic DNA lost the ability to produce β-lactamase; therefore, only colonies that failed to grow on solid media containing carbenicillin were subjected to further analysis. A total of 3,000 isolates were individually inoculated into 96-well, white, clear-bottom microtiter plates and maintained without shaking in LB medium at 37°C for 16 h. Next, the *eps-lux* activity was assessed and normalized by the density of the cultures. Bacterial cultures showing 6-fold or more induction in *eps-lux* activity in comparison to the control (an *E. coli* strain carrying p*eps-lux* and pACYC) were reassayed in triplicate. Subsequently, the inserted genomic DNA isolated from 50 colonies was sequenced. The cloned open reading frames (ORFs) clustered into several conserved regulatory pathways, including genes involved in activation of the RpoE (σ^E)-dependent cell envelope stress response (18 ORFs), phosphate metabolism (7 ORFs), and the production of the second-messenger molecule 3′,5′-cyclic diguanylic acid (c-di-GMP) (VC2697 and VC1372). In addition, we identified a

A.

B.

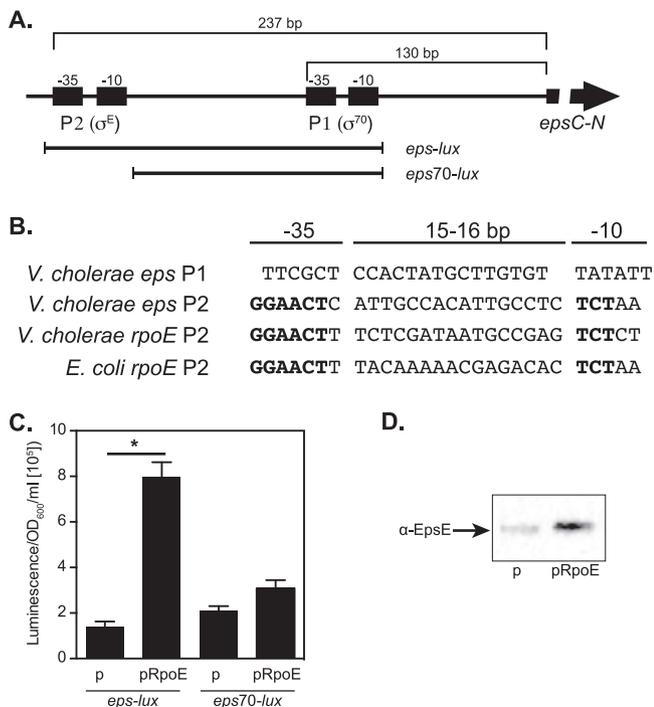|	-35	15-16 bp	-10
V. cholerae eps P1	TTCGCT	CCACTATGCTTGTGT	TATATT
V. cholerae eps P2	**GGAACT**C	ATTGCCACATTGCCTC	**TCT**AA
V. cholerae rpoE P2	**GGAACT**T	TCTCGATAATGCCGAG	**TCT**CT
E. coli rpoE P2	**GGAACT**T	TACAAAAACGAGACAC	**TCT**AA

C.   D.

FIG 7 Deletion studies of the *V. cholerae eps* regulatory region. (A) Diagram illustrating the distances between the *eps*P1 and *eps*P2 promoters from the *epsC* start codon, as well as constructs for deletion analysis of the *eps* regulatory region (not to scale). (B) Nucleotide sequences of the −35 and −10 regions of the promoters. Base pairs within the *V. cholerae eps*P2 promoter identical to those in *rpoE*P2 in *V. cholerae* and *E. coli* are indicated in boldface. (C) Effect of overproduction of RpoE on transcriptional activity of *eps*. The *V. cholerae* strains harboring plasmid pairs— empty pMMB67-EH (p) and p*eps-lux*, pRpoE and p*eps-lux*, pMMB67-EH and p*eps70-lux*, or pRpoE and p*eps70-lux*—were grown in LB medium at 37°C until the turbidity of the cultures reached an OD$_{600}$ of about 0.5. Subsequently, IPTG (at 100 μM final concentration) was added to each culture. The expression of *eps-lux* and *eps70-lux* was measured 30 min posttreatment and normalized by the OD$_{600}$. The bars represent means and SEM from five independent experiments (*, $P < 0.05$). (D) Overproduction of RpoE results in elevated levels of the T2S component EpsE. *V. cholerae* N16961 carrying either the empty vector pMMB67-EH (p) or pRpoE were grown in LB medium until the mid-logarithmic phase of growth (OD$_{600}$ = 0.5). The expression of *rpoE* was induced by the addition of 100 μM IPTG, and the cultures were harvested after 30 min. Samples containing the whole-cell lysates were matched by equivalent OD$_{600}$ units, resolved in 4 to 20% Tris-glycine gels, and transferred, and immunoblotting analysis was performed using rabbit antiserum against recombinant EpsE.

homolog of a transcriptional activator, UhpA (VCA0682), and two LysR transcriptional regulators, AphB (VC1049) and VCA0830 (data not shown). These results suggested that expression of T2S might be regulated by multiple endogenous factors, which could act either directly or via interplay between different regulatory circuits.

Organization of the *eps* regulatory region. Supporting our genetic-screening experiments, a link between the T2S pathway and the extracytoplasmic stress response governed by σ^E was suggested previously (17, 18, 35). Inspection of the chromosomal fragment containing the entire regulatory region of the *epsC-N* genes (17) revealed the presence of two putative promoters, P1 and P2, located 74 and 193 nucleotides (nt) upstream of the ATG in the *epsC* gene, respectively (Fig. 7A). The P1 promoter, with the −35 sequence TTCGCT, had three mismatches with the consen-

sus TTGACA, and the −10 sequence, TATATT, showed only one mismatch with the consensus TATAAT in the σ^{70} signature (Fig. 7B). The putative P2 promoter appeared to possess σ^E consensus. The −35 sequence, GAACTC, disclosed only a single mismatch with the *V. cholerae* and *E. coli* σ^E-dependent promoters, and the −10 sequence, TCTAA, perfectly matched the corresponding sequence in *E. coli*. In addition, the spacing between the two regions, 16 bp, was the same for these promoters (Fig. 7B). The *rpoE* genes in both *V. cholerae* and *E. coli* also possess similar regulatory arrangements. However, the σ^E-regulated promoter is localized 93 nt upstream of the ATG start codon, while the σ^{70}-dependent promoter is located 309 to 310 nt upstream of *rpoE* (36). These findings further suggested that expression of the T2S complex is controlled by both a housekeeping σ^{70} and an alternative σ^E factor.

RpoE positively regulates expression of *eps*. We decided to dissect the link between the RpoE and T2S pathways. First, to determine whether σ^E responds to the same environmental signals that induced expression of *eps-lux*, we utilized the *rpoEP2-lux* promoter fusion, containing the P2 promoter, whose expression is controlled by σ^E (17, 36). The presence of bile salts did not have any effect on *rpoEP2-lux*, whereas iron starvation and exposure to polymyxin B resulted in 4- and 6-fold increases, respectively, in transcriptional activity (Fig. 6B). Next, to directly assess the roles of P1 and P2 sequences in *eps* transcription and to test whether RpoE regulates expression of T2S genes, we constructed a truncated reporter fusion, *eps70-lux*, which lacked the σ^E-dependent P2 promoter (Fig. 7A). Subsequently, plasmids carrying individual promoter fusions, p*eps-lux* and p*eps70-lux*, were introduced into *V. cholerae* N16961 harboring either the empty vector pMMB67-EH or pMMB67-EH containing the *rpoE* gene cloned under the control of an IPTG-inducible promoter, P*tac* (pRpoE). The overproduction of RpoE resulted in over 4-fold induction of expression of *eps-lux* in comparison to *V. cholerae* harboring the empty plasmid. In contrast, overexpression of *rpoE* had no statistically significant effect on the transcriptional activity of *eps70-lux* (Fig. 7C). The immunoblotting analyses with anti-EpsE antisera additionally showed that RpoE positively regulated the expression of T2S at the protein level (Fig. 7D).

Finally, to confirm that σ^E plays a role in regulating expression of *eps*, in subsequent experiments, we utilized the Δ*rpoE* mutant and its parent strain, O395 (37). p*eps-lux* was introduced into both strains, which were then subjected to treatment with polymyxin B as described above. There was a statistically significant 2-fold induction of *eps-lux* activity in the wild-type *V. cholerae*, while the activity remained unchanged in the Δ*rpoE* mutant in comparison to the untreated cultures (Fig. 8A). As expected, the ectopic overexpression of *rpoE* led to increased *eps-lux* transcriptional activity in both strains in comparison to the control cultures carrying an empty vector (Fig. 8B). In summary, these experiments demonstrated that the alternative sigma factor σ^E stimulates expression of genes encoding the T2S system in *V. cholerae*.

DISCUSSION

Significant research in the T2S field has been dedicated to elucidating the architecture and functions of individual constituents of the T2S system. In contrast, specific regulators of expression of the T2S complex and environmental conditions, as well as signaling required for its induction/inhibition, so far remain elusive in many bacterial species. During the nearly 20 years of research on
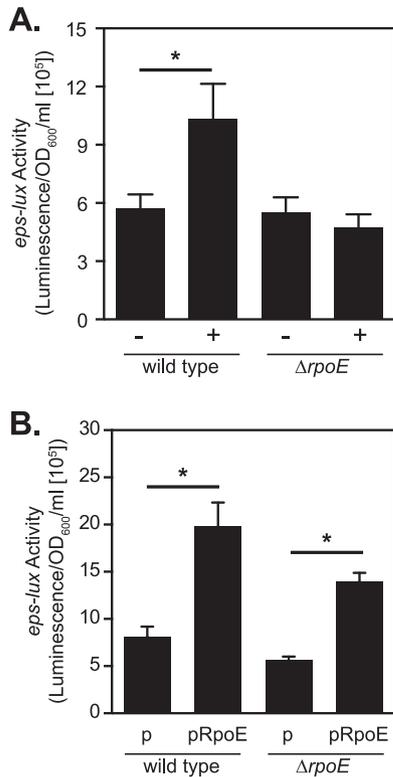
FIG 8 Induction of transcriptional *eps* expression occurs in an *rpoE*-dependent manner. (A) The *V. cholerae* O395 wild-type and isogenic Δ*rpoE* knockout strains carrying a p*eps-lux* reporter fusion were cultured in LB medium at 37°C to an OD_{600} of 0.5. Then, the cultures were divided into separate test tubes and either treated (+) or not treated (−) with polymyxin B (at 50 U/ml). The *eps-lux* activity was determined at 30 min postexposure and normalized by the turbidity of the cultures. (B) The *V. cholerae* Δ*rpoE* mutant and its parent O395 strain harboring the p*eps-lux* reporter fusion and either pMMB67-EH (p) or pRpoE were cultured in LB medium at 37°C until the mid-logarithmic phase of growth (OD_{600} = 0.5). IPTG was added to all cultures at a 100 μM final concentration, and the *eps-lux* activity, as well as the OD_{600}, was examined 30 min posttreatment. The means and SEM from five independent experiments are presented (*, $P < 0.05$).

the function of T2S in *V. cholerae*, regulation of the expression of this pivotal secretion pathway has not been addressed.

Here, we began to define the regulation of expression of T2S and several of its recently identified exoprotein genes. Our experiments, coupled with detailed statistical analyses, demonstrated common patterns of *eps* transcriptional activity between El Tor and classical *V. cholerae* isolates. T2S was constitutively expressed at 37°C in all the *V. cholerae* strains examined, while it followed bacterial growth during culture at the environmental temperature (Fig. 1 and 2). We concluded that quorum sensing did not directly contribute to the growth phase-dependent *eps* expression observed in *V. cholerae* at 25°C because, among the analyzed strains, only C6706 produces an active quorum-sensing master regulator, HapR (29, 30). On the other hand, genetic inactivation of *hapR* caused a 2.6-fold decrease in *eps-lux* activity in the C6706 strain, and, consistently, the overproduction of HapR resulted in almost 2-fold induction of *eps* expression in strains N16961 and O395 (Fig. 2). Together, these results suggest that quorum sensing may influence the transcription of T2S genes in *V. cholerae*. A positive effect of quorum sensing on expression of the genes encoding the

T2S pathway was also observed in *Erwinia carotovora*, *Pseudomonas aeruginosa*, and *Burkholderia cenocepacia* (27, 28).

Our experiments revealed that the lower temperature had a stimulatory effect on *eps* transcriptional activity (Fig. 1). Similarly, transcription of T2S genes was induced in *Yersinia enterocolitica* cultured at 26°C in comparison to those cultured at 37°C (38), whereas in enterotoxigenic *E. coli*, expression of the T2S gene cluster was repressed at lower temperature, which was mediated by the global regulatory proteins H-NS and StpA (39). Considering the recently revealed phylogenetic differences between the T2S secretins, it is possible that there is a broader bifurcation of T2S gene expression control across bacterial species (40). It is also tempting to speculate that the temperature-dependent T2S transcription patterns and the stimulatory effect of HapR in vibrios could contribute to their environmental fitness. In a nutrient-poor environment, it might be more cost-effective to delay energetically expensive secretion until a high density of the bacterial community is reached. In addition, the environmental temperature may also affect the pool of the intracellular signaling molecule c-di-GMP and thus activate expression of *eps*. *V. cholerae* has a plethora of proteins engaged in regulating expression of c-di-GMP levels, and in our genetic screen, we identified two of them, VC2697 and VC1372 (41). Global transcriptome analysis has shown that 4.5% of the genes of *V. cholerae* are differentially regulated upon exposure to elevated c-di-GMP concentrations. The genes encoding the T2S pathway were among the ones upregulated in both El Tor and classical biotypes of *V. cholerae* (42).

Proteomic profiling of the T2S secretomes in *Dickeya dadantii*, *V. cholerae*, and *Legionella pneumophila* revealed as many as 12, 21, and 25 exoproteins, respectively (14, 43, 44). A rigorous analysis of the T2S-dependent secretory traffic has not been undertaken, and it remains largely unknown when certain exoproteins are made, how long the cargo proteins reside in the periplasm, when they are secreted, and which of the exoproteins remain attached to the bacterial outer membrane, as well as for how long. The *V. cholerae* T2S secretome is large and diverse and could serve as a great model for studying the kinetics of this evolutionarily conserved secretion pathway. Among the recognized T2S-dependent exoproteins are cholera toxin, hemagglutinin protease (Hap), sialidase, lipase, several proteins participating in chitin utilization, aminopeptidases (LapA and LapX), Tarp, cytolysin, RbmC, serine proteases (VesA, VesB, and VesC), and three hypothetical proteins encoded by VCA0583, VCA0738, and VC2298 (14). To begin addressing the T2S-dependent secretory traffic, we investigated the expression of genes encoding the recently identified serine proteases and Tarp (14). Our studies demonstrated that the cargo genes examined displayed a growth-dependent expression pattern. Activation of expression of *vesB*, *vesC*, and *tarp* occurred in the mid-logarithmic phase of *V. cholerae* growth, while *vesA* peaked in the stationary phase (Fig. 4; see Fig. S4 in the supplemental material). This phenomenon might have important consequences for *V. cholerae* pathophysiology, particularly when a functional interplay between cargo proteins is considered. This scenario is supported by studies conducted on a pair of T2S substrates, GbpA and HapA. A current model of this interaction proposes that GbpA is secreted during the initial phase of infection, where it aids the attachment of *V. cholerae* to human intestinal cells, while the secretion of HapA protease occurs later in infection, during "mucosal escape." HapA is responsible for the degradation of GbpA, which may enhance the detachment of *V. cholerae* from the intestinal epithe-

lium and facilitate its exit into the aquatic environment (31, 32, 45, 46). Another exciting possibility arising from our studies is that expression of the T2S complex is dictated by the secretory traffic in order to support the transport of different cargo proteins under various growth conditions and at distinct stages of bacterial growth. This line of research is currently being pursued in our laboratory.

Finally, our genetic-screening experiments suggested that the expression of the T2S pathway in *V. cholerae* might be regulated by several endogenous factors, including RpoE, the phosphate regulon, c-di-GMP, AphB, and the putative transcriptional regulators UhpA (VCA0682) and VCA0830. We focused our attention on dissecting whether σ^E plays a role in controlling the expression of T2S genes, because the link between the σ^E cell envelope stress response and T2S pathway in *V. cholerae* has been suggested previously. Specifically, the T2S knockout strains displayed compromised outer membrane integrity, altered levels of several outer membrane proteins, increased sensitivity to membrane-perturbing agents, and a dramatic induction of a σ^E-driven cell envelope stress response (17, 18). Here, we discovered that the regulatory region of T2S genes includes two promoters, P1 and P2, which resemble the σ^{70} and σ^E promoters, respectively (Fig. 7A and B) (36). Interestingly, a putative σ^E-dependent promoter near the start of *gspC* (*ecfA*), the first gene of the T2S operon, has also been identified in *E. coli* K-12 (47). Our studies showed that the removal of the σ^E consensus (P2 promoter) prevented activation of *eps* expression by RpoE (Fig. 7C). Additionally, ectopic overexpression of *rpoE* resulted in the elevated expression of genes encoding the T2S system at both the transcriptional and translational levels (Fig. 7 and 8). Corroborating these findings, genome-wide expression analysis revealed that the Δ*rseA* knockout strain, which overexpresses σ^E, displayed an approximately 3-fold increase in the mRNA levels of the *epsC* and *epsE* genes in comparison to those in wild-type *V. cholerae* (35). In addition, our studies presented in Fig. 6A and 8A showed that *eps-lux* expression increased significantly upon treatment with polymyxin B, an antimicrobial peptide, which is a known activator of the σ^E pathway (18, 48). Together, these data strongly suggest that σ^E may regulate expression of T2S in *V. cholerae*.

The use of alternative transcription start sites recognized by either σ^{70} or σ^E factors could provide the bacteria a fine-tuning mechanism for T2S that fits best with the conditions encountered in a particular ecological niche. As illustrated in Fig. 9, we propose that the expression of genes encoding the T2S pathway in *V. cholerae* remains under the control of a housekeeping σ^{70} and that σ^E takes over in response to specific exogenous stimuli (e.g., the presence of antimicrobial peptides or iron deprivation) or endogenous conditions (e.g., accumulation of misfolded proteins or a buildup of cargo proteins in the periplasm). This occurs by cascades of reactions, which lead to degradation of RseA and release of σ^E to allow it to bind to the core RNA polymerase (RNAP). T2S is a unique secretion pathway that, in addition to transporting soluble exoproteins, handles membrane-anchored proteins (6). In this respect, linking the T2S pathway with a sigma factor that deals with cell envelope stress may have important functional implications.

Taken together, our results suggest that the T2S pathway is characterized by growth phase-dependent expression of genes encoding cargo proteins and requires a multifactorial regulatory net-
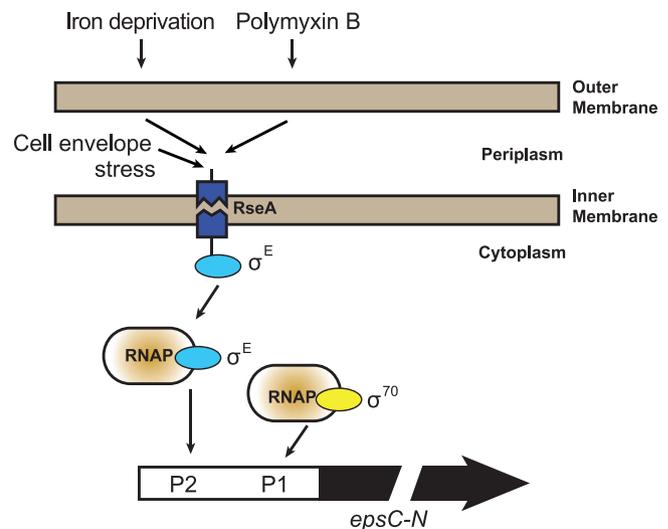

FIG 9 Model showing a link between the T2S pathway and the alternative sigma factor σ^E.

work to ensure appropriate kinetics of the secretory traffic and fitness of *V. cholerae* during its dual life cycle.

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