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The *virR* Gene, a Member of a Class of Two-Component Response Regulators, Regulates the Production of Perfringolysin O, Collagenase, and Hemagglutinin in *Clostridium perfringens*

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The perfringolysin O (theta-toxin) gene (*pfoA*) of *Clostridium perfringens* was cloned into an *Escherichia coli-C. perfringens* shuttle vector, and the *pfoA* gene was expressed in mutants of *C. perfringens* 13 which lacked the production of perfringolysin O. One group (SI117) could express the *pfoA* gene, and the other (SI112) could not. A mutation in the regulatory system for *pfoA* gene expression was suspected in SI112. A chromosomal DNA library constructed from strain 13 was transformed into strain SI112 to identify the regulatory gene(s) for the *pfoA* gene. Five strains of 10,000 transformants restored perfringolysin O production. All contained a 2.5-kb DNA fragment. This fragment activated the transcription of the *pfoA* gene and also restored the production of collagenase (kappa-toxin) and hemagglutinin in strain SI112. Deletion analysis showed that a 1.25-kb region was sufficient for the *trans* activity, and sequence analysis disclosed that open reading frame 2 (ORF2) was located in this region. A homology search for the deduced amino acid sequence revealed that ORF2 was homologous to a response regulator in a two-component signal transduction system. ORF2 was designated *virR*, and it is suggested that the *virR* gene plays an important role in the pathogenicity of *C. perfringens*.

The gram-positive anaerobe *Clostridium perfringens* produces a variety of exotoxins which are responsible for its pathogenicity (9, 14, 25). The structure and function of these exotoxins have been extensively studied, and some of these toxin genes were cloned and sequenced elsewhere (19). We have previously cloned and sequenced the alpha-toxin gene (*plc*), the perfringolysin O (theta-toxin) gene (*pfoA*), and its regulatory gene (*pfoR*) (16, 22). Regulation of these virulence genes appears to be complex, and little is known about its molecular mechanism.

In many pathogenic bacteria, expression of virulence genes is regulated by certain factors which enable the organisms to produce virulence factors in response to certain environmental stimuli during the course of infection (15). Some evidence for a possible regulatory mechanism for the alpha-toxin gene in *C. perfringens* has been reported previously (12, 27). Imagawa et al. reported that production of a set of exotoxins, perfringolysin O, collagenase (kappa-toxin), protease (lambda-toxin), and hemagglutinin (HA), in *C. perfringens* is regulated by a *trans*-acting diffusible factor termed substance A (11). Although these observations suggest the presence of a global regulatory mechanism for the production of exotoxins, no direct evidence for such a regulatory mechanism has been confirmed in *C. perfringens*.

In a previous report, we described the *pfoR* gene, a positive regulator of the *pfoA* gene (22). In this study, we isolated a mutant (strain SI112) whose structural genes seem to be intact but do not produce perfringolysin O, collagenase, or HA. The mutant was thought to be defective in a coordinating regulatory factor for the expression of these toxin genes. A chromosomal DNA fragment which was cloned from the parent strain into an *Escherichia coli-C. perfringens* shuttle vector, pJIR418 (24), gave rise to the production of these toxins in strain SI112. Deletion and sequence analysis revealed that the cloned fragment carried a *trans*-acting regulatory gene (*virR*) for the production of these toxins and that the *virR* gene appears to be a response regulator of the two-component signal transduction systems characteristic of many bacteria.

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MATERIALS AND METHODS

Strains, media, and plasmids. *C. perfringens* 13 (13) was used as a host for plasmid pJIR418 (24) derivatives. *E. coli* JM109 (30) was used for both pJIR418 and pUC19 (30) derivatives. *C. perfringens* strains were cultured in Gifu anaerobic medium (GAM) broth or GAM agar plates (Nissui Co., Ltd., Tokyo, Japan) at 37°C under anaerobic conditions by using AnaeroPack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). *E. coli* strains were cultured in Luria-Bertani broth at 37°C. Chloramphenicol-containing media (25 μg/ml) were used for strains harboring pJIR418 derivatives. Ampicillin-containing media (50 μg/ml) were used for strains containing pUC19 derivatives. Tetracycline (2.5 μg/ml) was also used for some experiments.

Chemicals and enzymes. All the chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), except that PZ peptide (4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg) chloramphenicol, ampicillin, and tetracycline were from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and modifying enzymes were obtained from Nippon Gene Co., Ltd. (Toyama, Japan); Toyobo Co., Ltd. (Osaka, Japan); and Takara Shuzo Co., Ltd. (Kyoto, Japan). The Geneclean kit was purchased from Bio 101, Inc. (La Jolla, Calif.). Nylon filter membranes (GeneScreen Plus membrane) were purchased from Dupont, NEN Research Products (Boston, Mass.).

Isolation of mutants. *C. perfringens* 13 was grown in 10 ml of GAM broth at 37°C, until the optical density at 600 nm (OD_{600}) reached 0.8. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was added to the culture to a final concentration of 0.02%, and

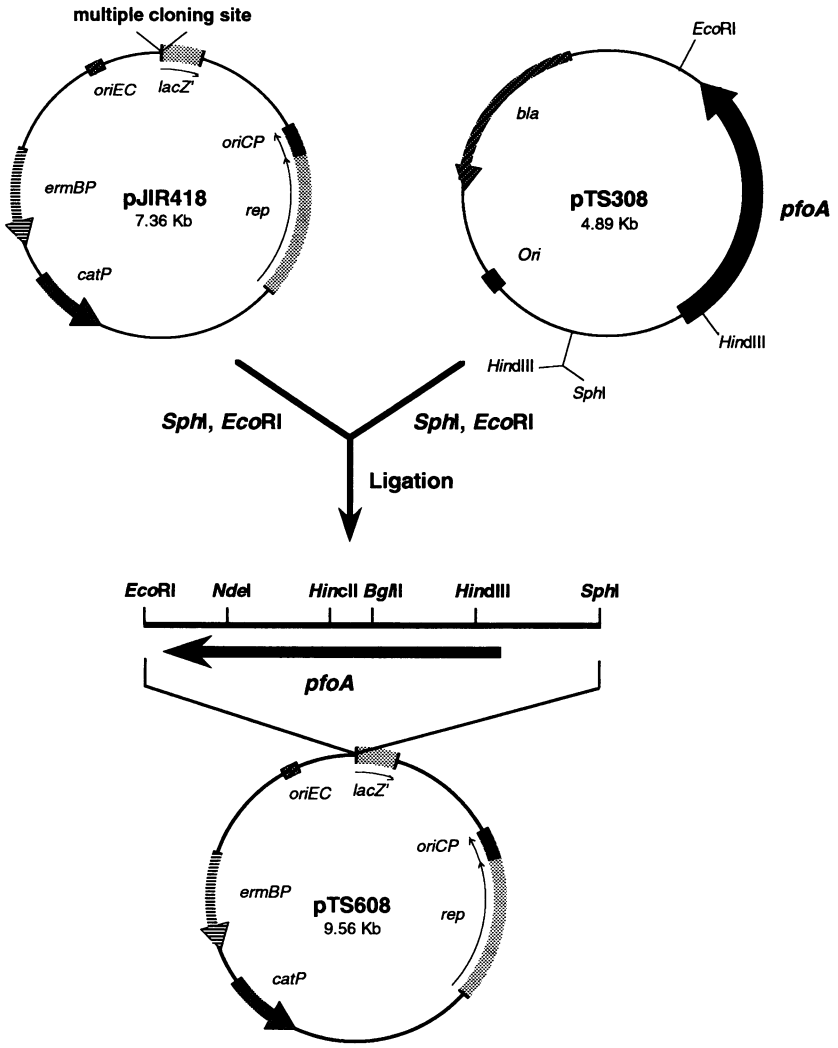

FIG. 1. Construction of a shuttle plasmid carrying the *pfoA* gene. The 2.2-kb *Sph*I-*Eco*RI fragment of pTS308 (22) was ligated to the *Sph*I-*Eco*RI site of pJIR418 (24). The resulting plasmid, pTS608, contained the *pfoA* gene and its possible promoter region.

the mixture was further incubated for 1 h at 37°C. The culture was centrifuged, and the cells were washed three times with 10 ml of phosphate-buffered saline and suspended in 10 ml of GAM broth. The suspension was cultured at 37°C overnight, and aliquots were spread onto GAM-sheep blood agar to screen for hemolysis-negative colonies.

DNA techniques. Chromosomal DNA was prepared from *C. perfringens* by the method described previously (16). Plasmids were isolated from *C. perfringens* according to the method of Roberts et al. (18). Transformation of *C. perfringens* with plasmid DNA was performed by the electroporation method of Allen and Blaschek (3, 4) with some modifications. Briefly, *C. perfringens* was cultured in GAM broth until the OD_{600} reached 3.0. The cells were harvested, washed twice, and suspended in 1/10 volume of 15% glycerol. Then 80 μl of cell suspension was mixed with 1 to 5 μg of plasmid DNA in a 2-mm gap cuvette and placed on ice for 10 min. Cells were pulsed at a field strength of 12.5 kV/cm and pulse length of 5.28 ms, using a Cellject Basic electroporator (EquiBio S.A., Angleur, Belgium). After electric shock, cells were placed on ice for 10 min, then suspended in 500 μl of GAM broth, and incubated for 2 h at 37°C. Finally, the cells were plated on appropriate selective media. All other recombinant DNA techniques were performed according to the method of Sambrook et al. (20), unless otherwise noted.

Construction of a *C. perfringens* chromosomal DNA library. Chromosomal DNA from *C. perfringens* 13 was partially di-

TABLE 1. Hemolytic activities of *C. perfringens* 13 and mutant strains with pTS608

Strain	Perfringolysin O activity[a] (U/μg of protein [mean ± SD])
13	4.6 ± 0.8
13(pJIR418)	4.1 ± 0.2
13(pTS608)	8.9 ± 1.3
SI112	<0.3 ± 0.1
SI112(pJIR418)	<0.3 ± 0.1
SI112(pTS608)	0.5 ± 0.1
SI107	ND
SI107(pJIR418)	ND
SI107(pTS608)	4.7 ± 0.2

[a] Each value was calculated from triplicate experiments. ND, not detected.

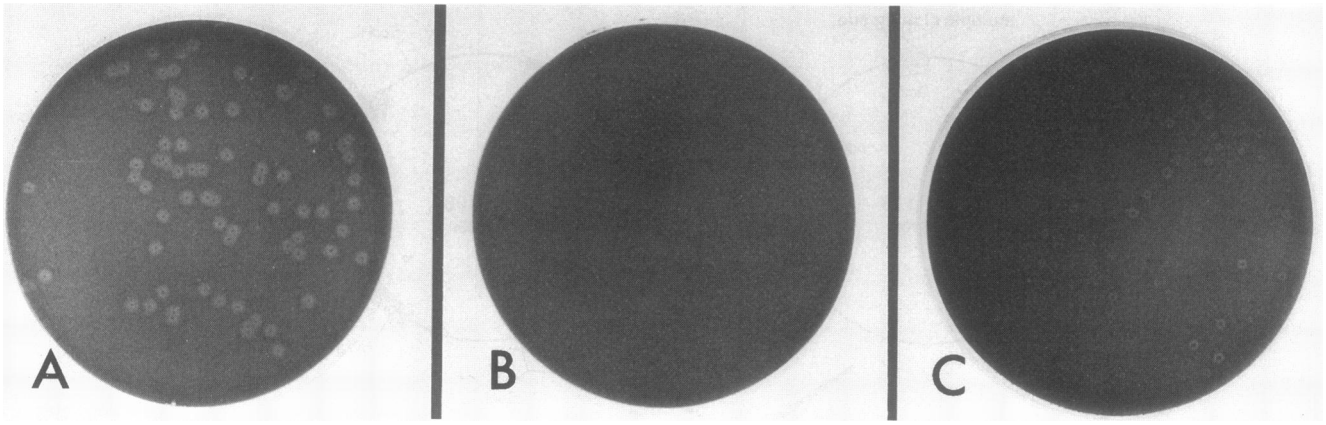

FIG. 2. Hemolysis of *C. perfringens* strains on sheep blood agar plates. Each strain was plated on GAM-sheep blood agar containing 25 μg of chloramphenicol per ml and cultured overnight at 37°C under anaerobic conditions. (A) 13(pJIR418); (B) SI112(pJIR418); (C) SI112(pBT404).

gested with *Hin*dIII, and 3- to 7-kb fragments were recovered from the agarose gel with a Geneclean kit. The recovered fragments were ligated to the dephosphorylated *Hin*dIII site of plasmid pJIR418 and transformed to *E. coli* JM109. The colonies of transformants on the plates were collected in Luria-Bertani broth, and an aliquot was inoculated into 100 ml of Luria-Bertani broth and cultured overnight. The plasmid library was obtained by large-scale plasmid preparation.

Northern and Southern hybridization. For Northern (RNA) hybridization, strains of *C. perfringens* were grown in GAM broth at 37°C and total RNA was recovered by the sodium dodecyl sulfate-phenol method (1) when the culture reached an OD_{600} of 0.8. The DNA probe for the *pfoA* gene (*Hin*dIII-*Nde*I 1.2-kb fragment) was prepared from pTS308 (22). The probe was labeled with [α-^{32}P]dCTP (~3,000 Ci/mmol; ICN Biomedicals Inc., Costa Mesa, Calif.) by the random-primer method (7). Northern hybridization was performed as described previously (20), and the autoradiogram was analyzed with BAS 2000 Bio-Imaging Analyser (Fuji Photo Film Co., Ltd., Kanagawa, Japan). Southern hybridization was performed basically according to the method of Southern (25a). The DNA probe was labeled with digoxigenin-11-dUTP, and the hybridized fragments were detected with a digoxigenin DNA labeling and detection kit (Boehringer GmbH, Mannheim, Germany). All the procedures were performed according to the instructions of the manufacturer.

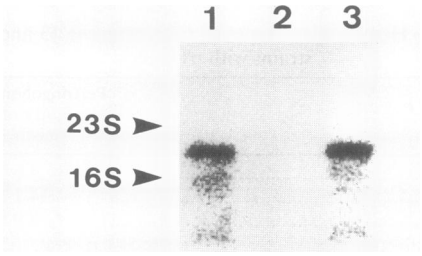

FIG. 3. Northern blot of *pfoA* mRNA from *C. perfringens* strains. RNA was extracted from logarithmic-phase *C. perfringens* grown in GAM broth, and 3 μg of each RNA preparation was denatured, electrophoresed on a formaldehyde denaturing gel, and blotted onto a nylon filter membrane. A ^{32}P-labeled *pfoA* fragment (1.2 kb) was used for a probe. Lanes: 1, 13(pJIR418); 2, SI112(pJIR418); 3, SI112 (pBT404). The 23S rRNA (2.9 kb) and 16S rRNA (1.5 kb) were used as size markers (on the left).

Assays. Titration of hemolysis of the culture supernatant was performed according to the method of Yamakawa and Sato (29), except that we used GAM broth and 5-h cultures. Hemolytic activity was expressed as the reciprocal of the dilution which showed 50% hemolysis of 0.5% sheep erythrocytes. The collagenase activity was measured by the PZ-peptide hydrolyzing method (28). Briefly, 100 μl of the culture supernatant was mixed with 1 ml of PZ-peptide solution (0.2 mM PZ peptide, 40 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.1). The mixture underwent incubation at 37°C for 30 min followed by the addition of 0.5 ml of 1.5 M citrate buffer (pH 4.5) and extraction with 2.5 ml of ethyl acetate. The collagenase activity was expressed as the A_{320} per milligram of protein of the supernatant. HA in the culture supernatant was detected by the method described previously (11), using chicken erythrocytes. DNase activity was detected on DNA agar plates (Nissui Co., Ltd.) by the change of color around the colonies. Alpha-toxin activities were assayed by the egg yolk agar plate method described elsewhere (16). The protein concentration of the culture supernatant was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Nucleotide sequence determination. Nucleotide sequencing of both strands was performed by the dideoxy-chain termination method (21). Unidirectional deletions of plasmids were made with a Kilo-Sequence deletion kit (Takara Shuzo Co., Ltd.). Appropriately deleted plasmids were subjected to nucleotide sequencing, using a Sequenase version 2.0 kit (Stratagene, La Jolla, Calif.) and [α-^{35}S]dCTP (Amersham International plc,

TABLE 2. Activities of various toxins in *C. perfringens* 13 and the mutants carrying plasmids[a]

Strain	Perfringolysin O (U/μg of protein)[b]	Collagenase (OD units/mg of protein)[b]	HA
13	4.6 ± 0.8	30.4 ± 0.5	+
13(pJIR418)	4.1 ± 0.2	15.2 ± 0.3	+
SI112	<0.3 ± 0.1	5.1 ± 0.2	−
SI112(pJIR418)	<0.3 ± 0.1	4.8 ± 0.4	−
SI112(pBT404)	2.3 ± 0.1	18.6 ± 0.5	+

[a] All activities were assayed as described in Materials and Methods. All strains were positive for both alpha-toxin and DNase.

[b] Each value was calculated from triplicate experiments (mean ± standard deviation).

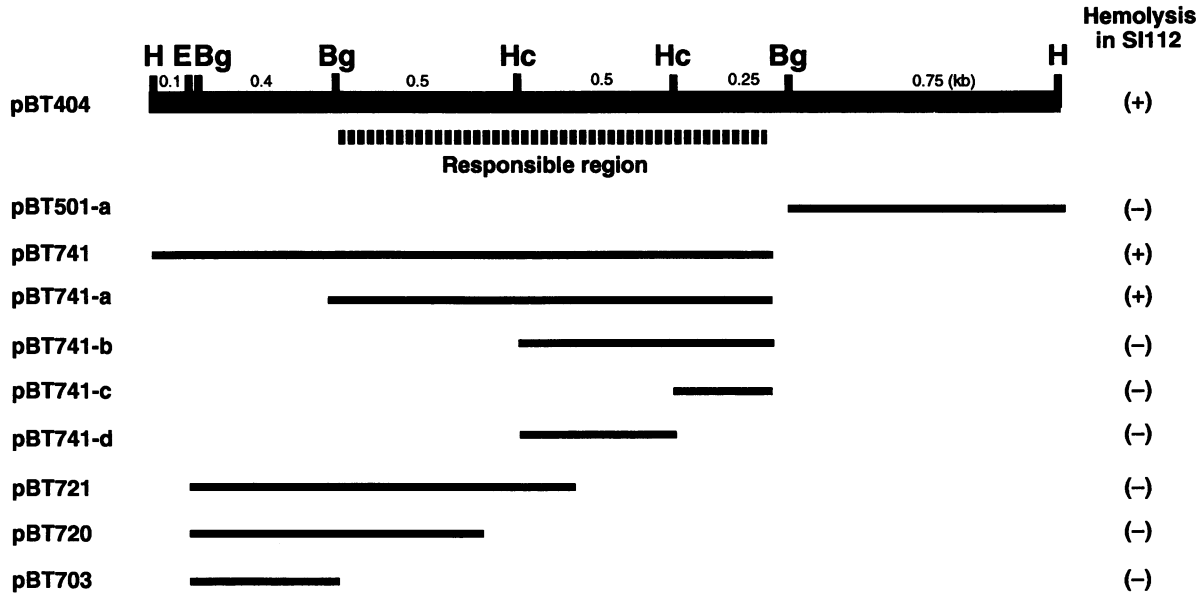

FIG. 4. Restriction mapping and deletion analysis of pBT404. The cloned 2.5-kb fragment with restriction sites is indicated at the top, and the deleted fragments are represented by lines. Strain SI112, as the recipient, was transformed with each deleted plasmid. The *trans* activity of each fragment was determined by determining the hemolytic activities (perfringolysin O hemolysis) of strain SI112 on sheep blood agar plates. Hemolysis is expressed as positive (+) or negative (−). The region responsible for *trans* activity for hemolysis is indicated by the thick broken line. Abbreviations for restriction sites: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII.

Buckinghamshire, England). Some synthetic oligonucleotide primers were used for some sequences.

Nucleotide sequence accession number. The sequence data used in Fig. 5 will appear in the DDBJ/GenBank/EMBL nucleotide sequence data library with accession number D14877.

RESULTS

Expression of the *pfoA* gene in *C. perfringens* strains. The 2.2-kb *Sph*I-*Eco*RI fragment of pTS308 comprising the *pfoA* gene (22) was ligated to the *Sph*I and *Eco*RI site of the *E. coli-C. perfringens* shuttle vector, pJIR418. The resulting plasmid was designated pTS608 (Fig. 1) and transformed to *C. perfringens* 13 and the mutant strains by electroporation.

The expression of the *pfoA* gene in perfringolysin O-deficient mutants showed two distinctive patterns. One group of mutants (group I) successfully expressed the *pfoA* gene with transformation of pTS608, while another group (group II) failed to express the *pfoA* gene on pTS608. Strains SI107 (group I) and SI112 (group II) were selected as the prototypes and were subjected to further studies.

Strain SI107 harboring pTS608 showed almost the same hemolytic activity as strain 13 (Table 1), indicating that the *pfoA* gene on pTS608 was successfully expressed. On the other hand, strain SI112 harboring pTS608 hardly expressed the *pfoA* gene (Table 1). The restriction pattern of the plasmid isolated from each transformant was identical to that of the original plasmid. Furthermore, when the plasmids isolated from strain SI112 transformants were transformed to strain SI107, the *pfoA* gene was expressed in the same manner as in strain SI107 with pTS608. This suggested that the lack of expression of the *pfoA* gene in strain SI112 was not due to a mutation of the *pfoA* gene on pTS608.

Isolation of a DNA fragment that restored hemolytic activity in SI112. Assuming that strain SI112 has a mutation in the regulatory gene for the *pfoA* gene expression, strain SI112 was randomly transformed with the constructed gene library of *C. perfringens* 13. The transformants were selected on GAM-sheep blood agar plates containing 25 μg of chloramphenicol per ml. Hemolytic activities were restored in 5 colonies of 10,000. The hemolysis was completely inhibited by cholesterol at a concentration of 50 μg/ml as well as anti-perfringolysin O antibody (data not shown), indicating that the hemolysis was due to the production of perfringolysin O. All five clones contained the same 2.5-kb *Hin*dIII-*Hin*dIII fragment in the same direction on pJIR418. When this fragment was inserted in the opposite direction, the transformant showed the same hemolytic activity. When strain SI112 was again transformed with one of these plasmids (designated pBT404), all the transformants regained hemolytic activity (Fig. 2). Furthermore, the fragment on pBT404 did not hybridize with the *pfoA* nor the *pfoR* gene (data not shown). These data strongly indicate that pBT404 carries the gene coding for a novel *trans*-acting factor required for the expression of the *pfoA* gene in strain SI112. Northern blotting analysis using the internal 1.2-kb DNA fragment of the *pfoA* gene as a probe showed that an mRNA for the *pfoA* gene (ca. 2,000 nucleotides in length) was significantly increased with transformation by pBT404 in strain SI112 (Fig. 3). This suggests that the gene on pBT404 activates the expression of the *pfoA* gene at the transcriptional level in strain SI112.

Effects of pBT404 on the production of other toxins in strain SI112. The effect of pBT404 on the production of other toxins, i.e., collagenase (kappa-toxin), HA, alpha-toxin, and DNase, was examined. Although strain SI112 scarcely produced collagenase and HA, their activities were significantly increased with the transformation of pBT404 (Table 2), while pBT404 had no effect on the production of alpha-toxin and DNase. This indicates that pBT404 carries the gene for a *trans*-acting regulatory factor which coordinately regulates the production of perfringolysin O, collagenase, and HA in *C. perfringens*.

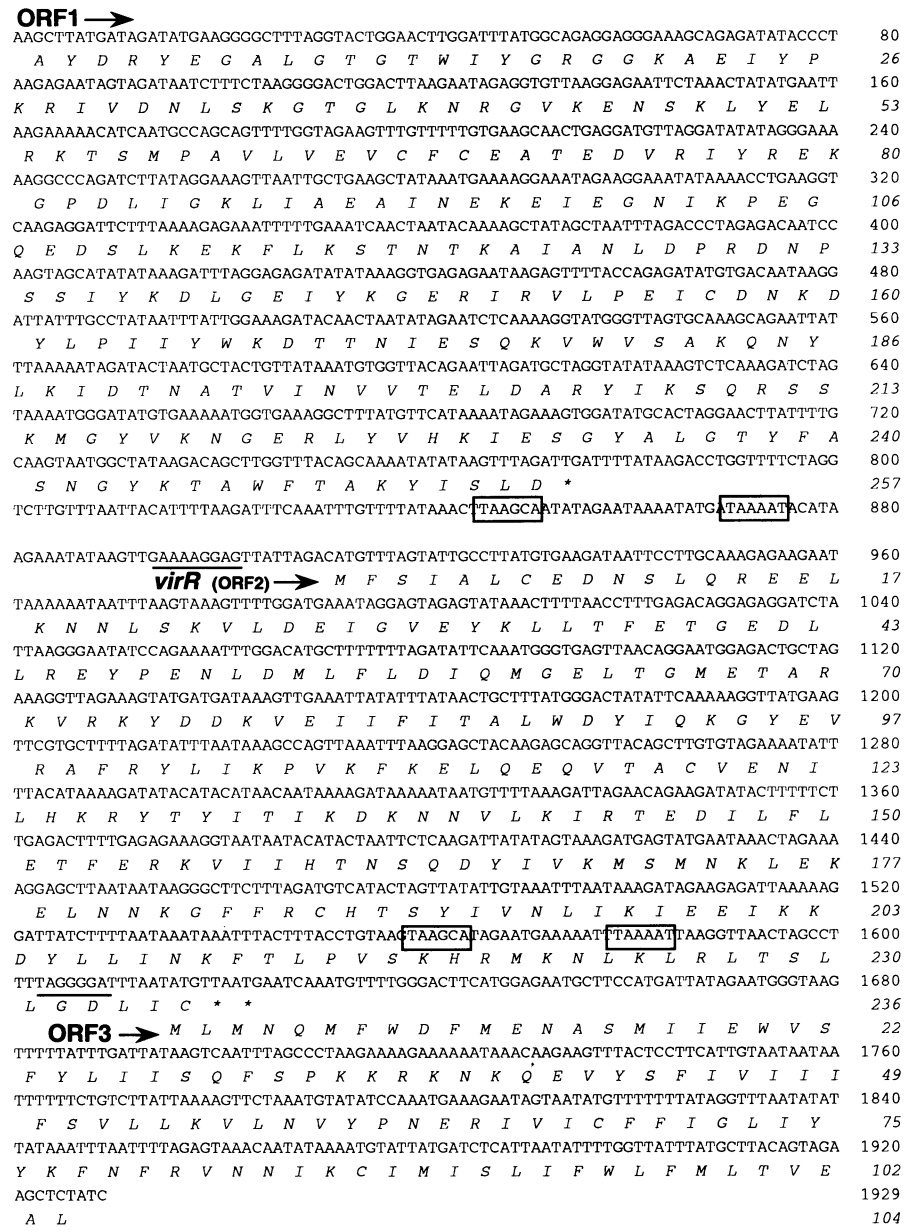

FIG. 5. Nucleotide sequence and deduced amino acid sequence of the fragment on pBT 741. The numbers for the nucleotides and deduced amino acids are shown at the right, and each ORF (ORF1, *virR*, and ORF3) is indicated. The putative ribosome binding sites and possible promoter sequences are underlined and boxed, respectively.

Restriction mapping, deletion analysis, and nucleotide sequence analysis of pBT404. The restriction map of the 2.5-kb *Hin*dIII-*Hin*dIII fragment is shown in Fig. 4. Nested deletions were generated in the cloned fragment, and the deleted fragments were transformed to strain SI112. The region responsible for the *trans* activity was identified in the 1.25-kb region of pBT741 (Fig. 4), indicating that the gene coding for the *trans*-acting regulator is located in this region. Therefore, we determined the complete nucleotide sequence of the fragment on plasmid pBT741.

The fragment on pBT741 consisted of 1,929 bp with 27.4% dG+dC content. Three major open reading frames (ORFs) were found in the sequenced region (Fig. 5). One ORF (designated ORF1) was located from positions 1 to 773 without a translation initiation codon (ATG), which indicates that ORF1 was truncated in this fragment. The second ORF (ORF2) was located from positions 912 to 1619 (total 708 bp in length) and coded for 246 amino acids. It had a putative ribosome binding site (23) just 9 bp upstream of the starting codon. The third ORF (ORF3) was found in the end of ORF2 (position 1616) and seemed to continue to the downstream region of this fragment. Although ORF3 overlapped with the coding frame of ORF2, the putative ribosome binding site was seen 7 bp upstream of the initiation codon. The identical putative promoter sequences (TAAGCA and TAAAAT) were seen at 5′-flanking regions of both ORF2 and ORF3 (Fig. 5).

The deduced amino acid sequence of each ORF product was subjected to a homology search at the National Center of

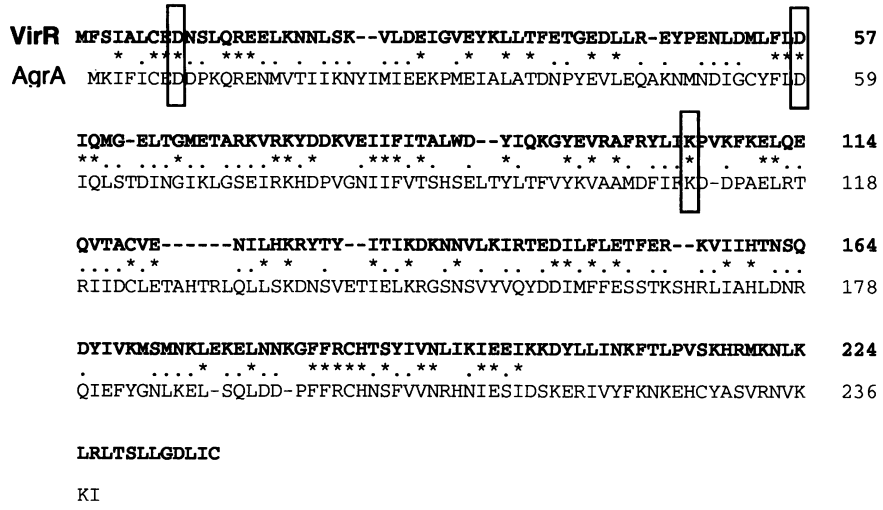

FIG. 6. Amino acid similarities of the *agrA* product of *S. aureus* (17) and the *virR* product. Asterisks and single dots indicate identities and conserved amino acid substitutions, respectively. Amino acids which are highly conserved within the family of response regulators (26) are indicated by the boxes.

Biotechnology Information, National Institutes of Health, by using the BLAST computer network service (5). The deduced amino acid sequence of the ORF1 product had significant similarity with the hypothetical 38.4-kDa protein (ORF10 product) coded on the bacteriocinogenic plasmid pIP404 of *C. perfringens* (8). Since the function of the ORF10 product of pIP404 remains unclear, we also cannot predict the function of the ORF1 product. The ORF2 product was proved to be highly homologous to the regulatory proteins of the two-component regulatory system in bacteria (26). It had high similarities at the N-terminal sequence with the MrkE protein of *Klebsiella pneumoniae* (2), the AlgR protein of *Pseudomonas aeruginosa* (6), and the AgrA protein of *Staphylococcus aureus* (17). The ORF2 product was especially similar to the AgrA (accessory gene regulator) protein in its whole sequence (27.6% identity and 67.3% similarity) (Fig. 6). Since ORF2 is located just in the region required for *trans* activity on *pfoA* expression, which was predicted from the deletion assay (Fig. 4), ORF2 proved to be responsible for the transcriptional activation of the *pfoA* gene in strain SI112 and belonged to a class of response regulators of the two-component regulatory systems. Thus, we designated ORF2 the *virR* gene.

Inactivation of the *virR* gene and complementation studies. The internal 0.5-kb *Hin*cII-*Hin*cII fragment of the *virR* gene on pBT741 was removed and replaced with the 2.7-kb fragment containing a tetracycline resistance determinant, which was cloned from *C. perfringens* isolated from soil (21a) (Fig. 7A). The resulting plasmid (pTS613) was transformed to *C. perfringens* 13 by electroporation, and the transformants were selected on GAM agar plates containing tetracycline (2.5 μg/ml). Several tetracycline-resistant colonies were seen on the plates. Since pTS613 did not have a replication origin for *C. perfringens*, these transformants were thought to have integrated the tetracycline resistance determinant into their *virR* locus by homologous recombination (Fig. 7A). Hemolytic activities of these transformants were checked on GAM-sheep blood agar plates, and one strain (TS133) which showed no distinctive hemolysis on blood agar plates was selected. Southern blotting analysis showed that the *virR* gene on the chromosome of strain TS133 was mutated by the integration of pTS613 (Fig. 7B). We introduced pBT404 into strain TS133, and the activities of perfringolysin O, collagenase, and HA were assayed. Strain TS133 scarcely produced these toxins, while strain TS133 carrying pBT404 significantly regained its ability to produce these toxins (Table 3). These data clearly indicate that the *virR* gene on pBT404 complemented the defect of the *virR* gene of strain TS133, suggesting that the *virR* gene plays an important role in the regulation of the expression of perfringolysin O, collagenase, and HA in *C. perfringens*.

DISCUSSION

In this report, we succeeded in cloning the *virR* gene, which plays a regulatory role in the production of perfringolysin O, collagenase, and HA in *C. perfringens*. Nucleotide sequence analysis of the *virR* gene revealed that the *virR* gene product belongs to a response regulator protein family of the two-component regulatory system (26).

The two-component signal transduction system in bacteria consists of histidine kinase and response regulator protein (26). These two components transmit various signals from the environment by means of phosphorylation and regulate the expression of various genes in response to environmental changes. If the *virR* gene is involved in the regulation of toxin production through the two-component system in *C. perfringens*, there should be another component (histidine kinase) in this regulatory system. In strain SI112, however, the gene for the histidine kinase seemed to be intact, since the existence of intact *virR* gene was sufficient for the restoration of *pfoA* gene expression. Although we could find no protein which is homologous to the ORF3 product in a data base search, the N-terminal sequence of the ORF3 product is highly hydrophobic and could form many membrane-spanning regions. Since histidine kinase commonly has membrane-spanning domains at its N terminus and the gene is located adjacent to the response regulator gene (26), ORF3 is a good candidate for a counterpart of the *virR* gene. The remaining sequence of ORF3 is now under investigation.

We previously reported that the expression of the perfringolysin O (*pfoA*) gene was positively regulated by the adjacent regulatory gene, *pfoR*, which acted on the *pfoA* gene in a *cis*-dominant manner in *E. coli* (22). The relationship between

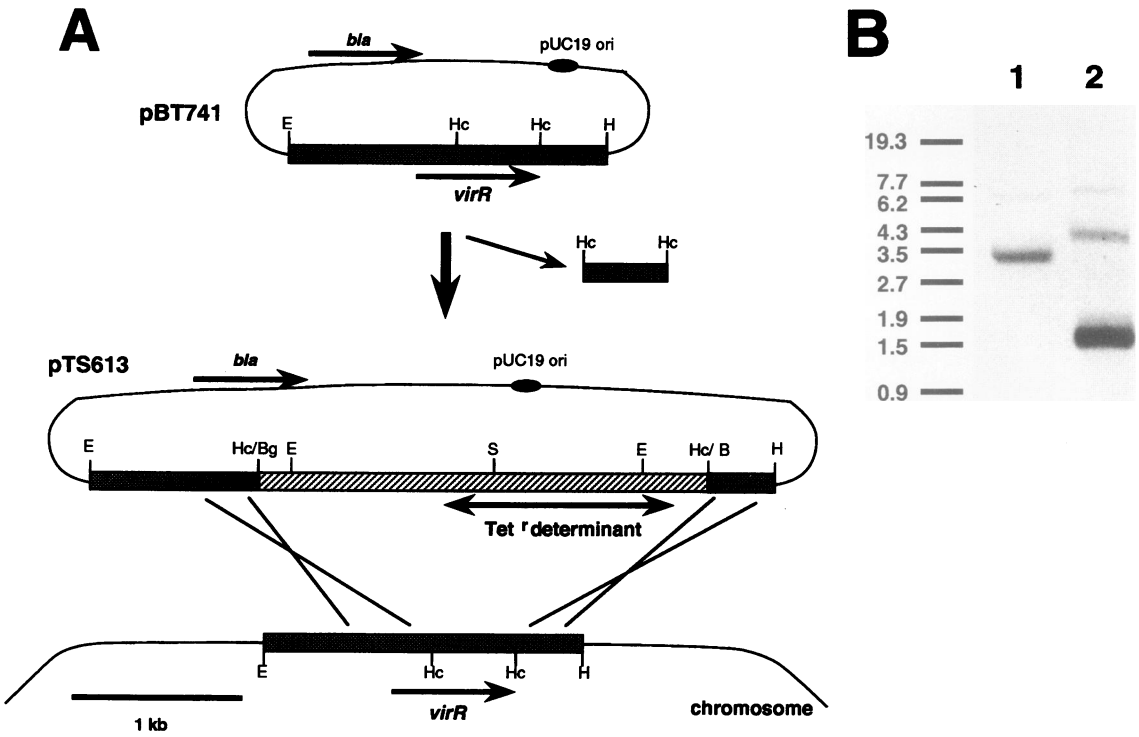

FIG. 7. (A) Construction of pTS613 and inactivation of the chromosomal *virR* gene of *C. perfringens* 13. The internal 0.5-kb *Hin*cII-*Hin*cII fragment of the *virR* gene was removed from pBT741 and was replaced with blunt-ended 2.7-kb *Bgl*II-*Bam*HI fragment of the tetracycline resistance determinant (21a). The resulting plasmid, pTS613, was transformed into *C. perfringens* 13 by electroporation. The integration of pTS613 into chromosome of strain 13 is schematically represented. Abbreviations for restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; S, *Sph*I. (B) Southern hybridization of chromosomal DNA of *C. perfringens* strains with a *virR* probe. Each chromosomal DNA (10 μg) was digested with *Eco*RI, electrophoresed, blotted onto nylon filters, and probed with the cloned 2.5-kb fragment of pBT404 labeled with digoxigenin-11-dUTP. Lanes: 1, 13; 2, TS133. DNA size markers (in kilobases) are indicated on the left.

the *virR* gene and the *pfoR* gene is still unclear. Since pTS608, which was used for the expression of the *pfoA* gene in *C. perfringens*, did not contain the *pfoR* gene, the *virR* gene appeared to directly regulate the expression of the *pfoA* gene, bypassing the *pfoR* control. Alternatively, if the *pfoR* gene product activates the *pfoA* gene in *trans* in *C. perfringens*, the *pfoA* gene on pTS608 could be activated by the *pfoR* product whose expression is regulated by the *virR* gene. This is an important point for an understanding of the regulatory system of the *pfoA* gene expression, and further experiments should be done to clarify this point.

Imagawa et al. previously reported that a small molecule produced by *a* type mutant strains of *C. perfringens* induced the production of perfringolysin O, collagenase, protease, and HA in *b* type mutant strains of *C. perfringens* (10, 11). They named the small molecule substance A, the molecular weight of which is estimated to be less than 2,000. Although no genetic information about this complementation was described, this phenomenon seems to be closely related to our findings. Since the *virR* gene also activated perfringolysin O, collagenase, and HA in *C. perfringens*, the same regulatory system would be involved in the phenomenon reported by them. Therefore, how the *virR* gene is related with the production of substance A is of great interest.

To our knowledge, this is the first report that has elucidated the presence of a global regulatory gene for the production of a group of toxins in *C. perfringens*. We believe that the identification of the *virR* gene will contribute not only to an understanding of the pathogenicity of this organism but also to understanding of the signal transduction systems of other clostridial species which are important for industrial purposes. Further studies are necessary to get a more concise picture of the global regulation of the virulence factors of *C. perfringens*.

TABLE 3. Activities of toxins in *C. perfringens* 13 and TS133 carrying plasmids[a]

Strain	Perfringolysin O (U/μg of protein)[b]	Collagenase (OD units/mg of protein)[b]	HA
13(pJIR418)	4.3 ± 0.9	27.8 ± 0.6	+
TS133(pJIR418)	<0.3 ± 0.1	5.4 ± 0.3	−
TS133(pBT404)	2.6 ± 0.2	19.5 ± 0.5	+

[a] All activities were assayed as described in Materials and Methods.
[b] Each value was calculated from triplicate experiments (mean ± standard deviation).

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