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Characterization of Genes Regulated Directly by the VirR/VirS System in *Clostridium perfringens*[▽]

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Analysis of the complete sequence of the genome of *Clostridium perfringens* strain 13 resulted in identification of five genes, including *pfoA* (encoding theta toxin) and *vrr* (encoding VirR/VirS-regulated RNA), with consensus VirR-binding sequences upstream of the open reading frame (ORF), suggesting that expression of these genes may be regulated directly by the two-component VirR/VirS system. To test this possibility, we examined VirR/VirS system-mediated transcriptional regulation of three genes, *virT*, *ccp* (encoding alpha-clostripain), and *virU*, with the novel VirR-binding sequences. Northern analysis revealed that the steady-state levels (increases or decreases in the amounts of RNA expressed) of *virT*, *ccp*, and *virU* mRNAs were lower in a *virR* mutant strain than in the wild-type strain, as were the levels of the *pfoA* and *vrr* transcripts. The consensus VirR-binding sites were located similarly relative to the transcription start sites in the *virT*, *ccp*, and *virU* promoters. Mutation and overexpression analyses with *virT* and *virU* revealed that the *virT* gene product has a negative effect on expression of *pfoA* and *ccp*, whereas the *virU* gene product positively affects expression of *pfoA*, *virT*, *ccp*, and *vrr*. Nonsense and frameshift mutations in the *virT* or *virU* putative ORF did not affect the regulatory functions, suggesting that *virT* and *virU* may encode RNA regulators rather than proteins. These results suggest that a complex regulatory network, perhaps involving several regulatory RNA molecules, governs the expression of the VirR/VirS regulon in *C. perfringens*.

The gram-positive anaerobic bacterium *Clostridium perfringens* produces numerous extracellular toxins that are believed to play important roles in the pathogenicity of various diseases, including gas gangrene, which is also known as clostridial myonecrosis (9, 21). Because the toxins are thought to act synergistically in the development of gas gangrene (2), knowledge of the mechanisms that regulate expression of toxin genes is critical for understanding the pathogenesis of myonecrosis.

Bacterial two-component systems, consisting of a sensor histidine kinase and a response regulator, enable bacteria to respond to various environmental conditions through a phosphorelay between the sensor and the regulator. The two-component VirR/VirS system comprising the VirR response regulator and the VirS sensor protein is known to be involved in global regulation of the production of theta-toxin (also known as perfringolysin O), kappa-toxin (or collagenase), alpha-toxin (or phospholipase C), sialidase, protease, and hemagglutinin in *C. perfringens* (13, 24). The VirR/VirS system regulates the mRNA levels of *plc* (alpha-toxin), *pfoA* (theta-toxin), and *colA* (kappa-toxin) (4). Primer extension analysis revealed both VirR/VirS-dependent and independent promoters for *pfoA* and *colA* and a single VirR/VirS-dependent promoter for *plc* (4). The absence of a consensus binding site for phosphorylated VirR protein in the promoters of the *colA* and *plc* genes (4) suggests that complex regulatory networks might be involved in *C. perfringens* toxin production (26).

Four targets of the VirR/VirS system have been identified through differential display analyses. The VirR/VirS system was found to promote expression of *ptp* (encoding protein tyrosine phosphatase), *cpd* (encoding 2′,3′-cyclic nucleotide phosphodiesterase), and *hyp7* (encoding a hypothetical 7-kDa protein) (3) and to inhibit expression of the *ycgJ-metB-cysK-luxS* (*ygaG*) operon (3, 20). It was suggested previously that *hyp7* acts as a secondary regulator that positively regulates the levels of *colA* and *plc* mRNAs but not the level of *pfoA* mRNA (3). However, we reported previously that VirR/VirS-regulated RNA (VR-RNA) (encoded by *vrr*) transcribed from the Hyp7 coding region is a regulatory RNA that mediates the signal from the VirR/VirS system to control the expression of *colA*, *plc*, *ptp*, *cpd*, and *ycgJ-metB-cysK-luxS*, whereas *pfoA* is regulated directly by the VirR/VirS system (28). The VirR/VirS–VR-RNA cascade was also found to affect levels of plasmid-borne *cpb2* (encoding beta2 toxin) and *cna* (encoding a possible collagen adhesin) mRNAs positively and negatively, respectively (19).

Two repeated sequences have been found upstream of the *pfoA* promoter (4), and it was reported previously that the VirR protein binds independently to these two repeats (CCC AGTTNTNCAC) (6). Interestingly, a monomeric repeat similar but not identical to the *pfoA* VirR-binding site has also been found in the promoter of *vrr*, the gene encoding VR-RNA (28). A CCAGTTNNNCAC core motif was highly conserved in both genes. These findings suggest that the VirR protein may bind to the *vrr* promoter, activating transcription of VR-RNA, which in turn activates *colA* and *plc* transcription,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
C. perfringens strains		
13	Wild-type strain (type A)	14
TS133	Strain 13 *virR*::Tet^r	24
TS140	Strain 13 Δ*vrr* Em^r	28
TS190	Strain 13 *virT*::Em^r	This study
E. coli DH5α	*supE*44 Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Takara Bio Inc.
Plasmids		
pBT405	pJIR418 Ω(PstI 4.3-kb strain 13 genomic library) (*virR*$^+$ *virS*$^+$ complementation vector), Amp^r	Shimizu, unpublished data
pJIR418	*E. coli-C. perfringens* shuttle vector, Cm^r Em^r	29
pSB1031	pJIR418 Ω(PCR-amplified 637-bp fragment) (*vrr*$^+$ complementation vector)	4
pTS930	pJIR418 Ω(PCR-amplified 1,400-bp fragment) (*virT*$^+$ complementation vector)	This study
pTS931	pJIR418 Ω(PCR-amplified 539-bp fragment) (*virU*$^+$ complementation vector)	This study
pTS932	pTS930 (585T→A)	This study
pTS933	pTS931 (182A→T)	This study
pTS934	pUC118 Ω(PCR-amplified 334-bp fragment) (*virT*$^+$ suicide vector), Em^r	This study
pTS935	pTS930 (1-bp frameshift at position 33)	This study
pTS936	pTS931 (1-bp frameshift at position 15)	This study
pUC19	Cloning vector, Amp^r *lacZ′* pMB 1 *ori*	Takara Bio Inc.
pUC118	Cloning vector, Amp^r *lacZ′* pMB 1 *ori*	Takara Bio Inc.

[a] Tet^r, resistance to tetracycline; Em^r, resistance to erythromycin, Cm^r, resistance to chloramphenicol; Amp^r, resistance to ampicillin.

thus forming the basis for a regulatory cascade in the VirR/VirS regulon (28).

The complete genomic sequence of *C. perfringens* strain 13 has been reported (25). On the basis of sequence similarities with other known virulence genes, more than 20 candidate virulence genes were identified. By screening the genome for the previously identified VirR-binding consensus sequence, we identified genes potentially regulated by the VirR/VirS system. Five genes were found to have the consensus VirR-binding site in their putative promoter regions (25). In the present study, regulation of expression of these novel target genes was analyzed to improve our understanding of the VirR/VirS regulon in *C. perfringens*.

MATERIALS AND METHODS

Strains, plasmids, medium, and culture conditions. The strains and plasmids used in this study are listed in Table 1. All *C. perfringens* strains were cultured in Gifu anaerobic medium (Nissui, Japan) at 37°C under anaerobic conditions as described previously (24). A single-crossover mutation was introduced into the *virT* gene of *C. perfringens* strain 13 with a pUC19-based suicide vector containing a 334-bp internal PCR fragment of *virT* and the *ermB* gene from pJIR418 (29). *Escherichia coli* DH5α was cultured as described previously (22). Plasmids pUC19 and pUC118 were used for cloning in *E. coli*, and pJIR418 (29) was used as an *E. coli-C. perfringens* shuttle vector.

DNA manipulation. Recombinant DNA was manipulated as described previously (22), unless otherwise noted. *C. perfringens* strains were transformed by electroporation as previously described (24).

Northern hybridization. Total RNA from *C. perfringens* was extracted and Northern blotting was performed as previously described (1) with an AlkPhos-direct kit and CDP-*star* chemiluminescence (GE Healthcare). DNA probes were prepared from genomic DNA of *C. perfringens* strain 13 by performing PCR with the appropriate primer sets (Table 2). In some situations, the signal densities of the mRNA bands were measured with a densitometer. All Northern hybridization experiments were performed at least three times, and the reproducibility was confirmed. The results described below are representative results from the repeated experiments.

Primer extension analysis. Primer extension was carried out as described previously (17) by using an Amersham 5′ oligolabeling fluorescence kit and the Promega primer extension system. Oligonucleotide primers 0845-PE, 0846-PE, and 0920-PE used to determine the transcription start sites of CPE0845 (*virT*), CPE0846 (*ccp*), and CPE0920 (*virU*), respectively, are shown in Table 2. Signals were detected with a FluorImager analyzer (GE Healthcare).

Assays for perfringolysin O and alpha-clostripain. The perfringolysin O activity in the *C. perfringens* culture supernatant was measured by the horse erythrocyte hemolysis method described previously (2). *C. perfringens* cells were cultured for 3 h to mid-log phase (see Fig. 2A) and collected by centrifugation. The supernatant was used for the hemolytic assay. Hemolytic activity was expressed as the reciprocal of the dilution that resulted in 50% hemolysis of 0.5% horse erythrocytes. The proteolytic activity of alpha-clostripain in the culture supernatant was determined with azocasein (Sigma Aldrich Japan) and the cysteine protease-specific inhibitors leupeptin and antipain (Wako Pure Chemicals) as previously described (11, 27). In brief, *C. perfringens* cells were cultured for 2 h to early log phase (see Fig. 2A) and collected by centrifugation, and 500 μl of the supernatant was mixed with an equal volume of an azocasein solution (5 mg/ml azocasein in 25 mM Tris-HCl [pH 7.5]–5 mM dithiothreitol) with or without 10 μM leupeptin or antipain. The mixture was incubated for 2 h at 37°C with gentle shaking, intact azocasein was removed by 3% trichloroacetic acid precipitation, and the absorbance at 450 nm of the supernatant was determined.

Site-directed mutagenesis and frameshift mutagenesis. Site-directed mutagenesis of the *virT* gene harbored by pTS930 was performed with an LA PCR in vitro mutagenesis kit (Takara Bio) with the mutagenic primer virT-NM (Table 2) to obtain pTS932. The *virU* gene on pTS931 was mutated by using a QuikChange site-directed mutagenesis kit (Stratagene) with primers virU-NM-F and virU-NM-R (Table 2) to obtain pTS933. Similarly, 1-base deletion frameshift mutations at positions 33 and 15 in the *virT* and *virU* coding regions, respectively, in the complemented plasmid vectors pTS930 and pTS931 (Table 1), respectively, were obtained by using a QuikChange site-directed mutagenesis kit (Stratagene) with primers virT-FM-F and virT-FM-R for *virT* and primers virU-FM-F and virU-FM-R for *virU* to construct pTS935 and pTS936 (Table 1). All procedures were performed according to the manufacturers' instructions.

RESULTS

Screening for VirR-binding sites in the *C. perfringens* genome. We scanned the genomic sequence of *C. perfringens* (25) for VirR-binding sites (CCAGTTNNNCAC) located upstream of the open reading frame (ORF). Only five genes, *pfoA*, CPE0845 (*virT*), CPE0846 (*ccp*), CPE0920 (*virU*), and *vrr*, were found to have sequences similar to the VirR-binding site in their putative promoter regions (Fig. 1) (25). With the exception of *virT* and *ccp*, which are located back to back and are

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′to 3′)	Use
virT-F	GAAGGTAAGTACCAAGATGA	Northern blotting
virT-R	TGATATTGCCACCCCAACTT	Northern blotting
ccp-F	GAGGCCGAAAAGACTGAAGG	Northern blotting
ccp-R	GCCCAATGTGGTATTGCTTG	Northern blotting
virU-F	CGATTCGTTTTTGATAGAAATGG	Northern blotting
virU-R	TTTATTTCTAGTTTTTCCTTTGATGA	Northern blotting
vrr-F	GACCAGTTACGCACAAAC	Northern blotting
vrr-R	GGACAGTTCTATTTCTAGG	Northern blotting
pfoA-F	GCAAGTATTGCAATGGCTTT	Northern blotting
pfoA-R	GTAAGTAATACTAGATCCAGGGT	Northern blotting
colA-F	GGTCTAGAGGATAGTGGAAGA	Northern blotting
colA-R	GTTCCTCTCATATCGTAAGT	Northern blotting
0845-PE	TTTATCAATGGGTAACGTAAAGAAAGTACCAGATA	Primer extension
0846-PE	GTTGAAACTCCTCCTAATAGAACGCTACCAATGGT	Primer extension
0920-PE	TCGGTATAATAAAAATGATATTGTTACTAATATG	Primer extension
virT-NM	GAAAGTACCAGATTAAATAGAAATAG	Site-directed mutagenesis
virU-NM-1	TTGGTATTATATATTAATATTTTCGTC	Site-directed mutagenesis
virU-NM-2	GACGAAAATATTAATATATAATACCAA	Site-directed mutagenesis
virT-FM-F	ATCGCTTTAAGTTTATCATTTCTATTTTATCTGGT	Site-directed mutagenesis
virT-FM-R	ACCAGATAAAATAGAAATGATAAACTTAAAGCGAT	Site-directed mutagenesis
virU-FM-F	GATATGAAAGACGAAAAATTAATATAAAATACCAA	Site-directed mutagenesis
virU-FM-R	TTGGTATTTTATATTAATTTTTCGTCTTTCATATC	Site-directed mutagenesis

transcribed on opposite strands, these five genes are not clustered (Fig. 1). The deduced amino acid sequence of the putative protein encoded by *ccp* was highly similar to that of alpha-clostripain, a cysteine proteinase from *Clostridium histolyticum* (7). The expression of *ccp* was previously shown to be positively regulated by the VirR/VirS system (27). The putative proteins encoded by *virT* and *virU* showed no significant similarity to known proteins, and their functions remain unclear. Identification of the previously uncharacterized putative VirR-binding sites in *virT*, *ccp*, and *virU* led to an investigation of whether these three genes, like *pfoA* and *vrr*, are targets for direct regulation by the VirR/VirS system.

Transcriptional regulation of *virT*, *ccp*, and *virU* by the VirR/VirS system. We performed Northern analyses of RNA of *C. perfringens* strains with different mutant backgrounds at different growth stages to look for changes in the steady-state levels

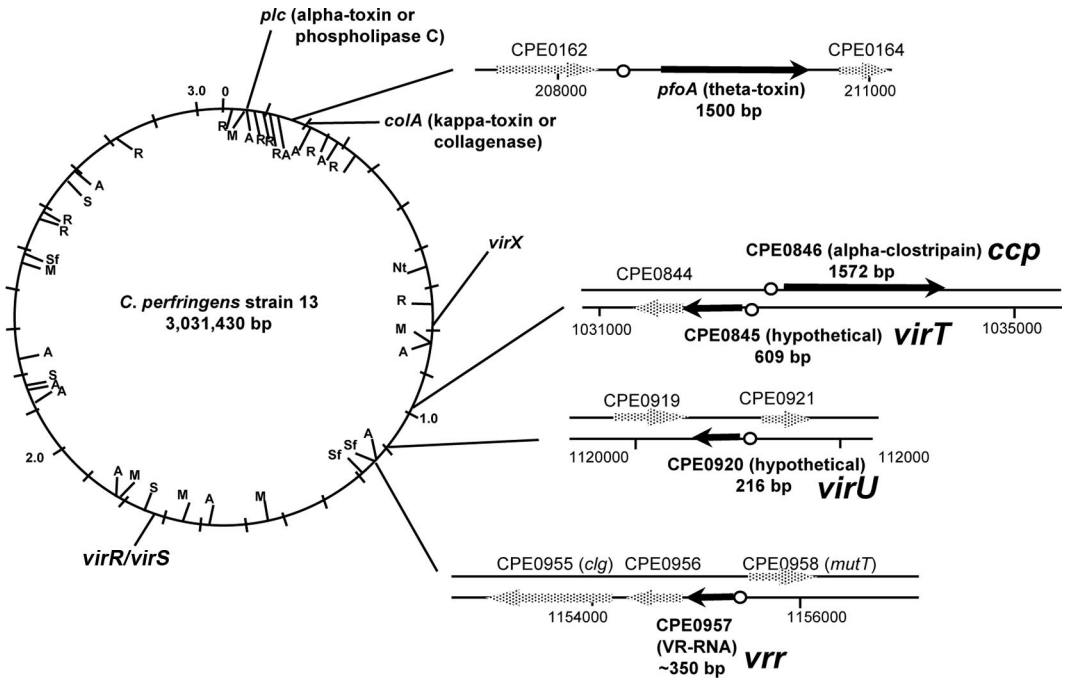

FIG. 1. Schematic diagram of the locations of putative VirR-binding sites in five genes (*pfoA*, *virT*, *ccp*, *virU*, and *vrr*) on the chromosome of *C. perfringens* wild-type strain 13. The solid and cross-hatched arrows represent genes with VirR-binding sites and their flanking genes, respectively. Open circles indicate putative VirR-binding sites. The chromosomal locations of other genes mentioned in this paper are also indicated. The nucleotide numbers are the numbers for the chromosomal sequence of *C. perfringens* strain 13 (GenBank accession number BA000016).

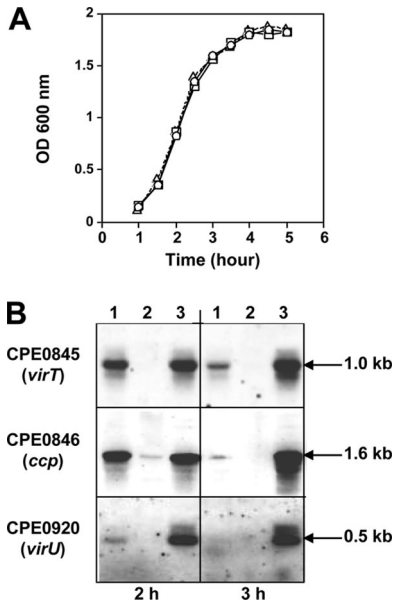

FIG. 2. Growth curves and Northern blot analyses for the *virR* mutant (TS133) of *C. perfringens*. (A) Growth curves for *C. perfringens* strains with a 1% inoculum. All three *C. perfringens* strains grew with a doubling time of ~20 min in Gifu anaerobic medium. ○, strain 13(pJIR418); □, strain TS133(pJIR418) (*virR*); △, strain TS133 (pBT405) (*virR*$^+$ *virS*$^+$). OD 600 nm, optical density at 600 nm. (B) Total RNA was prepared from each culture at the indicated times (2 and 3 h). Either 10 μg (for *virT* and *ccp*) or 40 μg (for *virU*) of total RNA was resolved by agarose electrophoresis, blotted onto a nylon membrane, and hybridized with probes for *virT* (CPE0845), *ccp* (CPE0846), and *virU* (CPE0920). Lane 1, strain 13(pJIR418); lane 2, strain TS133 (pJIR418); lane 3, strain TS133(pBT405).

of *virT*, *ccp*, and *virU* that confirmed that that there is regulation by the VirR/VirS system. In *C. perfringens* wild-type strain 13, a 1.0-kb transcript of *virT* was clearly detected after 2 h of growth (early to mid-exponential phase) (Fig. 2B, upper left panel), and the level had decreased after 3 h of incubation (late exponential phase) (Fig. 2B, upper right panel). Similar patterns were observed for expression of the 1.6-kb alpha-clostripain (*ccp*) transcript (Fig. 2B, center panels) and the 0.5-kb *virU* transcript (Fig. 2B, lower panels) in the wild-type background. Even with 40 μg of total RNA blotted onto the membrane, the *virU* transcript signal was very low for strain 13, indicating that the level of expression of *virU* was significantly lower than the level of expression of *virT* or *ccp*. The time points of accumulation for *virT*, *ccp*, and *virU* mRNAs were similar to those for *pfoA* and *vrr*; all of the molecules were most abundant during the early exponential to mid-exponential growth phase (3, 4). Importantly, transcripts of *virT*, *ccp*, and *virU* either were undetectable or the bands were very weak for the *virR* mutant strain TS133 (Fig. 2B, lane 2). Expression of these genes was increased by transformation of intact *virR* and *virS* genes into TS133 (Fig. 2B, lane 3). These data clearly indicate that the steady-state RNA levels for *virT*, *ccp*, and *virU* were regulated positively by the VirR/VirS system in *C. perfringens* in a manner similar to the manner of regulation of *pfoA* and *vrr* (3, 4, 28). The higher levels of the *virT*, *ccp*, and *virU* transcripts in the complemented strains were likely due to the high copy number of the pBT405 complementation plasmid.

Promoter analysis of the VirR/VirS-regulated genes. To analyze the promoter regions of the three VirR/VirS-regulated genes, we identified transcription initiation sites for *virT*, *ccp*, and *virU* by performing a primer extension experiment with wild-type and *virR* mutant RNA templates. The *virT*-specific primer generated a single extension product with wild-type strain 13 RNA, whereas no product was obtained with the *virR* mutant strain TS133 (Fig. 3A, left panel). Similarly, both the *ccp* and *virU* gene-specific primers (Fig. 3A, middle and right panels, respectively) yielded single extension products with the wild-type RNA template, whereas no products were obtained with *virR* mutant strain RNA. These results indicate that transcription initiation from single sites in *virT*, *ccp*, and *virU* is dependent on the VirR/VirS system because little mRNA for these genes was present in the *virR* mutant strain.

The length of each primer extension product was used to assign the position 1 site of transcription initiation for *virT*, *ccp*, and *virU* and to identify conserved elements (−35 and −10) in the promoter of each gene (Fig. 3B). We compared the locations of the VirR-binding sequences (CCAGTTWTNCA), the consensus promoter sequences (−35 and −10), and the mRNA start sites with those determined for *pfoA* and *vrr* in previous studies (4, 28) (Fig. 3B) and found that the relative distances between these elements were highly conserved in the five promoter regions. In particular, all five genes have a 42-bp interval between the VirR-binding sequences and the mRNA start site (Fig. 3B). The locations of the consensus promoter sequences (−35 and −10) were almost identical in all five genes.

In previous studies, two repeated sequences (VB1 and VB2) in the promoter region of *pfoA* (Fig. 3B) were identified as independent binding sites for the VirR protein (5, 6). The promoter regions of *vrr*, *virT*, *ccp*, and *virU* contained sequences similar to the VB2 sequence of *pfoA* (Fig. 3B). Although more divergent than the similarities between these four genes and *pfoA* in the VB2 consensus sequence, sequences similar to VB1 were found in these four genes (Fig. 3B), suggesting that the VB1 region may also be important for regulation of transcript by the VirR protein. The promoter structures of these five VirR-regulated genes are highly conserved and are distinct from the promoter structures of previously analyzed VirR/VirS-regulated genes (4, 19). The ability of the VirR protein to bind to the conserved sequences upstream of *virT*, *ccp*, and *virU* was confirmed in a previous study (5). Moreover, VirR has been reported to bind to some VirR boxes found in genes of two other strains of *C. perfringens*, ATCC13124 and SM101 (16), which suggests that the VirR-dependent regulatory system is present in various types of *C. perfringens* strains. Binding of a glutathione *S*-transferase–VirR fusion protein to the VirR-binding sequence was also examined using gel mobility shift assays, and this analysis confirmed that the VirR protein bound specifically to the conserved sequences in the promoter regions of *pfoA*, *vrr*, *virT*, *ccp*, and *virU* (data not shown).

Functional analysis of the *virT* and *virU* genes. We were unable to predict the putative function of either *virT* or *virU* using the results of computer-based searches for sequence similarities. To explore the functional roles of these genes, we

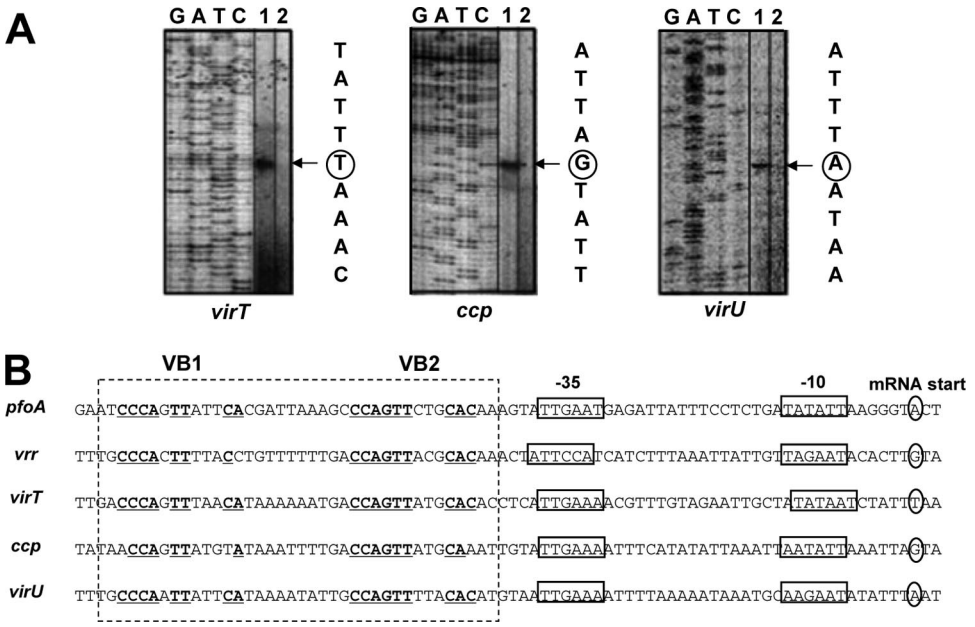

FIG. 3. Identification of the transcription initiation sites of the *virT*, *ccp*, and *virU* genes in *C. perfringens*. (A) Primer extension products derived using the oligonucleotide primers listed in Table 2 with template RNA prepared from 2-h cultures of *C. perfringens* strains (lane 1, wild-type strain 13; lane 2, *virR* strain TS133) were separated by electrophoresis on acrylamide gels. Sequencing reactions with the same primers and appropriate DNA templates were run on the same gel. The positions of extended products obtained with the *virT*, *ccp*, and *virU* primers are indicated by arrows, and the putative mRNA start sites are indicated by circles. (B) Deduced promoter sequences (−35 and −10) and consensus VirR-binding sites of the five VirR/VirS-regulated genes. The putative promoter sequences and mRNA start sites are indicated by boxes and circles, respectively. The deduced VirR-binding sequence of each gene is indicated by a dotted box, and conserved nucleotides are underlined. The promoter sequences of the theta-toxin (*pfoA*) and VR-RNA (*vrr*) genes are aligned, and the VB1 and VB2 regions are shown.

constructed *virT* isogenic mutants of strain 13 (see Materials and Methods). The resulting *virT* mutation in strain TS190, which was confirmed by Southern hybridization with a *virT* gene probe (data not shown), was used to examine expression of VirR/VirS-regulated genes. Compared to wild-type strain 13, in mutant strain TS190 there were at least 2.5-fold increases in the levels of both *pfoA* and *ccp* mRNAs during the logarithmic growth phase (2 and 3 h) (Fig. 4A). When mutant strain TS190 was complemented with the *virT*$^+$ pTS930 plasmid [resulting in TS190(pTS930)], the level of each of the transcripts was reduced to the wild-type level, although complementation was not complete until the 3-h time point for unknown reasons (Fig. 4A). These data suggest that the *virT* gene product acts as a negative regulator of *pfoA* and *ccp* in the wild-type strain. However, no significant change in *plc*, *colA*, *vrr*, or *virU* expression was observed in TS190 (Fig. 4A), indicating that the negative effect of the *virT* gene product was specific to *pfoA* and *ccp*.

For unknown reasons, repeated attempts to construct a strain 13 *virU* mutant using single-crossover or double-crossover recombination methods failed. As an alternative approach to test the function of *virU*, we introduced a *virU* overexpression plasmid, pTS931, into wild-type strain 13 to measure the effect of *virU* on the steady-state levels of putative VirR/VirS target genes. In 3-h cultures, the *pfoA*, *ccp*, *vrr*, and *virT* mRNA levels were all increased in response to the higher number of *virU* copies (Fig. 4B), suggesting that the *virU* gene encodes a positive regulator of *pfoA*, *ccp*, *vrr*, and *virT* expression. Although the abundance of *plc* and *colA* transcripts also increased slightly following transformation of pTS931 into strain 13 (Fig. 4B), we believe that this may have been a secondary effect of increased VR-RNA (Fig. 4B, *vrr* panels), which is known to enhance expression of *plc* and *colA*.

In addition to measuring changes in the levels of the *pfoA* and *ccp* mRNAs, we also measured the activities of the secreted gene products, perfringolysin O and alpha-clostripain, respectively, in supernatants from cultures of wild-type and mutant *C. perfringens* strains. Perfringolysin O activity was measured by determining the hemolytic activities of the culture supernatants with horse erythrocytes. The hemolytic activities of *virT* mutant strain TS190, TS190 with the plasmid expressing *virT* (pTS930), and wild-type strain 13 with the *virU* overexpression plasmid (pTS931) were 3.9-, 1.2- and 4.3-fold higher, respectively, than the hemolytic activity of wild-type strain 13 (Table 3). These results, which correspond well with the results of the Northern analyses, indicate that the activity encoded by wild-type *virU* stimulates the production of perfringolysin O, whereas the wild-type *virT* gene product inhibits the production of perfringolysin O.

Azocasein, a colorimetric substrate of alpha-clostripain and other cysteine proteinases, was used to measure alpha-clostripain activity specifically in culture supernatants containing two inhibitors of cysteine proteinases, leupeptin and antipain. In the presence of these inhibitors, the proteolytic activity of each strain, as measured by absorbance at 450 nm, decreased between 25 and 60% (Fig. 5A). The difference in activity represents alpha-clostripain-specific proteolysis of azocasein (Fig. 5B). The alpha-clostripain activity was increased in the *virT* mutant strain TS190, and when the mutation was complemented with pTS930 (*virT*$^+$), the activity decreased to a level

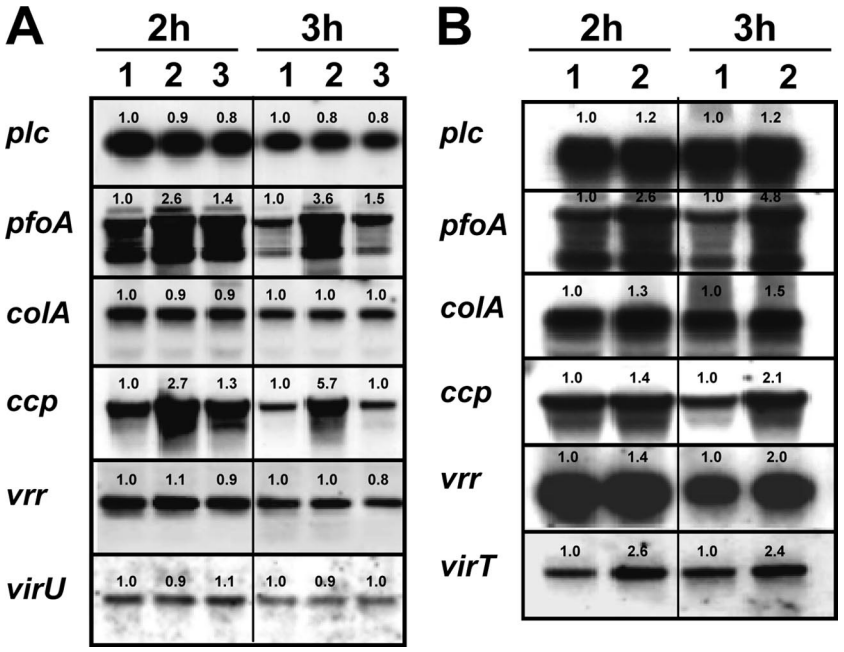

FIG. 4. Northern blot analyses of the *virT* mutant and wild-type strains transformed with the *virU*$^+$ expression plasmid (pTS931). Total RNA was prepared from each culture at the times indicated (2 and 3 h), and 10 μg of each RNA preparation (40 μg for hybridization with *virU* probe) was resolved by agarose electrophoresis, blotted onto nylon membranes, and hybridized with *plc*, *pfoA*, *colA*, *ccp*, *vrr*, *virT*, and *virU* gene probes as indicated. The band densities relative to those for wild-type strain 13 are indicated above the bands. (A) Lane 1, wild-type strain 13(pJIR418); lane 2, strain TS190(pJIR418) (Δ*virT*); lane 3, strain TS190(pTS930) (Δ*virT virT*$^+$). (B) Lane 1, wild-type strain 13(pJIR418); lane 2, wild-type strain 13(pTS931) (*virU*$^+$).

similar to the level in the wild-type strain (Fig. 5B), indicating that expression of alpha-clostripain is negatively regulated by *virT*. Similarly, the alpha-clostripain activity was higher in the *virU*-overexpressing strain than in the wild-type strain (Fig. 5B), suggesting that the *virU* gene product stimulates alpha-clostripain production. The similarities between the changes in perfringolysin O and alpha-clostripain activities and the changes in the levels of mRNAs for these genes in different genetic backgrounds (wild-type strain versus *virT* mutant or *virU*-overexpressing strain) suggest that the *virT* gene product negatively controls expression of *pfoA* and *ccp* and that the *virU* gene product enhances expression of *pfoA* and *ccp* in *C. perfringens*.

Mutational analyses of *virT* and *virU*. It has been reported that in *C. perfringens* regulatory RNA molecules control expression of several genes, including toxin genes (17, 28). Con-

TABLE 3. Perfringolysin O activities of various *C. perfringens* strains

Strain	Genotype	Perfringolysin O titer ($\log_2$)[a]	Difference (fold) compared to strain 13
13	Wild type	7.4 ± 0.5	1
TS133	Strain 13 *virR*::Tet^r	2.0 ± 0.1	<0.1
TS190	Strain 13 Δ*virT*	9.4 ± 0.5	3.9
TS190(pTS930)	Strain 13 Δ*virT* (*virT*$^+$)	7.7 ± 0.4	1.2
13(pTS931)	Strain 13 (*virU*$^+$)	9.5 ± 0.7	4.3

[a] Each value was calculated by using the results of triplicate independent experiments, and the values are means ± standard deviations.

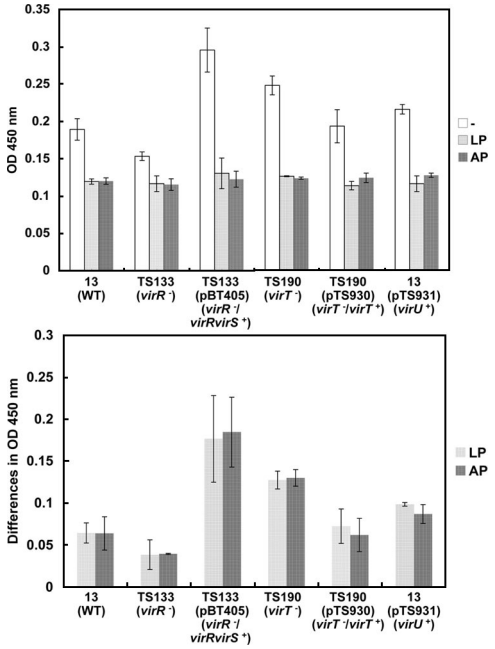

FIG. 5. Alpha-clostripain activities of various *C. perfringens* strains. (A) The alpha-clostripain activity of each *C. perfringens* strain (indicated at the bottom) was determined with azocasein as a substrate under conditions with no inhibitor (−), with leupeptin (LP), or with antipain (AP). For each strain and treatment combination, the mean absorbance and standard deviation (error bar) calculated from three independent experiments are shown. (B) The difference in mean proteolytic activity between assays without inhibitors and assays with the leupeptin or antipain inhibitor (shown in panel A), which represents alpha-clostripain-specific proteolytic activity, was plotted for each *C. perfringens* strain. WT, wild type; OD 450 nm, optical density at 450 nm.

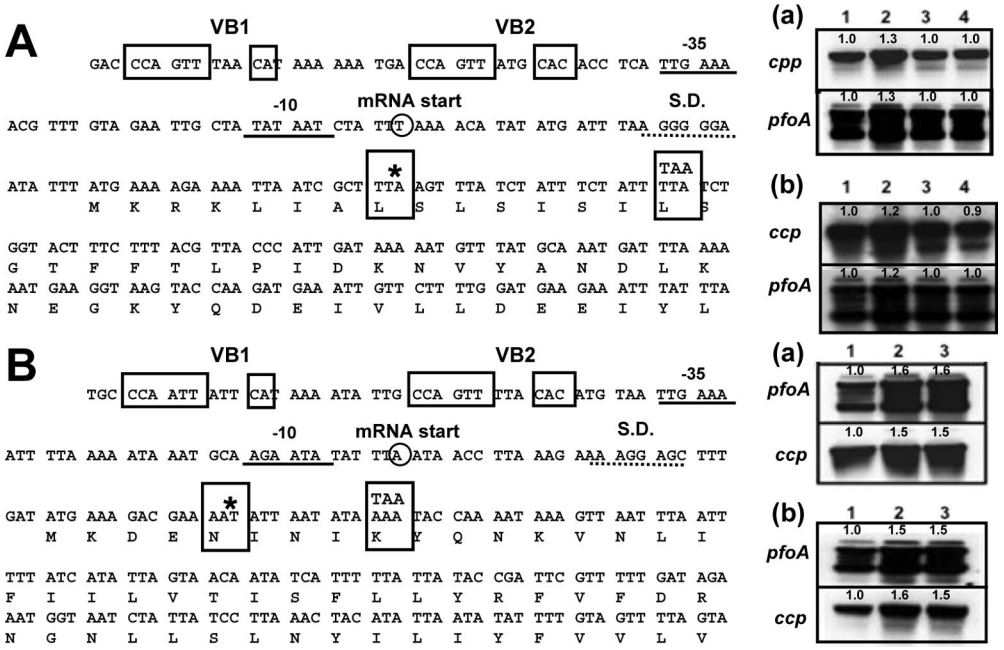

FIG. 6. Site-directed mutagenesis of the *virT* (A) and *virU* (B) genes and the effects of the mutations on steady-state levels of VirR-regulated gene mRNAs. The nonsense and frameshift codons engineered in the *virT* and *virU* coding regions (left panels) are indicated by large boxes. The positions of the nonsense mutation (NM) and frameshift mutation (FM) are indicated by TAA and an asterisk, respectively. Plasmids carrying mutated *virT* and *virU* genes (designated pTS932 and pTS935 for *virT* and pTS933 and pTS935 for *virU*) were transformed into different *C. perfringens* strains. The band densities relative to those for wild-type strain 13 are indicated above the bands. (A) (Panel a) Lane 1, wild-type strain 13(pJIR418); lane 2, strain TS190(pJIR418) (Δ*virT*); lane 3, strain TS190(pTS930 (Δ*virT virT*$^{+}$); lane 4, strain TS190(pTS932) (Δ*virT virT*^{NM}). (Panel b) Lane 1, wild-type strain 13(pJIR418); lane 2, strain TS190(pJIR418) (Δ*virT*); lane 3, strain TS190(pTS930) (Δ*virT virT*$^{+}$); lane 4, strain TS190(pTS935) (Δ*virT virT*^{FM}). (B) (Panel a) Lane 1, wild-type strain 13(pJIR418); lane 2, wild-type strain 13(pTS931) (*virU*$^{+}$); lane 3, wild-type strain 13(pTS933) (*virU*^{NM}). (Panel b) Lane 1, wild-type strain 13(pJIR418); lane 2, wild-type strain 13(pTS931) (*virU*$^{+}$); lane 3, wild-type strain 13(pTS9360 (*virU*^{FM}). Northern analyses were performed with the indicated probes (right panels).

sidering the relatively small size of the *virT* and *virU* ORFs (609 and 216 bp, respectively), it is possible that these genes, like *vrr* and *virX*, encode regulatory RNAs. To test this hypothesis, nonsense mutations were introduced into the protein-encoding regions of *virT* and *virU* with plasmid vectors (pTS932 and pTS933, respectively), and then differences between the *ccp* and *pfoA* steady-state mRNA levels in samples of wild-type and mutant cell total RNAs were determined (Fig. 6). When the *virT* nonsense mutant gene in pTS932 was introduced into the isogenic *virT* mutant strain TS190, the relative levels of *ccp* and *pfoA* transcripts in TS190(pTS932) were not different from the levels in TS190(pTS930) containing an intact *virT* gene (Fig. 6A, panel a, lanes 3 and 4). Similarly, a nonsense mutation was constructed in the coding region of *virU* in plasmid pTS933, which was transformed into wild-type strain 13 (Fig. 6B). The results indicated that there was a difference in the level of *vrr*, *pfoA*, or *ccp* mRNA between wild-type strain 13 overexpressing intact *virU* (pTS931) and wild-type strain 13 overexpressing mutated *virU* (pTS933) (Fig. 6B, panel a, lanes 2 and 3). Furthermore, one-base deletions at positions 33 and 15 in the *virT* and *virU* coding regions (Fig. 6) were introduced to generate frameshift mutations, resulting in plasmids pTS935 and pTS936, respectively. Plasmid pTS935 was transformed into the isogenic *virT* mutant strain TS190 and wild-type strain 13, and the mRNA levels for *pfoA* and *ccp* were determined by Northern hybridization. As shown in Fig. 6, the relative levels of *ccp* and *pfoA* transcripts in TS190(pTS935) with frameshifted *virT* were not different from the levels in TS190 (pTS930) containing an intact *virT* gene. Similarly, the transcript levels of *ccp* and *pfoA* were not different in strain 13 harboring pTS936 (with frameshifted *virU*) and strain 13 harboring pTS931. Taken together, these results indicate that there was no difference in the regulatory functions of *virT* and *virU* whether the ORFs were intact or not intact. These data strongly suggest that both the *virT* and *virU* genes encode regulatory RNA molecules, not proteins, that regulate the VirR/VirS regulon. The promoters of *pfoA*, *virT*, *ccp*, *vrr*, and *virU* were screened, and no conserved sequence motifs (besides the VirR consensus sequence) were found, indicating that it is unlikely that there are other regulatory RNAs or proteins that are shared by these VirR/VirS-regulated genes.

DISCUSSION

A genome-wide search for promoter-proximal VirR-binding sites previously identified the theta-toxin-encoding gene *pfoA* (6) and the *vrr* gene, which encodes regulatory VR-RNA (28), and also identified three new genes, *virT*, *ccp*, and *virU*, as potential members of the VirR/VirS regulon (25). To test the veracity of this in silico identification, genetic and molecular analyses of *virT*, *ccp*, and *virU* functions and regulation by the VirR/VirS system were performed.

Comparative Northern analyses of wild-type and *virR* mutant strains of *C. perfringens* revealed that *virT*, *ccp*, and *virU*

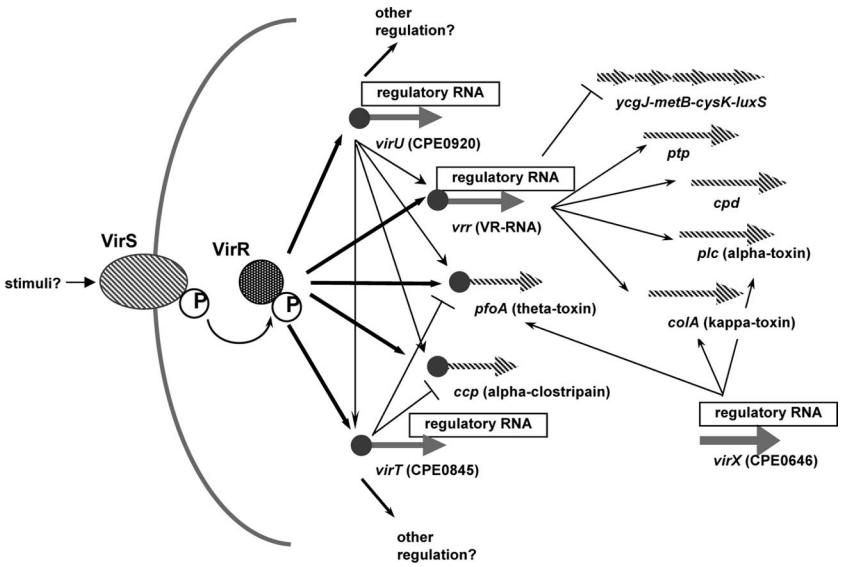

FIG. 7. Schematic diagram of the VirR/VirS regulon. The diagram was constructed by using the results of this and previous studies (3, 4, 12, 17, 18, 20, 24, 25, 28).

are positively regulated by the VirR/VirS system at the RNA level. The sequence and location of a putative VirR-binding consensus site (VB1/VB2) and its location (from position −40 to position −80 upstream of the initiation site) were conserved in the *pfoA*, *vrr*, *virT*, *ccp*, and *virU* promoters, suggesting that VirR may bind directly to these sites to activate transcription (5). Gel shift assays confirmed that the VirR protein binds specifically to the conserved promoter sequences. These data led us to conclude that the VirR/VirS regulon involves five genes regulated directly by the VirR protein in *C. perfringens* (Fig. 7).

The deduced amino acid sequence encoded by *ccp* is highly similar to the amino acid sequence of alpha-clostripain from *C. histolyticum* (EC 3.4.22.8) (7), which is a heterodimeric cysteine endopeptidase with specificity for Arg-X peptidyl bonds (7, 8). The two polypeptide chains (termed light and heavy) of the native enzyme are encoded by a single 1,581-bp gene, and the junction between the two polypeptide DNA sequences encodes a linker nonapeptide (7). Alpha-clostripain has been implicated in damage of cells in fetal rat calvaria (10) and may contribute to the virulence of other clostridial infections (23). Detection of alpha-clostripain protein and the enzymatic activity in the supernatants of *C. perfringens* cultures (27) suggest that alpha-clostripain may be a virulence factor. The *C. perfringens* genome lacks many genes needed for amino acid biosynthesis, and alpha-clostripain may participate in degradation of host proteins that yields nutrients required for *C. perfringens* survival and growth (25). Further studies are needed to elucidate the roles of alpha-clostripain in *C. perfringens* nutrient uptake and pathogenicity.

Although we were unable to predict the functions of the molecules encoded by *virT* and *virU* based on homology with other genes (25), the activities of these molecules clearly influence VirR/VirS gene regulation; the *virT* product acts as a negative regulator of expression, and the *virU* product acts as a positive regulator of expression (Fig. 4). The inability to alter these effects by nonsense mutations in *virT* and *virU* suggests that these genes, like *vrr* (28) and *virX* (17), encode regulatory RNA molecules rather than proteins (Fig. 6). A possible secondary structure was examined for the predicted *virT* and *virU* RNA molecules. The predicted secondary structures of the whole *virT* and *virU* RNAs were tight and compact overall, similar to the structure predicted for VR-RNA (data not shown) (28). The transcriptional terminator downstream of the *virT* and *virU* regions was also searched, and only *virU* was found to have inverted repeat sequences. Furthermore, Northern analyses were performed with 50-mer synthetic sense and antisense oligonucleotide probes to look for changes in the steady-state levels of *virT* and *virU* mRNAs. Both *virT* and *virU* transcripts were detected in wild-type strain 13 with antisense probes, whereas no signals were obtained with the sense *virT* and *virU* probes (data not shown).

Unexpectedly, the number of regulatory RNA molecules found to be involved in regulation of virulence (and other) genes in *C. perfringens* is increasing. Based on recent reports of the importance of small RNA molecules in regulation of transcription and/or translation in both prokaryotes and eukaryotes (12, 15), many RNA molecules involved in other aspects of *C. perfringens* gene regulation may still be unknown. In the case of the *virT* and *virU* RNAs, the absence of a putative consensus sequence for direct annealing of these RNAs to the promoters of the *virT*- and *virU*-regulated genes *pfoA*, *virT*, *ccp*, *virU*, and *vrr* suggests that these regulatory RNAs may affect the activity of other proteins or RNA regulators for these five genes. Because the effects of *virT* and *virU* mutations on transcription were much more subtle than those of the VirR/VirS system or VR-RNA, *virT* and *virU* may fine-tune transcription of VirR/VirS-regulated genes to maintain balanced gene expression. Future studies of the effects of *virT* and *virU* on gene regulation, such as DNA microarray analyses of *C. perfringens* cultured under changing environmental con-

ditions, may provide a more detailed view of the overall effects of these regulatory genes.

The conclusion that the VirR/VirS system directly regulates only five genes (*pfoA*, *vrr*, *virT*, *ccp*, and *virU*) in *C. perfringens* via VirR binding is somewhat surprising. It has been reported that the VirR/VirS system influences expression of many other genes, including *plc* (encoding alpha-toxin), *colA* (encoding kappa-toxin), *cpd* (encoding 2′,3′-cyclic nucleotide phosphodiesterase), *ptp* (encoding protein tyrosine phosphatase), *ycgJ* (encoding a hypothetical protein), *metB* (encoding cystathionine gamma-lyase), *cysK* (encoding cysteine synthase), and *luxS* (encoding the autoinducer 2 production protein) (3, 4, 18, 20). However, for *plc*, *colA*, *cpd*, *ptp*, and *ycgJ-metB-cysK-luxS*, VR-RNA has been shown to be a secondary RNA regulator (3, 28) (Fig. 7). Another RNA regulator, *virX*, controls the levels of *pfoA*, *plc*, and *colA* mRNAs independent of the VirR/VirS regulatory cascade (17) (Fig. 7). Furthermore, cell-cell signaling by autoinducer 2 synthesized by the *luxS* gene product, which may be mediated through an unidentified two-component system (18), also plays an important role in the regulation of toxin production.

It is clear that the VirR/VirS regulon consists of two classes of genes, the genes that are regulated directly by the VirR/VirS system and the genes that are regulated indirectly (Fig. 7). The mechanism of regulation of the VirR/VirS regulon, including the actions of the *virT* and *virU* regulatory RNA molecules, should be clarified by comprehensive DNA microarray-based analyses of gene expression.

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