Regulation of *Vibrio cholerae* Genes Required for Acid Tolerance by a Member of the “ToxR-Like” Family of Transcriptional Regulators

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The ability of the intestinal pathogen *Vibrio cholerae* to undergo an adaptive stress response, known as the acid tolerance response (ATR), was previously shown to enhance virulence. An essential component of the ATR is CadA-mediated lysine decarboxylation. CadA is encoded by the acid- and infection-induced gene *cadA*. Herein, *cadA* is shown to be the second gene in an operon with *cadB*, encoding a lysine/cadaverine antiporter. *cadC*, which is 5' of *cadB*, encodes an acid-responsive, positive transcriptional regulator of *cadBA*. Unlike in *Escherichia coli*, *V. cholerae* *cadB* and *cadA* are also transcribed monocistronically. Of note, bicistronic *cadBA* is transcribed at low constitutive levels in an acid- and CadC-independent manner. CadC represents a new member of the “ToxR-like” family of transcriptional regulators in *V. cholerae* and, in addition, exhibits extensive amino acid and functional similarity to *E. coli* CadC. The amino-terminal, putative DNA binding domains of ToxR and CadC are highly conserved, as are the putative promoter elements recognized by these transcription factors.

Vibrio cholerae is the causative agent of the endemic and epidemic diarrheal disease cholera. Since the natural environmental reservoir for this intestinal pathogen is aquatic, it stands to reason that ingestion by a human and subsequent colonization of the relatively sterile small intestine involve the expression of genes that are crucial for survival and adaptation in this new environment. Several research strategies designed to identify pathogen genes that are upregulated during infection have been used to show that adaptation/stress systems are often induced upon entry of a pathogen into its host environment (5, 13, 21, 40, 42). Although much emphasis has been placed on the identification and characterization of *V. cholerae* virulence factors, such as those within the ToxR/ToxT regulon (37), very little is known about the survival and adaptation systems employed by this gram-negative bacterium during infection.

We recently used recombinase-based in vivo expression technology to identify *V. cholerae* genes that are transcriptionally induced within two separate animal models of cholera. One such gene was *cadA*, which encodes a lysine decarboxylase; *cadA* was subsequently shown to be essential for *V. cholerae*'s ability to undergo an adaptive stress response known as the acid tolerance response (ATR) (25). This stress response has been well characterized in the two closely related enteric pathogens, *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, and has been shown to be necessary for pathogenicity of the latter (4, 23, 24, 29). We have found that *V. cholerae* cells that are acid adapted are more virulent than cells grown at a neutral pH. This finding suggests that the *V. cholerae* ATR may play an important role in the fitness of this pathogen, with respect to both infectivity of a single host and rapid epidemic spread within populations (25).

Here we extend our characterization of the *V. cholerae* *cadA* locus and show that, as in *E. coli*, it is the downstream gene in

and acid-inducible operon, *cadBA*. However, in contrast to what is observed in *E. coli*, the *V. cholerae* *cadA* also possesses an independent promoter. In addition, we have identified a positive transcriptional regulator of the *cadBA* operon, CadC, which shows significant homology to both ToxR and the *E. coli* CadC protein. Mutational analyses revealed that CadC is essential for acid induction of *cadBA* but is not needed for basal-level transcription of *cadBA*. The basal-level expression of *cadBA* was insufficient for a functional ATR, as *cadC* mutants were unable to mount either an inorganic or organic ATR.

MATERIALS AND METHODS

Sequence analysis. DNA sequences were analyzed for open reading frames (ORFs) by using DNA Strider version 1.2 (C. Marck). *E. coli* CadB, CadC, and Lcd amino acid sequences were used to search for putative homologs in the *V. cholerae* genome sequence (The Center for Genomic Research [TIGR]) using the BLAST algorithm (1). Multiple alignments of conserved protein domains and hydropathy profile predictions were performed using MacVector 6.0.1 (Oxford Molecular). *cadC*, *-B*, and *-A* are located on the *V. cholerae* large chromosome (replicon 1) beginning at positions 306411, 308670, and 310117, respectively (TIGR database).

Strain and plasmid construction. All strains, plasmids, and primers used in this study are listed in Tables 1 and 2. Mutagenesis of *cadB* and *cadC* was by insertional inactivation of a suicide plasmid, pGP704. The mutagenic plasmids were constructed as follows. A 253-bp internal fragment of *cadB* was amplified with *Pfu* polymerase (Stratagene) by PCR using primers BF1 and BR1. *Sfi*I adapters were ligated onto the PCR product, and the resulting DNA fragment was ligated into the *Sfi*I-digested pAC212 backbone as previously described (25) to generate pDSM373. A 288-bp internal fragment of *cadC* was amplified with *Taq* polymerase using primers CadCF1 and CadCR1 and was subsequently cloned into pCR-Script Amp SK(+) (Stratagene) according to the manufacturer's directions, generating pDSM582. The *cadC* fragment was liberated from pDSM582 by *Sac*I/*Sal*I double digestion and cloned into similarly digested pGP704 (26) to generate pDSM588. A recombinant PCR method, gene splicing by overlap extension (15) using primers BAΔF1, BAΔR1, BAΔF2, and BAΔR2, was used to generate strain DSM-V783, which contains a deletion of the entire *cadBA* coding sequence.

All plasmids used for construction of insertion mutations were mobilized into *V. cholerae* from *E. coli* SM10 λ pir as previously described (25), and all integration mutations were subsequently verified by Southern blot analysis (data not shown). The loss of the ability to decarboxylate lysine was shown by utilization of lysine decarboxylation indicator broth (Difco) as previously described (25).

Growth conditions. All strains were maintained at -80°C in Luria-Bertani (LB) broth containing 30% glycerol. All strains were grown at 37°C in LB broth.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype	Source or reference(s)
<i>E. coli</i>		
SM10 λ pir	<i>thi recA thr leu tonA lacY supE</i>	Laboratory strain
DH5 α pir	RP4-2-Tc::Mu λ :: <i>pir</i> F ⁻ Δ (<i>lacZYA-argF</i>)U169 <i>recA1</i> <i>endA1 hsdR17 supE44</i> <i>thi-1 gyrA96 relA1</i> λ :: <i>pir</i>	11, 18
<i>V. cholerae</i>		
C6709-1	El Tor biotype, Sm ^r	33
AC-V66	C6709-1 <i>lacZ</i> :: <i>res1-tet-res1</i> Tc ^r <i>LacZ</i> ⁻	5
AC-V168	C6709-1 <i>lacZ</i> ::pGP704, <i>LacZ</i> ⁻	5
DSM-V376	AC-V66 <i>cadA</i> ::pGP704	25
DSM-V387	C6709-1 <i>cadB</i> ::pGP704	This work
DSM-V591	C6709-1 <i>cadC</i> ::pGP704	This work
DSM-V783	C6709-1 Δ <i>cadBA</i>	This work
Plasmids		
pAC212	pGP704::SfiI- <i>neo</i> -SfiI	25
pDSM-373	pGP704:: <i>cadB</i> '	This work
pDSM582	pCR-script Amp SK(+):'cadC'	This work
pDSM588	pGP704::'cadC'	This work
pDSM667	pGemT:: <i>cadBA</i>	This work
pDSM673	pGemT::'cadC'	This work

The pH of the medium was adjusted with HCl. Ampicillin and streptomycin were used at concentrations of 100 μ g ml⁻¹. RNA was harvested from strains grown in the following manner. Overnight cultures of each test strain were grown in LB broth containing ampicillin and then diluted 1:150 into 30 ml of fresh medium plus ampicillin. The diluted cultures were grown with aeration until they reached an optical density (600 nm) of approximately 0.16 to 0.20. At this point, cells were pelleted at 5,000 $\times$ g for 5 min at 24°C, and the supernatants were removed by aspiration. Cells were resuspended in 1 ml of LB broth (pH 7.0), and then 10 and 90% of the cells were placed into two microcentrifuge tubes. The cells were pelleted at 12,000 $\times$ g for 1 min at 24°C, and the supernatants were removed by aspiration. The 10% cell pellet was resuspended in 1 ml of LB broth (pH 7.0), and the 90% cell pellet was resuspended in 1 ml of LB broth (pH 5.7); then both were transferred to culture tubes and grown at 37°C with aeration for 1 h. After 1 h, all of the pH 7.0-grown cells and half of the pH 5.7-grown cells were pelleted and then flash frozen in a -80°C isopropanol bath. The remainder of each pH 5.7 culture was resuspended in LB (pH 4.5) and incubated at 37°C for 15 min. These cells were then pelleted and flash frozen as above. Strains which were exposed to organic acids were grown exactly as above except that pH 5.7 LB and

pH 4.5 LB were supplemented with 0.075 $\times$ and 0.1 $\times$ organic acid cocktail, respectively (1 $\times$ cocktail was 87 mM acetic acid, 25 mM butyric acid, and 37 mM propionic acid). Cell pellets were then used to collect total RNA.

RPAs and primer extension. RNase protection assays (RPAs) were conducted on total RNA isolated from AC-V168 and DSM-V591 as previously described (25). A 318-bp probe 1 and 732-bp probe 2 fragment were generated by PCR using *Taq* polymerase and primer pairs AF1-AR1 and CadBOF-CadAOR, respectively. The amplification products were ligated to pGemT (Promega), proper orientation was confirmed, and riboprobes were synthesized using a Maxiscript kit (Ambion) and 50 μ Ci of [³²P]UTP (NEN) as previously described (25). In each case, riboprobes transcribed from the pGemT SP6 or T7 promoters contain pGemT-specific sequence that results in riboprobes that are slightly larger than the original probe 1 or probe 2 *V. cholerae*-specific PCR fragments. Therefore, hybridization of the probe with its target mRNA followed by digestion with RNase results in a smaller protected fragment as the nonhybridizing pGemT-specific transcript is cleaved. RPAs were conducted with an RPAII kit using 1 to 3 μ g of RNA as described by the manufacturer (Ambion). The products of RNase protection were separated on 5% denaturing polyacrylamide gels and exposed to phosphor screens (Kodak). Quantification and peak analysis of bands was conducted using a PhosphorImager and the ImageQuant program (Molecular Dynamics).

Primer extension to map the 5' ends of *cadA*, *cadB*, and *cadC* mRNA transcripts produced after acid challenge was conducted using primers CadAPE (and CadAP2), CadBPE, and CadCPE, respectively. Approximately 10 pmol of each primer was end labeled and used for primer extensions with a primer extension system-avian myeloblastosis virus reverse transcriptase kit as described by the manufacturer (Promega). pDSM567, which contains the entire *cadBA* promoter region and coding sequences, and pDSM673, which contains the *cadC* promoter region, were generated by PCR using primer pairs Cad8F1-Cad8R1 and CadCCF-CadCRI, respectively. Amplification products were ligated into pGemT, and the plasmids were used as templates for sequencing reactions using the appropriate end-labeled primer. Sequencing reactions were conducted by denaturation of template DNA as described elsewhere (16), followed by utilization of the denatured template in manual sequencing reactions using a Sequenase DNA sequencing kit (version 2.0) as described by the manufacturer (Amersham).

Products of primer extensions and sequencing reactions were resolved in 7% denaturing polyacrylamide gels, dried, and exposed to phosphor screens. Products were analyzed using a PhosphorImager.

ATR and competition assays. ATR and competition assays were conducted as previously described (25). The output ratio from each in vivo and in vitro competition was corrected for any deviations in the inoculum ratio from a value of 1:1. The competitive index was calculated by dividing the in vivo output ratio by the in vitro output ratio.

RESULTS

Identification of CadB and CadC homologs. In an earlier study, *cadA* was identified as an infection-induced gene of *V. cholerae* and shown to encode an inducible lysine decarboxylase (25). Searches against the *V. cholerae* genome sequence revealed the presence of *V. cholerae* DNA sequences 5' of *cadA* on the large chromosome coding for putative polypeptides with 67 and 27% identity to *E. coli* CadB and CadC, respectively. Due to the high degree of identity of the putative CadB and the similar location of its gene immediately upstream of *cadA*, we designated this gene *cadB*.

In *E. coli*, CadB acts as an antiporter that is responsible for the transport of cadaverine, the by-product of lysine decarboxylation, from the bacterial cell, concomitant with import of lysine (24, 30). The *V. cholerae* *cadB* ORF consists of 1,338 bp and encodes a predicted protein of 446 amino acids (46.8 kDa). It lies upstream of, and in a putative bicistronic operon with, the previously identified *cadA* (Fig. 1). To verify the requirement of the *cadBA* operon for lysine decarboxylation and cadaverine export, a plasmid insertion mutation within *cadB* was constructed; the resulting mutant was unable to decarboxylate lysine in a lysine decarboxylase indicator medium. Although this result does not distinguish between loss of CadB function and a polar effect of the mutation on *cadA*, given that *cadB* represents the only lysine/cadaverine antiporter homolog in the *V. cholerae* genome, and given data below that substantiate bicistronic transcription of *cadBA*, it is likely that both effects occur.

CadC has been shown to act as a positive transcriptional

TABLE 2. Sequences of primers used in this study

Primer	DNA sequence (5'-3')
AF1.....	AAGATCGTCGGTATGTTT
AR1.....	GTCGAAGTGGATGTGTTT
BA Δ F1.....	CCTATTCCTCCCAAGTCAAA
BA Δ F2.....	GATGTTATGTAAGCCAGCTTTACCCATA
BA Δ R1.....	GCTGGCTTACATAACATCTCCAATCG
BA Δ R2.....	CACCCAAGCCAGCACCAA
BF1.....	ACTGCACTAGCGTGTTTC
BR1.....	GTCAGCAGAACCGCATCG
Cad8F1.....	CTCATTGGCATTCTGTGC
Cad8R1.....	GACGCTTTTACCTAAAT
CadAOR.....	GTAACCGGCTTTTCTAA
CadAP2.....	GCAGTTGGCGAAGAGGCTCTTCTTTAAAGA
CadAPE.....	ACACACCCATGTGGTTCAAGATAGCGAAAA
CadBOF.....	GGACTGCGCCATTCTGTG
CadBPE.....	CCACTGCCCATCATGTTGCCAGCCACCACA
CadCCF.....	TGAAGAGCTGACCCGTAT
CadCF1.....	TCGGCAAGATAGGGAGGTC
CadCR1.....	CTTGGCGAGAAACATAGGG
CadCPE.....	CTATCTTGGCGATACAGCTTATTCTCATCA

FIG. 1. Schematic depiction of the *V. cholerae* *cadCBA* locus. Arrows above the designated ORFs depict transcriptional orientation and represent promoters based on 5' end mapping of mRNA species by primer extension. The locations of primers used for primer extension analysis are depicted by small arrows within the ORFs. Riboprobes used for RPAs and corresponding to the *V. cholerae*-specific component of the probe are represented by hatched boxes labeled probe 1 and probe 2. Relative sizes of *cadBA*, *cadB*, and *cadA* transcripts are represented by black lines beneath the ORFs. The locations of putative transcriptional terminators sequences (*t*) are also shown.

regulator of the *cadBA* operon in *E. coli* (24, 41). The putative *V. cholerae* *cadC* ORF consists of 1,560 bp and encodes a predicted protein of 519 amino acids (58.6 kDa). CadC lies upstream of, and in the same transcriptional orientation as, *cadB* but is separated from the *cadB* open reading frame by region of 699 bp (Fig. 1). The *V. cholerae* CadC also shares a high degree of identity with the N-terminal portion of *V. cholerae* ToxR (Fig. 2).

ToxR is a transcriptional activator localized within the inner membrane that is required for proper expression of virulence factors such as cholera toxin and the toxin coregulated pilus (reviewed in reference 37). The N-terminal domain of ToxR is cytoplasmic and encodes an OmpR-like DNA binding domain that is necessary for both positive and negative transcriptional regulation of ToxR-regulated genes. In addition, ToxR possesses a transmembrane segment and periplasmic domain that are believed to be involved in receiving and transmitting proper signals to elicit ToxR-mediated regulation (26, 27, 31). Based on amino acid sequence similarity at the N-terminal end of *V. cholerae* ToxR and CadC and a conserved hydrophathy profile throughout (data not shown), CadC is predicted to be a transmembrane transcriptional activator with topological and localization features similar to those of ToxR.

Transcription of *cadA* is regulated by CadC. Since CadC has previously been shown to be a positive transcriptional regulator of the *cadBA* operon in *E. coli* (24, 41), we sought to determine if CadC was similarly required for *cadA* expression in *V. cholerae*. *V. cholerae* strain DSM-V591, which contains an insertion mutation in *cadC*, was unable to decarboxylate lysine upon inoculation into lysine decarboxylase indicator medium whereas the wild-type parental strain did, suggesting that *cadC* was necessary for proper expression of *cadBA*.

To determine whether the inability of the *cadC* strain to decarboxylate lysine was due to an inability to properly regulate transcription of *cadA*, RPAs were conducted with probe 1,

a riboprobe specific for *cadA* transcripts (Fig. 1). Total RNA was collected from isogenic strains AC-V168 and DSM-V591 after growth in a rich medium at pH 7.0, 5.7, or 4.5. As previously reported (25), a large induction of *cadA* transcription occurs in wild-type *V. cholerae* cells upon exposure to pH 4.5 (Fig. 3A). In contrast, the *cadC* strain showed no induction of *cadA* transcription upon exposure to low pH. Of note, a basal level of *cadA* transcript is seen which appears both pH and CadC independent (Fig. 3A and B).

To determine if CadC was also necessary for the previously reported induction of *cadA* transcription upon exposure to low pH plus organic acids (25), similar RPAs were conducted using total RNA collected from AC-V168 and DSM-V591 grown in the presence of organic acids. As expected, AC-V168 showed an increase in *cadA* transcription when grown in medium at pH 5.7 and 4.5 that had been supplemented with organic acids (Fig. 3B and reference 25). In contrast, no increase in *cadA* transcript was evident in samples prepared from DSM-V591, indicating that CadC is required for organic acid-induced transcription of *cadA* at pH 5.7.

Transcription of the *cadBA* operon. Since *cadA* was shown to play an essential role in the ATR of *V. cholerae* (25) but not of *E. coli* (34), we sought to develop a better understanding of the differences in regulation of *cadA* expression in these two organisms. Previous studies of *E. coli* have shown that *cadA* exists as part of a bicistronic operon with *cadB*. Transcription of this operon occurs from a promoter that has been designated pCad (41). pCad has been shown to be stimulated in a CadC-dependent manner by low pH, oxygen limitation, and high lysine concentrations (2, 23, 29, 35, 39, 41). To determine if *cadB* and *cadA* were cotranscribed in *V. cholerae*, RPA analysis was performed using probe 2, a riboprobe designed to span the entire *cadB-cadA* intergenic region and portions of *cadB* and *cadA* (Fig. 1). Three possibilities for potential transcripts were considered: (i) bicistronic *cadB* and *cadA* transcripts, (ii) specific

<i>E. c.</i> CadC	36	L	L	V	F	F	A	Q	H	S	G	E	V	L	S	R	D	E	L	I	D	N	V	W	K	-	-	R	S	I	V	T	N	H	V	V	T	Q	S	I	S	E	L	R	K	S	L	K	D	N	D	83
<i>V. c.</i> CadC	36	L	L	H	F	L	A	E	H	A	G	E	V	F	G	R	E	E	L	I	Q	H	V	W	D	-	-	G	A	I	V	T	D	Q	V	V	T	Q	S	I	F	E	L	R	K	L	L	R	D	G	R	83
<i>V. c.</i> ToxR	54	I	L	W	L	L	A	Q	R	P	N	E	V	I	S	R	N	D	L	H	D	F	V	W	R	E	Q	G	F	E	V	D	D	S	S	L	T	Q	A	I	S	T	L	R	K	M	L	K	D	S	T	103
Consensus		.	L	.	.	.	A	.	.	.	.	E	V	.	.	R	.	.	L	.	.	.	V	W	.	.	.	.	V	.	.	.	.	T	Q	.	I	.	.	L	R	K	.	L	.	D	.	.				

<i>E. c.</i> CadC	84	E	D	S	P	V	Y	I	A	T	V	P	K	R	G	Y	K	L	M	V	P	V	I	W	Y	S	108
<i>V. c.</i> CadC	84	E	E	N	L	S	Y	V	V	T	V	P	K	R	G	Y	K	L	V	A	N	V	Q	R	L	D	108
<i>V. c.</i> ToxR	104	K	-	S	P	Q	Y	V	K	T	V	P	K	R	G	Y	Q	L	I	A	R	V	E	T	V	E	128
Consensus		.	.	.	.	Y	.	.	T	V	P	K	R	G	Y	.	L	.	.	V	.	.	.	.	.	.	

FIG. 2. Multiple sequence alignment of amino-terminal portions of *E. coli* (*E. c.*) CadC and *V. cholerae* (*V. c.*) CadC and ToxR. Identical amino acids are highlighted by a gray background; conservative changes are outlined in black. A consensus sequence is depicted below the multiple alignment.

FIG. 3. RPA for *cadA* transcription in wild-type (WT) and *cadC* mutant strains. Total RNA was prepared from bacteria grown in either low-pH media (A) or low-pH media supplemented with organic acids (B). The lanes corresponding to undigested probe represent the size of probe 1 used in the assays. Negative controls lacking bacterial RNA (–) show no protected bands. The protected *cadA* transcript is indicated. Relative molecular sizes are indicated on the left.

cadB and *cadA* transcripts, and (ii) both bicistronic and single gene-specific transcripts. Based on probe 2, we expected that bicistronic *cadBA* would result in protection of a 732-bp fragment. A monocistronic *cadB* transcript would result in protection of a fragment that spanned the 3' end of the *cadB* ORF (532 bp) as well as downstream sequences, depending on the site(s) of transcriptional termination. A monocistronic *cadA* transcript would result in a protected fragment that encompassed the 5' end of the *cadA* ORF (101 bp) as well as upstream sequences, depending on the site of transcriptional initiation.

Total RNA was prepared from AC-V168 grown at pH 7.0, 5.7, and 4.5, and RPAs using probe 2 revealed protected fragments that correspond to sizes expected for a bicistronic transcript. Therefore, *cadB* and *cadA* are transcribed as a bicistronic operon in *V. cholerae* (Fig. 4; depicted in Fig. 1). Upon shift to pH 4.5, a condition known to increase transcription of *cadA*, an increase in the amount of bicistronic *cadBA* message was seen. In addition, two protected fragments that correspond to the sizes expected of transcripts terminated at the end of the *cadB* ORF appeared. A minor band that would correspond to the expected size of a *cadA*-specific transcript also appeared (Fig. 4).

Since additional fragments of unexpected sizes were protected in each lane, we wished to confirm the identities of the indicated transcripts. An isogenic *V. cholerae* strain that contained a deletion of the entire *cadBA* coding sequence was constructed and used in similar RPAs. As shown in Fig. 4, deletion of the *cadBA* coding sequence resulted in loss of bicistronic *cadBA* and of monocistronic *cadB* and *cadA* bands, thus confirming that the indicated bands are specific for *cad* transcripts.

In Fig. 4, the protected fragments corresponding to the *cadB*-specific transcript, which would result from termination or processing downstream of the *cadB* ORF, was shown to be visible only upon shift to pH 4.5. Analysis of the nucleotide sequence immediately downstream of *cadB* revealed two large

FIG. 4. RPA for *cadBA* transcription in wild-type (WT) *cadC* mutant, and Δ *cadBA* strains. Total RNA was prepared from strains grown at the pH values indicated. The lanes corresponding to undigested probe represents the size of synthesized probe 2 used in the assays. Bands corresponding to undigested full-length probe and *cadBA*, *cadB*, and *cadA* transcripts are indicated. Molecular weight markers are shown with relative sizes. The undigested probe and molecular weight markers are shown from a shorter exposure, which was necessary to prevent overexposure in comparison to protected fragments.

inverted repeats, designated as IR 1 and IR 2, which could potentially code for factor-independent transcriptional terminators (see Fig. 6). IR 1 is a perfect inverted repeat whose predicted stem and loop structure is followed by three uracil nucleotides. IR 2 is an imperfect repeat; the spacing of the run of uracil nucleotides is four positions distal to the base of the predicted hairpin structure. Termination at IR 1 would result in a 562-bp *cadB* transcript, while termination at IR 2 would result in a 621-bp *cadB* transcript. These sizes are in good agreement with the sizes for *cadB* transcripts indicated in Fig. 4. The presence of only three uracil nucleotides at the base of IR 1 and the spacing of uracil nucleotides distal to the stem of IR 2 would be consistent with low efficiency termination at these sites (8).

Since we had shown that CadC was required for low-pH-induced transcription of *cadA* (Fig. 3), we investigated whether regulation by CadC occurred at the bicistronic *cadBA* promoter, the internal *cadA* promoter, or both. Total RNA was collected from DSM-V591, and RPA analysis using probe 2 was carried out. No significant increase in *cadBA* (or *cadB* or *cadA*) transcript was seen upon shift of DSM-V591 to lower pHs (Fig. 4). Furthermore, basal-level transcription of *cadBA* is CadC independent (Fig. 4). These results indicate that CadC regulation of both *cadA* and *cadB* expression occurs at the *cadBA* and *cadA* promoters (Fig. 4).

Identification of the transcription start sites of *cadC*, *cadB*, and *cadA*. Primer extension analysis was carried out to map the 5' termini of the *cadB*, *cadA*, and *cadC* transcripts to begin to define the promoter elements within this region. Total RNA was extracted and used for primer extension as described in Materials and Methods. For *cadB*, two primer extension products separated by three nucleotides were detected using RNA collected after acid challenge (Fig. 5A). This suggests a transcriptional start site(s) that is located 96 to 99 bp upstream of the translational start codon. Putative promoter elements were deduced according to the apparent transcriptional start site(s). The predicted –35 element (TATTTT) bears little resemblance to the *E. coli* σ^{70} consensus sequence (TTGACA). In

FIG. 5. (A) Primer extension analysis of *cadB* transcripts. The two primer extension products obtained with primer CadBPE are depicted in lane 1. Lane 2 represents a negative control where no RNA was added. The sequencing ladder obtained with the same primer is shown to the left. The +1 sites are indicated by arrows, and a likely -10 box is shown. The length of exposure of the sequencing ladder was reduced to prevent overexposure in comparison to primer extension products. (B) Nucleotide sequence of the *cadB* promoter region. Direct (DR) and inverted (IR) repeats are indicated by arrows. Transcription start sites are indicated by asterisks, and a likely -10 and a -35 site are underlined. The probable ribosomal binding site (RBS) is shaded, and the translational start is indicated.

addition, the -10 region (TACTCT) varies from the consensus (TATAAT) by three bases (Fig. 5B). Interestingly this -10 region shows greater similarity to the σ^S consensus sequence (TATACT) in that it varies by only two nucleotides. A mutation in *rpoS* that encodes σ^S , though, has previously been shown not to affect *cadA* transcription under the tested conditions (25). The predicted weakness of these -10 and -35 elements may account for the low level of basal transcription seen in the absence of the positive regulator CadC.

Analysis of the region upstream of the *cadB* transcription start sites revealed regions rich in A and T nucleotides. Two direct repeats of TGATAT₅NG are located starting at position -180, and two smaller direct repeats of T₇NG overlap the -35 region (Fig. 5B). These repeats represent potential CadC binding sites and show striking similarity to A/T-rich regions that ToxR has been shown to bind (7, 14, 20, 27). In particular, the TGATAT₅NG repeat is similar to the consensus repeat sequence TG(a/T)₃TTTNN bound by ToxR within the *ompT* promoter (20).

Primer extension of *cadA* transcripts was conducted using two separate primers, and in each case a single primer extension product was detected (Fig. 6A and data not shown). This

probable transcriptional start site is located 73 bp upstream of the translational start codon. The predicted -35 element (TTGACC) differs from the *E. coli* σ^{70} consensus sequence by only one nucleotide, but the -10 region (CAGCTT) bears little similarity to the consensus (Fig. 6B). Comparison of these regions to consensus sequences utilized by other sigma factors revealed no other significant sigma factor motifs. Two inverted repeats of 8 and 11 bp are found upstream of the *cadA* translational start site and, as mentioned above, potentially represent the site(s) of inefficient termination of transcripts originating from the *cadB* promoter.

A single primer extension product was detected for *cadC* (Fig. 7A). This product suggests a transcriptional start site that is located 75 bp upstream of the translational start codon. A poorly conserved -35 element was predicted (TAGGTG), but a consensus -10 was evident (Fig. 7B).

CadC does not regulate other genes required for acid tolerance or genes essential for virulence. Since CadC acts as a transcriptional regulator of the *cadBA* operon and shares a high degree of similarity to ToxR, which directly or indirectly regulates as many as 17 genes, we sought to determine whether CadC regulates additional pathways, other than lysine decar-

FIG. 6. (A) Primer extension analysis of *cadA* transcript. The primer extension product obtained with primer CadAPE is depicted in lane 2. Lane 1 represents a negative control where no RNA was added. The sequencing ladder obtained with the same primer is shown to the right. The +1 site is indicated by an arrow, and a likely -10 box is shown. The length of exposure of the sequencing ladder was reduced to prevent overexposure in comparison to primer extension products. (B) Nucleotide sequence of the *cadA* promoter region. The transcription start site is indicated by an asterisk, and a likely -10 and a -35 site are underlined. IR 1 and IR 2 are indicated by arrows. The probable ribosomal binding site (RBS) is shaded, and the translational start is indicated.

boxylation, that are required for acid tolerance. This possibility was tested by assessment of the ability of DSM-V591 to undergo an attenuated ATR. We reasoned that if CadC regulated additional pathways required for ATR, the ATR defect exhibited by this *cadC* strain should be more severe than the ATR defect exhibited by a *cadA* strain. ATR assays using AC-V168 (wild type), DSM-V591 (*cadC*), and DSM-V376 (*cadA*) were performed as previously described (25). All three strains, when unadapted, were quickly killed when exposed to pH 4.5 (data not shown). Acid-adapted AC-V168 was able to mount a productive ATR and showed complete survival out to 60 min (Fig. 8). However, both DSM-V591 and DSM-V376 exhibited attenuated ATRs, and the rates of acid killing for both of these adapted strains were virtually identical (Fig. 8). Note that the rates of death of adapted DSM-V376 and DSM-V591 were not as severe as that for the unadapted wild-type strain, suggesting that some acid adaptation is occurring in these mutant strains. Similar results were obtained in organic ATR assays (data not shown). The sum of these experiments supports the hypothesis that CadC does not regulate additional genes (besides *cadBA*) that are required for the ATR of *V. cholerae*.

The ability of DSM-V591 (*cadC*) to colonize the infant mouse intestine was tested in a competition assay. DSM-V591 and the virulent, isogenic strain AC-V66 were mixed to attain an input ratio of approximately 1:1, and the mixture was used in competition assays as previously described (25). DSM-V591 exhibited no detectable defect in colonization, giving a competitive index of 0.96, which is essentially identical to the com-

petitive index reported for a *cadA* mutant (25). These results suggest that in this model system, CadC is not required for regulation of factors essential for *V. cholerae* intestinal colonization.

DISCUSSION

The *cadA* gene of *V. cholerae* was previously identified as an infection-induced gene in two different animal models of *V. cholerae* (25). In vitro transcription analysis revealed that the gene was induced by low pH, high lysine concentration, and oxygen limitation. It was shown that *cadA* plays an essential role in the ATR of *V. cholerae*. Additionally, it was found that *V. cholerae* cells that were acid adapted prior to intragastric inoculation into animals greatly outcompeted unadapted cells, suggesting interesting implications for ATR in the epidemic spread of *V. cholerae*. In the present study, we examined the gene arrangement, transcription, and regulation of the *cadA* locus in more detail. The regulation of *cadA* has not been investigated in *S. enterica* serovar Typhimurium, the only other organism known to require lysine decarboxylation by *cadA* for ATR (30).

We found that *V. cholerae* contains *cadB* and *cadC* homologs, which are linked to *cadA* and arranged similarly as in *E. coli*. CadB in *E. coli* functions as a lysine/cadaverine antiporter, and its structural gene, *cadB*, is transcribed in a bicistronic operon with *cadA*. The *cadBA* operon is positively regulated by CadC (2, 23, 29, 35, 39, 41). RPA analysis revealed that *cadB* and *cadA* are also transcribed as a bicistronic operon

FIG. 7. (A) Primer extension analysis of *cadC* transcript. The primer extension products obtained with primer CadCPE are depicted in lanes 2 and 3. Lane 3 represents threefold less RNA than lane 2. Lane 1 represents a negative control where no RNA was added. The sequencing ladder obtained with the same primer is shown to the right. The +1 site is indicated by an arrow, and a likely -10 site is shown. The length of exposure of the sequencing ladder was reduced to prevent overexposure in comparison to primer extension products. (B) Nucleotide sequence of the *cadC* promoter region. The transcriptional start is indicated by an asterisk, and a likely -10 and a -35 site are underlined. The probable ribosomal binding site (RBS) is shaded, and the translational start is indicated.

in *V. cholerae*. However, unlike in *E. coli*, *cadB*- and *cadA*-specific transcripts are also present in *V. cholerae*.

Neely and Olson (29) previously determined the kinetics of *cadBA* expression in *E. coli* and found that a *cadBA* transcript

is not detectable at neutral pH but becomes apparent upon shift to pH 5.8. Similarly, in *V. cholerae*, *cadBA* transcription was greatly increased upon shift from neutral to acidic pH, although pH 4.5 was necessary to see induction (or pH 4.5 and 5.7 plus organic acids [data not shown]). In addition, *cadA* monocistronic transcript appears upon shift to low pH. Together, these results suggest that our previous demonstration of low-pH induction of *cadA* transcription (25) occurs at the level of increased bicistronic *cadBA* and monocistronic *cadA* mRNA. pH-regulated induction of *cadBA* in *E. coli* is completely dependent on CadC in that a *cadC* null mutant does not express *cadBA* under any conditions tested (28). Unlike the case for *E. coli*, though, *V. cholerae* shows basal-level expression of *cadBA* transcript at neutral pH. In addition, this basal-level transcription is CadC independent, as mutations in *cadC* resulted in no decrease in transcription at any pH tested.

The location of *cadC* adjacent to *cadBA* on the *V. cholerae* large chromosome, and the similarity of the predicted CadC primary sequence to that of *E. coli* CadC, suggested that CadC might act as a transcriptional regulator required for low-pH induction of *cadBA*. Indeed, a greater than fivefold increase in *cadBA* mRNA was detected upon exposure of wild-type cells to low pH, and this increase was completely ablated in a *cadC* mutant. Again, however, basal-level transcription of *cadBA* still occurs in a *cadC* mutant, suggesting that functional lysine decarboxylase may be produced at a low level.

What might be the role of low constitutive levels of CadA and CadB in *V. cholerae*? Amino acid decarboxylases contribute to either biosynthetic or biodegradative pathways and have been shown to play important roles in polyamine synthesis or pH homeostasis, respectively (23, 24, 30). Biosynthetic amino acid decarboxylases are generally expressed constitutively, while biodegradative amino acid decarboxylases require low-pH induction for expression (3, 23, 24, 35, 38). *E. coli* is known to possess both biosynthetic and biodegradative lysine decarboxylases, Lcd and CadA, respectively. Lcd is responsible for the constitutive, low-level biosynthesis of cadaverine from lysine (17, 19). However, after acid induction, the bulk of lysine decarboxylase activity is due to the induction of *cadA* (23, 29). Extensive searches conducted against the *V. cholerae* genome sequence reveal no other potential lysine decarboxylase-encoding genes, suggesting that CadA is the sole enzyme responsible for the decarboxylation of lysine by *V. cholerae* (D. S. Merrell and A. Camilli, unpublished data). We hypothesize that the CadC-independent, basal-level expression of *cadBA* results in

FIG. 8. ATR assays of wild-type (WT; AC-V168), *cadA* (DSM-V376), and *cadC* (DSM-V591) *V. cholerae* strains. Strains were acid adapted or unadapted as described in Materials and Methods, and percent survival was calculated as a function of time after resuspension of bacteria in acid challenge medium, pH 4.5.

low levels of lysine decarboxylase necessary for biosynthetic decarboxylation, but at a level that is undetectable in our broth indicator medium assay. Upon exposure of *V. cholerae* to low pH, oxygen limitation, and high lysine concentrations, high levels of CadA and CadB are expressed in a CadC-dependent manner to assist with maintenance of pH homeostasis.

The N-terminal domain of CadC shares a high degree of identity with the N-terminal DNA binding domain of ToxR. ToxR is responsible for transcriptional activation of the ToxR/ToxT virulence gene regulon. Upon receiving unknown signals in the host intestine, ToxR stimulates transcription of *toxT* and several other genes; ToxT subsequently acts as a major transcription factor for a large set of virulence genes (37). *V. cholerae* also contains TcpP, an additional inner membrane protein that shares homology with ToxR in both sequence and function (6, 12). Recent studies have shown that both TcpP and ToxR are required for maximal expression of ToxT (12). *toxR* and *tcpP* are cotranscribed with *toxS* and *tcpH*, respectively; ToxS and TcpH are inner membrane proteins required for maximal activity of ToxR and TcpP, respectively (9, 12, 32). The *V. cholerae* and *E. coli* CadC proteins, though similar to ToxR in their N-terminal domains and cellular locations, appear not to require a ToxS-like component for activity (20). This suggests that the *V. cholerae* CadC protein is independently capable of receiving and transmitting signals. However, the possibility remains that an unidentified protein is capable of associating with CadC and facilitating its function.

It has long been known that the ability to respond and adapt to low-pH environments is an important aspect of the life cycle of many pathogenic organisms that colonize within the gastrointestinal tract. For instance, mutant strains of *S. enterica* serovar Typhimurium, *Listeria monocytogenes*, and *Helicobacter pylori* that are compromised in their ability to survive acid exposure have been shown to exhibit attenuated virulence (4, 10, 22, 36). Interestingly, the CadC homolog of *S. enterica* serovar Typhimurium was previously identified using in vivo expression technology as an infection-induced gene expressed within both the small intestines and spleens of BALB/c mice (13). The fact that two genes within the *cad* locus have been shown to be upregulated in two pathogens (*V. cholerae* and *S. enterica* serovar Typhimurium) upon entry into the host suggests that homologs of these genes may play crucial roles in the survival and adaptation of other pathogenic organisms as well.

Studies of the mechanism of transcriptional regulation by ToxR have shown that it binds to promoter elements exhibiting high degrees of A/T richness (7, 14, 20, 27). Analysis of the region upstream of the *cadB* promoter revealed the presence of direct repeats that are strikingly similar to ToxR-recognized elements in the *ompT*, *ompU*, *ctxAB*, and *toxT* promoters (20). Despite this sequence similarity, we found that a *toxR* null mutant shows normal levels of *cadA* expression (25), suggesting that ToxR does not regulate transcription of the *cad* locus under the conditions tested. Instead, these findings suggest that ToxR and CadC recognize very similar A/T-rich sequences but with a high degree of fidelity.

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