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The *virR/virS* Locus Regulates the Transcription of Genes Encoding Extracellular Toxin Production in *Clostridium perfringens*

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Extracellular toxin production in *Clostridium perfringens* is positively regulated by the two-component regulatory genes *virR* and *virS*. Northern (RNA) blots carried out with RNA preparations from the wild-type strain 13 and the isogenic *virR* and *virS* mutants TS133 and JIR4000 showed that the *virR* and *virS* genes composed an operon and were transcribed as a single 2.1-kb mRNA molecule. Primer extension analysis led to the identification of two promoters upstream of *virR*. Hybridization analysis of the mutants and their complemented derivatives showed that the *virR/virS* system positively regulated the production of alpha-toxin (or phospholipase C), theta-toxin (perfringolysin O), and kappa-toxin (collagenase) at the transcriptional level. However, the modes of regulation of these genes were shown to differ. The theta-toxin structural gene, *pfoA*, had both a major and a very minor promoter, with the major promoter being *virR/virS* dependent. The *colA* gene, which encodes the kappa-toxin, had two major promoters, only one of which was *virR/virS*-dependent. In contrast, the alpha-toxin structural gene, *plc*, had only one promoter, which was shown to be partially regulated by the *virR* and *virS* genes. Comparative analysis of the *virR/virS*-dependent promoters did not reveal any common sequence motifs that could represent VirR-binding sites. It was concluded that either the *virR/virS* system modulates its effects via secondary regulatory genes that are specific for each toxin structural gene or the VirR protein does not have a single consensus binding sequence.

Clostridium perfringens is the causative agent of several human and animal diseases, including clostridial myonecrosis, or gas gangrene (5, 14). The organism produces many extracellular enzymes and toxins, several of which have been implicated in the disease process. These toxins include the alpha-toxin (or phospholipase C), theta-toxin (or perfringolysin O), and kappa-toxin (or collagenase), which are encoded by the *plc*, *pfoA*, and *colA* genes, respectively. Recent studies have shown that the alpha-toxin is an essential virulence factor in gas gangrene (2, 27).

Recently, we have cloned and sequenced two regulatory genes, *virR* and *virS*, which control the expression of these toxins as well as protease and sialidase production (8, 19). Virulence studies carried out with mice have revealed that the *virR/virS* regulon is important in the pathogenesis of clostridial myonecrosis (8). Comparative sequence analysis has shown that the VirR and VirS proteins have significant amino acid sequence similarity to response regulator and sensor histidine kinase proteins, respectively, of two-component regulatory systems that are found in many bacteria (22).

In our previous studies, a *virR* mutant, TS133, was isolated by homologous recombination (19) and a chromosomal *virS* mutant was isolated by Tn*916* mutagenesis (8). Both mutants were derived from a transformable *C. perfringens* strain, 13 (18), and could be complemented in *trans* by their respective wild-type genes (8, 19). In this study, we have used these mutants to examine the transcriptional regulation of the virulence factors controlled by the *virR/virS* system in *C. perfringens.*

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MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. *C. perfringens* strains were grown in GAM medium (19) or in TPG, BHI, FTG medium, or nutrient agar as previously described (8) at 37°C under anaerobic conditions. When necessary, antibiotics were used at the following concentrations: chloramphenicol, 25 μg/ml; erythromycin, 50 μg/ml; tetracycline, 10 μg/ml; and nalidixic acid, 25 μg/ml. *Escherichia coli* strains were grown under standard conditions (17) in the presence of ampicillin (50 or 100 μg/ml) or erythromycin (300 μg/ml). Molecular methods were used as previously described (8, 17, 19).

Northern (RNA) blot and RNA slot blot analysis. Total RNA from logarithmic-phase cultures of the *C. perfringens* strains 13, TS133, and TS133(pBT404) was prepared as described before (1). The probes were labeled with [α-^{32}P]dCTP (ICN Biomedicals, Inc., Costa Mesa, Calif.) by the random primer method (4) with the Ready-To-Go DNA labeling kit (Pharmacia P-L Biochemicals, Inc., Milwaukee, Wis.). Ten micrograms of each RNA preparation was denatured, separated by electrophoresis, and blotted onto a nylon filter membrane. The filter was subjected to Northern hybridization as described previously (19) with various gene probes. Filters were hybridized for 2 h at 55°C in Rapid-hyb buffer (Amersham International, Plc., Buckinghamshire, United Kingdom) and washed twice for 5 min at room temperature in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na_2HPO_4, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate (SDS) followed by two washes of 15 min each in 0.7× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C. The resultant autoradiogram was analyzed with a BAS2000 Bio-Imaging Analyzer (Fuji Photo Film Co., Ltd., Kanagawa, Japan) or by autoradiography.

For slot blots, RNA was extracted from *C. perfringens* cells grown to mid-logarithmic phase in TPG broth (100 ml). The cells were washed and resuspended in 0.5 ml of ice-cold AE buffer (20 mM sodium acetate, 1 mM EDTA [pH 5.5]) and then added to a preheated (60°C) solution containing 6 ml of phenol-chloroform, 60 μl of 25% SDS, and 2.5 ml of AE buffer. Samples were incubated for 20 min at 60°C and vortexed every 5 min. Total RNA was then extracted as described before (11). Slot blot analysis was carried out as described previously (17) with restriction fragments labeled with [α-^{32}P]dATP (Amersham International, Plc.). Filters were hybridized overnight at 65°C and washed for 20 min at room temperature in 1× SSC–0.1% SDS, followed by three washes of 20 min each in 0.2× SSC–0.1% SDS at 65°C.

Primer extension analysis. The synthetic oligonucleotide primers colA-1 (5′-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
13	Wild type	9
TS133	13*virR*	19
JIR325	13 Nal^r Rif^r	8
JIR4000	JIR4025 *virS*::Tn*916*	8
JIR4025	JIR325Rif^s	8
JIR4086	JIR4000(pJIR751)	8
JIR4087	JIR4000(pJIR870)	8
JIR4089	JIR4000(pJIR872)	8
Plasmids		
pUC19	Ap^r	28
pTOX6	pTZ18 Ω(*Hin*dIII [2.7 kb] *plc*$^+$)	16
pTS302	pUC19 Ω(3.9 kb, *pfoR*$^+$ *pfoA*$^+$)	20
pKY3135	pUC18 Ω(*Xba*I-*Bgl*II [1.6 kb] *colA*$^+$)	10
pJIR418	Shuttle plasmid, Cm^r Em^r	21
pJIR751	Shuttle plasmid, Em^r	3
pJIR870	pJIR751 Ω(*Hin*dIII [2.7 kb] *virR*$^+$ Δ*virS*)	8
pJIR872	pJIR751 Ω(*Pst*I [4.3 kb] *virR*$^+$ *virS*$^+$)	8
pBT404	pJIR418 Ω(*Hin*dIII [2.6 kb] *virR*$^+$Δ*virS*)	19
pOT201	pUC19 Ω(*Bgl*II [1.2 kb] Δ*colA*$^+$ from strain 13)	This study
pKB300	pUC19 Ω (*Hin*dIII [3.1 kb] *plc*$^+$ from strain 13)	This study

TCTCCCCTTTTTAAGTTTTTCTT-3′), pfoA-2 (5′-AATTTTTTCTTTTCCC TAAACTCTC-3′), plc-1 (5′-CAAATAAGCGCCTTACAAA-3′), and virR-2 (5′-TCTCTTTGCAAGGAATTATCTTCACATA-3′), which were complementary to the *colA* (10), *pfoA* (20), *plc* (12), and *virR* (8, 19) genes, respectively, were used for the primer extension analyses. Ten to 50 pmol of each primer was end labeled with 50 μCi of [γ-^{32}P]ATP (>7,000 Ci/mmol; ICN Biomedicals, Inc.) and 10 U of T4 polynucleotide kinase. After purification of the labeled primer with a Bio-Spin 30 column (Bio-Rad Laboratories, Richmond, Calif.), the primer was annealed to 50 μg of total RNA at 80 to 90°C for 5 min and slowly cooled to 42°C over 30 min. The annealed primer was extended by 4 U of avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) per μl with 0.25 mM deoxynucleoside triphosphates at 42°C for 30 min, and the reaction mixture was treated with RNase. The extension products were separated by electrophoresis on a sequencing gel together with a nucleotide sequence reaction which had been carried out with the appropriate plasmid and with the same primer.

RESULTS

Transcriptional analysis of the *C. perfringens virR* and *virS* genes. Sequence analysis of the *virR/virS* locus suggested that these overlapping genes were cotranscribed (8). Northern blotting was carried out to analyze the transcripts derived from the *virR* and *virS* genes. A single 2.1-kb mRNA molecule was detected in *C. perfringens* 13 by use of either *virR*-specific or *virS*-specific probes (Fig. 1). The same 2.1-kb transcript was also detected in the wild-type strain by using a mixture of the two probes, indicating that the *virR* and *virS* genes were transcribed as a single 2.1-kb mRNA. In the *virR* mutant TS133, no transcripts were detected with the *virS* probe (Fig. 1), suggesting that recombination into the *virR* gene (19) had disrupted the integrity of the *virR-virS* transcript and had the expected polar effect on the expression of *virS*.

Further evidence that these genes were cotranscribed was obtained from the analysis of the Tn*916*-derived *virS* mutant JIR4000 (8). When RNA from JIR4000 or JIR4000 containing the shuttle plasmid pJIR751 was hybridized with a *virR*-specific probe, a 1.2-kb transcript was detected (Fig. 2). This smaller transcript presumably resulted from transcription from the putative promoter located upstream of *virR*, which terminated within the Tn*916* sequence. No *virS*-specific transcript was detected (data not shown), because the probe used was located downstream of the Tn*916* insertion site. Similar experiments

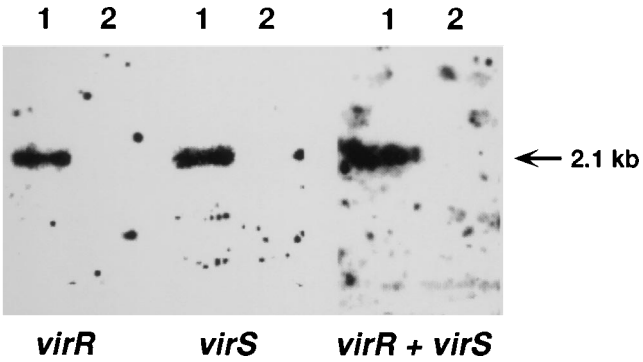

FIG. 1. Northern analysis of the *virR* and *virS* genes. Ten micrograms of total RNA prepared from *C. perfringens* was denatured, subjected to electrophoresis, and blotted onto nylon filters. The blot was hybridized with the following ^{32}P-labeled probes as indicated: *virR* (0.5-kb *Hin*cII fragment from pBT404), *virS* (0.7-kb *Bgl*II-*Hin*dIII fragment from pBT404), or both probes. The size of the hybridizing fragment was calculated from the mobilities of 23S and 16S rRNA and is indicated to the right. Lanes 1, strain 13; lanes 2, strain TS133.

were carried out with RNA derived from JIR4000(pJIR870) and JIR4000(pJIR872). The plasmid pJIR872 contains a complete copy of the *virR/virS* locus, whereas pJIR870 has a truncated *virS* gene (8). Northern blots (Fig. 2) of RNA from JIR4000(pJIR872) revealed both the chromosomal 1.2-kb transcript and the plasmid-derived 2.1-kb transcript when probed with the *virR* fragment, while only the 2.1-kb transcript

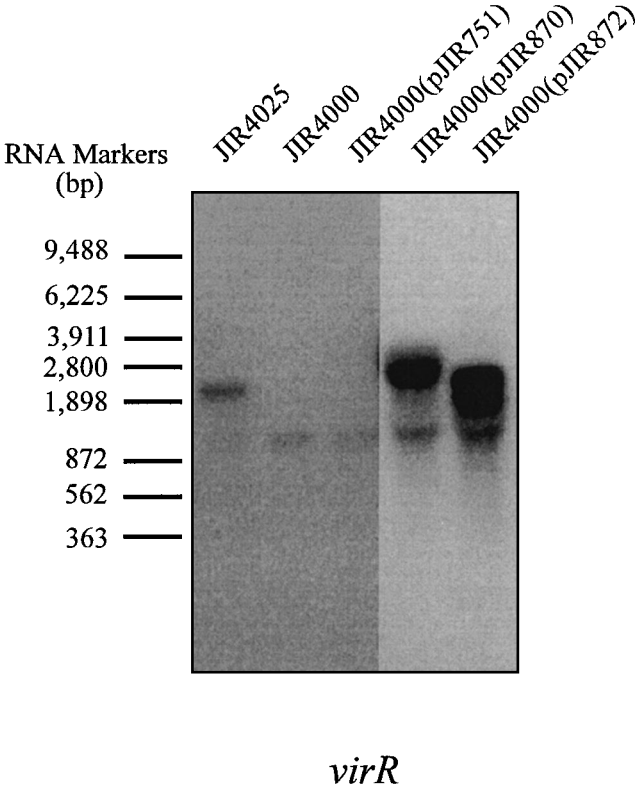

FIG. 2. Northern analysis of the *virS* mutant JIR4000 and its derivatives. Total RNA was isolated from JIR4025 and derivatives of JIR4000 and probed with a *virR*-specific 0.6-kb *Bgl*II-*Eco*RI fragment from pJIR872 (8). The filter was hybridized as described in Materials and Methods for the RNA slot blots. Molecular size markers are shown.

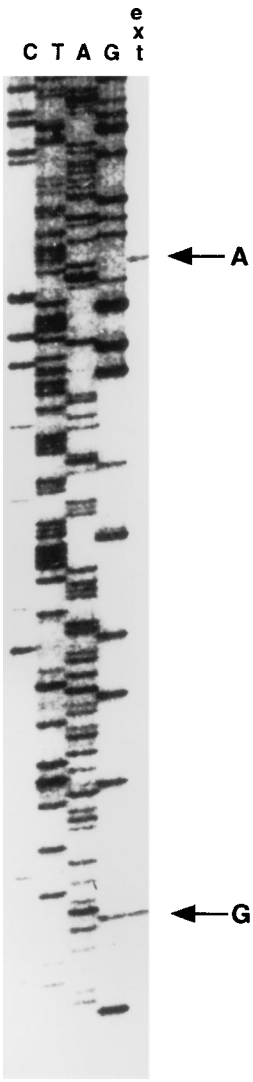

FIG. 3. Primer extension analysis of the *virR*/*virS* operon. RNA from strain 13 was analyzed with primer virR-2. The extension products (ext) were examined in parallel with sequence ladders (loaded in the order CTAG) generated with the same primer with pBT404. The arrows indicate the transcription start sites.

hybridized to the *virS* probe (data not shown). The same *virR*-specific 1.2-kb transcript was detected in RNA from JIR4000 (pJIR870). However, a larger plasmid-derived transcript with a size of ca. 2.5 kb was detected with both the *virR* and *virS* fragments. These results are consistent with the presence of a single *virR-virS* transcript, the deletion of the normal transcriptional terminator in pJIR870, and the extension of the transcript into the plasmid vector. Taken together, the results from the analysis of the *virR* and *virS* mutants provide convincing evidence that the *virR* and *virS* genes are transcribed as a single mRNA molecule and therefore make up an operon.

Identification of the *virR*/*virS* promoter. The *virR*/*virS* promoter region was tentatively identified by primer extension analysis, and two specific products were observed (Fig. 3). The apparent transcription start points were located 31 (P1) and 133 (P2) bp upstream of the start codon (ATG) of the *virR* gene (Fig. 4). Sequences with similarity to σ^{70} promoters were found upstream of each of these start sites. However, the putative P1 promoter had a suboptimal spacing of 19 bp between the −35 and −10 regions (Fig. 4).

Transcriptional analysis of genes regulated by *virR* and *virS*. To determine whether the products of the *virR* and *virS* genes act at the transcriptional level, Northern blots were carried out with the *virR* mutant TS133, and RNA slot blots were done with the *virS* mutant JIR4000 and its derivatives. With a *pfoA*-specific probe, three mRNA species (2.0, 1.7, and 0.8 kb) were detected in strain 13 (Fig. 5). These bands were not detected in RNA preparations from TS133 but were present in TS133 (pBT404). RNA slot blots revealed that the amount of *pfoA*-specific RNA was reduced significantly in JIR4000 compared with that in its parent strain, JIR4025 (Fig. 6). These RNA levels were restored in the presence of the *virR*$^+$ plasmid pJIR870 and the *virR*$^+$ *virS*$^+$ plasmid pJIR872 (Fig. 6). On the basis of these data, it was concluded that the transcription of the *pfoA* gene was positively regulated by the *virR*/*virS* system and that a multicopy *virR* gene could partially complement strains defective in *virS*, as previously proposed (8).

Previous studies suggested that the product of the *pfoR* gene is a positive regulator of *pfoA* (20). Similar Northern and RNA slot blot experiments therefore were carried out with a *pfoR*-specific probe. In both of our laboratories, *pfoR*-specific mRNA was barely detectable. A very faint 1.6-kb mRNA molecule was observed in RNA from strain 13 but not in that from strain TS133 (Fig. 5). Similarly, slot blot experiments revealed that increased *pfoR*-specific transcript was observed when the *virS* mutant was complemented by plasmids carrying the *virR* and *virS* genes, although the original *virS* mutation appeared to have little effect on *pfoR* expression in JIR4000 (Fig. 6). Because of the extremely small amount of the *pfoR* transcript detected in the wild-type and mutant strains, these signals were very close to the background level. When the studies with the recombinant plasmids are considered, it can be tentatively concluded that the low level of transcription of the *pfoR* gene was positively regulated by the *virR*/*virS* system.

Quantitative determination of both alpha-toxin (8) and kappa-toxin (19) levels revealed that the production of both enzymes was partially regulated by the *virR* and *virS* genes. Constitutive, but lower-level, expression of both toxins was still observed in the *virR* and *virS* mutants. RNA analyses carried out with both *plc*-specific and *colA*-specific probes confirmed that the regulation of these genes also occurred at the level of transcription (Fig. 5 and 6). Consistent with the enzyme assay data, mRNA levels appeared to be only partially reduced in both the *virR* and *virS* mutants. Complementation by multicopy *virR* and *virS* genes restored mRNA synthesis to at least wild-type levels.

The conclusions derived from the qualitative examination of the slot blot data were validated by scanning the blots with a densitometer and determining the relative amounts of each specific transcript by comparison with that of a 16S rRNA

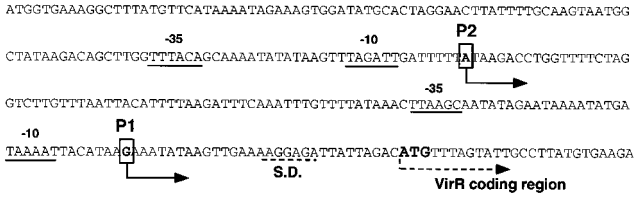

FIG. 4. Promoter region of the *virR*/*virS* operon. The consensus promoter sequences (−35 and −10) are underlined, and the P2 and P1 transcription start sites are boxed. The putative ribosome binding site (S.D. [Shine-Dalgarno]) is indicated by the broken line. The *virR* gene is indicated by the broken arrow.

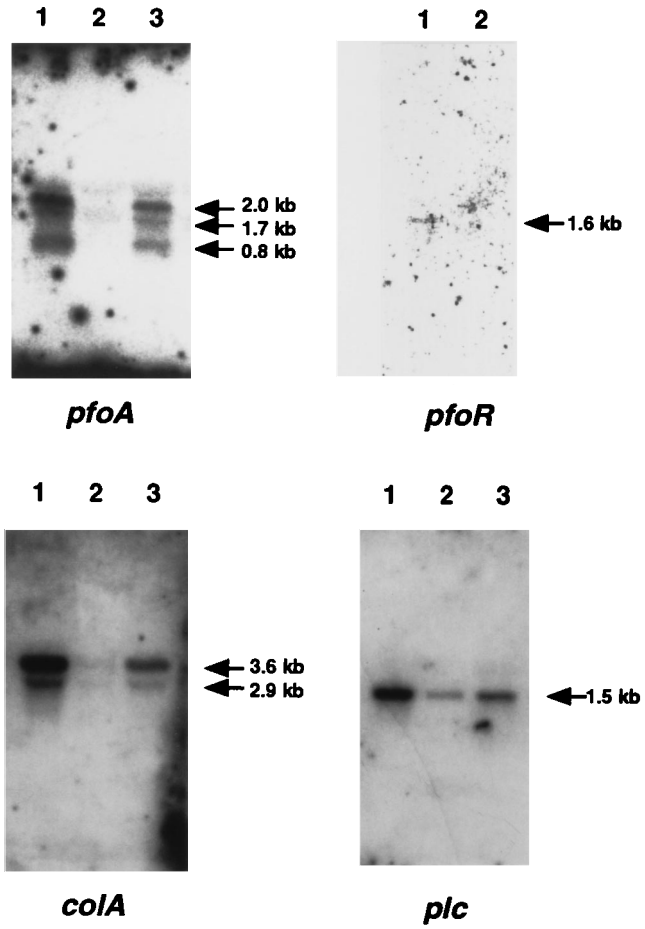

FIG. 5. Northern analysis of the *C. perfringens pfoA*, *pfoR*, *colA*, and *plc* genes. RNA blots prepared as described before were hybridized with the following probes as indicated: *pfoA* (1.5-kb *Hin*dIII-*Eco*RI fragment from pTS302), *pfoR* (0.9-kb *Hin*dIII-*Apa*LI fragment from pTS302), *colA* (1.5-kb *Xba*I-*Sma*I fragment from pKY3135), and *plc* (1.1-kb *Bam*HI-*Hin*dIII fragment from pKB300). The sizes of the hybridizing fragments were calculated from the mobilities of 23S and 16S rRNA and are indicated by the arrows. Lanes 1, strain 13; lanes 2, TS133; lanes 3, TS133(pBT404).

control (Table 2). The results confirmed that the *virS* mutation led to reductions in *pfoA*, *plc*, and *colA* mRNA levels, which were restored to various degrees (but at least to wild-type levels) by the plasmid-encoded *virR* and *virS* genes. They also confirmed that the *virS* mutation had a much more dramatic effect on the transcription of the *pfoA* gene than on the other toxin genes that were examined, which again correlates with the previous observation that no theta-toxin was detected in the *virS* mutant (8).

TABLE 2. Comparative analysis of RNA slot blots

Strain	Relative RNA level[a]				
	plc	*colA*	*pfoA*	*pfoR*	16S
JIR4025	1.00	1.00	1.00	1.00	1.00
JIR4000	0.10	0.10	0.00	0.65	1.00
JIR4000(pJIR751)	0.47	0.20	0.00	0.44	1.00
JIR4000(pJIR870)	3.15	2.45	1.28	1.16	1.00
JIR4000(pJIR872)	6.42	5.34	4.91	2.53	1.00

[a] Values represent the ratio of specific mRNA levels to those in the 16S rRNA blot.

Primer extension analysis of *virR*/*virS*-regulated genes. We have shown that the response regulator VirR positively regulates the transcription of at least four different structural genes. If the activated VirR protein directly regulates the expression of these genes, then it may bind to sequences located near the respective promoters. To identify these promoters, a series of primer extension experiments were performed with total RNA from strains 13 and TS133.

Examination of the *pfoA*-specific mRNA revealed that there were two apparent transcriptional start sites, P1 and P2, which were located 273 and 301 bp upstream of the start codon of the *pfoA* gene, respectively (Fig. 7A). When a primer which annealed to a region downstream of the start codon was used, no other apparent transcriptional start sites were detected (data not shown). The major extension product, P2, was observed only in strain 13, whereas the minor product, P1, was present in both strains 13 and TS133. This result clearly indicates that P1 and P2 transcripts are derived from *virR*/*virS*-independent and *virR*/*virS*-dependent promoters, respectively. On the basis of the relative densities of the primer extension products and previous toxin assay data (8, 19), it was concluded that the VirR-regulated P2 transcript was the transcript primarily responsible for the production of theta-toxin.

The *colA* apparent transcriptional start sites were also divided into two categories (Fig. 7B). Two major (P1 and P2) and three minor (P3, P4, and P5) primer extension products were observed both in strain 13 and in strain TS133. The minor products may reflect premature termination or stalling of the primer extension reaction. It was apparent that the P1/P2 transcripts were derived from a *virR*/*virS*-independent promoter. In contrast, another pair of primer extension products (P6 and P7) was observed only in strain 13 and not in strain TS133. It was concluded that the promoter responsible for these transcripts was *virR*/*virS* dependent and that the observed reduction in kappa-toxin production in the *virR* mutant resulted from decreased transcription from this promoter.

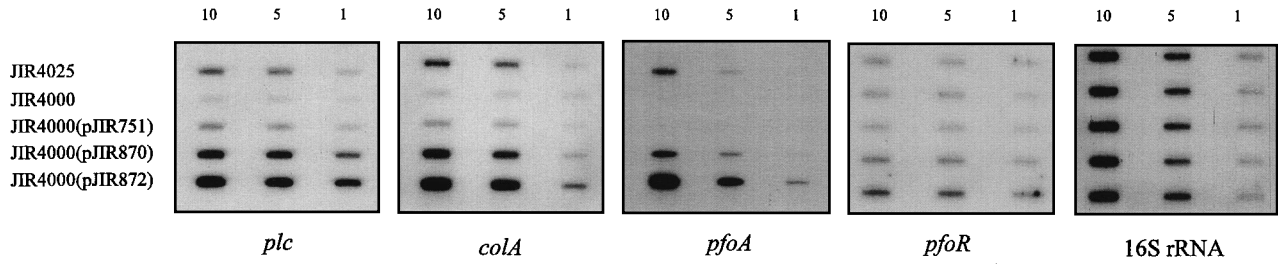

FIG. 6. RNA slot blots of JIR4025 and its derivatives. Various amounts of RNA (10, 5, or 1 μg as shown at the top) from the *C. perfringens* strains indicated were hybridized with the probes specific for the *plc*, *colA*, *pfoA*, *pfoR*, and 16S rRNA genes.

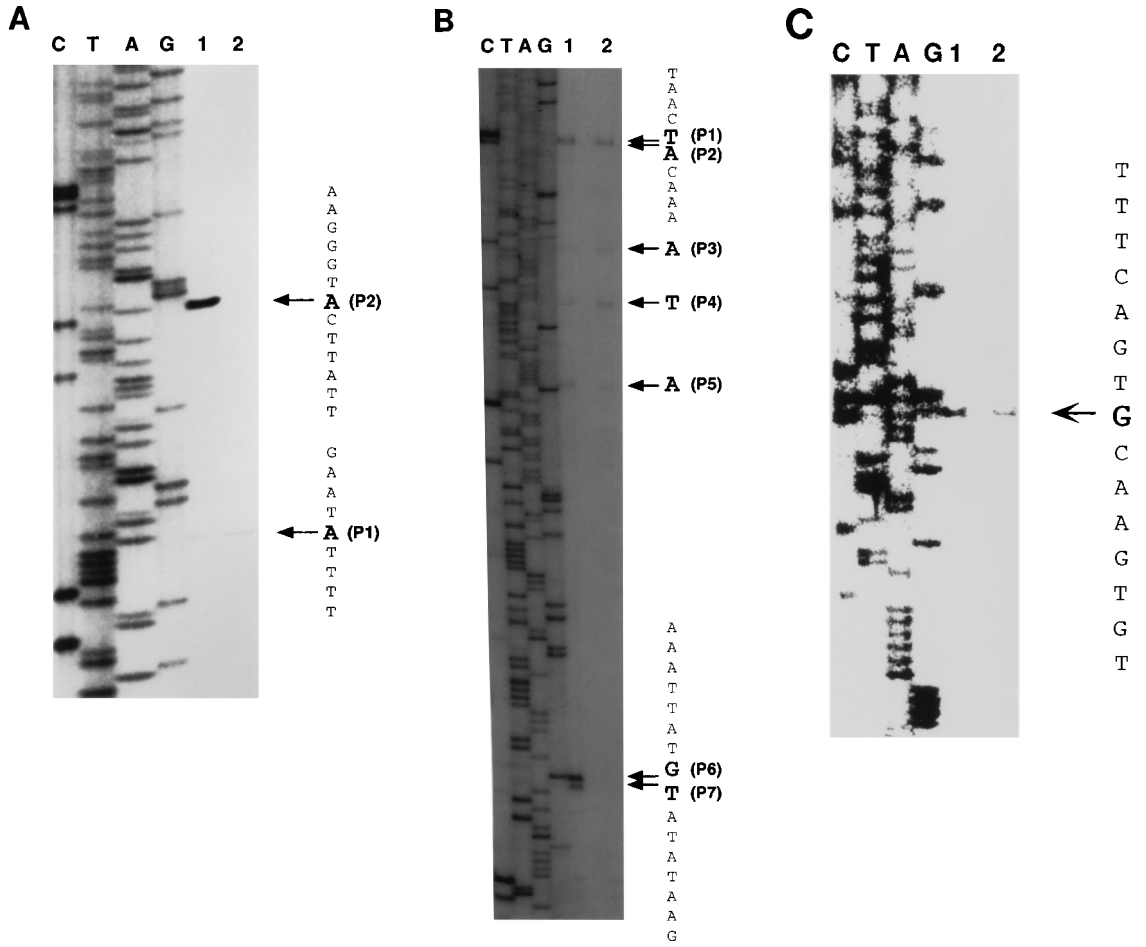

FIG. 7. Primer extension analysis of toxin structural genes. Primer extension products and corresponding sequencing ladders (loaded in the order CTAG) are shown. (A) *pfoA* reactions with primer pfoA-2 and pTS302. (B) *colA* reactions with primer colA-1 and pOT201. (C) *plc* reactions with primer plc-1 and pKB300. The arrows indicates the primer extension products. Lanes 1, strain 13; lanes 2, TS133.

A different situation was observed for the *plc* gene. A single primer extension product was detected in strain 13 with a *plc*-specific primer (Fig. 7C). This product was also detected in strain TS133, but there was much less transcript than in strain 13. These data indicated that the *plc* gene was transcribed from a single promoter and that although transcription from this promoter was positively regulated by VirR protein, there was a basal level of transcription from this promoter, even in the absence of a functional *virR* gene.

Comparative analysis of the promoter regions of *virR/virS*-regulated genes. The consensus promoter sequences for the strain 13 *pfoA*, *colA*, and *plc* genes were deduced from the results of the primer extension experiments. Potential σ^{70} promoter sequences were found upstream of both the P1 and P2 *pfoA*-mRNA start sites (Fig. 8). These putative *pfoA* promoters were located a considerable distance upstream of the start codon of the *pfoA* gene coding region (301 and 273 bp for P2 and P1, respectively). Direct repeats (CCCAGTTNTNCAC) were found in the region upstream of the P2 promoter sequence.

There were no directly repeated or palindromic sequences in the flanking regions of either of the *colA* promoters. The putative *virR/virS*-dependent promoter upstream of the P6/P7 transcriptional start sites had σ^{70}-like binding sequences (Fig. 8), but an atypical −35 box was associated with the P1/P2 sites.

The strain 13 *plc* promoter sequence was almost identical to the deduced promoter sequence reported previously (12, 23), except for the final nucleotides in the −35 region, which were C in NCTC8237 and G in strain 13 (Fig. 8). The strain 13 *plc* transcription start site was identical to the start site previously reported in NCTC8237 (24). Although the promoter sequence was the same as that reported in strain 8-6 by other workers (16), the transcription start point was different from that of strain 8-6. Finally, the (dA-dT)n regions, which are thought to be involved in DNA bending and the regulation of *plc* gene expression (25), were present in the region adjacent to the *plc* promoter of strain 13.

DISCUSSION

In many bacterial two-component regulatory systems, the genes encoding the response regulator and the sensor histidine kinase are transcribed as a single mRNA molecule (22). Examination of the wild-type strain and derivatives of the *virS* mutant JIR4000 revealed that the overlapping *virR* and *virS* genes were transcribed as a single 2.1-kb mRNA molecule and therefore composed an operon. These genes appeared to be transcribed from two promoters that were 102 bp apart. Previous studies showed that the *virS* mutation in JIR4000 could be partially complemented by a multicopy $virR^+$ gene (8).

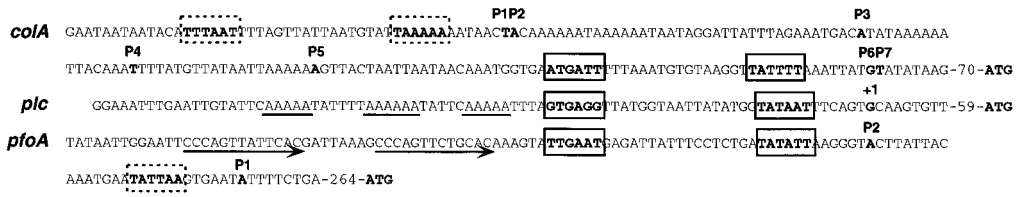

FIG. 8. Comparison of the promoter regions of the strain 13 *colA*, *plc*, and *pfoA* genes. The consensus promoter sequences (−35 and −10) of *virR*/*virS*-dependent and independent promoters are boxed with solid and broken lines, respectively. The directly repeated sequences upstream of the *pfoA* promoter are indicated by the arrows. The dA-rich regions upstream of the *plc* gene are underlined.

Consistent with these results, transcription of the *virR*/*virS*-regulated genes (*pfoA*, *colA*, and *plc*) in TS133 was complemented partially by transformation with pBT404, which only carried the wild-type *virR* gene. The mechanism by which activation of the VirR protein occurs in the absence of VirS is not known but may involve cross talk from other histidine kinases (26) or nonspecific phosphorylation by low-molecular-weight phospho-donors (7), as discussed previously (8).

The *virR*/*virS* system is important in the pathogenesis of *C. perfringens* infections because it controls the expression of virulence-associated genes in a global fashion. Specifically, it is known to be involved in the regulation of alpha-toxin, theta-toxin, kappa-toxin, sialidase, and protease production (8, 19). In this study, we have shown that this regulatory system controls the expression of the *pfoA*, *pfoR*, *colA*, and *plc* genes at the transcriptional level.

A major finding of these experiments was that the mode of regulation of the various toxin structural genes by the *virR*/*virS* system differed from gene to gene. The gene most stringently regulated by VirR, the *pfoA* gene, appeared to be transcribed from the major promoter, P2, and a minor promoter, P1. Transcription from P2 and expression of theta-toxin activity required a functional VirR protein. Despite the observation that the P1 promoter was functional in a *virR* mutant, no *pfoA*-specific mRNA or theta-toxin activity was detected in the mutants TS133 and JIR4000. It is concluded that transcription from the putative P1 promoter makes a negligible contribution to the expression of the *pfoA* gene under the conditions used. In strain 13, at least three *pfoA*-specific mRNA molecules were detected. The two smaller molecules presumably represent degraded or incompletely transcribed mRNA species, since these bands were absent in TS133 but were detected in TS133 (pBT404).

The *colA* gene also appeared to be transcribed from two promoters, one of which was *virR*/*virS* dependent. Both promoters (P1/P2 and P6/P7) appeared by primer extension analysis to lead to the production of major transcripts, although the amount of the transcripts derived from the *virR*/*virS*-dependent P6/P7 promoter was greater than that from the *virR*/*virS*-independent P1/P2 promoter. The results indicate that activation of P6/P7 by VirR is required for optimal expression of the *colA* gene in *C. perfringens.*

In contrast to the other target genes, the *plc* gene appeared to be expressed from a single promoter. The VirR protein enhanced the transcription of *plc* from this promoter, but significant levels of *virR*-independent *plc*-specific mRNA were detected by both Northern and primer extension analyses in TS133 and by RNA slot blot analysis in JIR4000. These data are in agreement with the observations that alpha-toxin is still produced in TS133 (19) and JIR4000 (8), albeit at reduced levels. It is also consistent with the findings of other workers who performed in vitro transcription experiments with a *plc* template and purified RNA polymerase from *C. perfringens* and showed (16) that the *plc* gene was expressed in vitro in the absence of VirR. Taken together, the data show that the *plc* gene appears to be transcribed at a basal constitutive level in *C. perfringens.*

Upstream of the *pfoA* gene, a divergently transcribed positive regulatory gene, *pfoR*, has been identified by a series of deletion analyses carried out with *E. coli* (20). The deduced PfoR protein contained typical DNA binding motifs and could activate the expression of the *pfoA* gene in *cis*. In this study, the *pfoR* transcript in strain 13 was identified by Northern analysis, although the amount of the *pfoR*-specific mRNA detected was extremely small. Northern and slot blot analyses indicated that the VirR protein also positively regulated the transcription of the *pfoR* gene in *C. perfringens*, but to a lesser extent. It is possible that *pfoA* gene expression is positively regulated by the product of the *pfoR* gene, which is in turn regulated by VirR. That is, the observed effects of both the *virR* and *virS* mutations on the expression of *pfoA* may not be mediated by the direct action of VirR on the *pfoA* promoter region but may be mediated by the PfoR protein. Alternatively, the VirR protein may regulate the expression of both the *pfoA* and *pfoR* genes, with its effects on the latter amplifying its ability to positively regulate the production of theta-toxin from *pfoA*. The role of the *pfoR* gene in *pfoA* regulation needs to be examined further to clarify the precise mechanism that regulates *pfoA* gene expression.

Comparative analysis of the *virR*/*virS*-dependent promoters of the *pfoA*, *colA*, and *plc* genes did not reveal any sequence motifs common to these promoter regions. It was not possible to identify shared sequences which had the potential to act as the sites to which the putative phosphorylated VirR protein could bind and positively regulate gene expression. VirR may therefore resemble the OmpR response regulator, which does not have a single consensus binding sequence (13). Alternatively, by analogy with the postulated interaction between *pfoR* and *pfoA*, the results may imply that there are as yet unidentified regulatory factors which independently control the expression of the *colA* and *plc* genes. It seems unlikely that the *pfoR* gene also regulates the *colA* and *plc* genes, considering its postulated *cis*-dominant action (20). There is preliminary evidence that some *C. perfringens* isolates have a gene whose product binds to the *plc* gene and positively regulates *plc* gene expression (6). This putative regulatory gene has been designated as *plcR* (8, 15). The effects of VirR on *plc* gene expression may be modulated via the putative *plcR* gene. Similarly, the *virR*/*virS* system may regulate the expression of the *colA* gene through another unidentified regulatory factor, which we would postulate to be the product of the *colR* gene.

In summary, it is clear that we have much to learn about the regulation of extracellular toxin production in *C. perfringens*. Hopefully, the continued application of the now considerable tools available for genetic analysis and manipulation of this

organism will lead to the elucidation of the precise mechanism of action of this complex regulatory network.

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