pepA, a Gene Mediating pH Regulation of Virulence Genes in *Vibrio cholerae*

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ToxT, a member of the AraC family of transcriptional regulators, controls the expression of several virulence factors in *Vibrio cholerae*. In the classical biotype of *V. cholerae*, expression of *toxT* is regulated by the same environmental conditions that control expression of the virulence determinants cholera toxin and the toxin coregulated pilus. Several genes that activate *toxT* expression have been identified. To identify genes that repress *toxT* expression in nonpermissive environmental conditions, a genetic screen was used to isolate mutations which alter the expression of a *toxT-gusA* transcriptional fusion. Several mutants were isolated, and the mutants could be divided into two classes. One class of mutants exhibited higher expression levels of *toxT-gusA* at both the nonpermissive pH and temperature, while the second class showed elevated *toxT-gusA* expression only at the nonpermissive pH. One mutant from the second class was chosen for further characterization. This mutant was found to carry a Tn*phoA* insertion in a homolog of the *Escherichia coli pepA* gene. Disruption of *pepA* in *V. cholerae* resulted in elevated levels of expression of cholera toxin, *tcpA*, *toxT*, and *tcpP* at the noninducing pH but not at the noninducing temperature. Elevated levels of expression of *toxT* and *tcpP* at the nonpermissive pH in the *pepA* mutant were abolished in *tcpP toxR* mutant and *aphB* mutant backgrounds, respectively. A putative binding site for PepA was identified in the *tcpPH-tcpI* intergenic region, suggesting that PepA may act at the level of *tcpPH* transcription. Disruption of *pepA* caused only partial deregulation at the noninducing pH, suggesting the involvement of additional factors in the pH regulation of virulence genes in *V. cholerae*.

Vibrio cholerae is a gram-negative bacillus that is the etiologic agent for the diarrheal illness cholera. Most epidemics of cholera are caused by strains belonging to serotype O1, which can be divided into classical and El Tor biotypes (28). Cholera toxin and the toxin-coregulated pilus (TCP) are among the important *V. cholerae* virulence factors. Cholera toxin is a heterodimeric secreted protein which consist of two subunits, A and B. The A subunit is enzymatically active and causes elevation of intracellular cyclic AMP (cAMP). A pentamer of B subunits, associated with a single A subunit, binds the holotoxin to the ganglioside GM1 receptor on eukaryotic cells (17, 18, 35). The genes that encode cholera toxin, *ctxA* and *ctxB*, are arranged as an operon and are contained within the genome of a filamentous bacteriophage, CTXϕ, that lysogenizes *V. cholerae* (36, 56). The second major *V. cholerae* virulence factor, TCP, is a type IV pilus. A cluster of 12 genes are involved in the processing and assembly of the pilus on the surface of the bacterium. The major subunit of TCP is encoded by *tcpA* and the *tcp* gene cluster is expressed as an operon from a promoter upstream of *tcpA* (3, 50). The genes of the *tcp* operon, as well as adjacent genes, have recently been shown to be encoded by another filamentous bacteriophage, VPIϕ (29).

ToxT, a member of the AraC family of transcriptional regulators, controls the transcription of the *ctxAB* and the *tcp* operons (15, 24). The *toxT* gene is contained within the *tcp* gene cluster, and *toxT* transcription occurs as part of the *tcp* operon, as well as from a promoter immediately upstream of *toxT* itself (3). Activation of *toxT* transcription depends on two pairs of proteins, ToxR-ToxS and TcpP-TcpH, which act synergistically at the *toxT* promoter (7, 22). ToxR is a transmembrane protein with an amino-terminal, cytoplasmic DNA-binding domain that acts as a transcriptional activator, while ToxS encodes a periplasmic protein that facilitates dimerization and activation of ToxR (14, 41, 42, 44). TcpP is a transmembrane protein, which, like ToxR, has homology with members of the bacterial two-component family of response regulators (22). TcpH, which is encoded by a gene that forms an operon with *tcpP*, enhances the activity of TcpP as a transcriptional activator of *toxT* (7). Recently, expression of the *tcpPH* operon has been shown to be positively regulated by the proteins AphA and AphB. AphA has no known homolog in the database, while AphB is homologous to members of the LysR family of transcriptional regulators (30, 47).

Due to the important role of ToxR in activating virulence genes and because it was the first regulator identified, the regulatory circuit controlling virulence gene expression in *V. cholerae* is called the ToxR regulon. The current model for controlling virulence gene expression in *V. cholerae* is that of a regulatory cascade (13). According to this model, AphA and AphB activate expression of TcpP and TcpH which, in turn, act synergistically with ToxR and ToxS to positively regulate *toxT* expression. Finally, ToxT activates expression of its dependent genes, which are collectively called the ToxT-dependent branch of the ToxR regulon, leading to production of cholera

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toxin and TCP. A different branch of the ToxR regulon involves the direct control of expression of the outer membrane proteins OmpU and OmpT by ToxR, and this branch is called the ToxT-independent branch (9, 11, 15, 32).

Expression of cholera toxin and TCP, and of their activator ToxT, in classical strains of *V. cholerae* is strongly regulated by environmental signals, such as pH, temperature, amino acid concentration, and osmolarity (43, 44). The environmental conditions that activate expression of *V. cholerae* virulence genes are called ToxR-inducing conditions (30°C and pH 6.5), whereas conditions of pH 8.4 and 37°C, termed ToxR-noninducing conditions, result in repression of the regulon (43). Expression of the *tcpPH* operon in the classical cholera strain is regulated by the same environmental conditions that regulate expression of the ToxT-dependent branch of the ToxR regulon, whereas expression of *toxR* has been shown to be constitutive in different environmental conditions. These observations have led to the proposal that the regulated expression of *tcpPH* couples environmental signals to the expression of *toxT* and ToxT-dependent genes (7).

As described above, significant knowledge exists about the role of transcriptional activators of virulence genes in *V. cholerae*. However, surprisingly little information is available about the mechanisms used by the bacterium to sense its environment and the regulatory pathways that transduce these signals to affect regulation of the ToxR regulon in different environmental conditions, particularly the regulatory signals that repress expression in nonpermissive environmental conditions. Recently, it was reported that disruption of the genes, *cya* and *crp*, which encode adenylate cyclase (cAMP) and the cAMP receptor protein (CRP), respectively, in a classical *V. cholerae* strain derepresses the expression of cholera toxin and TCP at the nonpermissive pH of 8.4 (46). A putative consensus binding site for the cAMP-CRP complex overlaps the −35 sequence of the *tcpA* promoter, and in the *crp* mutant, derepression of *ctx* expression also occurs in the *toxR* background. Therefore, the authors of that study hypothesized that the cAMP-CRP complex acts as a repressor at the level of *toxT* expression, perhaps by modulating activity at the *tcpA* promoter. According to this model, disruption of *cya* or *crp* would derepress *tcpA* from pH regulation, causing increased expression of *toxT*, and consequently cholera toxin and TCP, as a result of increased readthrough transcription from the upstream *tcpA* promoter (46).

The present study was undertaken to identify additional negative regulators of the ToxR regulon. To isolate mutants showing altered expression of the *toxT* promoter in the noninducing environmental conditions, we performed transposon mutagenesis on a classical strain of *V. cholerae* carrying a *toxT-gusA* transcriptional fusion. By inserting the *toxT-gusA* fusion within *lacZ*, separate from the rest of the *tcpA* operon, we hoped to identify novel genes exerting direct transcriptional control over either *toxT* expression or one of its regulators upstream in the regulatory cascade, and not genes such as *cya* or *crp*, that act at the *tcpA* promoter. Here, we report the identification of several mutants causing deregulated *toxT-gusA* expression at either the noninducing pH or the noninducing temperature, and we present a detailed characterization of one of them, a homolog of *pepA* from *Escherichia coli*

that mediates negative regulation of the ToxR regulon at the noninducing pH in *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are shown in Table 1. All strains were maintained at −70°C in Luria-Bertaini (LB) medium containing 15% glycerol. LB medium contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. Ampicillin (25 or 100 μg/ml), streptomycin (100 μg/ml), tetracycline (15 μg/ml), or kanamycin (45 μg/ml) were added as appropriate.

Bacteria were grown in either LB medium at pH 6.5 and 30°C to activate expression of ToxR-dependent virulence gene expression (inducing conditions) (43) or in LB medium at pH 6.5 and 37°C (noninducing temperature), LB medium at pH 8.4 and 30°C (noninducing pH), or LB medium at pH 8.4 and 37°C (noninducing temperature and pH) to repress virulence gene expression. Cultures were examined for the presence of autoagglutination, which reflects expression of TCP, after overnight growth with moderate aeration as described elsewhere (52).

In vitro manipulation of DNA. Restriction analyses and cloning were done by standard techniques as described earlier (2). Enzymatic reagents were purchased from New England Biolabs (Beverley, Mass.) or Boehringer Mannheim (Indianapolis, Ind.) and used as specified by the manufacturer. Amplification of DNA by PCR was carried out as previously described (26).

Construction of a chromosomal *toxT-gusA* fusion. A 761-bp *toxT* promoter-containing fragment that extends from within the *tcpF* gene to the start codon of *toxT* was PCR amplified from O395 using the primer pair RT19B (5′-AAAGGATCCTATGATATTGTGAATGTTGGTGGTG-3′, *Bam*HI site underlined) and PAC59 (5′-AAACAGGATTTCTATATACATTAGTTTGAAAAG-3′; *Pst*I site underlined) and cloned into the *Bgl*II and *Pst*I sites of plasmid pUJ10. The *phoA* gene in pUJ10 was replaced with the promoterless *gusA* gene, encoding β-glucuronidase, from plasmid pWM2 (37), to create plasmid pJB4. The DNA fragment containing the *toxT-gusA* fusion was excised by digestion with *Xba*I and *Not*I and cloned into pT689, to yield plasmid pJB6. Plasmid pJB6 was introduced into O395 by electroporation, and the *toxT::gusA* fusion was exchanged by double homologous recombination into the *lacZ* gene as previously described (6), resulting in strain JB29. Insertion of the fusion into the chromosome at the *lacZ* locus was confirmed by Southern blot analysis.

Tn*phoA* mutagenesis and determination of sequences adjacent to transposon insertions. A library of random transposon insertion mutants derived from strain JB29 was constructed by Tn*phoA* mutagenesis as previously described (51). Appropriate dilutions of the mutant library were plated onto LB agar plates, at pH 8.4, containing 40 μg of X-glucuronidase (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated for 48 h at 30°C. Colonies that appeared blue on plates, reflecting elevated *toxT-gusA* expression under these noninducing conditions, were isolated for further characterization by quantitative β-glucuronidase assays.

To identify the DNA region that harbored the transposon in a given mutant, the Tn*phoA*-chromosomal junction was amplified by three rounds of PCR amplification with a set of nested Tn*phoA*-specific primers (PAC35, 5′-TATCGCC CTGAGCAGCCCGG; JB15L, 5′-AGCGGCAGTCTGATCACCCG; and JB16L, 5′-CTTCGGCATAATTACGTGCG) and random primers containing restriction site-specific sequences for 4- or 6-bp cutters at their 3′ end (JB11R, 5′-NNNNNNNNNNGATC; JB12R, 5′-NNNNNNNNNNTCGA; JB13R, 5′-NNNNNNNNNNGGATCC; and JB14R, 5′-NNNNNNNNNNGAATTC), as previously described (21). The amplicons were gel purified, recovered with a Compass kit (American Bioanalytical, Natick, Mass.), and sequenced with a Tn*phoA*-specific primer (JB28L, 5′-TTCCAGAACAGGGCAAAACG) that lies 5′ of the primer used in the third round of amplification.

DNA sequencing was performed at the Massachusetts General Hospital Department of Molecular Biology in the DNA Sequencing Core Facility by using ABI Prism DiTerminator Cycle sequencing with AmpliTaq DNA polymerase FS and an ABI377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). The sequences obtained were analyzed by using the University of Wisconsin Genetics Computer Group package, version 8.0 (Madison, Wis.) and at the National Center for Biotechnology Information via the BLAST program (1).

Strain constructions. To construct a polar mutation in the *pepA* gene, a 938-bp *Xba*I-*Eco*RI fragment was amplified by PCR from the O395 chromosome with the primer pair JB29R (5′-GCGGTGATCTAGAGGGTAAAC-3′; *Xba*I site underlined) and JB30L (5′-CAACGGGAATTCAGTACGTCACACA-3′; *Eco*RI site underlined), ligated to *Xba*I-*Eco*RI-digested suicide vector pGP704, and transformed into *E. coli* DH5α λpir to create plasmid pJB10. The recombinant plasmid was mobilized into JB29 and YM2-34 by conjugation, using the *E. coli*

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype[a]	Source or reference
V. cholerae		
O395	Wild type (classical Ogawa); Sm^r	36
C6709	Wild type (El Tor Inaba); Sm^r	5
JB29	O395 *lacZ::toxT-gusA*; Sm^r	This study
JB62	JB29 *eno*::Tn*phoA*; Sm^r Km^r	This study
JB65	JB29 *rhlB*::Tn*phoA*; Sm^r Km^r	This study
JB66	JB29 *cadB*::Tn*phoA*; Sm^r Km^r	This study
JB69	JB29 *pepA*::Tn*phoA*; Sm^r Km^r	This study
JB70	JB29 *yhdA*::Tn*phoA*; Sm^r Km^r	This study
JB73	JB29 *argH*::Tn*phoA*; Sm^r Km^r	This study
JB98	JB29 *pepA*::pJB10; Sm^r Ap^r	This study
JB104	YM2-34 *pepA*::pJB10; Sm^r Ap^r	This study
JB115	JB29 *lap*::pJB12; Sm^r Ap^r	This study
JB123	JB29 Δ*pepA*; Sm^r	This study
JB129	JB123 *toxR*::pVM55; Sm^r Ap^r	This study
JB131	JB123 *tcpP*::pYM2-16; Sm^r Ap^r	This study
JB132	YM2-34 Δ*pepA*; Sm^r	This study
JB134	JB29 *toxR*::pVM55; Sm^r Ap^r	This study
JB135	JB29 *tcpP*::pYM2-16; Sm^r Ap^r	This study
JB148	YM2-34 *aphB*::pJB15; Sm^r Ap^r	This study
JB150	JB132 *aphB*::pJB15; Sm^r Ap^r	This study
JB152	JB123 Δ*pepA*::pJB14; Sm^r Ap^r	This study
YM2-34	O395 *lacZ::tcpP-gusA*; Sm^r	45
E. coli		
DH5α	F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169 (φ80*dlacZ*ΔM15)	20
DH5α λ*pir*	λ*pir* lysogen of DH5α	Laboratory strain
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir* R6K	43
Plasmids		
pUJ10	Plasmid for the construction of divergent fusions to *lacZ/phoA* reporters; Ap^r	12
pGP704	Suicide vector; pBR322 derivative with *oriR6K mobRP4*; Ap^r	19
pT689	A p6891MCS derivative which contains the *rrnB* transcriptional terminator from plasmid pKK223-3; Ap^r	45
pCVD442	Positive selection suicide vector; Ap^r	16
pYM2-16	pGP704 derivative carrying a 230-bp internal *tcpP* fragment; Ap^r	Y. M. Murley and S. B. Calderwood, unpublished data
pVM55	Suicide plasmid pJM703.1, containing an internal fragment of *toxR*; Ap^r	43
pJB4	pUJ10 derivative carrying the *toxT-gusA* fusion; Ap^r	This study
pJB6	pT689 derivative carrying the *toxT-gusA* fusion; Ap^r	This study
pJB10	pGP704 derivative carrying an internal *pepA* fragment; Ap^r	This study
pJB12	pGP704 derivative carrying an 800-bp internal fragment of *lap*; Ap^r	This study
pJB13	pCVD442 derivative carrying a *pepA* allele containing an in-frame deletion; Ap^r	This study
pJB14	pGP704 derivative carrying a wild-type copy of *pepA*; Ap^r	This study
pJB15	pGP704 derivative carrying a 584-bp internal fragment of *aphB*; Ap^r	This study

[a] Sm, streptomycin; Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

donor strain SM10λ*pir*, to create strains JB98 and JB104, respectively. Disruption of the *pepA* locus was verified by Southern blot analysis.

To construct a nonpolar deletion in *pepA*, we first created pJB13, a derivative of the positive selection suicide vector pCVD442 carrying an in-frame deletion in *pepA*. To engineer the in-frame deletion in *pepA*, we performed overlap extension PCR using O395 chromosomal DNA as the template. The first round of PCR was performed with the primer pairs JB33R (5′-TTGCAGCAA<u>GTCGAC</u>ACTGTTTTCTGC-3′; *Sal*I site is underlined) and JB34L (5′-<u>CACCGCGCTG</u> <u>ATGTGATGGCCC</u>ACACCGGGTACTTGATGCAG-3′; bases complementary to the 3′ fragment are underlined) to generate the 852-bp 5′ fragment; and with the primer pair JB35R (5′-<u>CTGCATCAAGTACCCGGTGTGGGCCATC</u>ACATCAGCGCGGTG-3′; bases complementary to the 5′ fragment are underlined) and JB36L (5′-AGTCTAC<u>GAGCTC</u>GGGTAAAGGTACGC-3′; *Sac*I site is underlined) to amplify the 800-bp 3′ fragment. The amplicons of the first round were purified and used as templates in a second PCR with the outer primer pairs JB33R and JB36L to generate a 1,610-bp fragment encompassing the *pepA* gene but containing a 957 bp in-frame deletion in its coding sequence. The 1.6-kbp PCR product was digested with *Sal*I and *Sac*I and cloned into

similarly digested pCVD442 to generate pJB13. Plasmid pJB13 was mobilized into JB29 or YM2-34 by conjugation, and allelic replacement of the chromosomal copy of *pepA* was achieved as previously described (16). The deletion and the loss of vector sequences from the chromosome were confirmed by PCR amplification of the *pepA* region with flanking primers and sequencing across the deletion junction.

To complement the *pepA* mutation in JB123, a wild-type copy of *pepA* was amplified from O395 using the primers JB31R (5′-TGGTAGG<u>GAATTC</u>CACCG TCAG-3′; *Eco*RI site underlined) and JB32L (5′-TGCGGTAG<u>AGATCT</u>TGG TCTC-3′; *Bgl*II site underlined). The 1,935-bp amplicon, which also included the upstream ORF-*pepA* and *pepA*-*holC* intergenic regions (see Fig. 2), was digested with *Eco*RI and *Bgl*II and cloned into pGP704. The recombinant plasmid, called pJB14, was conjugated into JB123 to force its integration at the *pepA* locus based on the homology between the wild-type and mutant copies of *pepA*, creating the merodiploid strain, JB152.

Disruptions in *tcpP*, *aphB*, and *lap* were made as described above for *pepA* with the pGP704 derivative vectors pYM2-16, pJB15, and pJB12, respectively, which

carry internal fragments of these genes. Plasmid pVM55 was used for creating *toxR* disruptions as previously described (43).

Assays. β-Glucuronidase assays were performed as described previously, with some minor modifications (27). For *toxT-gusA* fusion-containing strains, overnight cultures (approximately 16 h of incubation) were used for β-glucuronidase assays. Assays were performed by harvesting 500 μl of bacterial culture, pelleting the cells by centrifugation, washing them once with 50 mM phosphate buffer (pH 7.0), and resuspending the cells in 500 μl of phosphate buffer. Cell were lysed (20 to 250 μl, depending on the expected β-glucuronidase activity) by vortexing them for 20 s after the addition of 10 μl of toluene. The volume of the reaction tubes was brought up to 900 μl with 50 mM phosphate buffer, and samples incubated at 37°C for 10 min. *p*-Nitrophenyl-β-D-glucuronide (100 μl; Sigma) was added to start the reactions, and the samples were incubated at 37°C for 20 to 60 min. Reactions were stopped by the addition of 400 μl of 3 M 2-amino-2-methylpropanediol (Sigma), vortexed for 20 s, and centrifuged for 10 min, and the absorbance of the supernatants at 420 nm was measured. Similar assays were performed with the *tcpP-gusA* fusion-containing strains, except that overnight cultures in LB medium were subcultured the next day and samples harvested after 6 h of incubation at the inducing or noninducing conditions (expression of the fusion in inducing conditions was highest at this time point). Cholera toxin was assayed in culture supernatants after overnight growth in inducing or noninducing conditions, using GM-1 enzyme-linked immunosorbent assay (ELISA) as previously described (25).

Protein analysis. Total cell lysates prepared from *V. cholerae* cells grown to stationary phase overnight were diluted 1:100 in distilled water, and the protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). For immunoblot analysis, 40 μg of each protein sample was subjected to electrophoresis on a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was probed with a polyclonal rabbit anti-TcpA antibody (a gift of John J. Mekalanos) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, N.J.) for 1 h. The blot was developed using the ECL kit (Amersham Pharmacia) exposed to film for up to 1 min.

Nucleotide sequence accession number. The sequence of the *V. cholerae* O395 *pepA* gene has been deposited in the GenBank database under accession no. AF282267.

RESULTS

Isolation of mutants showing deregulated expression of *toxT* in noninducing environmental conditions. To isolate mutants of *V. cholerae* exhibiting enhanced *toxT* expression in noninducing growth conditions, we constructed a pool of approximately 50,000 independent Tn*phoA* insertion mutants derived from the parental strain JB29. Subsequently, the mutant pool was plated onto LB agar plates, with the pH adjusted to 8.4 and containing 40 μg of the chromogenic substrate X-glucuronidase per ml, and incubated at 30°C for 48 h. Normally, growth of the parental strain JB29 at pH 8.4 and 30°C represses expression of *toxT-gusA* and colonies are white on the indicator medium for the first 24 to 48 h. We were interested in isolating colonies that appeared blue, since they might represent mutants exhibiting elevated *toxT* expression in noninducing conditions. Of the several dozen mutants initially isolated from plates, 16 reproducibly showed increased β-glucuronidase activity in noninducing conditions in quantitative liquid assays and were chosen for further analysis.

To determine the insertion sites of the transposon in the various mutants, we amplified the DNA region upstream of the junction with the transposon by PCR by using a series of nested primers specific to Tn*phoA* and a set of random primers containing restriction site-specific sequences at the 3′ end as described in Materials and Methods. After three rounds of PCR amplification, the amplicons were gel purified and sequenced with a Tn*phoA*-specific primer that read outward from the transposon toward the adjacent unknown chromosomal sequence. Six mutants—JB62, JB65, JB66, JB69, JB70, and JB73—yielded sufficient sequence information to enable us to identify the site of transposon insertion by similarity searches.

These mutants were characterized further with regard to *toxT-gusA* expression in different environmental conditions (Fig. 1). Based on the β-glucuronidase activities at either the noninducing pH or temperature, the six mutants could be divided into two groups. The first group, which included JB65, JB66, and JB70, showed slightly higher β-glucuronidase activities than the control at the noninducing pH but significantly higher activities at the noninducing temperature. For reasons that are unclear, JB70 reproducibly expressed very low levels of *toxT-gusA* in inducing conditions. All three strains in this group showed significant growth defects and, with the exception of the growth of JB66 at pH 8.4, grew too poorly in all conditions tested for us to assess their agglutination phenotypes.

The second group, including JB62, JB69, and JB73, exhibited higher *toxT-gusA* expression at the noninducing pH compared to the control. At the noninducing temperature, JB69 expressed the fusion at levels comparable to that of the parent, while JB62 and JB73 failed to grow reproducibly; JB73 also had nearly twofold-higher *toxT-gusA* expression in inducing conditions than the parental strain. Elevated *toxT-gusA* expression at pH 8.4 in JB62, JB69, and JB73 was accompanied by agglutination of cultures after overnight growth, suggesting that these mutants were also expressing elevated levels of the TCP in these normally nonpermissive conditions. The mutant JB69, which showed defective pH regulation of *toxT-gusA* expression and had no obvious growth defect, was selected for further detailed characterization.

Sequence analysis of the *pepA* gene. We utilized PCR amplification and sequencing of the junctional fragment with Tn*phoA* in JB69 and demonstrated that the transposon had inserted within *pepA* (Fig. 2). We searched the *V. cholerae* genome in the TIGR database and located the complete sequence of *pepA* and the adjacent chromosomal region. Analysis of an approximately 6-kb fragment in the vicinity of *pepA* revealed four open reading frames (ORFs) (Fig. 2). We identified the 447-bp coding region of a putative protein 82 bp downstream of *pepA* and transcribed in the same orientation, which was 41% identical (49% similar) to the χ subunit of DNA polymerase III holoenzyme from *E. coli* encoded by *holC* (accession no. P28905) (8). A third ORF, located 92 bp downstream of the *holC* homolog and oriented similarly, encoded a 953-amino-acid putative protein with 75% amino acid identity to valyl-tRNA-synthetase from *E. coli* (accession no. P07118). A fourth ORF, located upstream of *pepA* and transcribed divergently, encoded a putative protein with 42% identity to a 40.4-kDa hypothetical transmembrane protein from *E. coli* (accession no. P39340). The organization of these four ORFs in *V. cholerae* is strikingly similar to that of *E. coli*. Based on the significant similarity to *E. coli* genes and on their conserved arrangement on the chromosome, we have named these ORFs *pepA*, *holC*, and *valS*.

We designed oligonucleotide primers to amplify overlapping segments of the *pepA* gene from the classical biotype strain O395 and the El Tor strain C6709 by PCR. Direct sequencing of both strands of PCR amplified products revealed 30 nucleotide differences between the *pepA* coding sequences from the classical and the El Tor strains. However, all of these base
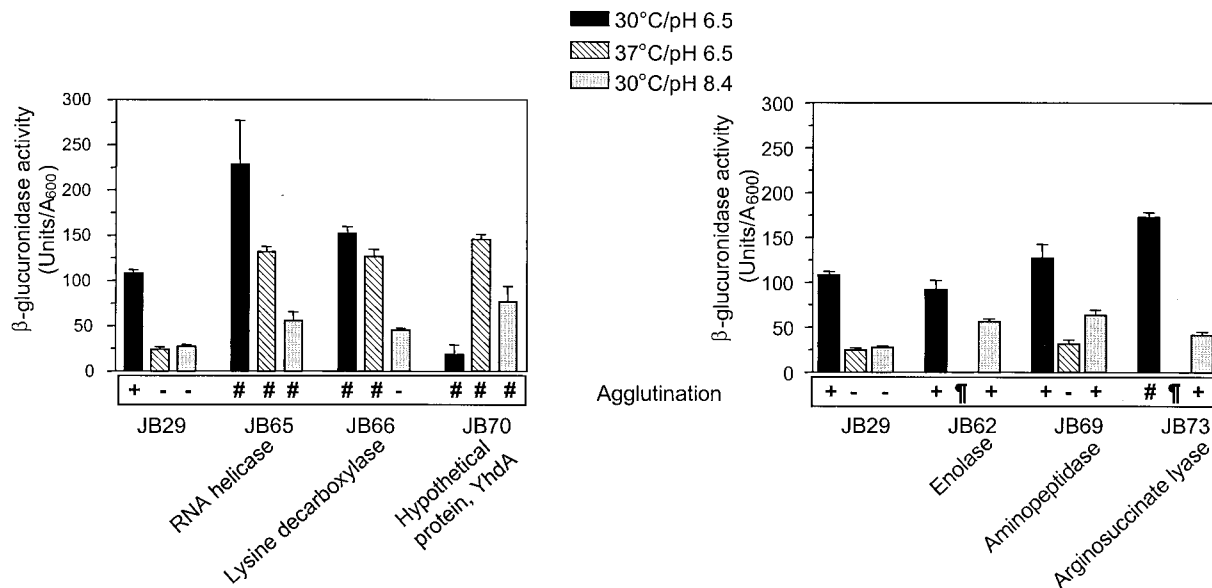
FIG. 1. β-Glucuronidase activity in three different growth conditions of *V. cholerae* O395 carrying a *toxT-gusA* fusion and six transposon-generated mutant strains. The left panel shows the control strain compared with three mutants exhibiting defective regulation of *toxT-gusA* expression in response to both temperature and pH. The right panel shows the control strain and three mutants exhibiting defective regulation in response to pH. The agglutination phenotypes are shown at the bottom of each graph. The symbols used for agglutination in the figure represent the following: +, agglutination; −, no agglutination; ¶, strain failed to grow; and #, strain grew too poorly to allow analysis of agglutination. Also shown in the figure are the proteins in the database showing the highest degree of homology to the disrupted sequences in each of the mutants. The data represent means plus the standard deviations (SDs) of two independent experiments, each done in duplicate.

changes were silent, resulting in identical deduced amino acid sequences of PepA from the two strains, which were also identical to that of El Tor strain N16961 in the TIGR database. The mole percent G+C content of *pepA* from O395 was 50%, which is consistent with that previously observed in *V. cholerae* (33). A putative ribosome binding site, AGGAG, is centered 8 bp upstream of the apparent *pepA* initiation codon. The *pepA* ORF is predicted to encode a protein of 503 amino acids with a calculated M_r of 54,617 and pI of 6.37. The insertion of the transposon in JB69 was found to have occurred immediately before a guanine nucleotide at position 1085 of the *pepA* ORF, resulting in deletion of the terminal 141 residues of the *pepA* gene product. The deduced amino acid sequence of PepA had a very high degree of similarity to PepA from *E. coli* (81%

identity, 87% similarity; accession no. X86443), PhpA from *Pseudomonas aeruginosa* (55% identity; accession no. AF054622), PepA from *P. putida* (53% identity; accession no. AJ010261), and PepA from *Haemophilus influenzae* (61% identity; accession no. U32843), including perfect conservation of the residues believed to be involved in the active site of the enzyme based on crystallographic studies of bovine lens leucine aminopeptidase (4) (Fig. 3).

Effect of *pepA* disruption on *toxT-gusA* expression. To determine whether the phenotype of JB69 was actually due to the insertion of Tn*phoA* into the *pepA* gene or to an unlinked second-site mutation or polar effect on a downstream gene, we constructed additional *pepA* mutations in the *toxT-gusA* fusion strain JB29. Strain JB98 was created by forcing integration of
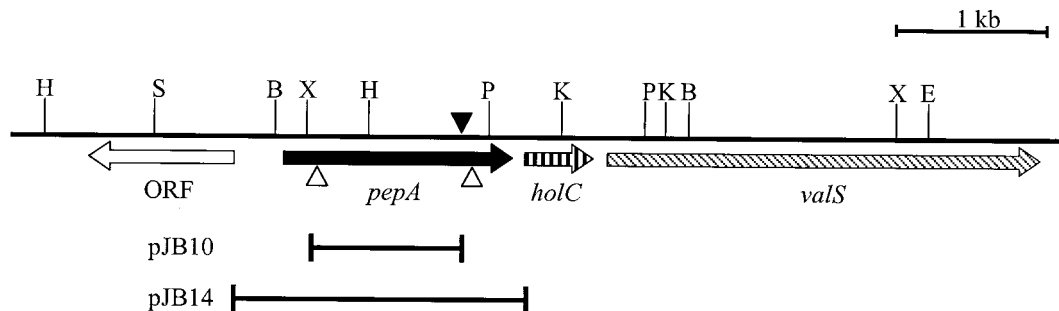

FIG. 2. Schematic representation of the *V. cholerae pepA* locus and its surrounding region. ORFs are shown as arrows pointing in the direction of transcription. The site of insertion of Tn*phoA* in JB69 is marked by a solid arrowhead. The open arrowheads indicate the fragment of *pepA* deleted in JB123. The 938-bp insert in plasmid pJB10 and the 1,935-bp insert in pJB14 used to complement the mutation in *pepA* are shown below the *pepA* ORF as black bars. H, *Hin*dIII; B, *Bam*HI; X, *Xba*I; P, *Pst*I; K, *Kpn*I; E, *Eco*RI; S, *Sal*I.

```
             1
V. chol   MEFSVKSGSP  EKQRSACIVV  GVFEPRRLSP  VAEQLDKISD  GYISSLLRRG  DLEGKPGQML  LLHQVPGVLS  ERVLLVGCGK  ERELGERQYK  EIIQKTINTL
E. coli   MEFSVKSGSP  EKQRSACIVV  GVFEPRRLSP  IAEQLDKISD  GYISALLRRG  ELEGKPGQTL  LLHHVPNVLS  ERILLIGCGK  ERELDERQYK  QVIQKTINTL
P. aeru   MEFLVKSVRP  ETLKTATLVL  AVGEGRKLGA  SAKAVDDATG  GAISAVLKRG  DLAGKVGQTL  LLQSLPNLKA  ERVLLVGAGK  ERELGDRQYR  KLASAVLSTL
H. infl   MKYQAKN.TA  LSQATDCIVL  GVYENNKFSK  SFNEIDQLTQ  GYLNDLVKSG  ELTGKLAQTV  LLRDLQGLSA  KRLLIVGCGK  KGELTERQYK  QIIQAVLKTL
P. puti   MELVVKSVAA  ASVKTATLVI  PVGENRKLGA  VAKAVDLASE  GAISAVLKRG  DLAGKPGQTL  LLQNLQGLKA  ERVLLVGSGK  DEALGDRTWR  KLVASVAGVL
            *     *                 *     *           *           *           *  **            * * **  *       *    *    *

             101
V. chol   NETGSMEAVC  FLTELHVKGR  DTYW.KVRQA  VEATKDGLYI  FDQFKSVKPE  IRRPLRKLVF  NVPTRRELNL  GERAITHGLA  ISSGVKACKD  LGNMPPNIAN
E. coli   NDTGSMEAVC  FLTELHVKGR  NNYW.KVRQA  VETAKETLYS  FDQLKTNKSE  PRRPLRKMVF  NVPTRRELTS  GERAIQHGLA  IAAGIKAAKD  LGNMPPNICN
P. aeru   KGLAGADAAL  ALGDLAVKGR  DAH.AKARLL  VETLADGLYV  FDRYKSQKAE  PLK.LKKLTL  .LADKADSAA  VEQGSKEAQA  IANGMALTRD  LGNLPPNVCH
H. infl   KETNTREVIS  YLTEIELKDR  DLYW.NIRFA  IETIEHTNYQ  FDHFKSQKAE  .TSVLESFIF  NT....DCAQ  AQQAISHANA  ISSGIKAARD  IANMPPNICN
P. puti   KGLNGADAVL  ALDDVAVNNR  DAHYGKYRLL  AETLLDGEYV  FDRFKSQKVE  PRA.LKKVTL  .LADKAGQAE  VERAVKHASA  IATGMAFTRD  LGNLPPNLCH
                     *            *              * *   *  **   *          **              *   *   *     *    *   *    ** *

             201
V. chol   PAYLASQARR  LADDY.ESIT  TKIIGEEEME  KLGMASYLAV  GRGSRNESMM  SVIEYKGNPD  PEAKPIVLVG  KGLTFDSGGI  SLKPGEGMDE  MKYDMCGAAS
E. coli   AAYLASQARQ  LADSYSKNVI  TRVIGEQQMK  ELGMHSYLAV  GQGSQNESLM  SVIEYKGNAS  EDARPIVLVG  KGLTFDSGGI  SIKPSEGMDE  MKYDMCGAAA
P. aeru   PTFLGEQAKG  LAKEF.KGLK  VEVLDEKKLR  ELGMGSFLAV  AQGSDQPPRL  IVLQYNGA.K  KDQAPHVLVG  KGITFDTGGI  SLKPGLGMDE  MKFDMCGAAS
H. infl   PAYLAEQAKN  LAEN.STALS  LKVVDEEEMA  KLGMNAYLAV  SKGSENRAYM  SVLTFNNAPD  KNAKPIVLVG  KGLTFDAGGI  SLKPAADMDE  MKYDMCGAAS
P. puti   PSFLAEQAKE  LGKAH.KALK  VEVLDEKKIK  DLGMGAFYAV  GQGSDQPPRL  IVLNYQGG.K  KADKPFVLVG  KGITFDTGGI  SLKPGAGMDE  MKYDMCGAAS
            *  **          *            *   *  ***  *      *           *            * ****  ** *** *** * *  ***  ** ******

             301
V. chol   VFGTMKAIAK  LGLPLNVIGV  LAGCENMPGS  NAYRPGDILT  TMSGQTVEVL  NTDAEGRLVL  CDVLTYVERF  EPECVVDVAT  LTGACVIALG  HHISAVMSNH
E. coli   VYGVMRMVAE  LQLPINVIGV  LAGCENMPGG  RAYRPGDVLT  TMSGQTVEVL  NTDAEGRLVL  CDVLTYVERF  EPEAVIDVAT  LTGACVIALG  HHITGLMANH
P. aeru   VFGTFRAVLE  LQLPINLVGL  LACAENMPSG  GATRPGDIVT  TMSGQTVEIL  NTDAEGRLVL  CDALTYAERF  KPQSVIDIAT  LTGACIVALG  SNTSGLMGNN
H. infl   VFGTMKTIAQ  LNLPLNVIGV  LAGCENLPDG  NAYRPGDILT  TMNGLTVEVL  NTDAEGRLVL  CDTLTYVERF  EPELVIDVAT  LTGACVVALG  QHNSGLVSTD
P. puti   VFGTLRAVLE  LQLPVNLVCL  LACAENMPSG  GATRPGDIVT  TMSGQTVEIL  NTDAEGRLVL  CDTLTYAERF  KPQAVIDIAT  LTGACIVALG  SHTTGLMGNN
            * *         *   * *      ** ** *         * ****  ** * *** *  **********  ** *** ***  * * * **  *****  ***

             401
V. chol   NPLAHELVNA  SEQSSDRAWR  LPLADEYHEQ  LKSPFADMAN  IGGRPGGAIT  AACFLSKFAK  KYNWAHLDIA  GTAWKS.GAA  KGSTGRPVSL  LVQFLLNRSG
E. coli   NPLAHELIAA  SEQSGDRAWR  LPLGDEYQEQ  LESNFADMAN  IGGRPGGAIT  AGCFLSRFTR  KYNWAHLDIA  GTAWRS.GKA  KGATGRPVAL  LAQFLLNRA.
P. aeru   EALVRQLLKA  GEFADDRAWQ  LPLFDEYQEQ  LDSPFADIAN  IGGPKAGTIT  AGCFLSRFAK  KYHWAHLDIA  GTAWISGGKD  KGATGRPVPL  LTQYLLERAK
H. infl   NNLANALLQA  ATETTDKAWR  LPLSEEYQEQ  LKSPFADLAN  IGGRWGGAIT  AGAFLSNFTK  KYRWAHLDIA  GTAWLQ.GAN  KGATGRPVSL  LTQFLINQVK
P. puti   DDLVGQLLDA  GKRADDRAWQ  LPLFDEYQEQ  LDSPFADMGN  IGGPKAGTIT  AGCFLSRFAK  AYNWAHMDIA  GTAWISGGKD  KGATGRPVPL  LTQYLLDRAG
            *  *  *       * **   ***  ** ** * * ***  * ***   * ** *   * *** *     * *** *** ****   *   ** *****  * * * *

             501
V. chol   GLDAEE
E. coli   GFNGEE
P. aeru
H. infl
P. puti   A
```

FIG. 3. Alignment of the amino acid sequences of the PepA homologs from *V. cholerae* (V. chol), *E. coli*, *P. aeruginosa* (P. aeru), *H. influenzae* (H. infl), and *P. putida* (P. puti). An asterisk indicates that the residue at that position is conserved among all the sequences shown. The residues shown in boldface are in the active site of the enzyme based on the crystal structure of bovine lens leucine aminopeptidase (4).

a *pepA* internal fragment cloned in suicide vector pGP704, into the chromosome of JB29 at the *pepA* locus, thereby disrupting the ORF after codon 362. Analysis of *toxT-gusA* expression in JB98 revealed the same phenotype as the transposon mutant JB69, with approximately threefold-higher expression of the fusion in cultures grown at the nonpermissive pH of 8.4 (Fig. 4). It is worth noting that JB69 and JB98 still exhibited some residual regulation at pH 8.4, with *toxT-gusA* expression levels at pH 8.4 approximately 50% of those at pH 6.5, suggesting that classical *V. cholerae* may have regulatory pathways mediating pH regulation of *toxT* expression independently of *pepA*. Importantly, the disruption of *pepA* resulted in neither any difference in the maximal levels of *toxT-gusA* expression in inducing conditions (pH 6.5, 30°C) compared to the parent strain, nor did it influence the negative regulation of the fusion by the nonpermissive temperature. Similarly, when cultures were incubated at the nonpermissive conditions of 37°C and pH 8.4, expression was downregulated to the same extent as with the wild-type strain. These results suggest that *pepA* mediates negative regulation of *toxT* expression at the noninducing pH but not at the noninducing temperature.

As mentioned above, the *pepA* gene lies immediately upstream of the *V. cholerae holC* and *valS* homologs, therefore raising the possibility that the three genes might be expressed as a polycistronic message from the *pepA* promoter. The disruption of *pepA* by insertional mutagenesis in JB98 could result in polar effects on the two downstream genes, confounding the results obtained above. To address this possibility, we engineered an in-frame *pepA* deletion in JB29, extending from codon 69 to 387, creating strain JB123. Similar to the results
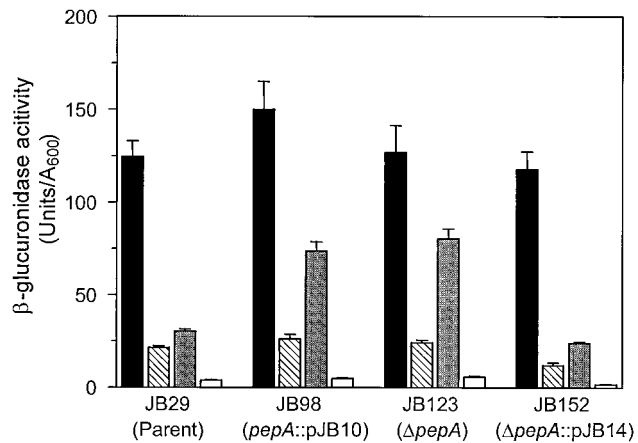

FIG. 4. β-Glucuronidase activity in different growth conditions of various *V. cholerae* strains carrying a transcriptional *toxT-gusA* fusion in the *lacZ* locus. The data represent the means + the SDs of two independent experiments, each done in triplicate. Columns: ■, 30°C, pH 6.5; ▨, 37°C, pH 6.5; ▦, 30°C, pH 8.4; □, 37°C, pH 8.4.

obtained with JB98, *toxT-gusA* expression in JB123 was comparable to that of JB98 and was partially derepressed at the noninducing pH. We therefore concluded that the phenotype observed with strains JB69, JB98, and JB123 was the result of *pepA* disruption and not due to polar effects on downstream genes.

We also asked whether it was possible to rescue the *pepA* phenotype by introducing a single copy of the wild-type allele into strain JB123. We inserted a complete copy of *pepA*, including its upstream region, into the suicide vector pGP704 and introduced it into JB123 by conjugation. Integration of the plasmid at the *pepA* locus as a result of sequence homology between the introduced copy of *pepA* and the partially deleted *pepA* allele on the chromosome resulted in the merodiploid strain, JB152. This strain was found to have normal regulation of *toxT-gusA* expression when grown in medium at pH 8.4 (Fig. 4), confirming the role of *pepA* in pH regulation of *toxT*.

Deregulated expression of cholera toxin and TcpA at pH 8.4 in *pepA* mutants. ToxT controls the expression of the two major virulence factors of *V. cholerae*, cholera toxin and the TCP (15). Since disruption of *pepA* caused elevated *toxT* expression at the noninducing pH, we hypothesized that deregulated expression of ToxT at pH 8.4 should also result in elevated expression of the genes controlled by it. To test whether the deregulated *toxT* expression in *pepA* mutants was similarly reflected in regulation of cholera toxin production, we performed cholera toxin ELISAs on culture supernatants of the *pepA* mutants grown in various conditions (Fig. 5A). Cholera toxin production in strains JB98 and JB123, containing the polar and in-frame *pepA* deletions, respectively, was only slightly elevated in inducing conditions or at the noninducing temperature. However, when the strains were grown at the noninducing pH, cholera toxin expression was three- to fourfold higher in the *pepA* mutants than in the control. Interestingly, the expression of cholera toxin was not completely deregulated in the *pepA* mutants, a finding that is consistent with our results from *toxT-gusA* expression assays.

We also measured cholera toxin production in strain JB152, in which an in-frame *pepA* deletion was complemented with a wild-type *pepA* allele. Similarly to the parent strain, cholera toxin production in JB152 showed an approximately 10-fold downregulation at the nonpermissive pH. Also, the level of cholera toxin production at pH 8.4 was similar to that of JB29, demonstrating that the wild-type copy of *pepA* had successfully restored pH regulation in this mutant (data not shown).

Like cholera toxin, expression of TCP in the classical biotype is also downregulated dramatically when *V. cholerae* is grown either at 37°C, at pH 8.4, or both. To test whether the *pepA* mutants showed elevated levels of TcpA at a noninducing pH, we performed immunoblot analysis with cell extracts prepared from cultures grown at pH 6.5 or at pH 8.4 (Fig. 5B). The parent strain showed approximately 10-fold lower levels of TcpA protein at pH 8.4 than at pH 6.5, while the *pepA* mutants JB98 and JB123 showed only twofold repression of TcpA at pH 8.4.

Expression of TCP in liquid cultures can be inferred by monitoring the agglutination of cells (52), which results in the formation of a cell pellet at the bottom of the test tube accompanied by almost complete clearing of the supernatant. Consistent with the results obtained from the immunoblot analysis,
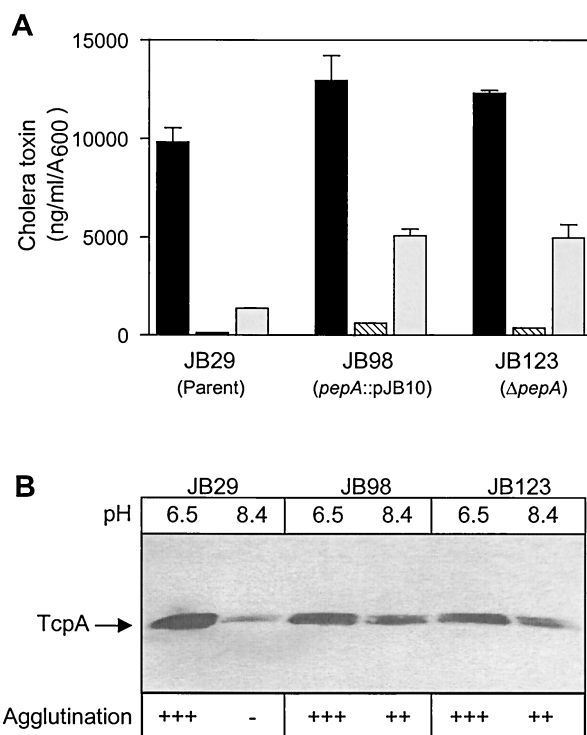
FIG. 5. Effect of *pepA* disruption on expression of virulence factors. (A) Cholera toxin production in various *V. cholerae* strains. Supernatants were assayed after overnight growth in LB medium in the conditions indicated. The data represent the means + the SDs of two separate experiments, each done in duplicate. Columns: ■, 30°C, pH 6.5; ▨, 37°C, pH 6.5; □, 30°C, pH 8.4. (B) Immunoblot of TcpA production and agglutination phenotype in various *V. cholerae*. Strains were grown overnight at 30°C in LB medium adjusted either to pH 6.5 or 8.4, as indicated above each lane. A total of 40 μg of total protein was loaded into each lane. The position of the TcpA band is indicated on the left. The agglutination phenotype of each culture is shown at the bottom of the gel. The symbols used in the figure indicate the following: +++, agglutination with the formation of a large cell pellet, with almost complete clearing of the supernatant; ++, agglutination with a moderately sized pellet, with some residual turbidity of the supernatant; and −, no agglutination.

JB98 and JB123 cultures agglutinated at the noninducing pH, whereas the control strain failed to do so. Perhaps as a function of slightly lower levels of TcpA expression, the pellets formed by the *pepA* mutants JB98 and JB123 were smaller, and the supernatant was more turbid, at pH 8.4 compared to those formed at pH 6.5. None of the strains agglutinated at the noninducing temperature (data not shown), again suggesting that *pepA* mediates pH but not temperature regulation of *toxT* expression and the genes regulated by it.

Mutation in *pepA* does not change the requirement for ToxR and TcpP for *toxT* expression. As mentioned above, the expression of *toxT* is dependent on the transcriptional activators ToxR and TcpP. The results suggesting that the *pepA* gene product functions as a negative regulator of virulence gene expression led us to question whether disruption of *pepA* could cause elevated expression of *toxT* at pH 8.4 in the absence of ToxR or TcpP. We tested this possibility directly by constructing disruptions in either *toxR* or *tcpP* in the wild-type and *pepA* backgrounds and then assaying *toxT-gusA* expression in differ-
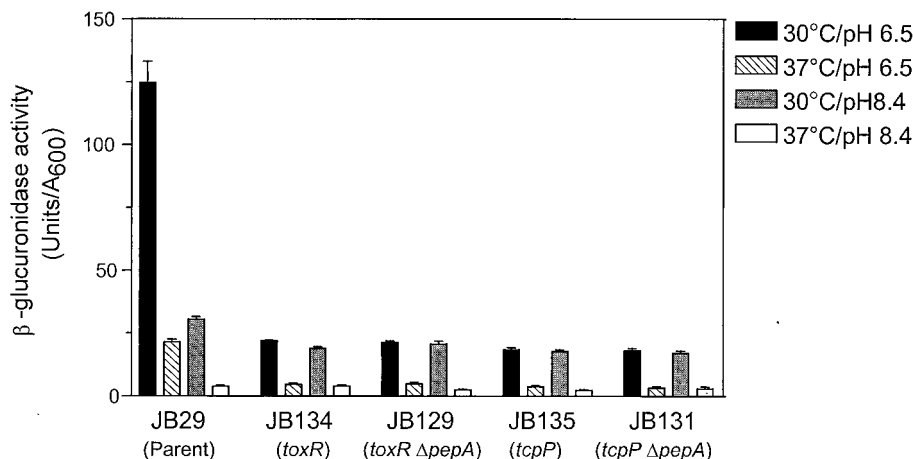
FIG. 6. Comparison of *toxT-gusA* expression in parent and single and double *toxR*, *tcpP*, and *pepA* disruption mutants. β-Glucuronidase activity was assayed after overnight growth of cultures in different growth conditions. The data represent the means + the SDs of two independent experiments, each done in triplicate.

ent conditions of pH and temperature. Disruption of either *toxR* or *tcpP* in the wild-type background resulted in dramatically lower levels of expression of the fusion in all four growth conditions (Fig. 6). Expression levels in the double mutants were also very low and comparable to those of the single mutants. These results suggest that loss of PepA function does not change the requirement for the transcriptional activators ToxR and TcpP for *toxT* expression in either inducing or noninducing conditions and raise the possibility that PepA may act indirectly on *toxT*, through either *toxR* or *tcpP*. However, our results do not exclude the possibility that PepA may act at more than one level in the virulence gene regulatory cascade.

Disruption of *pepA* partially relieves negative regulation of *tcpP* expression by noninducing pH. Recent reports from our own and other laboratories have established the important role of TcpP and TcpH in transcription of *toxT* in classical *V. cholerae* (7, 22). Transcription of *tcpPH* in classical *V. cholerae* is regulated by pH and temperature, while the expression of *toxR* has been shown to be constitutive in different environmental conditions. These observations have led to the hypothesis that regulated expression of TcpP and TcpH may couple environmental growth conditions to transcription of *toxT* and ToxT-dependent virulence genes in *V. cholerae* (7).

Our observations that *pepA* mutants showed partially defective pH regulation of *toxT* and its dependent genes raised the possibility that *pepA* may be necessary for negative regulation of *tcpPH* expression at pH 8.4. To test this hypothesis, we constructed a polar *pepA* disruption and an in-frame *pepA* deletion in the strain YM2-34 (45), which contains a *tcpP-gusA* fusion cloned in the *lacZ* locus in a classical *V. cholerae* background, resulting in the strains JB104 and JB132, respectively. When these *pepA* mutant strains were grown in LB medium, pH 6.5, no significant difference in the levels of β-glucuronidase activity were found compared to the parental control (Fig. 7). However, when cultures were grown at the noninducing pH of 8.4, JB104 and JB132 expressed β-glucuronidase activity at levels approximately twofold higher than did YM2-34, suggesting that *pepA* disruption partially relieves the negative regulation of *tcpPH* expression at the noninducing pH. These same

two mutants also agglutinate in the nonpermissive pH, similar to the results described above.

Recently, two proteins, AphB and AphA, have been shown to function synergistically to activate *tcpPH* expression in *V. cholerae*, although the expression of neither *aphA* nor *aphB* is strongly regulated by environmental conditions (30, 47). To test whether the elevated *tcpP* expression at pH 8.4 in the *pepA* mutant strain could be observed in the absence of the *tcpP* transcriptional activator, AphB, we engineered an *aphB* disruption by insertional mutagenesis in JB123 (Δ*pepA*) and in the parental strain, JB29. As shown in Fig. 7, disruption of *aphB* dramatically reduced expression of the *tcpP-gusA* fusion at both the inducing and the noninducing pH in the *aphB* single mutant, as well as in the *pepA aphB* double mutant, suggesting that AphB is required for transcriptional activation of *tcpPH* even in the absence of the negative regulator, PepA. These results, along with those presented above, lead us to conclude that *pepA* mediates pH regulation of the ToxT-dependent branch of the ToxR regulon by acting as a negative regulator at a level upstream of *tcpPH* transcription in the virulence gene regulatory cascade in classical *V. cholerae*.

The *V. cholerae lap* gene does not mediate pH regulation of the ToxR regulon. Recently, Toma and Honma reported the identification of a *V. cholerae* gene, *lap*, encoding a 501-amino-acid protein with homology to the *Vibrio proteolyticus* aminopeptidase and showed that this was an active leucine aminopeptidase by the ability of the recombinant *lap* gene product to cleave the substrate leucyl-*p*-nitroanilide (53). The *lap* gene was found by PCR analysis to be widely distributed among *V. cholerae* strains but was absent in other bacterial species examined. While the *pepA* gene identified in our screen encodes a protein with homology to leucine aminopeptidases from *E. coli* and other species, there is no sequence similarity between it and the *V. cholerae lap* gene. To test whether the *lap* gene, like *pepA*, also mediates environmental regulation of virulence gene expression in *V. cholerae*, we engineered a disruption in *lap* by insertional mutagenesis with the suicide plasmid pJB12, creating the strain JB115. Analysis of *toxT-gusA* expression and agglutination of JB115 in cultures grown at either the nonper-
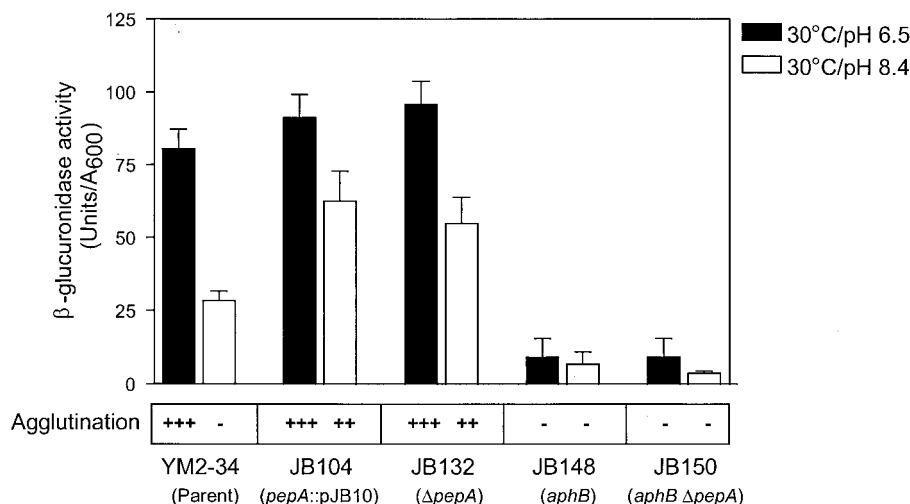
FIG. 7. β-Glucuronidase activity of *V. cholerae* parent and *pepA* and *aphB* single and double disruption mutants. All strain carry a transcriptional *tcpP-gusA* fusion in the *lacZ* locus. Overnight cultures in LB broth were subcultured into LB medium adjusted to either pH 6.5 or pH 8.4 and incubated at 30°C for 6 h before the samples were harvested for assays. The data represent the means + the SDs of two independent experiments, each done in duplicate. The agglutination phenotypes were assessed after overnight growth of cultures (see Fig. 5 for symbols).

missive pH or temperature revealed no difference between JB115 and the parental strain JB29 (data not shown). Thus, the *lap* gene product, unlike that of *pepA*, does not appear to have a role in pH regulation of the ToxR regulon.

DISCUSSION

The expression of virulence determinants in classical strains of *V. cholerae* is coordinately regulated by several regulatory proteins and is strongly influenced by signals derived from the host and the environment. We report here the identification of two classes of transposon-generated mutants showing altered regulation of *toxT* and its dependent genes when grown at a noninducing pH or noninducing temperature. The successful isolation of several different mutants exhibiting deregulated pH and temperature control suggests that environmental regulation of virulence genes in *V. cholerae* is a complex process involving multiple genes and regulatory pathways. While several positive regulators of the ToxR regulon in *V. cholerae* have been identified, we know little about negative regulation of this regulon. We demonstrate here that *pepA* acts as a negative regulator of virulence determinants at the noninducing pH, suggesting that downregulation of gene expression in nonpermissive conditions may also play an important role in controlling the ToxR regulon. The fact that disruption of *pepA* deregulates pH but not temperature regulation of *toxT* also suggests that these two environmental signals are perceived differently by the organism and have separable signaling pathways in the bacterium.

It is now well established that many pathogenic bacteria have evolved from related nonpathogenic species by acquiring virulence genes that remain clustered together on the bacterial chromosome, forming pathogenicity islands (31). Since these horizontally acquired genes are often regulated by the same environmental signals that regulate non-virulence-associated genes, it is possible that horizontally acquired genes may utilize or adapt to existing regulatory circuits modulating gene expression in response to environmental cues. Therefore, it is not surprising that the horizontally acquired, phage-encoded *ctxAB* and *tcp* operons in *V. cholerae* have acquired regulation by genes such as *toxR* that are located elsewhere on the primordial bacterial chromosome. Evidence for the regulation of accessory genetic elements by ancestral chromosomal components, such as cAMP and CRP (46), AphA and AphB (30, 47), and the NQR complex (23), points toward a significant interplay between the metabolic and virulence functions of this bacterium. PepA appears to be another example of an ancestral chromosomal gene that is involved in the regulation of phage-encoded virulence genes and presumably has other cellular metabolic functions besides regulation of virulence genes, as has been hypothesized for ToxR and cAMP-CRP.

The *pepA* gene product in *E. coli* is a multifunctional protein. It has been shown to be an active leucine aminopeptidase (55), a member of the multiprotein complex that resolves plasmid multimers into monomers to result in heritable stability of plasmid ColE1 (48), and a repressor in the pyrimidine-mediated negative regulation of the *carAB* operon that encodes carbamoylphosphate synthetase (10). Recently, a role for PhpA, the *P. aeruginosa* homolog of PepA, was demonstrated in the regulation of virulence determinants. Disruption of *phpA* in an *algB* genetic background resulted in increased expression of the alginate biosynthetic operon, suggesting that *phpA* may act as a negative regulator of virulence-associated genes in this pathogenic bacterium as well (57).

Considering the fact that PepA homologs serve a remarkably wide range of functions, there are several possible ways in which PepA could exert a negative regulatory effect on the ToxR regulon in *V. cholerae*. The regulatory role of PepA could be dependent on its enzymatic activity and could depend on an exopeptidase activity to activate a target repressor protein, or inactivate a transcriptional activator of *tcpPH*, at the noninducing pH. For example, the removal of an N-terminal methionine residue results in the activation of glutamine phosphoribosylpyrophosphate aminotransferase that requires an N-
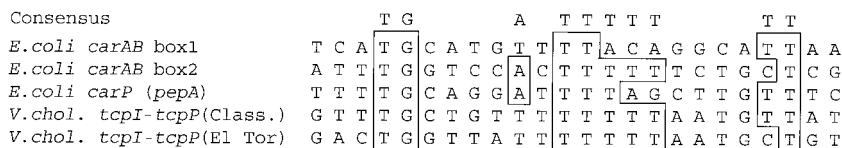
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Consensus                          T G        A   T T T T T        T T
E.coli carAB box1      T C A|T G|C A T G|T|T|T T A|C A G G C A|T|T A A
E.coli carAB box2      A T T|T G|G T C C|A|C|T T T T|T C T G|C|T C G
E.coli carP (pepA)     T T T|T G|C A G G|A|T|T T T|A G|C T T G|T|T T C
V.chol. tcpI-tcpP(Class.)  G T T|T G|C T G T T|T|T T T T T|A A T G|T|T A T
V.chol. tcpI-tcpP(El Tor)  G A C|T G|G T T A T T|T T T T T|A A T G C|T|G T
```

FIG. 8. Sequence alignment of the DNA-binding sites for PepA in the control regions of the *E. coli carAB* operon and the *E. coli pepA* gene was used to generate an AT-rich consensus shown above the sequences (10). Bases conserved in the classical and El Tor *V. cholerae tcpI-tcpP* intergenic region with the consensus sequence are boxed.

terminal cysteine residue for its activity (54). AphA and AphB have been shown to activate *tcpPH* expression but do not appear to be strongly regulated themselves by the environmental signals that modulate *tcpPH* transcription (30, 47). It is conceivable that PepA could act directly or indirectly to modify AphA or AphB activity. However, by analogy with *E. coli*, it appears less likely that the role of aminopeptidase as a pH regulator in *V. cholerae* is dependent on its enzymatic activity. Aminopeptidases have broad substrate specificity and can cleave N-terminal amino acids from peptides of various sizes and sequences (38, 39), making it less likely that there are specific targets for PepA activity. Moreover, it has been demonstrated that the aminopeptidase activity of PepA in *E. coli* is separable from its regulatory function and that the aminopeptidase activity is not required for either plasmid ColE1 Xer site-specific recombination or for the repression of the *carAB* operon (10, 34). However, it is possible that the regulatory role of PepA may be dependent on the degradation or modification of a peptide that functions as an inducer or repressor of the ToxR regulon or as a signaling molecule in specific environmental conditions.

Another intriguing possibility is that PepA may mediate pH regulation by functioning as a DNA-binding protein and directly affecting transcription. In *E. coli*, PepA has been conclusively shown to be a sequence-specific DNA-binding protein involved both in the regulation of *carAB*, the carbamoylphosphate synthetase operon, and as an autorepressor at the *pepA* promoter (10). Sequence alignment of five experimentally identified DNA-binding sites of PepA, three of which are shown in Fig. 8, has revealed that a number of positions that are AT-rich are strongly conserved (10). There are two PepA target sites, 25 to 30 bp in length and 65 nucleotides apart, in the *carAB* operon in *E. coli* and *Salmonella enterica* serovar Typhimurium and in the ColEI *cer* site but only one site in the promoter region of the *pepA* gene itself. Based on the consensus sequence proposed by Charlier et al. (10), we have identified a putative PepA target site in the *tcpPH-tcpI* intergenic region (Fig. 8). This site is centered 133 bp upstream of the *tcpPH* transcriptional start site and 49 bp upstream of the *tcpI* transcriptional start (45). In the classical biotype, the putative target sequence matches the consensus in 9 of 10 positions, while in the El Tor biotype, it matches the consensus in 8 of 10 positions (Fig. 8).

We speculate that PepA could act by binding to this putative target site in the *tcpPH* control region and interfering with transcriptional regulation. It is also possible that binding of PepA to the target site could interfere with the expression of *tcpI*, which in turn may be involved in regulating other genes in the ToxR regulon. Whether, in fact, PepA can directly bind to

the *tcpPH* promoter and repress its activity either by inducing changes in DNA topology or by potentially competing with AphA or AphB for target sites in the *tcpI-tcpPH* intergenic region is currently under investigation. We are also investigating whether expression of *pepA* itself is regulated by environmental cues in *V. cholerae*.

Our results showing that disruption of the *lap* gene, encoding another *V. cholerae* leucine aminopeptidase with no sequence homology to PepA, does not alter regulation of *toxT* are consistent with the conclusion that PepA has distinct regulatory roles not shared by other cellular aminopeptidases. *E. coli* and serovar Typhimurium have several different aminopeptidases (39, 40). In serovar Typhimurium, metabolic analysis of strains carrying mutations in the genes encoding aminopeptidases A, B, D, and N suggests that the enzymes function to break down exogenously supplied peptides for use as nutrients. In addition, they degrade endogenous peptides generated from cleavage of cellular protein. However, only PepA appears to have additional regulatory functions in Xer recombination and regulation of pyrimidine biosynthesis (10, 38, 39).

We have isolated several mutants that show deregulated *toxT* expression at the noninducing pH alone or at both the noninducing pH and the noninducing temperature. As yet, we do not know the precise role of the genes disrupted in these deregulated mutants. It is possible that one or more of these genes may participate in the same control pathway as PepA. In *E. coli*, *cer* site-specific recombination involves a multiprotein complex in which PepA appears to serve an accessory role (49). Therefore, it is possible that PepA may play a role as a member of a protein complex that controls gene regulation at the noninducing pH, and disruption of *pepA* would interfere with the regulation mediated by this complex. This model would also explain why only partial deregulation at the noninducing pH is observed in *pepA* mutants, since the loss of PepA would result in defective pH sensing but perhaps not the total loss of function of the pH-sensing complex.

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