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Structural Analysis of the *acfA* and *acfD* Genes of *Vibrio cholerae*: Effects of DNA Topology and Transcriptional Activators on Expression

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The *Vibrio cholerae acfA, B, C,* and *D* genes are involved in the synthesis of a colonization factor; their expression is under the control of ToxR, the cholera toxin transcriptional activator. By a combination of Southern blot analysis, cloning, and nucleotide sequence analysis, we determined that the *acf* genes are clustered on a 5-kb region, the *acfA* and *acfD* genes are transcribed divergently, and the translation start sites of the two genes are separated by only 173 bp. Expression from the *acfA* and *acfD* promoters in *V. cholerae* was studied by using *acfA:phoA* translational and *acfD-lacZ* transcriptional fusions; when carried by the chromosome, the *acfA-acfD* intergenic region flanked by the two reporter genes was found to contain the *cis*-acting element(s) necessary for the environmental regulation of the two promoters. However, this regulation was almost completely abolished when the same construction was carried by a low-copy-number plasmid. These results suggested that differences in DNA topology between the plasmid versus the chromosomal constructs might influence the expression of the *acfA* and *acfD* promoters. Support for this conclusion was obtained by showing that ToxR-dependent but not basal expression of both promoters was strongly inhibited by nalidixic acid and novobiocin, two DNA gyrase inhibitors, suggesting that the activation of these promoters is affected by changes in DNA supercoiling. Expression of the *acfA* and *acfD* promoters was also investigated in the heterologous host *Escherichia coli* harboring plasmids expressing either ToxR or ToxT, two transcriptional activators of the *V. cholerae* virulence genes. ToxR activated the *acfD* promoter 2.5-fold but inhibited the *acfA* promoter 2-fold. In contrast, the expression of the *acfA* promoter was activated 10-fold and that of the *acfD* promoter was activated 3-fold by ToxT, supporting the previously proposed cascade model for organization of the ToxR regulon.

Vibrio cholerae is the causative agent of the diarrheal disease Asiatic cholera. This diarrhea is largely induced by the action of cholera toxin, a heat-labile enterotoxin composed of two different subunits encoded by the *ctxA* and *ctxB* genes (4, 14). In addition, *V. cholerae* adheres to and colonizes the small intestine of the host, a virulence property that has been associated with the synthesis of the toxin-coregulated pilus (TCP) (8, 23, 28).

Expression of the *ctxAB* operon is under the control of the transcriptional activator encoded by the *toxR* locus (17). This locus is composed of two genes, *toxR* and *toxS*, that are clustered in an operon (16). *toxR* encodes a transmembrane DNA-binding protein that recognizes a heptanucleotide sequence tandemly repeated in the *ctx* regulatory region (19). The product of *toxS*, a membrane protein, was shown to act in conjunction with ToxR to activate *ctxAB* expression (16).

The ToxR regulon, defined as the set of genes whose expression is under the control of ToxR, comprises, in addition to *ctxAB*, the *tcp* genes required for the production of the TCP, the tag genes (ToxR-activated genes), which have not yet been associated to a definite virulence property, and the *acfA, B, C,* and *D* genes involved in the synthesis of an accessory colonization factor (22, 23). These genes were characterized by Tn*phoA* mutagenesis of the *V. cholerae* chromosome and the screening for the active alkaline phosphates hybrid proteins whose expression was modulated by the environmental growth conditions known to regulate toxin production. In addition, the genes were shown to be under the direct or indirect control of ToxR, inasmuch as they were not expressed in a *toxR* mutant (23). Recently, a third regulatory gene, *toxT*, which can directly activate several ToxR-regulated Tn*phoA* fusions that cannot be activated by ToxR alone, was identified (2). The *toxT* gene product is an AraC homolog whose expression is controlled by ToxR and which therefore occupies a downstream position in the regulatory cascade controlling virulence in *V. cholerae* (1a, 2).

To understand the structure and regulation of expression of the *acf* genes, we determined the organization of the *acf* cluster and cloned the *acfA:phoA* fusion from *V. cholerae* KP9.62. Expression from the *acfA* and *acfD* divergent promoters was studied by using *acfA:phoA* and *acfD-lacZ* fusions. In *V. cholerae*, whereas expression of these two promoters was environmentally regulated and under the control of ToxR when present in the chromosome, both the regulation and the ToxR control were lost when the genes were expressed from a plasmid. Our analysis suggests that DNA topology may influence the regulation and expression of these two virulence genes.

MATERIALS AND METHODS

Bacterial strains and growth media. Derivatives of *V. cholerae* El Tor O395 and *Escherichia coli* K-12 were maintained at −70°C in LB medium (15) containing 25%

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(vol/vol) glycerol or on LB plates. Antibiotics were used at the following concentrations: ampicillin, 50 mg/ml; chloramphenicol, 30 mg/ml; kanamycin, 30 mg/ml; streptomycin, 100 mg/ml; tetracycline, 15 mg/ml. *E. coli* GT869 [*thrB1004 pro thi rpsL hsdS lacZΔM15* F'(*lacZDM15 lacI*^{q} *traD36 proA*$^+$ *proB*$^+$)] (20) was used for cloning experiments. *E. coli* SM10 (*thi thr leu tonA lacY supE recA*::RP4-2Tc::Mu Km) (26) was used to transfer plasmids to *V. cholerae* by conjugation. *E. coli* KS272 (F$^-$ Δ*lacX74 galE galK thi rpsL* Δ*phoA*) (27) was used to assay the alkaline phosphatase activity expressed from the recombinant plasmids. *E. coli* MC1061 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK hsdR rpsL*] (1) was used as the recipient for the integration of pVC19 into the *E. coli* chromosome. *V. cholerae* O395 Sm is a streptomycin-resistant derivative of wild-type O395, and *V. cholerae* JJM43 is a *toxR* derivative of O395 Sm (28). Construction of O395 Sm derivatives carrying Tn*phoA* insertions in *acfA* (KP9.62), *acfB* (KP3.51), *acfC* (KP3.44), *acfD* (KP8.11), *ctxA* (KP1.25), and *tcpA* (KP9.79) was described previously (23).

Molecular cloning procedures. Plasmid DNA purification, DNA restriction and separation by gel electrophoresis, transfer and hybridization, and ligation and transformation of *E. coli* were performed by the standard methods of Maniatis et al. (12). Nucleotide sequences were determined by the dideoxy-chain termination procedure (24) on double-stranded plasmid DNA.

β-Galactosidase and alkaline phosphatase assays. β-Galactosidase activity was assayed as detailed by Miller (15) with the substrate *o*-nitrophenol-β-D-galactoside; alkaline phosphatase activity was assayed as detailed by Peterson and Mekalanos (23) with the substrate *p*-nitrophenyl phosphate.

RESULTS

Characterization of the Tn*phoA* insertion sites in the *acf* genes. The Tn*phoA* insertions in the *acfA*, *B*, *C*, and *D* genes were previously mapped to a 24-kb *Xba*I fragment (23). To further characterize the Tn*phoA* insertions in the *acf* genes, we performed a Southern blot analysis with two restriction enzymes on the chromosomal DNA isolated from these mutants. Chromosomal DNAs from KP9.62 (*acfA*::Tn*phoA*), KP3.51 (*acfB*::Tn*phoA*), KP3.44 (*acfC*::Tn*phoA*), and KP8.11 (*acfD*::Tn*phoA*) were digested by *Bam*HI, which cuts once in Tn*phoA*, and by *Bam*HI plus either *Eco*RV, *Sca*I, *Stu*I, or *Xba*I, restriction enzymes that do not recognize the transposon sequence. The probe used to screen the Southern blot was the 1.6-kb *Hin*dIII-*Bam*HI fragment of Tn*phoA* (Fig. 1); this DNA fragment contains part of IS*50* and therefore hybridizes to Tn*phoA* on both sides of the *Bam*HI site, giving a strong hybridization signal with the fragment containing the *phoA* sequence upstream from the *Bam*HI site and a lighter signal with the fragment located downstream from the *Bam*HI site (23).

The sizes of the upstream *Bam*HI, *Bam*HI-*Stu*I, and *Bam*HI-*Xba*I fragments increased from KP3.51 (*acfB*::Tn*phoA*) to KP3.44 (*acfC*::Tn*phoA*) and KP8.11 (*acfD*::Tn*phoA*), confirming that the Tn*phoA* insertions are in the same orientation in these three strains (Fig. 1). In contrast, the pattern obtained for the downstream fragments (i.e., those giving a lighter hybridization signal) in KP9.62 (*acfA*::Tn*phoA*) was similar to that obtained for the upstream fragments in KP8.11 (*acfD*::Tn*phoA*), suggesting that the Tn*phoA* insertions are in opposite orientations in KP9.62 and KP8.11. A detailed analysis of these results led to the restriction map of the *acf* cluster shown in Fig. 1. Since the Tn*phoA* insertions in *acfB* and *acfC* are in the same orientation and only 1 kb apart, the *acfB* and *acfC* genes might belong to the same operon. The *acfA* gene is located between *acfC* and *acfD* and is transcribed in the opposite orientation. It thus appears that the *acf* genes span a 5-kb region and belong to at least three transcription units.

Cloning of the *acfA*:*phoA* fusion. Since the Tn*phoA* insertions in *acfA* and *acfD* are localized in opposite orientation and on the same side of a *Bam*HI site that should be located within the *acfD* coding sequence (Fig. 1), we decided to clone the 5.4-kb *Bam*HI fragment from KP9.62; this fragment should contain 0.4 kb of DNA upstream from the *acfA*:*phoA* junction as well as 5 kb of Tn*phoA* (including the *aphA-2* gene confering resistance to kanamycin). KP9.62 chromosomal DNA was digested by *Bam*HI and ligated with *Bam*HI-linearized pUC18 DNA (30), and the ligation mixture was used to transform *E. coli* GT869; transformants were selected on LB plates containing ampicillin and kanamycin. The recombinant plasmid pVC10 thus obtained (Fig. 2) was used to transform the *E. coli phoA* strain KS272, and assay of the alkaline phosphatase activity indicated that this plasmid encodes an active PhoA hybrid protein (data not shown).

Nucleotide sequence of the *acfA-acfD* intergenic region. The nucleotide sequence of the 0.4-kb DNA fragment located upstream from the *acfA*:*phoA* junction in pVC10 was determined from both strands of DNA and is presented in Fig. 3 along with the deduced N-terminal sequences of the AcfA and AcfD polypeptides.

The *acfA* open reading frame (ORF), identified as the one in frame with the *phoA* coding sequence, extends from nucleotide 331 to nucleotide 400 at the junction with Tn*phoA*. Within this ORF, the ATG codon at nucleotide 334 is preceded by a potential ribosome binding site (5'-AG GAG-3') and appears as the likely *acfA* translation start site. The AcfA part of the hybrid protein would thus be only 22 amino acid residues long, which is consistent with previous immunoblot analysis (23) that identified the AcfA:PhoA fusion to a polypeptide of about 49 kDa (of which 48 kDa make up the PhoA part).

Upstream from and in the opposite orientation to *acfA*, only one significant ORF, extending from nucleotide 262 to the *Bam*HI site (nucleotide 1), was detected. The Tn*phoA* insertion site in KP8.11 (*acfD*::Tn*phoA*) has been localized immediately upstream from this *Bam*HI site (Fig. 1), so this ORF should correspond to the *acfD* reading frame. The first potential translation start within the *acfD* ORF is the ATG codon at nucleotide 160, which is preceded by the sequence 5'-AGG-3'. This proposed translation start site is in line with the estimated size of 53 kDa for the AcfD:PhoA hybrid protein (23).

Examination of the N-terminal sequences deduced for the *acfA* and *acfD* gene products reveals the presence of stretches of hydrophobic and nonpolar amino acid residues that are likely to represent the signal sequences responsible for the membrane or periplasmic localization of the fusion proteins. Such a localization is assumed, since the AcfA:PhoA and AcfD:PhoA fusions are endowed with alkaline phosphatase activity (13). No statistically significant similarity between the N-terminal sequences of AcfA or AcfD and any protein sequence contained in the National Biomedical Research Foundation library was detected with the FASTP computer program of Lipman and Pearson (11). At the end of the AcfD putative signal sequence, we detected the motif Leu-X-Gly-Cys, which is common to the processing sites of lipoproteins (29). [^{3}H]palmitate labelling studies have confirmed that *acfD* encodes a lipoprotein (21, 22).

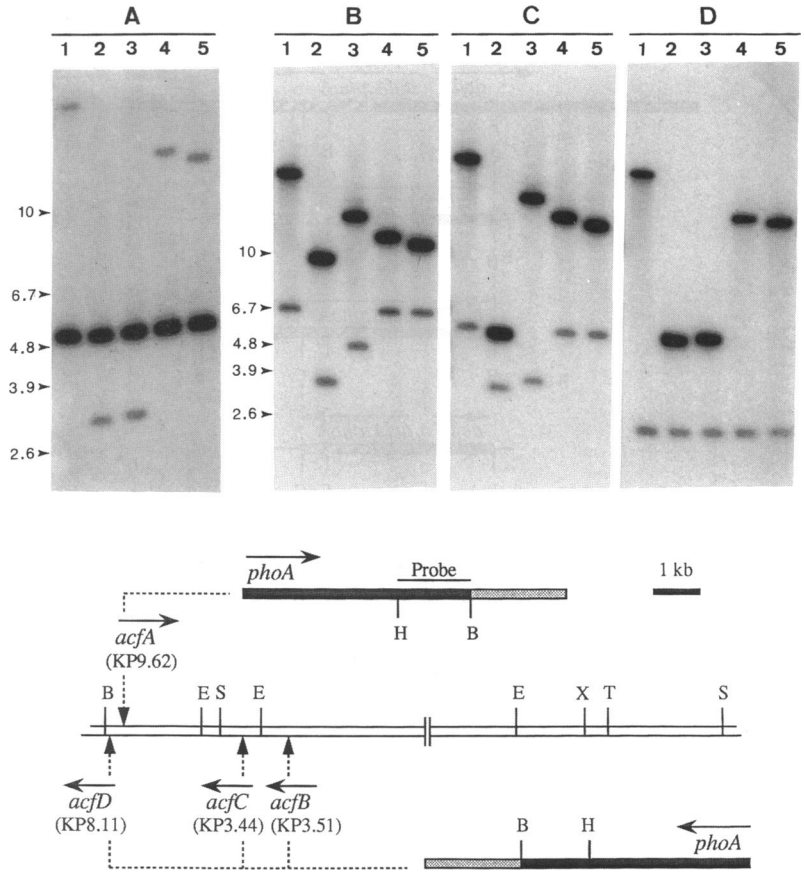

FIG. 1. Southern blot analysis of the TnphoA insertion sites in the acf genes. Chromosomal DNAs of strains KP9.62 (acfA::TnphoA) (A), KP3.51 (acfB::TnphoA) (B), KP3.44 (acfC::TnphoA) (C), and KP8.11 (acfD::TnphoA) (D) were digested by BamHI (lanes 1), BamHI plus EcoRV (lanes 2), BamHI plus ScaI (lanes 3), BamHI plus StuI (lanes 4), and BamHI plus XbaI (lanes 5), electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to the 1.6-kb HindIII-BamHI fragment of TnphoA. Since the probe contains part of IS50L, it hybridizes to TnphoA on both sides of the BamHI site, giving a strong hybridization signal with the upstream fragment and a lighter signal with the downstream fragment (upstream and downstream refer to the direction of transcription of phoA). Numbered arrows show the positions and sizes in kilobases of standard DNA fragments. The restriction map of the acf cluster deduced from this analysis is shown in the bottom part of the figure, where the TnphoA insertion sites are indicated by dashed arrows. The parts of TnphoA that give strong and light hybridization signals are represented by bold and dashed bars, respectively. Restriction sites: B, BamHI; E, EcoRV; H, HindIII; S, ScaI; T, StuI; X, XbaI.

Expression from the cloned *acfA* and *acfD* promoters in *V. cholerae*. To study the expression from the *acfA* promoter, the 5.4-kb BamHI insertion of pVC10 was cloned into the BamHI site of the low-copy-number vector pLAFR2 (Tet^r) (5) to give rise to the recombinant plasmids pVC11 and pVC12, representing the two possible orientations of the BamHI insertion (Fig. 2). These plasmids were then mobilized by conjugation into *V. cholerae* O395 Sm (wild type) and JJM43 (*toxR*), and the alkaline phosphatase activity was assayed in the transconjugants inoculated in LB at pH 6.5 or 8.4. These growth conditions are known to modulate the expression of the chromosomal *acfA:phoA* fusion by a factor of over 20-fold (23). Expression of the *acfA:phoA* fusion cloned in pVC11 was not regulated by the starting pH of the growth medium, and the levels of expression were almost the same in the *toxR* and wild-type strains (Table 1). Similar results were obtained with pVC12 (data not shown). This was quite unexpected, since the 0.4-kb DNA fragment located upstream from *phoA* contains the beginning of both the *acfA* and *acfD* genes and presumably the promoters and regulatory regions involved in the expression of these genes.

To investigate the expression from the *acfD* promoter, we constructed an *acfD-lacZ* transcriptional fusion on the plasmid already carrying the *acfA:phoA* translational fusion. The *lacZ* reporter gene (lacking its own promoter) from the pMLB1010 vector (25) was isolated as a SmaI-DraI fragment and cloned into the filled-in XbaI site located immediately downstream from the beginning of the *acfD* coding sequence on pVC11. The pVC16 plasmid thus constructed (Fig. 2), carrying the *acfA:phoA* and *acfD-lacZ* fusions, was then mobilized into O395 Sm (wild type) and JJM43 (*toxR*), and the β-galactosidase and alkaline phosphatase activities were assayed in transconjugants grown under either inducing (pH 6.5) or repressing (pH 8.4) conditions. Expression of the *acfD-lacZ* fusion was modulated by a factor of twofold in response to the starting pH of the growth medium (Table 1). This modulation was not observed in the *toxR* mutant, suggesting that it was under the control of ToxR. However, the amplitude of this regulation is much less than the 20-fold factor previously observed for the chromosomal *acfD:phoA* fusion (23).

Effect of the cloned *acfA-acfD* intergenic region on the

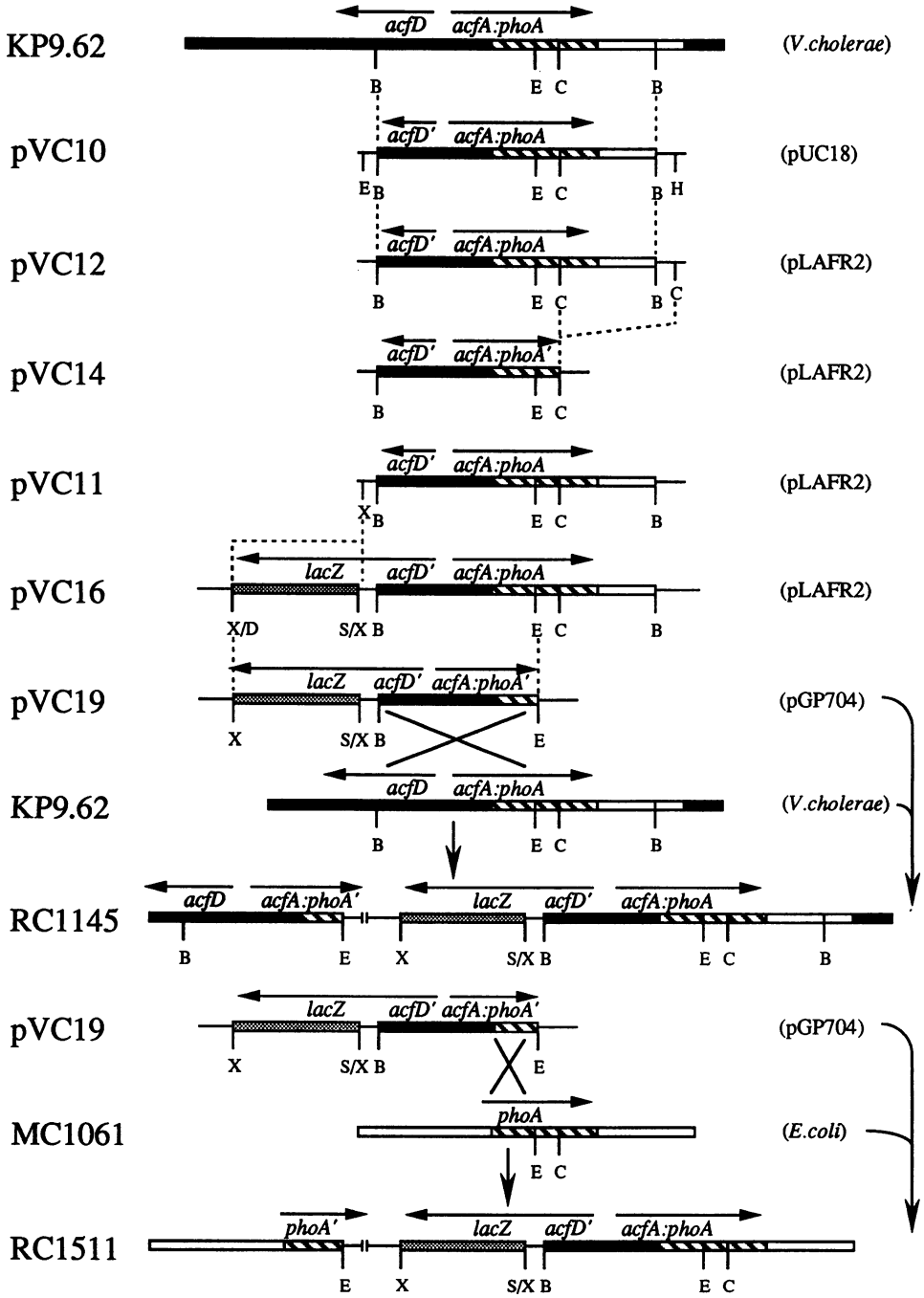

FIG. 2. Schematic representation of the chromosome- and plasmid-encoded *acfA:phoA* and *acfD-lacZ* fusions. The *Bam*HI fragment from strain KP9.62, carrying the *acfA:phoA* fusion and part of Tn*phoA*, was first cloned into pUC18 to give rise to pVC10. This *Bam*HI fragment was then cloned into pLAFR2 to construct pVC11 and pVC12. The pVC14 plasmid was obtained after digestion of pVC12 by *Cla*I and relegation, and pVC16 was constructed by inserting the *lacZ* reporter gene into the filled-in *Xba*I site of pVC11. The *Xba*I-*Eco*RI fragment of pVC16 was then cloned in between the *Xba*I and *Eco*RI sites of pGP704 to give pVC19. Homologous recombination between the identical sequences carried by the pVC19 plasmid and the chromosome of *V. cholerae* KP9.62 gave rise to strain RC1145. Strain RC1511 was similarly constructed by integration of pVC19 into the chromosome of *E. coli* MC1061. The *V. cholerae* DNA is shown by a bold bar, the *E. coli* and Tn*phoA* DNAs are shown by open bars, on which the *phoA* coding sequences are indicated by dashes, the *lacZ* reporter genes are indicated by grey bars, and the vector parts are indicated by thin lines. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I; X, *Xba*I. The shill in between two letters indicates that the corresponding restriction sites have been filled in during the construction. The positions and directions of transcription of *acfA*, *acfD*, *lacZ*, and *phoA* are indicated by arrows.

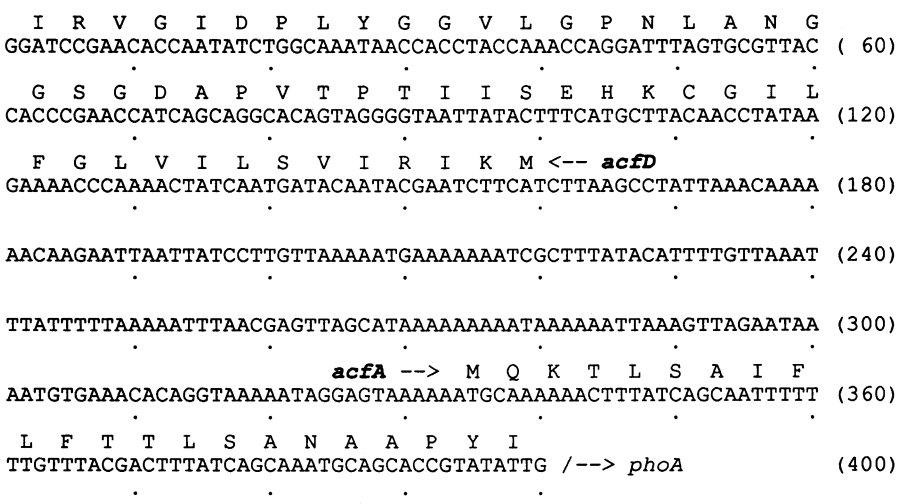

FIG. 3. Nucleotide sequence of the *acfA-acfD* intergenic region. The nucleotide sequence is shown from 5′ to 3′ in the direction of transcription of *acfA*, along with the deduced N-terminal sequences of the *acfA* and *acfD* gene products. *acfD* is oriented opposite to *acfA*, so the AcfD sequence should be read from right to left and from bottom to top.

expression of ToxR-regulated genes. The lack of regulation of the cloned *acfA* and *acfD* promoters could be due to a titrating effect of the plasmid on a transcriptional activator or repressor molecule(s). To test whether the presence of the *acfA-acfD* intergenic region cloned into pLAFR2 was interfering with the expression of chromosomal, ToxR-regulated genes, we constructed a deletion derivative of pVC12 that did not encode an active PhoA hybrid protein. The DNA of pVC12 was digested by *Cla*I and relegated to give rise to pVC14, in which most of the Tn*phoA* sequence, including the last 169 codons of *phoA*, has been deleted (Fig. 2). The pVC14 and pLAFR2 plasmids were then mobilized into *V. cholerae* strains harboring Tn*phoA* fusions in *acfA*, *acfB*, *acfC*, *acfD*, *ctxA*, or *tcpA*, and the alkaline phosphatase activity expressed from these chromosomally located fusions was assayed in cells grown under inducing (pH 6.5) or repressing (pH 8.4) conditions. No difference in alkaline phosphatase activities expressed from the *ctxA*:*phoA* and *tcpA*:*phoA* fusions between bacteria harboring pLAFR2 or pVC14 grown under either conditions was observed (Table 2). On the other hand, a 2.5-fold decrease in the expression of the *acfA*, *acfB*, *acfC*, and *acfD*:*phoA* fusions was observed in bacteria carrying pVC14 and grown under inducing conditions. These results indicate that pVC14 interferes with the expression of the *acf* genes, probably by titrating a transcriptional activator. However, such an effect does not account for the lack of activation of the cloned *acfA* and *acfD* promoters, since expression of the chromosomal *phoA* fusions is still regulated over a 10-fold range in the presence of pVC14.

Construction of a *V. cholerae* strain carrying the *acfA-acfD* intergenic region flanked by two reporter genes on the chromosome. To ascertain that the cloned *acfA-acfD* intergenic region carries all of the *cis*-acting sequence(s) necessary for the regulation of the *acfA* and *acfD* promoters, we reintroduced this fragment flanked by two reporter genes into the *V. cholerae* chromosome. First, the *Eco*RI-*Xba*I fragment of pVC16, carrying the 5′ part of *phoA*, the *acfA-acfD* intergenic region, and the entire *lacZ* reporter gene, was cloned between the *Eco*RI and *Xba*I sites of pGP704 (Amp^r) (22a), a derivative of the pJM703.1 suicide vector (18). The recombinant plasmid pVC19 thus constructed (Fig. 2) was mobilized into KP9.62 (*acfA*::Tn*phoA*), and the transconjugants were selected on LB plates containing ampicillin. Since pVC19 does not replicate, the Amp^r clones arose through

TABLE 1. Alkaline phosphatase and β-galactosidase activities expressed from the cloned *acfA*:*phoA* and *acfD-lacZ* fusions in *V. cholerae*[a]

Plasmid[b]	Fusion	*toxR*[c]	PhoA (U)		β-Gal (U)	
			pH 6.5	pH 8.4	pH 6.5	pH 8.4
pVC11	*acfA*:*phoA*	+	2,590	2,510	ND[d]	ND
pVC11	*acfA*:*phoA*	−	1,630	1,830	ND	ND
pVC16	*acfA*:*phoA* and *acfD-lacZ*	+	2,970	3,060	1,110	590
pVC16	*acfA*:*phoA* and *acfD-lacZ*	−	2,710	3,560	460	600

[a] Bacteria were grown at 30°C in LB at pH 6.5 or 8.4. Alkaline phosphatase and β-galactosidase (β-Gal) activities were assayed as detailed by Peterson and Mekalanos (23) and by Miller (15), respectively.
[b] See Fig. 2 for diagrams of pVC11 and pVC16.
[c] O395 Sm and JJM43 were used as the *toxR*$^+$ and *toxR* mutant *V. cholerae* strains, respectively.
[d] ND, not determined.

TABLE 2. Alkaline phosphatase activity expressed from chromosomal, ToxR-regulated *phoA* fusions in *V. cholerae* strains carrying the *acfA-acfD* intergenic region on a plasmid[a]

Strain	Fusion	PhoA (U)			
		pH 6.5		pH 8.4	
		pLAFR2	pVC14[b]	pLAFR2	pVC14
KP9.62	*acfA*:*phoA*	1,020	430	40	35
KP3.51	*acfB*:*phoA*	240	140	20	10
KP3.44	*acfC*:*phoA*	400	175	25	5
KP8.11	*acfD*:*phoA*	420	180	23	5
KP1.25	*ctxA*:*phoA*	790	680	80	80
KP8.56	*tcpA*:*phoA*	1,280	1,080	70	80

[a] Bacteria were grown in LB pH 6.5 or pH 8.4. Alkaline phosphatase activity was assayed as detailed by Peterson and Mekalanos (23).
[b] See Fig. 2 for a diagram of pVC14.

TABLE 3. Alkaline phosphatase and β-galactosidase activities expressed from the chromosomally encoded *acfA:phoA* and *acfD-lacZ* fusions in *V. cholerae*[a]

Strain[b]	Fusion	PhoA (U)		β-Gal (U)	
		pH 6.5	pH 8.4	pH 6.5	pH 8.4
KP9.62	*acfA:phoA*	1,360	80	40	45
RC1145	*acfA:phoA* and *acfD-lacZ*	1,240	90	620	90

[a] Bacteria were grown at 30°C in LB at pH 6.5 or 8.4 Alkaline phosphatase and β-galactosidase (β-Gal) activities were assayed as detailed by Peterson and Mekalanos (23) and by Miller (15), respectively.
[b] See Fig. 2 for diagrams of strains KP9.62 and RC1145.

TABLE 4. Effect of nalidixic acid on the expression of the chromosomal *acfA:phoA* and *acfD-lacZ* fusions in *V. cholerae* RC1511[a]

NAL (ng/ml)	PhoA (U)		β-Gal (U)	
	pH 6.5	pH 8.4	pH 6.5	pH 8.4
0	1,240	90	620	90
20	1,025	95	470	80
40	900	100	435	90
60	450	120	260	105
80	65	100	55	75

[a] Bacteria were grown at 30°C in LB pH 6.5 or 8.4 in the presence or absence of nalidixic acid (NAL), as indicated. Alkaline phosphatase and β-galactosidase (β-Gal) activities were assayed as detailed by Peterson and Mekalanos (23) and by Miller (17), respectively.

homologous recombination between the identical DNA fragments carried by the chromosome and the plasmid (Fig. 2). As a consequence, the 0.4-kb fragment carrying the *acfA-acfD* intergenic region is present twice in the chromosome of the recombinant strains; one copy is flanked by the *phoA* and *lacZ* reporter genes, and the other copy is located in between the wild-type *acfD* gene and a truncated *acfA:phoA* fusion that does not encode an active alkaline phosphatase hybrid protein. The alkaline phosphatase and β-galactosidase activities of one of these transconjugants (RC1145) and the parental KP9.62 strain, grown under inducing (pH 6.5) or repressing (pH 8.4) conditions, were assayed. Expression of both the *acfA:phoA* and *acfD-lacZ* fusions was regulated by the starting pH of the growth medium (Table 3). This result indicates that the 0.4-kb fragment between the reporter genes carries the *cis*-acting element(s) necessary for the regulated expression of the *acfA* and *acfD* promoters. However, the *acfD-lacZ* fusion carried by RC1142 shows only a 7-fold regulation, compared with the 20-fold regulation with the original *acfD:phoA* fusion carried by KP8.11 (23). This minor difference may be due to a difference in the transcriptional and translational natures of the two respective fusions.

Effect of nalidixic acid on the expression of the *acf* genes. When carried by the chromosome, the 0.4-kb fragment upstream from *phoA* was found to contain the *cis*-acting sequence(s) involved in the regulated expression of the *acfA* and *acfD* promoters (Table 3), whereas this regulation was not observed when the same construction was carried by the pVC16 plasmid (Table 1). These results suggested that the activities of the *acfA* and *acfD* promoters might be sensitive to differences that may exist between the chromosome and the plasmid in the topology of the DNA. This led us to investigate the effect of nalidixic acid, a DNA gyrase inhibitor, on the expression of the chromosomal *acfA:phoA* and *acfD-lacZ* fusions in *V. cholerae* RC1145. Nalidixic acid dramatically decreased the expression of both fusions under inducing conditions, whereas it had no effect on their expression under repressing conditions (Table 4). Similar results were obtained in KP9.62 (*acfA:phoA*) with novobiocin, another inhibitor of DNA gyrase, when the starting pH or the NaCl concentration of the medium was used as a modulator (data not shown). These results suggest that the ToxR-dependent activation of the *acfA* and *acfD* promoters is sensitive to changes in DNA superhelicity.

Expression of the *acfA:phoA* and *acfD-lacZ* fusions in *E. coli*. In *V. cholerae*, expression of the *acf* genes is under the control of ToxR (23). To investigate whether the expression of the *acfA:phoA* and *acfD-lacZ* fusions could be activated by ToxR in *E. coli*, we first constructed an *E. coli* strain carrying the two reporter genes on the chromosome. The integrative plasmid pVC19 (*acfD-lacZ acfA:phoA'*) was mobilized by conjugation into *E. coli* MC1061 (*rpsL ΔlacZ phoA*$^+$), and the recombinants were selected on LB plates containing streptomycin and ampicillin. Integration of pVC19 should occur through homologous recombination between the *phoA* sequences carried by the plasmid and the chromosome, leading to the disruption of the *E. coli phoA* gene and the concomitant reconstruction of the *acfA:phoA* fusion (Fig. 2). The alkaline phosphatase activity expressed by the recombinant strains was not regulated by the phosphate concentration of the growth medium (data not shown), thereby confirming that the recombination had taken place within the *phoA* gene. One of these recombinants (RC1511) was then transformed by either pACYC184 or pVM25 (*toxR*$^+$) (16), and the alkaline phosphatase and β-galactosidase activities were assayed after growth of the transformants in LB at 30°C. ToxR led to a 2-fold decrease in the expression of the *acfA:phoA* fusion and to a 2.5-fold increase in the expression of the *acfD-lacZ* fusion. Although these are slight variations, the reciprocal effect of ToxR on the expression of the two reporter genes strongly suggests that ToxR binds to the *acfA-acfD* intergenic region.

The *toxT* locus has been recently identified through its ability to activate the expression of a number of ToxR-regulated genes, including the *ctx* genes, in *E. coli* (2). To test whether the expression of the *acfA* and *acfD* promoters could be activated by ToxT in *E. coli*, the pGJ2.3 plasmid (*toxT*$^+$) (1b) was used to transform RC1511; ToxT enhanced the expression of the *acfA:phoA* and *acfD-lacZ* fusions by 10- and 3-fold, respectively (Table 5). It thus appears that in *E. coli*, whereas both ToxR and ToxT activate the expression of the *acfD-lacZ* fusion by similar ca. threefold factors

TABLE 5. Alkaline phosphatase and β-galactosidase activities expressed from the *acfA:phoA* and *acfD-lacZ* fusions in *E. coli*[a]

Plasmid[b]	PhoA (U)[c]	β-Gal (U)[d]
None	165	55
pACYC184	150	50
pVM25 (*toxR*$^+$)	80	135
pGJ2.3 (*toxT*$^+$)	1,550	150

[a] The *acfA:phoA* and *acfD-lacZ* fusions are carried by *E. coli* RC1511 (Fig. 2).
[b] The *toxR* gene is expressed from pVM25, a pACYC184 derivative (16), and the *toxT* gene is expressed from pGJ2.3 (2).
[c] Alkaline phosphatase activity was assayed as detailed by Peterson and Mekalanos (23).
[d] β-Galactosidase (β-Gal) activity was assayed as detailed by Miller (17).

only ToxT significantly activates the expression of the *acfA*:*phoA* fusion.

DISCUSSION

In *V. cholerae*, expression of the *acfA*, *B*, *C*, and *D* genes is under the control of ToxR, and the Tn*phoA* insertions in these genes affect the colonization properties of the mutant strains (23). We report here a detailed analysis of the Tn*phoA* insertion sites in the *acfA*, *B*, *C*, and *D* genes, and we show that these genes span a 5-kb DNA fragment. Cloning and nucleotide sequence analysis of the 0.4-kb region located upstream from Tn*phoA* in KP9.62 (*acfA*:: Tn*phoA*) confirmed that the *acfA* and *acfD* genes are in opposite orientations; the translation start sites of the two genes are separated by only 173 nucleotides.

Recent work has shown that the *toxT* gene product plays a direct role in the activation of some ToxR-regulated genes that ToxR cannot directly activate (2). Given that transcription of *toxT* is dependent on ToxR, a regulatory cascade apparently is responsible for the control of genes in the ToxR regulon (2, 23). Therefore, to identify the transcriptional activator directly involved in the regulation of the *acfA* and *acfD* promoters, we investigated the effects of ToxR and ToxT on the expression of the *acfA*:*phoA* and *acfD-lacZ* fusions introduced into the *E. coli* chromosome. ToxR decreased the expression of the *acfA*:*phoA* fusion by 2-fold and increased the expression of the *acfD-lacZ* fusion by 2.5-fold. Even though the effect of ToxR on the *acfA* and *acfD* promoters in *E. coli* does not reproduce the regulation seen in *V. cholerae*, this effect is interesting in terms of ToxR binding ability, especially since the *acfA-acfD* intergenic region does not exhibit the tandemly repeated heptanucleotide 5′-TTTTGAT-3′, which was shown to be the target of ToxR in the *ctx* regulatory region (19). ToxT activated the expression of the *acfA*:*phoA* fusion by a 10-fold factor in *E. coli*. The amplitude of this activation is thus very similar to the regulation observed in *V. cholerae*, suggesting that ToxT is the principal component involved in the regulated expression from the *acfA* promoter. On the other hand, ToxT led to only a threefold increase in the expression of the *acfD-lacZ* fusion. Whether a combination of ToxR and ToxT, or of these and another factor yet to be identified, is required to fully activate the *acfD* promoter remains to be elucidated. Overall, these data continue to support a cascade model for the organization of the ToxR regulon in which ToxR activates the expression of the genes for ToxT and other downstream regulators that go on to activate genes that are not responsive directly to ToxR (1a, 2).

When present in the *V. cholerae* chromosome, the *acfA-acfD* intergenic region flanked by the *lacZ* and *phoA* reporter genes was shown to contain the *cis*-acting sequence(s) involved in the regulated expression of the *acfA* and *acfD* promoters. However, this regulation was not observed when the same construction was carried by the pVC16 plasmid, a derivative of the low-copy-number vector pLAFR2. The lack of regulation of the cloned *acfA* and *acfD* promoters was unexpected, since, using very similar constructions, we previously observed that the regulations of *ctxA*:*phoA* and *tagA*:*phoA* fusions were the same, whether these fusions were carried by the chromosome or cloned into pLAFR2; expression of the cloned *ctxA*:*phoA* and *tagA*:*phoA* fusions was 10-fold higher than that of the chromosomal fusions, under both inducing and repressing conditions (21). In the case of the *acfA*:*phoA* fusion, there is a 30-fold increase in expression of the plasmid versus the chromosomal fusion under repressing conditions. The lack of regulation of the plasmid-encoded *acfA*:*phoA* fusion thus appears to be a consequence of a constitutive expression. This is not due to a titrating effect of the cloned *acfA-acfD* region on repressor molecule(s), since the presence of pVC14 did not lead to a higher expression of the chromosomal *acf*:*phoA* fusions under repressing conditions. The high expression of the cloned *acfA* promoter is not due to the transcription from a vector promoter either, since, in pVC16, the *acfA* promoter is separated from the vector by the entire *lacZ* sequence (in the opposite orientation). We have also shown that, even though the cloned regulatory region has a slight titrating effect on activator molecule(s) necessary for the full expression of the *acf* genes, this titrating effect alone cannot account for the lack of regulation of the cloned *acfA* and *acfD* promoters.

These results suggested that the activities of the *acfA* and *acfD* promoters might be sensitive to differences in the topology of the DNA, such as differences in DNA supehelicity, that may exist between the chromosome and the plasmid. Indeed, induction of the chromosomal *acfA* and *acfD* promoters was abolished DNA gyrase inhibitors such as nalidixic acid and novobiocin. This leads us to propose that, when present on the chromosome, the *acfA* and *acfD* promoters require some ancillary factor(s) to be active, whereas, when carried by the plasmid, the *acfA-acfD* intergenic region might be in such a conformation that the two promoters are transcribed constitutively. The effect of plasmid location on the expression and regulation of the *acfA* and *acfD* promoters and the effect of nalidixic acid and novobiocin on the expression of these promoters when chromosomally located provide the first evidence that DNA supercoiling or DNA topology plays a role in the regulation of some *V. cholerae* virulence genes. However, conclusions based on the effects of gyrase inhibitors alone are unreliable because of the known pleiotropic effects of these drugs. Thus, to confirm our proposal, it will be necessary to obtain additional evidence for the role of supercoiling in *acfA* and *acfD* expression that will involve either direct measurement of chromosomal supercoiling levels or the isolation of second site mutations that affect supercoiling levels (3a).

Recently, the expression of several virulence genes of *Salmonella typhimurium* and *Shigella flexneri* and the pyelonephritis-associated pilus genes of *E. coli* were shown to be affected by either gyrase inhibitors or mutations in the gene encoding the H-NS histonelike protein (3, 6, 7). It has been proposed that environmental signals and mutations in H-NS cause changes in DNA supercoiling that directly affect the expression of virulence genes responding to stimuli such as growth phase, anaerobiosis, osmolarity, temperature, and pH (9, 10). Indeed, expression of the *V. cholerae* ToxR regulon is modulated by such environmental parameters (23). However, an important difference between the *acf* genes and the *ctxA* and *tagA* genes is that regulation of the latter two is unaffected by cloning into a plasmid (21). This does not preclude a role for DNA supercoiling in the regulation of these latter genes as well but suggests that there are different hierarchies in regulatory responses to global changes in DNA topology and to specific regulators (ToxR, ToxT). This hierarchical response might be involved in growth-phase-specific or temporal gene expression, where certain ToxR-regulated genes would be expressed optimally during an early stage in the infection cycle and others would be expressed during a late stage. Alternatively, environmental factors that affect DNA topology and the activity of regulatory proteins in a differential manner could serve to

finely regulate the expression of the appropriate subsets of ToxR-regulated genes at various microanatomical sites (e.g., intestinal lumen, mucus gel, epithelial cell surface, etc.).

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