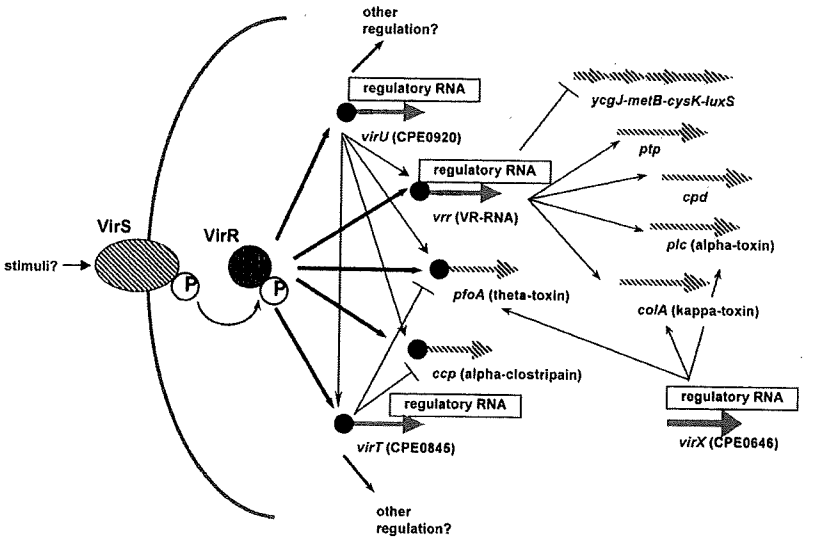

FIG. 7. Schematic diagram of the VirR/VirS regulon. The diagram was constructed by using the results of this and previous studies (3, 4, 12, 17, 18, 20, 24, 25, 28).

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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Carbon Catabolite Repression of Type IV Pilus-Dependent Gliding Motility in the Anaerobic Pathogen *Clostridium perfringens*▽†

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Clostridium perfringens* is an anaerobic, gram-positive, spore-forming bacterium responsible for the production of severe histotoxic and gastrointestinal diseases in humans and animals. In silico analysis of the three available genome-sequenced *C. perfringens* strains (13, SM101, and ATCC13124) revealed that genes that encode flagellar proteins and genes involved in chemotaxis are absent. However, those strains exhibit type IV pilus (TFP)-dependent gliding motility. Since carbon catabolite regulation has been implicated in the control of different bacterial behaviors, we investigated the effects of glucose and other readily metabolized carbohydrates on *C. perfringens* gliding motility. Our results demonstrate that carbon catabolite regulation constitutes an important physiological regulatory mechanism that reduces the proficiencies of the gliding motilities of a large number of unrelated human- and animal-derived pathogenic *C. perfringens* strains. Glucose produces a strong dose-dependent inhibition of gliding development without affecting vegetative growth. Maximum gliding inhibition was observed at a glucose concentration (1%) previously reported to also inhibit other important behaviors in *C. perfringens*, such as spore development. The inhibition of gliding development in the presence of glucose was due, at least in part, to the repression of the genes *pilT* and *pilD*, whose products are essential for TFP-dependent gliding proficiency. The inhibitory effects of glucose on *pilT* and *pilD* expression were under the control of the key regulatory protein CcpA (*c*atabolite *c*ontrol *p*rotein A). The deficiency in CcpA activity of a *ccpA* knockout *C. perfringens* mutant strain restored the expressions of *pilT* and *pilD* and gliding proficiency in the presence of 1% glucose. The carbon catabolite repression of the gliding motility of the *ccpA* mutant strain was restored after the introduction of a complementing plasmid harboring a wild-type copy of *ccpA*. These results point to a central role for CcpA in orchestrating the negative effect of carbon catabolite regulation on *C. perfringens* gliding motility. Furthermore, we discovered a novel positive role for CcpA in *pilT* and *pilD* expression and gliding proficiency in the absence of catabolite regulation. Carbon catabolite repression of gliding motility and the dual role of CcpA, either as repressor or as activator of gliding, are analyzed in the context of the different social behaviors and diseases produced by *C. perfringens*.

Motility is an important attribute utilized by many pathogenic and nonpathogenic bacteria to colonize new environments, to search for nutrients, and to allow the formation of complex architectural structures (e.g., biofilms and fruiting bodies) (14, 15, 25). Translocation in liquid medium (swimming) is mediated by flagella, and the swimming model has been traditionally used to describe bacterial motility. However, it is also true that in nature, most bacteria are associated with surfaces and therefore have evolved diverse mechanisms of translocation on solid or semisolid biotic and abiotic surfaces which are important for rapid dissemination and colonization during the course of an infection (21). Among the surface-associated mechanisms of motility that play key roles in cell-cell and cell-surface interactions, it is worth mentioning (i) swarming, a flagellum-dependent social form of translocation where planktonic cells differentiate into giant and hyperflagellated cells that move in groups ("swarms") to explore and colonize new habitats (10); (ii) twitching, a flagellum-independent but type IV pilus (TFP)-dependent form of intermittent and jerky surface motility (4, 14, 15), which has been demonstrated to occur in the opportunistic pathogen *Pseudomonas aeruginosa* (5), in the pathogen *Neisseria gonorrhoeae* (23), and recently in *Haemophilus influenzae*, which was previously considered a nonmotile bacterium (2); and (iii) gliding, which has been studied in detail in model organisms, such as *Myxococcus xanthus* and *Synechocystis* and *Anabaena* spp. (19). In *M. xanthus*, two types of mechanisms for gliding motility are utilized: adventurous gliding and social gliding (16). Adventurous gliding motility is observed when cells are isolated or in low numbers without contact with each other. In this sense, gliding motility is defined as a smooth movement of rod-shaped cells in the direction of their long axes on a surface. It has been proposed that the driving force for adventurous gliding motility in *M. xanthus* is generated by the action of a nozzle-like struc-

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ture that produces a polysaccharide slime trail. Social gliding motility, similar to twitching motility, is dependent on the active extension and retraction of TFP (16). For other bacteria (e.g., *Cytophaga* spp., *Flavobacterium* spp., and *Mycoplasma mobile*), gliding takes place in the absence of TFP and ATP consumption. Instead, they generate motility with motility proteins anchored to the membrane that in turn use proton motive force and/or polar polysaccharide extrusion to achieve cellular motility over surfaces; all these gliding examples point out the extreme diversity of gliding mechanisms (4, 14, 15, 22, 24). Extracellular TFP appendages are polymers of the small protein PilA, or pilin. In *P. aeruginosa*, PilA is initially translated as a prepilin with a short leader peptide cleaved by PilD (48); PilB provides energy for assembly. PilT and PilU are nucleoside triphosphate binding proteins that have been implicated in pilus retraction during twitching motility (4). In *P. aeruginosa*, almost 40 genes are involved in the biosynthesis and function of TFP (23, 47). Besides the key role of TFP in twitching and gliding, these appendages also mediate other important phenomena, such as adherence, fruiting body formation, bacteriophage absorption, DNA uptake, cytotoxicity, activation of host cell responses, and biofilm development (1, 14, 23, 24, 31).

Clostridium perfringens is a gram-positive, anaerobic, spore-forming bacterium that causes severe gastrointestinal and histotoxic infections in humans and animals (28, 32, 33). This pathogen has been traditionally described as a nonmotile bacterium, as no genes that encode flagellar proteins or genes involved in chemotaxis were identified in the complete genomic sequences of the three human-pathogenic *C. perfringens* strains 13, SM101, and ATCC13124 (26, 36). However, sequence analysis (data not shown) suggests that the three strains carry genes which code for TFP components (such as *pilA* to *pilD* and *pilT*) (26, 36). Recently, Varga et al. demonstrated that *C. perfringens* strains 13, SM101, and ATCC13124 exhibited social gliding motility on brain heart infusion agar (BHIA) plates, and TFP were detected on the surfaces of the bacteria (41). This study also demonstrated that *pilT* and *pilC* mutants failed to produce detectable pili and were nonmotile on BHIA (41). Other putative TFP biosynthesis genes found in *C. perfringens* remain to be characterized, and the exact mechanism of TFP assembly and its physiological role, importance, and regulation are currently unknown.

In this respect, diverse bacterial behaviors, such as biofilm development, sporulation, fruiting body formation, and surface-associated motility, are regulated by environmental, metabolic, and quorum-sensing signals (9, 10, 16, 28, 46). In particular, carbon catabolite regulation is a widespread phenomenon in bacteria where the expressions of a number of genes are regulated by the presence of a preferred carbon source, such as glucose or fructose (27, 39, 43). In *C. perfringens*, and many low-G+C-content, gram-positive bacteria, carbon catabolite control is under the regulation of CcpA (*c*atabolite *c*ontrol *p*rotein A), a pleiotropic transcriptional regulator belonging to the LacI/GalR family of transcription factors (27, 39, 43). CcpA functions as a DNA binding protein, either activating or repressing genes generally in the presence of a preferred carbon source (35, 43). More precisely, surface-associated motility is an interesting example of social bacterial behavior that could be regulated by nutrient (i.e., carbon) availability in nature. Interestingly, in *P. aeruginosa*, swarming (but not twitching) motility is carbon source regulated; poor swarming activity is observed in the presence of glucose (37). Therefore, the ability of the pathogen to carry out surface-associated social motility during the course of the infection is of crucial importance (2, 10, 14, 21, 24). In this work, we have investigated the effects of glucose and other readily metabolized carbohydrates on the TFP-dependent social gliding motilities of a collection of pathogenic *C. perfringens* strains isolated from human and animal sources. Our results clearly demonstrate that carbon catabolite repression is a general and common regulatory mechanism of gliding motility in *C. perfringens*, independent of the source of isolation (human or animal) or type of infection (diarrhea or myonecrosis). We demonstrate that the repressive effect of glucose on gliding motility is partially due to the CcpA-mediated down-regulation of TFP biosynthesis genes. In addition, we have discovered and analyzed a novel carbon catabolite-independent positive role for CcpA in *C. perfringens* gliding motility.

TABLE 1. *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Isolation source or phenotype	Source or reference
C. perfringens strains		
Human isolates		
Strain 13	Gas gangrene	36
KO13	*ccpA* mutant derivative of strain 13	This study
NCTC8239	Food poisoning	7
NCTC10239	Food poisoning	7
SM101	Food poisoning	49
191-10	Food poisoning	38
F4406	Sporadic diarrhea	7
F4969	Sporadic diarrhea	7
F5603	Sporadic diarrhea	7
B11	Antibiotic-associated diarrhea	7
B41	Antibiotic-associated diarrhea	7
NB16	Antibiotic-associated diarrhea	7
Animal isolates		
JGS1807	Diarrheic pig	45
JGS1818	Diarrheic pig	45
294442	Diarrheic dog	R. Carman
317206	Diarrheic dog	R. Carman
AHT327	Diarrheic horse	44
AHT2911	Diarrheic horse	44
Plasmids		
pMRS127	*sigK-gusA* in pJIR751	30
pEJZ2	*pilT* promoter fused with *gusA* in pMRS127	This study
p*PilD*gus-127	*pilD* promoter fused with *gusA* in pMRS127	This study
KO2		This study
pJIR750	*C. perfringens-E. coli* shuttle vector	3
pIH100		This study

MATERIALS AND METHODS

Strains and culture conditions. The *C. perfringens* strains used in this study are listed in Table 1. The growth media employed to propagate strains were fluid thioglycolate (FTG) and TGY (3% tryptic soy broth, 2% glucose, 1% yeast extract, and 0.1% cysteine) (8, 32). TY medium results from the omission of glucose from the TGY formula. All cultures were grown under anaerobic conditions in anaerobic jars containing GasPak (BD) at 37°C.

TABLE 2. Primers used in this study

Primer	Primer sequence[a]	Positions[b]	Gene	Use[c]
CPP53	5′ GCGTCGACGAGATATGGTCTTTTAGATGG 3′	−333 to −312	*pilT* promoter	GUS
CPP55	5′ GCTGCAGCAGATGCTCCTTCTTTAACTG 3′	+28 to + 48	*pilT* promoter	GUS
CPP230	5′ GCGTCGACCTTGAAGATTTAGATAAGCCTC 3′	+19 to + 41	*pilD* promoter	GUS
CPP231	5′ GCTGCAGCCTTCCAATTATTAATCCAAATAA 3′	−361 to −341	*pilD* promoter	GUS
CCP-F	5′ AACTAGGATATAGACCTAAT 3′	+172 to +192	*ccpA*	MP
CCP-R	5′ TGATCCCATATCATACATTG 3′	+876 to +896	*ccpA*	MP
KO-F	5′ CTGGAGTGTCAATAGCAAC 3′	+34 to +53	*ccpA*	PROBE
KO-R	5′ TCTCCTAGTGATGAACTCAT 3′	+366 to +386	*ccpA*	PROBE
CPP265	5′ ATATCCATCAAGTTCATCTATAGAG 3′	−505 to −481	*ccpA*	COMP
CPP266	5′ TATGTTACCTAATGATTATGCATT 3′	+1012 to +1036	*ccpA*	COMP
P1	5′ ATGCTGATTACTCAGAAGCT 3′	−774 to −754	*ccpA* upstream	PCR
P2	5′ CTCATAAGCCCTTGATGAAC 3′	+1461 to +1484	*ccpA* downstream	PCR
M13-F	5′ GTA AAA CGA CGG CCA GT 3′		pUC18 vector	PCR
M13-R	5′ CAGGAAACAGCTATGAC 3′		pUC18 vector	PCR

[a] Restriction sites that have been added are underlined.
[b] The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence (36).
[c] GUS, construction of plasmid for β-glucuronidase assay; MP, construction of mutator plasmid; PROBE, construction of DNA probe for southern blot analysis; COMP, construction of complementing plasmid.

Motility assays. *C. perfringens* strains were grown overnight in FTG at 37°C, and 300 μl of this culture was inoculated and propagated in TGY for 5 h at the same temperature. Next, 1 ml of culture was centrifuged and concentrated twofold. Five microliters of the concentrated cell suspension was spotted onto a predried (1 h at 37°C) BHIA, TGY agar (TGYA), or TY agar (TYA) plate. All plates were supplemented with 0.7% agar. The inoculated plates were then incubated anaerobically for 48 to 96 h at 37°C. Photographs of the plates were taken with a Canon Power Shot SD550 digital camera.

Construction of *gusA* fusion plasmids and the β-glucuronidase assay. The PCR-amplified product carrying the upstream region of *pilD* or *pilT* was first cloned into the pCR-XL-TOPO vector by using a TOPO-XL cloning kit (Invitrogen). Briefly, the DNA fragment carrying the promoter region of *pilT* or *pilD* from SM101 or strain 13 was amplified by PCR using primers CPP53/CPP55 or CPP230/CPP231, respectively (Table 2). The SalI site was incorporated into the forward primer and the PstI site into the reverse primer of each primer pair. These PCR products were then cloned into the pCR-XL-TOPO vector. The recombinant clones carrying the expected DNA fragment were confirmed by restriction enzyme digestion, PCR analysis, and then DNA sequencing. The SalI-PstI fragments carrying the promoter region of *pilD* or *pilT* from pCR-XL-TOPO clones were then recloned into the SalI/PstI sites of pMRS127 to create *pilD-gusA* or *pilT-gusA* fusion constructs derived from either strain SM101 or strain 13. These reporter plasmids were then introduced by electroporation and Cm^r selection (8) into the corresponding wild-type *C. perfringens* strains 13 and SM101 and the isogenic derivates deficient in CcpA.

The strains carrying the *gusA* fusions were grown overnight in FTG at 37°C, and 150 μl of each culture was inoculated into TY and TGY. At various time points, 1 ml of each culture was centrifuged for 2 min at 9,000 rpm, and the pellet was stored frozen at −20°C until used. The cell pellets were resuspended with 1 ml of buffer Z (8.54 g of Na_2HPO_4, 5.5 g of $NaH_2PO_4 \cdot H_2O$, 0.75 g of KCl, 0.25 g of $MgSO_4$, and 1.4 ml of β-mercaptoethanol · $7H_2O$, pH 7, per liter) (13, 28). Adequate aliquots of the resuspended cells were brought to a final volume of 730 μl with buffer Z. Next, 10 μl of 10 mg/ml lysozyme was added and the mixture was incubated for 30 min at 37°C, followed by the addition of 10 μl 10% Triton X-100 (Sigma). The enzymatic reactions were initiated by adding 100 μl of 6 mM 4-nitrophenyl β-D-glucuronide (Sigma). After 15 min of incubation at 37°C, the reaction was stopped by adding 150 μl of 1 M Na_2CO_3. The absorbance was measured at 405 nm, and the β-glucuronidase activity was calculated using the following equation: (A_{405} × 1,000)/(optical density at 600 nm [OD_{600}]pls cross chk inf or sup, edi file not provided × culture volume [in milliliters] × time [in minutes]) (19).

Insertional inactivation of *ccpA* gene in strain 13. To disrupt *ccpA* in strain 13, an internal 760-bp fragment of the *ccpA* gene was amplified by PCR using primers CCP-F and CCP-R (Table 2) and cloned into the SmaI site of pUC18 to create pKO1. For selection, an erythromycin resistance cassette (*ermBP*) was ligated into the HincII site of pKO1, creating pKO2. The plasmid pKO2 (which has no origin of replication for *C. perfringens*) was transformed into strain 13 by electroporation and Em^r selection. One Em^r clone (KO13) was analyzed (see Fig. S1 in the supplemental material) for correct integration by a single crossover event involving homologous recombination of the suicidal mutator plasmid (pKO2) on *ccpA* and utilized in the present study.

The insertional disruption of wild-type *ccpA* in KO13 was first demonstrated by PCR analysis of DNA isolated from the mutant (see Fig. S1 in the supplemental material). As expected, a 1.6-kb product was amplified from KO13 DNA by using primers P1 and M13F and a 2.5-kb product by using primers P2 and M13R. However, no PCR product was obtained with either P1 and M13R or P2 and M13F. These PCR results are consistent with the suicidal mutator plasmid pKO2 having been inserted into the wild-type *ccpA* gene in KO13. Southern blot analyses showed that a 2.4-kb HindIII DNA fragment from wild-type strain 13 hybridized with our *ccpA*-specific probe. However, two hybridizing bands, of 2.9 and 4.3 kb, were observed with DNA from mutant strain MO13. This profile is consistent with the expected result since the inserted pKO2 plasmid has a HindIII site.

Southern blot analysis. A 350-bp internal *ccpA* DNA fragment was amplified from strain 13 by PCR using primers KO-F and KO-R (Table 2) and labeled with alkaline phosphatase using the Gene Images AlkPhos direct labeling and detection system (Amarsham Bioscience) (28, 44). Isolated *C. perfringens* DNA samples, prepared as described previously (8, 38), were digested with HindIII, separated by electrophoresis on 1% agarose gels, and transferred by Southern blotting. The blot was hybridized with the AlkPhos-labeled *ccpA* probe, and the hybridized probe was then detected by CDPstar chemiluminescence (Amersham Bioscience).

Construction of the CcpA-complementing plasmid pIH100. A 1,539-bp fragment containing the *ccpA* open reading frame and its 450-bp upstream region was amplified by PCR using primers CPP265 and CPP266 (Table 2) and then cloned into pCR-TOPO-XL (Invitrogen) to generate pCcpA-comp-XL. Next, the ~1.5-kb KpnI/XbaII fragment was cloned into the KpnI/XbaI sites of shuttle vector pJIR750 to generate the *ccpA*-complementing plasmid pIH100.

RESULTS

Glucose represses the gliding motilities of wild *C. perfringens* strains isolated from human and animal infections. A recent study demonstrated TFP-dependent gliding motility in three human-derived *C. perfringens* strains for which genome sequences have been previously determined (41). TFP-dependent gliding motility constitutes an important bacterial behavior also known as twitching (14, 15, 24). Taking into account that the referred study (41) was limited to the analysis of only three strains, all of human origin, when there exists a great diversity of *C. perfringens* isolates causing diverse infections (i.e., gas gangrene, food poisoning, antibiotic-associated diarrhea, etc.) in human beings and animals, we considered it of

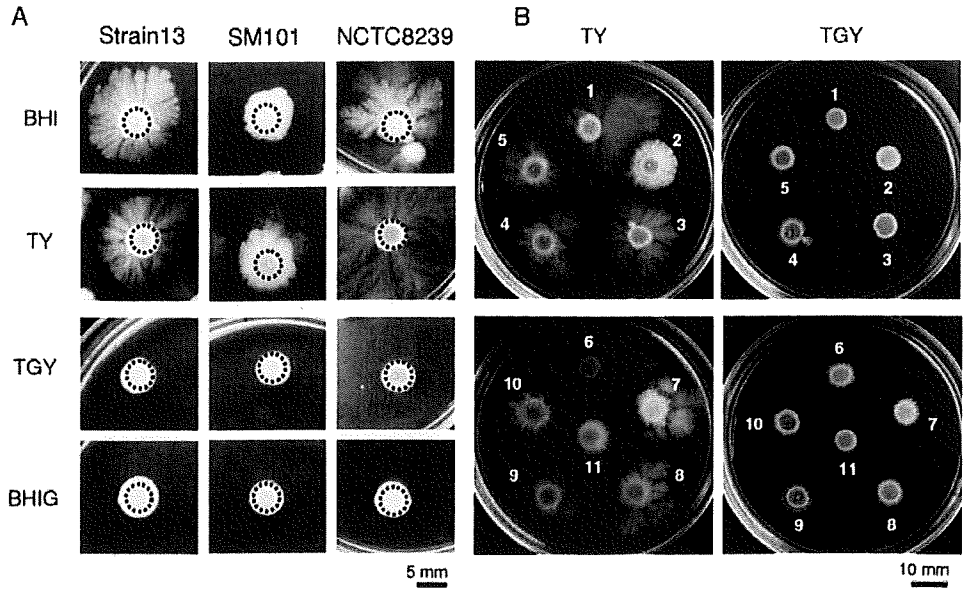

FIG. 1. Glucose represses the gliding motilities of *C. perfringens* strains isolated from different mammalian hosts. (A) Gliding phenotypes of the three genome-sequenced human pathogen *C. perfringens* strains 13, SM101, and NCTC8239. Gliding was developed after inoculation of a 5-μl drop of a concentrated middle-log-phase culture of the corresponding strain on BHIA or TYA medium with or without 2% glucose supplementation. Top-bottom photographs were taken after 72 h of anaerobic incubation at 37°C. BHIA, TYA medium without glucose; BHIG and TGY, media supplemented with 2% glucose. Black dotted circles show the diameters of the initial inoculation spots (see Materials and Methods for details). (B) Gliding phenotypes of a collection of human and animal pathogenic *C. perfringens* strains: 1, NB16; 2, JGS1818; 3, 294442; 4, NCTC 10239; 5, 317206; 6, AHT327; 7, B11; 8, B41; 9, F5603; 10, F4969; and 11, AHT2911 (see Table 1 for the origin and type of infection produced for each pathogenic strain).

interest to evaluate whether gliding motility was an intrinsic and general property of wild and undomesticated *C. perfringens* isolates. To this end, we performed a gliding motility analysis on a collection of 17 different pathogenic human- and animal-derived *C. perfringens* strains (including those strains whose gliding behavior was previously reported) (Table 1). In our study, motility is defined as the ability of cells to spread away from the edge of inoculation point by at least 0.4 cm in a curved flare pattern after 72 h at 37°C. When culture aliquots (see Materials and Methods for details) of strains 13, SM101, and NCTC8239 were spotted onto BHIA or TYA plates, the pattern of colony translocation was similar to that of the gliding motility previously observed (41), showing a distinctive curved flare pattern (Fig. 1A). Interestingly, both on TGYA and on BHIGA plates (which contained 2% glucose), the spotted cultures of strains 13, SM101, and NCTC8239 remained in the inoculation point, indicating that gliding motility was inhibited. In addition, we noted that the degree of motility was more evident on TYA than on the BHIA that was used in the previous study (41) (Fig. 1A and data not shown). Therefore, all subsequent motility experiments were conducted using TYA plates.

When the gliding motility assay was performed on the entire collection of *C. perfringens* isolates (Table 1), all the strains exhibited full proficiency in social gliding motility on TYA plates. Gliding motility was, in contrast, completely blocked in the presence of glucose (Fig. 1B and data not shown). Therefore, taking into consideration that glucose is a known inhibitor of other bacterial social behaviors, such as sporulation (28, 30) and biofilm formation (39), we concluded that the glucose added to the BHIA and TYA plates, yielding BHIG and TGYA, respectively, was responsible for the repressive effect on the gliding motilities of all the surveyed *C. perfringens* isolates.

To determine how gliding motility was affected by different glucose levels, a glucose gradient was generated on a TYA plate (see Materials and Methods). Five microliters of a concentrated middle-log phase culture of the food poisoning strain SM101 was inoculated at various positions distributed along the TYA glucose concentration testing plate (see Materials and Methods). As observed in Fig. 2A, the extent of gliding motility exhibited by the pathogenic food poisoning SM101 strain was inversely proportional to the glucose concentration; as the concentration of glucose decreases, the extent of gliding motility increases.

To determine the minimum concentration of glucose required to inhibit gliding motility, five different concentrations of the sugar were independently assayed: 0.1%, 0.25%, 0.5%, 1%, and 2%. As shown in Fig. 2B, at a glucose concentration of 1%, no gliding motility was observed in the gas gangrene producer strain 13 or in the food poisoning isolate NCTC8239. In contrast, these two strains were able to glide slightly on TYA plates supplemented with 0.5% glucose. No inhibition of gliding motility was observed when these strains were spotted on TYA plates supplemented with 0.25% and 0.1% glucose (Fig. 2B and data not shown). Therefore, glucose repression of social gliding motility in *C. perfringens* was concentration dependent and apparently triggered at a glucose concentration of 0.5%.

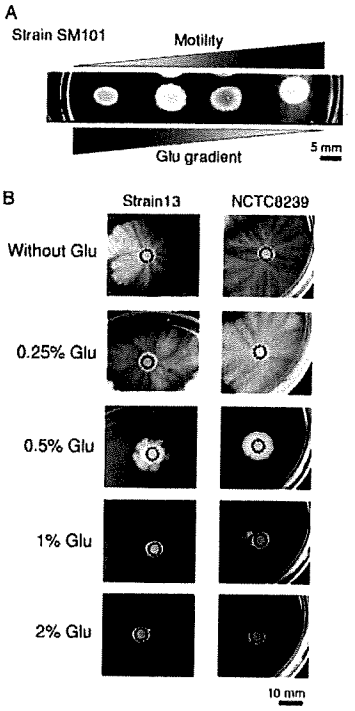

FIG. 2. Dose-dependent repressive effect of glucose (Glu) on *C. perfringens* gliding proficiency. (A) Glucose gradient effect on the gliding motility of the enterotoxigenic *cpe*$^+$ food poisoning *C. perfringens* strain SM101. One-third of a TYA plate was cut out and replaced with 7 ml of melted TGYA medium, which contained 2% glucose (see Materials and Methods and elsewhere in text for details). After solidification of the added TGYA, a natural glucose gradient was generated (shown from left to right) due to the diffusion of glucose molecules from the TGYA section (which contained 2% added glucose) to the TYA portion (which contained 0% added glucose) of the plate. (B) Evaluation of the minimum glucose concentration required to inhibit gliding proficiency of the food poisoning and gas gangrene producer strains NCTC8239 and 13. For panels A and B, strains were grown on TYA plates as indicated in Fig. 1 and supplemented with glucose as shown in the figure. Top-bottom photographs were taken after 72 h of anaerobic incubation at 37°C.

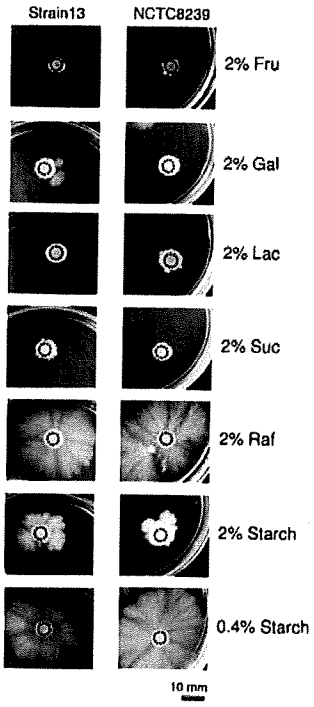

FIG. 3. Effects of simple and complex carbohydrates on *C. perfringens* gliding motility. Rapidly metabolized carbohydrates (Fru, fructose; Gal, galactose; Lac, lactose; Suc, sucrose) and complex carbohydrates (Raf, raffinose) commonly used to enhance sporulation and CPE production in Spo0A-proficient *C. perfringens* strains were assayed for their abilities to affect gliding motility. The gliding phenotypes of the gas gangrene producer and Spo0A-deficient strain 13 and the Spo0A-proficient and food poisoning strain NCTC8239 are shown. Top-bottom photographs were taken after 96 h of anaerobic incubation at 37°C on TYA plates supplemented with the corresponding concentrations of sugar as indicated.

Carbon catabolite repression of *C. perfringens* gliding motility. In order to determine if the observed inhibitory effect of glucose on gliding motility was a general phenomenon of carbon catabolite regulation (repression), other readily metabolized carbohydrates, such as galactose, fructose, lactose, and sucrose, were tested. The motilities of strains NCTC8239 (food poisoning) and 13 (gas gangrene) were inhibited when these strains were grown on plates containing 2% of either fructose, galactose, lactose, or sucrose (Fig. 3). Next, we analyzed the effects on gliding of complex carbohydrates (e.g., raffinose and starch), which are slowly metabolized but are required for efficient sporulation of *C. perfringens* and for *C. perfringens* enterotoxin (CPE) production (17, 18). When 2% raffinose was added to TYA plates, no inhibition of gliding motility was observed (Fig. 3). With 2% starch, the extents of motility in both strains (13 and NCTC8239) were suppressed to a minor degree, although gliding was not affected in the presence of 0.4% starch (Fig. 3). When similar experiments were performed on the rest of the *C. perfringens* isolates listed in this work (Table 1), no gliding motility was observed for all tested strains grown on TYA plates supplemented with 2% fructose, galactose, lactose, or sucrose, while gliding motility was observed with 2% raffinose supplementation. However, on TYA plates supplemented with 2% starch, all tested strains were nonmotile on the agar surface, with the exception of the strains NCTC10239 (food poisoning), which exhibited partial gliding motility, and JGS1807 (diarrheic pig), which was highly motile even in the presence of 2% starch (data not shown). The overall results indicated that the complex carbohydrates raffinose and starch did not affect gliding motility at the concentrations routinely used (2% and 0.4%, respectively) for *C. perfringens* spore formation and CPE production (17, 18, 28). More importantly, we demonstrate for the first time that *C. perfringens* gliding motility is subject to carbon catabolite repression.

Kinetics of TFP-dependent gliding motility in *C. perfringens*. Proficiency in surface-associated motility is an important feature of many human and animal pathogens (2, 10, 14, 21, 24). In the cases of virulent and invasive *C. perfringens* isolates, an efficient mechanism of translocation on solid or semisolid surfaces could be favorable for escape from host defenses and for rapid dissemination of the infection (i.e., an aggressive gan-

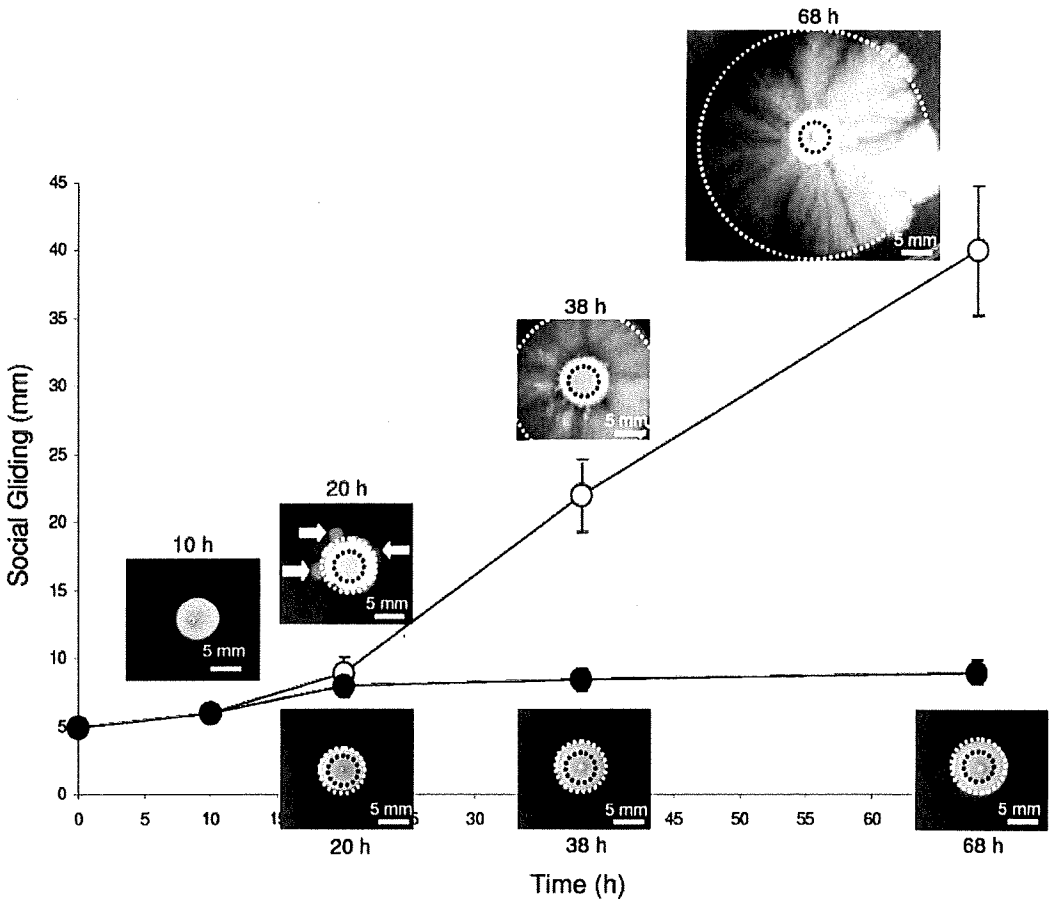

FIG. 4. Kinetics of gliding development of the gas gangrene producer *C. perfringens* strain 13 in the absence and presence of added glucose. Five microliters of a concentrated middle-log-phase culture of strain 13 grown in TY broth was inoculated in the center of each 100-mm petri dish, containing TYA medium with or without supplementation with 2% glucose. Inoculated petri dishes were anaerobically incubated at 37°C, and gliding proficiency was recorded at different times, measuring the distance traveled (in mm) from the initial inoculation point (black dotted circles) to the edge of the expanding colony (white dotted circles). Open and closed symbols represent the experiments performed in the absence and the presence of 2% added glucose, respectively. White arrows indicate the onset (under the influx of unknown signals; see text for details) of gliding development. The onset of gliding was observed only in TYA plates without glucose supplementation, while in the presence of glucose, gliding was never initiated. Photographs are representative of six independent experiments, and plotted values are the averages for those repetitions.

grene can progress at a rate of 1 cm h^{-1}). Therefore, we consider it of interest to characterize the kinetics and efficiency of the gliding motility of strain 13 (gas gangrene producer) on semisolid TYA plates in the absence and presence of added glucose. As observed in Fig. 4, it is possible to visualize two clear phases of colony growth and expansion. Initially, the growing colony expanded on the TYA surface from the inoculation point at a rate comparable to the speed of colony expansion expected from nonswimming bacteria. During the first 20 h of incubation, the size of the growing colony was evenly increased by a factor of 1.75 as a consequence of cell division, reaching a speed of colony spreading of approximately 125 μm h^{-1} (Fig. 4 and data not shown). After this initial phase of even colony spreading, a striking change occurred: social gliding was initiated by groups of cells located at the edge of the colony (see marked burgeoning cells in Fig. 4 at a developmental time of 20 h). After the appearance of these first signs of active surface gliding motility (the emergence of up-and-coming cells from the budding sites), a robust and uniform gliding was strongly induced, reaching a maximal speed of 600 to 700 μm h^{-1} (Fig. 4), a value for gliding speed comparable to the average values (200 μm h^{-1} to 1,000 μm h^{-1}) for social gliding and twitching speed previously reported for other microorganisms (10, 14, 24).

Interestingly, in the presence of 2% glucose, gliding motility was not generated even after long incubation times (Fig. 4 and data not shown). The morphology of the *C. perfringens* colony developed on TYA plates supplemented with glucose (gliding-deficient phenotype) was quite similar to the morphology of the colony developed on TYA plates without glucose supplementation just before the onset of active gliding; it was also very similar to that of the central part of a mature colony that has glided for more than 40 h on TYA plates (Fig. 4 and data not shown). These observations indicate that glucose and other sugars (data not shown) did not affect the initial phase of vegetative colony spreading. However, they do show that the sugar completely blocked (with carbon catabolite repression) the onset of the

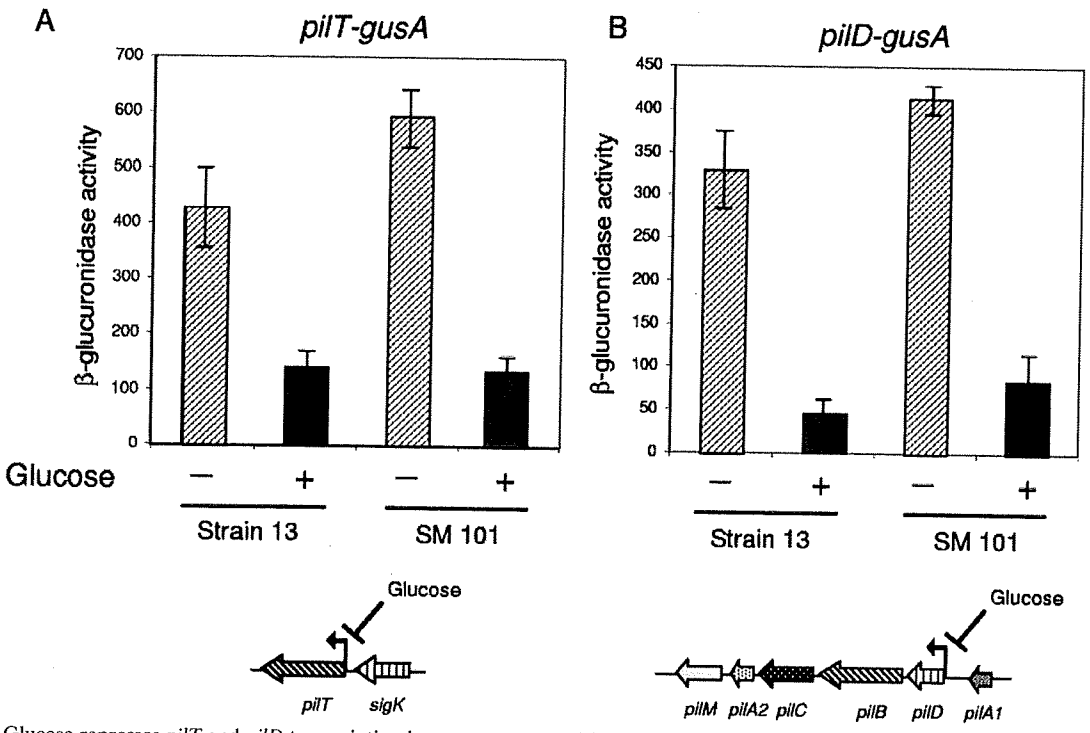

FIG. 5. Glucose represses *pilT* and *pilD* transcription in gas gangrene and food poisoning *C. perfringens* strains. Transcription of *pilT* (A) and *pilD* (B) promoters measured by a β-glucuronidase assay of *C. perfringens* strains 13 and SM101, harboring reporter *pilT-gusA* (A) and *pilD-gusA* (B) transcriptional fusions. Strains were grown on TY broth with or without the addition of 1% glucose as indicated in the figure (+ or −, respectively). Accumulated β-glucuronidase activity was measured after 30 h of growth. A representative result from three independent assays is shown. Gene arrangements of *pilT* (A) and *pilD* (B) chromosomal regions in *C. perfringens* strains 13 and SM101 are shown at the bottom of the figure, with the repressive effects of glucose on gene transcription indicated (see text for details).

second phase of surface translocation, which relied on the development of active gliding.

Effects of glucose on *pilT* and *pilD* expression of gas gangrene and food poisoning-producing *C. perfringens* strains. The recent study of Varga et al. demonstrated that the gliding of *C. perfringens* strain 13 depended on the products of *pilT* and *pilC*, which are required for TFP assembly (41). The *pilT* mutant of strain 13 does not spread out from the inoculation spot as the wild type does, and no pili were detected using field emission-scanning electron microscopy (41). Since glucose and other readily metabolized carbohydrates completely suppressed gliding motility in all the surveyed *C. perfringens* strains (Table 1) that we analyzed (Fig. 1 to 4 and data not shown), we considered the scenario where the transcription of *pil* genes might be affected by carbon catabolite repression. Therefore, to test this hypothesis, we measured the expression levels of two other genes required for TFP-dependent motility, *pilD* and *pilT*, in TY medium with and without glucose supplementation (Fig. 5). The β-glucuronidase *pilD-gusA* and *pilT-gusA* reporter fusions were introduced separately into strains 13 and SM101 by DNA electroporation, and *pil*-driven β-glucuronidase activity was assayed (see Materials and Methods for details). There were no significant differences in the growth of strains carrying the *pil-gusA* fusions in medium with or without added glucose (data not shown), although the final cellular yield was slightly higher in TY medium supplemented with glucose than in non-supplemented TY medium (data not shown). Interestingly, in the presence of 1% glucose, there were dramatic down-regulations of *pilD* and *pilT* promoter activities in both *C. perfringens* strains (Fig. 5). The expression levels of *pilT* were reduced by 60% in the gas gangrene producer strain 13 and by 75% in the food poisoning isolate SM101 when these strains were grown in the presence of 1% glucose (Fig. 5A). Similarly, significant reductions in *pilD-gusA* expression were observed in cultures of strains 13 (~80% reduction) and SM101 (~75% reduction) when these strains were grown in the presence of 1% glucose in comparison with their expression levels in the absence of glucose (Fig. 5B). These results demonstrated that glucose strongly down-regulates the expression levels of the *pilT* and *pilD* genes. They also suggest that the inhibitory effect of carbon catabolite regulation on gliding motility took place, at least partially, at the level of TFP expression.

Dual role of the carbon catabolite protein CcpA in *C. perfringens* gliding motility. It is known that catabolite control protein A (CcpA) plays a key role in low-G+C-content, gram-positive bacteria, interconnecting carbon metabolism with several cellular responses, such as virulence, spore formation, and biofilm development (39, 43). In *C. perfringens*, CcpA is required for efficient sporulation and expression of the enterotoxin (CPE) that is responsible for the symptoms of food poisoning and diarrhea in humans and animals (42). Due to the observed repressive effect of glucose on the expression levels of *pil* genes (Fig. 5) that are essential for the ability of *C. perfringens* to glide on a solid substrate (41), we were tempted to

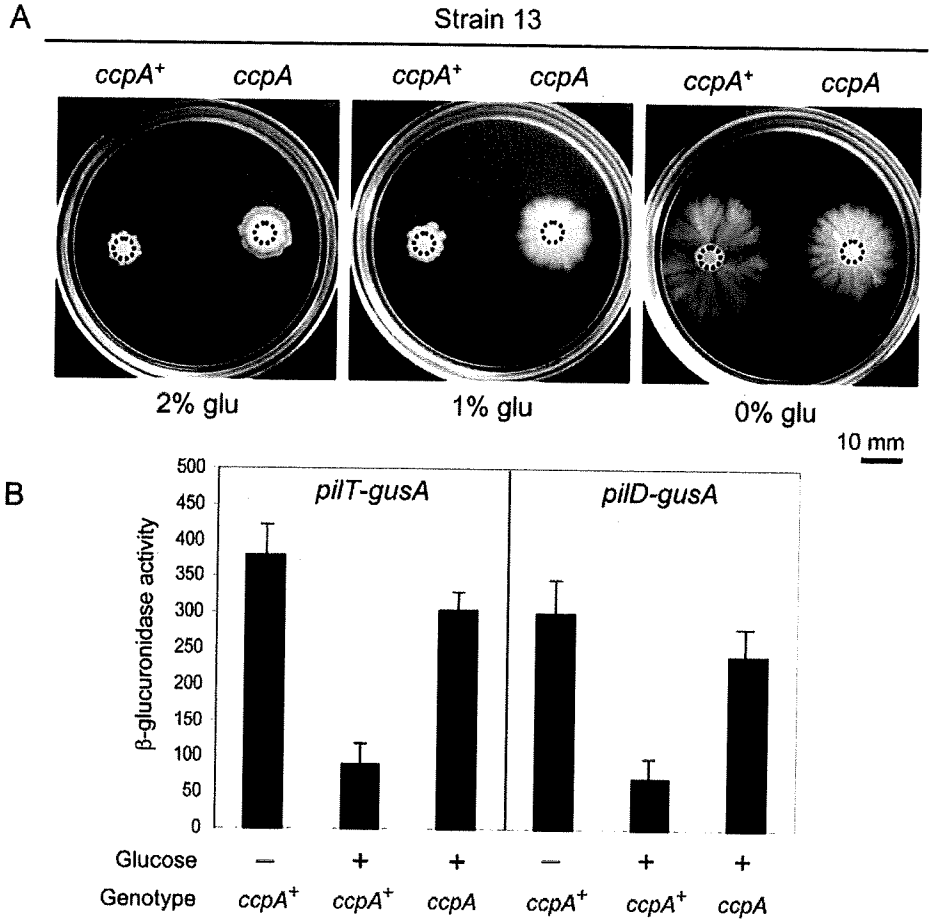

FIG. 6. CcpA mediates carbon catabolite repression of *C. perfringens* gliding motility. (A) Gliding motility phenotypes of the CcpA-proficient (*ccpA*$^+$) *C. perfringens* strain 13 and its isogenic CcpA-deficient derivate KO13 (see Materials and Methods for details). A motility assay was performed according to the protocol described in the above figure legends. Top-bottom photographs were taken after 40 h of anaerobic incubation on TYA plates supplemented or not supplemented with glucose (glu) as indicated in the figure. Black dotted circles indicate the initial sizes of the colonies immediately after drop inoculation. (B) Expression of *pilT-gusA* and *pilD-gusA* reporter fusions in *ccpA*$^+$ and *ccpA* cultures of isogenic *C. perfringens* strains 13 and KO13 grown for 30 h on TY broth in the absence (−) or presence (+) of 1% glucose. β-Glucuronidase activity was calculated as indicated in Fig. 5. For panels A and B, a representative set of results obtained from five independent experiments is shown.

analyze whether CcpA has a role in the carbon catabolite repression of *C. perfringens* gliding motility. To explore this possibility, we constructed a *C. perfringens ccpA* mutant strain (see Materials and Methods and Fig. S1 in the supplemental material) to assay the gliding phenotypes and the activities of TFP genes (*pilT* and *pilD*) of isogenic *ccpA*$^+$ and *ccpA C. perfringens* strains grown in the presence or absence of added glucose. When gliding proficiency was evaluated in the presence of 1% and 2% glucose, the CcpA-proficient strain 13 was, as expected, unable to move at both sugar concentrations, remaining situated at the point of inoculation (round and smooth colony morphology). In contrast, the isogenic *ccpA* mutant derivate KO13, deficient in CcpA production and inoculated on the same plates, was able to retain more than 50% of its gliding ability in the presence of 1% glucose and also showed a low but perceptible and reproducible level of gliding proficiency on TYA plates supplemented with 2% glucose (Fig. 6A). Under similar conditions, no gliding motility was observed in the presence of 1% glucose with the *ccpA* mutant strain harboring the CcpA-complementing plasmid pIH100 (Table 1 and data not shown), suggesting that functional CcpA was required for carbon catabolite repression of *C. perfringens* social gliding motility.

In order to further confirm the role of CcpA in carbon catabolite repression of *C. perfringens* gliding motility, we compared the expression levels of *pilT* and *pilD* in isogenic CcpA-proficient and CcpA-deficient *C. perfringens* cultures grown in TY broth plus/minus 1% glucose. As shown in Fig. 6B, the CcpA-deficient cultures, but not the cultures that express CcpA, showed active expression of *pilT* and *pilD* in the presence of glucose at almost the same level observed for wild-type cultures grown in the absence of glucose. Collectively, these results demonstrate that CcpA was actively involved in the carbon catabolite repression of *C. perfringens* gliding motility and the glucose-induced down-regulation of TFP biosynthetic genes.

An intriguing observation was that the gliding ability of the CcpA-deficient *C. perfringens* strain KO13 grown in the ab-

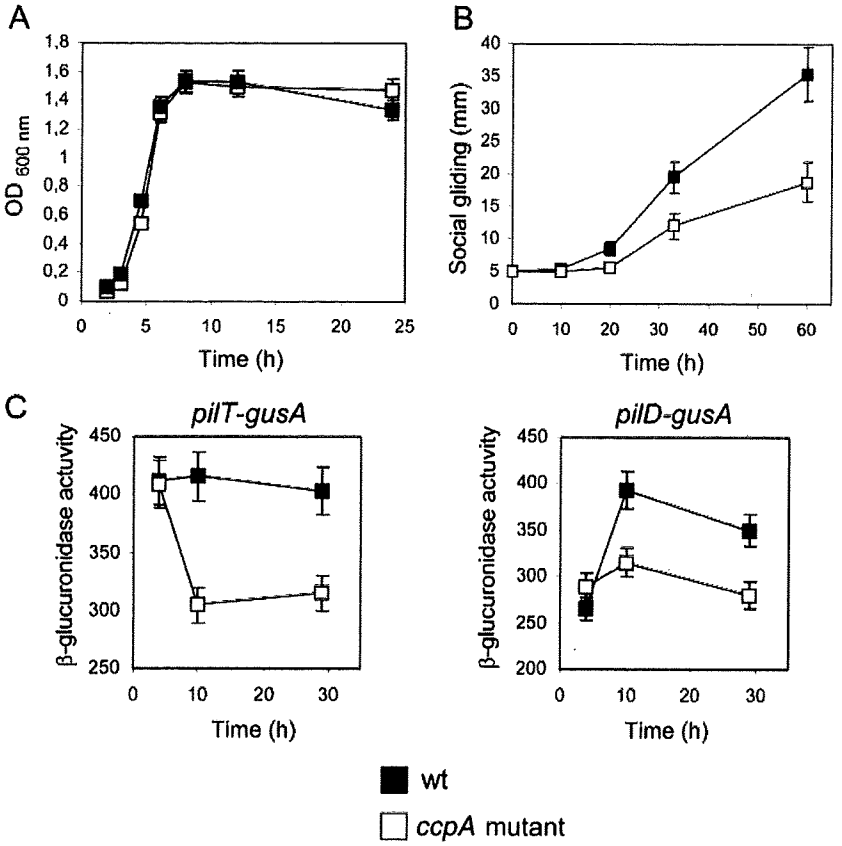

FIG. 7. CcpA has a novel catabolite-independent positive role in *C. perfringens* gliding motility. (A) Growth curves of the *ccpA*$^+$ *C. perfringens* strain 13 and its isogenic *ccpA* derivate KO13. Growth was monitored over time, measuring the OD_{600}s of both cultures developed on TY broth at 37°C. A representative set of results obtained from five independent experiments is shown. Closed symbols, wild type (wt); open symbols, *ccpA* mutant. (B) Kinetics of gliding motility of strain 13 (wt) and its isogenic CcpA-deficient derivate KO13 (*ccpA* mutant) developed on TYA plates without sugar supplementation. Gliding was recorded as indicated in Fig. 4. Average values of gliding obtained from five independent experiments are plotted. (C) Requirement of CcpA activity for full expression of *pilT* and *pilD* genes of *C. perfringens* strain 13 grown on TY broth without glucose supplementation. β-Glucuronidase activities driven from CcpA-proficient and CcpA-deficient *C. perfringens* cultures (strains 13 and KO13, respectively) harboring reporter *pilT-gusA* (left) and *pilD-gusA* (right) transcriptional fusions are shown. The four cultures were grown on TY broth without addition of glucose; accumulated β-glucuronidase activity was measured at the times indicated in the figure. Closed and open symbols represent CcpA-proficient and CcpA-deficient isogenic cultures, respectively. A representative set of results obtained from five independent experiments is shown.

sence of sugar supplementation was delayed in comparison with the gliding proficiency of the wild-type (*ccpA*$^+$) strain 13 (see right panel, no added glucose, in Fig. 6A). This observation could uncover an additional, and unexpected, carbon-independent positive requirement of CcpA for full proficiency of *C. perfringens* in gliding motility. It is worth indicating that the slight but reproducible catabolite-independent positive effect of CcpA on social behaviors has been previously documented during biofilm formation in *Bacillus subtilis* (39) and spore development in *C. perfringens* (42). In these bacteria, the absence of CcpA activity was reflected in a low level of proficiency in biofilm development and spore formation in comparison with the abilities of the wild-type (CcpA-proficient) strains (39, 42).

We confirmed our hypothesis by quantifying the gliding developed by the *ccpA*$^+$ (strain 13) and its isogenic *ccpA* mutant derivate on TYA plates in the absence of glucose supplementation. Previously, we determined that introduction of the *ccpA* mutation in strain 13 did not affect its growing ability and the final cellular yield after growth in liquid TY medium (Fig. 7A). In contrast to what occurred in broth, where the growth phenotypes of the CcpA-proficient and CcpA-deficient cultures were the same, we observed clear differences in the speed and kinetics of gliding between the *ccpA*$^+$ and *ccpA* strains. The gliding proficiency of the *ccpA* mutant strain showed a reduced speed compared with the velocity of gliding of the wild-type strain: 250 $\mu m\ h^{-1}$ and 670 $\mu m\ h^{-1}$ for the CcpA-deficient and CcpA-proficient strains, respectively (Fig. 7B). Also, the gliding of the *ccpA* mutant strain stopped before the gliding of the wild-type strain did (Fig. 6A, right, and 7B). These observations strongly suggest a novel and overlooked positive role for CcpA in the proficiency of gliding motility of *C. perfringens* in the absence of sugar supplementation. To confirm this conclusion, we measured the expression levels of *pilD* and *pilT* (whose products are essential for TFP-dependent gliding motility) in wild-type and CcpA-deficient *C. perfringens* cultures

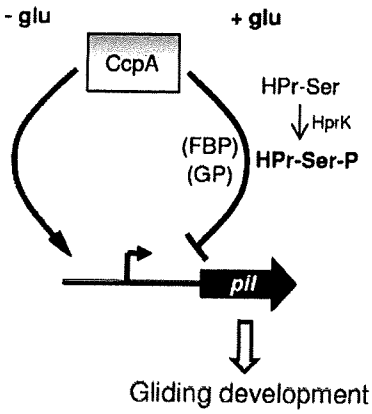

FIG. 8. A dual (positive and negative) role for CcpA in *C. perfringens* gliding development. The present cartoon depicts one hypothetical model that might explain our actual knowledge of the gliding phenotypes of CcpA-proficient and CcpA-deficient *C. perfringens* strains grown in the presence and absence of sugar supplementation. In the absence of glucose (−glu; left) or other catabolite-repressing sugars, CcpA by itself might be able to bind to the positive regulatory regions of genes (*pil*) involved in TFP expression (i.e., *pilT* and *pilD*), producing a positive effect on transcription and hence stimulating gliding proficiency. In support of this view, it has also been reported that in vitro and in vivo, CcpA-DNA mediated interactions do occur in the absence of added sugars (20, 29, 35). In the presence of catabolite-repressing amounts of glucose (right), the phosphotransferase enzyme of the sugar-specific phosphotransferase system Hpr-Ser would be phosphorylated by HprK (35). Hpr-Ser-P_i would bind to CcpA, and the newly formed Hpr-Ser-P_i::CcpA complex would interact with repressor sites located on the regulatory regions of *pil* (*pilT* and *pilD*) and therefore interfere with gliding proficiency. Also shown in the picture is the possibility that the coeffectors fructose 1,6-bisphosphate (FBP) and glucose 6-phosphate (GP) would function as adjunct corepressors to enhance and to fine-tune the response of CcpA to the metabolic needs of the cell (35, 43). Another possibility (an indirect effect of CcpA) that is not illustrated in this model is that CcpA would dually regulate an unidentified factor responsible for switching on and off the expressions of *pil* genes.

grown in TY broth without sugar supplementation. As observed in Fig. 7C, there are unambiguous down-regulations of *pilT* and *pilD* expression in the cultures deficient in CcpA production. This positive role of CcpA in TFP expression (Fig. 7C) and social gliding proficiency (Fig. 6A and 7B) in the absence of sugar supplementation and its opposite (negative) effect on the same social behavior (gliding motility) under conditions of carbon catabolite regulation (Fig. 6, presence of sugar) suggest a novel, dual (activating and repressing) role for CcpA in regulating *C. perfringens* gliding motility (Fig. 8).

DISCUSSION

Our current study shows several significant contributions toward the understanding of the physiology and regulation of TFP-dependent gliding motility in *C. perfringens*. First, we extended the analysis to a total of 17 different *C. perfringens* strains isolated from diverse infections (diarrhea, food poisoning, myonecrosis) produced not only in human beings but also from animal origins (Table 1). Interestingly, all the analyzed strains exhibited active proficiencies in social gliding motility on agar surfaces (Fig. 1 and data not shown). These results significantly consolidate and strengthen the idea that gliding motility is an intrinsic property of pathogenic *C. perfringens*, regardless of its origin of isolation (41).

Our understanding of the environmental and metabolic factors that control surface-associated translocation in pathogenic bacteria is very limited. Precisely, the main contribution of our work is the demonstration that carbon catabolite repression (20, 39, 43, 50) regulates social gliding motility in *C. perfringens*. In fact, all the surveyed isolates exhibited social gliding motility on BHIA plates (with no glucose supplementation) but not on TGYA medium which contained 2% glucose, suggesting that glucose is capable of inhibiting social gliding motility. The removal of glucose from TGYA allowed the cells to exhibit social motility, while the addition of glucose in BHIA resulted in the inhibition of gliding motility, confirming that glucose plays a crucial role in inhibiting gliding motility (Fig. 1 and data not shown).

In addition to glucose, gliding motility was also inhibited by other rapidly metabolized sugars, such as fructose, lactose, sucrose, and galactose (Fig. 3). This finding confirmed that the repression of gliding in *C. perfringens* was due to a general process of carbon catabolite repression (43). Interestingly, two complex carbohydrates, raffinose and starch, behaved differently from the single sugars: raffinose did not inhibit motility at any of the assayed concentrations, and starch inhibited gliding only at concentrations higher than 2% (Fig. 3 and data not shown). These results are consistent with previously reported findings that other social behaviors present in *C. perfringens*, such as sporulation and enterotoxin (CPE) production, were also repressed by rapidly metabolized single sugars, such as glucose and lactose (28, 42), while the complex carbohydrates raffinose and starch were found to induce both events (17, 18). The correlation between carbon catabolite repression of sporulation and surface-associated motility suggests that the two social processes might share, at least in part, a common regulatory network (Fig. 9).

We demonstrate that carbon catabolite repression of gliding motility in *C. perfringens* occurs through the repression of at least two genes involved in TFP production and functionality, namely, *pilD* and *pilT*. As observed in Fig. 5, the addition of 1% glucose to growing cultures of the gliding-proficient reference strains 13 and SM101 resulted in dramatic decreases in *pilD-gusA* and *pilT-gusA* expression. The maximum reduction in transcription due to the added glucose occurred approximately after 24 h of growth, which is consistent with the observation that gliding motility on agar plates begins only after 18 to 20 h of growth (Fig. 4 and data not shown).

In low-G+C-content, gram-positive bacteria, carbon catabolite regulation is under the control of the key transcription factor CcpA (*c*arbon *c*atabolite *p*rotein A), a member of the LacI-GalR family of transcriptional regulators (43). In the better-known cases of CcpA-mediated carbon catabolite regulation (i.e., in *Bacillus subtilis* and *Lactococcus lactis*), a complex and sophisticated signaling network is present (20, 29, 40, 50). Basically, the CcpA-dependent regulatory network utilizes sugar transporters, glycolytic enzymes, and an ATP-dependent, metabolite-activated protein kinase (HprK) and two small HprK target proteins: the phosphotransfer protein Hpr of the phosphotransferase system and the Hpr homologue Crh (35, 43). Moreover, a central role has been reserved for CcpA,

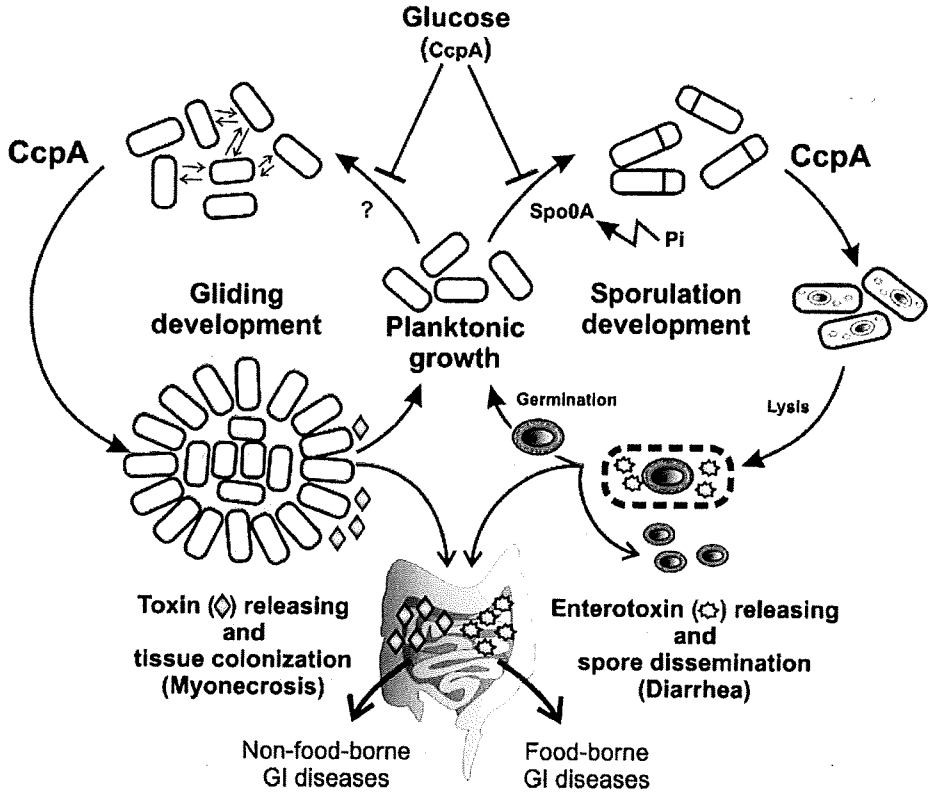

FIG. 9. A workable model linking carbon catabolite regulation of social behaviors (gliding, sporulation, and toxin production) with disease progression in *C. perfringens*. In this hypothetical but realistic scenario, toxigenic, vegetative *C. perfringens* cells that reach the lumen of a human or animal gastrointestinal (GI) tract, where the basal luminal concentrations of glucose are normally lower than 0.5% (12), have the possibility of undergoing at least two different differentiation pathways: sporulation and/or gliding development. In the first case, the activation of the key transcription factor Spo0A by inorganic phosphate (P_i) present in the intestinal lumen triggers spore morphogenesis and enterotoxin (CPE) production (28). In the case of gliding development (left), unknown signals that might be linked to cell-cell and cell-surface interactions (double arrows) orchestrate the spatial and temporal organization of the cells to the onset of gliding. The progression of either developmental program (sporulation or gliding) would not exclude the occurrence of the other alternative pathway: sporulation and CPE production would take place in the lumen of the GI tract while gliding motility and vegetative toxin synthesis (i.e., collagenase production) would take place in association with the intestinal mucosa. The key role of glucose (representing the occurrence of CcpA-mediated carbon catabolite regulation when the level of the sugar is at least 1%) as a repressor of sporulation (25, 28) and gliding (this study) development is indicated. This regulatory blockage derives from the inhibition of enterotoxin (25, 28) and vegetative-linked toxin (11, 42) production in *Clostridium* spp. The novel role of CcpA as an activator of sporulation (42) and gliding proficiency (this study) is also shown. The development of inhibitors (e.g., monosaccharide analogs) that block the onsets of gliding and/or sporulation or antagonists that interfere with the positive role of CcpA on toxin production would contribute to combating the outbreak and dissemination of clostridial diseases.

which binds to DNA sequences (*cis*-acting replication element sites) present on the regulatory regions of its target genes (20, 29, 35, 43). For the activation of CcpA binding to the *cis*-acting replication elements, it is necessary, although not essential (20, 29, 35), for CcpA to bind to the phosphorylated forms of Hpr and/or Crh produced by HprK (35, 43). In *C. perfringens*, orthologs of *ccpA*, *hpr*, and *hprK* (but not *crh*) are present on the chromosomes of all the sequenced strains, suggesting that the basic elements for CcpA-mediated carbon catabolite regulation are present in this pathogen (reference 43 and data not shown). In fact, we demonstrated that the repression of *C. perfringens* gliding motility by glucose was mediated, in large part, by the action of CcpA. As observed in Fig. 6, the inactivation of *ccpA* significantly restored gliding proficiency (Fig. 6A) and *pil* expression (Fig. 6B) in the presence of glucose. The reversion to the gliding-deficient phenotype of the *ccpA* mutant strain in the presence of glucose was obtained after the introduction (by DNA electroporation) of the plasmid pIH100, harboring a wild-type copy of *ccpA*, which provided direct genetic evidence supporting the strong linkage between CcpA expression and carbon catabolite repression of gliding motility in *C. perfringens*. These results suggest that CcpA could act as a transcriptional regulator of TFP biosynthesis genes. However, this effect might be indirect since no putative *cre* sites have been identified in any of the TFP biosynthesis genes analyzed so far (data not shown). It might be possible that other *cre*-like consensus sequences, different from the ones reported for *Bacillus* and other low-G+C-content, gram-positive bacteria, exist in clostridia (20, 42, 43). Another possibility is that, apart from CcpA, an unidentified intermediate factor might be involved in regulating TFP gene expression. This suggestion received support based on the observation that the *ccpA* mutant strain was not able to restore, in the presence of sugar supplementation, full gliding proficiency and *pil* expres-

sion as the levels reached that of the wild-type strain in the absence of added sugars (Fig. 6 and data not shown).

A final and unexpected finding of our study was the observation that, in the absence of added sugar, CcpA has a positive role in gliding motility. As observed in Fig. 6, in the absence of added glucose, the *ccpA* mutant strain glided on the agar plate to a lesser extent than the isogenic wild-type strain. As observed in Fig. 7B, the wild-type strain (CcpA proficient) reached a maximum speed of gliding of 630 to 670 $\mu m\ h^{-1}$, while its isogenic *ccpA* derivate (CcpA deficient) reached a maximal speed of gliding of 220 to 250 $\mu m\ h^{-1}$ only. Two observations argue strongly for a positive role for CcpA in gliding development: first, the *ccpA* mutant strain did not show any growth defect on liquid medium, reaching essentially the same final OD and viable-cell number as the wild-type strain (Fig. 7A); furthermore, the results for the initial phase of colony growth (before the onset of gliding) were very similar for both the $ccpA^+$ and *ccpA* strains (Fig. 7B and data not shown). This hypothesis was reinforced by the demonstration that CcpA production was required for efficient expression of *pilT* and *pilD* in growth media without sugar supplementation (Fig. 7C). These findings indicate that CcpA has a dual role in controlling gliding motility in *C. perfringens* (Fig. 8). In the presence of rapidly metabolized sugars (e.g., glucose), CcpA has a negative role on the onset of gliding, an effect that is partly mediated through repression of *pilT* and *pilD* expression (Fig. 6). In the absence of added sugars, CcpA switches to a positive role on gliding, a novel property that is uncovered by the deficient gliding phenotypes and poor *pilT* and *pilD* expression levels of CcpA-deficient cells cultured in the absence of added glucose (Fig. 7). In agreement with our finding, a similar positive role for CcpA in spore formation and *cpe* expression under conditions without catabolite regulation (in the absence of added sugars) has been reported for *C. perfringens* (42).

Excess glucose in the environment of *C. perfringens* not only affects stationary phase phenomena, such as sporulation-linked CPE production (25, 28, 42) and gliding motility (this study), but can also act as a catabolic repressor of collagenase production during vegetative growth (42). Moreover, in the other intestinal pathogenic *Clostridium* bacterium *C. difficile*, glucose represses toxin production (11). Importantly, within the context of the development of a clostridial infection, it is plausible to envision that proficiency in gliding associated with toxin production and tissue damage would contribute to the progression of the infectious process (Fig. 9). Luminal glucose concentrations in the small intestines of mammals are in the range of 0.006% to 0.4% (12). Interestingly, in our study the catabolite repression of gliding motility by glucose was concentration dependent; surface motility was observed only when the glucose concentration was less than 0.5% (Fig. 2). This finding opens the possibility that gliding willingly would occur during the course of a clostridial infection (Fig. 9). We are just grasping the regulatory network of surface-associated motility in pathogenic clostridia, and the understanding of how carbon catabolite repression inhibits known and potential virulence processes (sporulation, toxin production, and gliding motility) in *C. perfringens* (6–8, 21, 26, 36) will contribute to preventing and combating clostridial diseases (Fig. 9).

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Detection of a Group II Intron without an Open Reading Frame in the Alpha-Toxin Gene of *Clostridium perfringens* Isolated from a Broiler Chicken▽

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A DNA insertion of 834 bp, designated CPF-G2Im, was identified within the alpha toxin gene (*cpa*) of *Clostridium perfringens* strain CPBC16ML, isolated from a broiler chicken. Sequence analysis of CPF-G2Im indicated that it was integrated 340 nucleotides downstream of the start codon of *cpa*. However, the insertion did not abolish the phospholipase C and hemolytic activities of CPBC16ML. To investigate the expression of its alpha toxin, the intact copy of *cpa* was cloned into an expression vector and transformed into *Escherichia coli* M15 cells. Immunoblotting analysis showed that the protein expressed from the transformant as well as in the culture supernatant of *C. perfringens* strain CPBC16ML had the expected molecular weight detected in reference strains of *C. perfringens*. Northern hybridization and reverse transcriptase PCR (RT-PCR) analysis revealed that the entire CPF-G2Im insertion was completely spliced from the *cpa* precursor mRNA transcripts. The sequence of the insertion fragment has 95% and 97% identity to two noncoding regions corresponding to sequences that flank a predicted group II RT gene present in the pCPF4969 plasmid of *C. perfringens*. However, an RT was not encoded by the CPF-G2Im fragment. Based on the secondary structure prediction analysis, CPF-G2Im revealed typical features of group II introns. The present study shows that CPF-G2Im is capable of splicing in both *C. perfringens* and *E. coli*. To our knowledge, this is the first report that a group II intron without an open reading frame (ORF) is located in the *cpa* ORF of *C. perfringens*.

Clostridium perfringens is a gram-positive anaerobic organism which is widely distributed in the environment and in the gastrointestinal tracts of animals and is associated with enteric and histotoxic diseases in humans and animals (12, 22). It has been reported that *C. perfringens* produces a variety of toxins, and these toxins are considered the most important virulence factors of this organism (22). *C. perfringens* strains are classified into five types (A, B, C, D, and E) according to the production of four major toxins (alpha, beta, epsilon, and iota) (1, 22). The alpha toxin is produced by all five types of *C. perfringens* and is the first bacterial protein from *C. perfringens* reported to show both enzymatic and toxic properties (12). The mature alpha toxin, produced by a single *cpa* gene and consisting of one single polypeptide with a molecular mass of 42.5 kDa, is a zinc-dependent phospholipase C (PLC) with 370 amino acid residues (30, 31, 41). The PLC produced by *C. perfringens* exhibits various biological activities, such as hemolysis, necrosis, and lethality to the intracellular metabolism of eukaryotic target cells (6, 22, 32).

Mobile genetic elements have important impacts on eukaryotic and prokaryotic genomes (5, 13, 18). In the prokaryotic world, transposable elements, such as insertion (IS) elements and transposons, which can disrupt the inserted genes, are involved in large-scale genome rearrangement and in the transfer of accessory genes and thus usually have important impacts on genome evolution (4). Introns, another type of interesting mobile element, are classified into four major classes, namely, self-splicing group I and group II introns, tRNA and rRNA archaeal introns, and spliceosomal introns in nuclear pre-mRNA (8, 14, 29, 34, 37). Bacterial introns belong to either group I or II, among which group I introns are mostly found in tRNAs or in protein-encoding genes associated with DNA metabolism of bacteriophages (8, 10, 13), while most group II introns are inserted in or associated with other mobile genetic elements, such as IS elements, transposons, or plasmids (10, 19, 21, 35, 45). After DNA insertion, they can remove themselves by protein-assisted autocatalytic RNA splicing (19). During the past several years, introns have been detected in surprising numbers in bacterial genomes as a result of genome sequencing projects (17, 20, 42).

In the genus *Clostridium*, introns have been reported for *Clostridium difficile* (2, 13, 28, 36). Although group I and group II introns have not been identified in *C. perfringens*, the sequence of a predicted group II intron reverse transcriptase (RT) gene, which was from plasmid pCPF4969 of strain F4969 (26), was recently deposited in GenBank with accession number AB236336.1. However, the group II intron present in the plasmid has not been characterized fully. In our previous experiments involving PCR screening of the *cpa* genes from 110 *C. perfringens* strains isolated from broiler chickens (unpublished data), we detected one isolate (designated CPBC16ML) with a larger amplicon for the *cpa* gene (Fig. 1A). Interestingly, strain CPBC16ML showed both PLC and hemolytic activities, as detected in reference strains (Fig. 1B and C). It was re-

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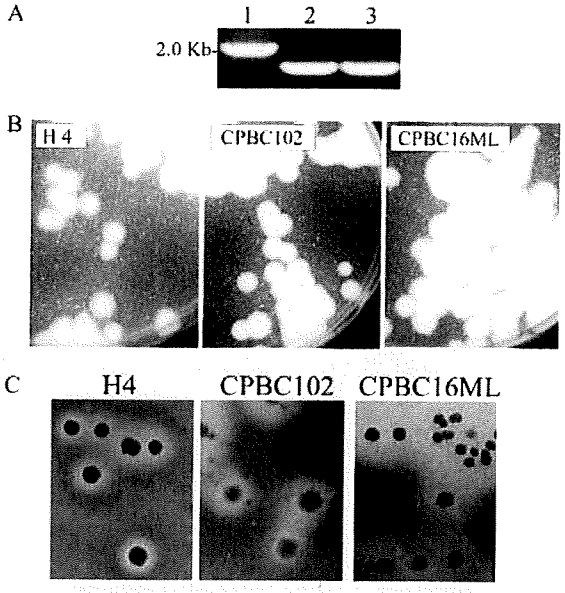

FIG. 1. (A) PCR amplification of the *cpa* gene, using primers cpalphatox1-L and -1-R. Strain designations for the lanes are as follows: lane 1, CPBC16ML; lane 2, H4; and lane 3, CPBC102. The molecular size marker is indicated to the left of the gel. The amplicon of CPBC16ML represents a larger size than the others. (B) Colonies of strains H4, CPBC102, and CPBC16ML on egg yolk-CW agar plates. CPBC16ML showed typical *C. perfringens* colonies with a surrounding zone of precipitation, similar to those of the wild-type strain CPBC102 and the reference strain H4. (C) Colonies of strains H4, CPBC102, and CPBC16ML on horse blood agar plates. CPBC16ML also showed hemolytic activity, as seen in strains H4 and CPBC102.

ported that the *cpa* gene is highly conserved, with an overall sequence difference of only about 1.5% (12, 16, 39). Therefore, we hypothesized that a mobile genetic element may be integrated into the *cpa* gene.

Our goals in this study were to determine the nucleotide sequence of the *cpa* gene of CPBC16ML, to clone it for expression in *Escherichia coli*, to determine its molecular size and reactivity against an anti-Cpa antibody, and to examine the expression of the alpha toxin. As a result of these studies, the presence of a group II intron without an open reading frame (ORF) integrated into the *cpa* gene of *C. perfringens* was demonstrated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 110 *C. perfringens* strains were isolated from the large intestinal contents of broiler chickens. Colonies indicating PLC activity on kanamycin-containing CW agar plates (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with egg yolk (EY-CW) were subjected to 16S rRNA gene sequence analysis for bacterial identification. PCR of the 16S rRNA gene was performed using bacterial universal primers F8 and R15 (Table 1), as described previously (25). The nucleotide sequences were determined directly from the PCR amplicons, using each universal primer. *C. perfringens* strains CPBC16ML and CPBC102 were selected from the 110 isolates and used for further studies. In addition to chicken isolates, *C. perfringens* strain H4 (type A, isolated from a human), which possesses a normally sized *cpa* gene, was used as a control. In addition to the PLC activity on EY-CW agar plates, the hemolytic activity of these strains was examined on GAM agar plates (Nissui) supplemented with 10% horse blood. *C. perfringens* strain 13, which has been sequenced completely (40), was used for Southern and Northern hybridization tests, as described below. *Escherichia coli* DH5α (TaKaRa, Tokyo, Japan) and M15 (QIAGEN Inc., Tokyo, Japan) were used for cloning and expression, respectively. *E. coli* DH5α was cultured on Luria-Bertani (LB) agar plates containing 100 μg/ml of ampicillin. Recombinant *E. coli* M15 was grown in LB broth or on LB agar plates containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin.

Genetic techniques. *C. perfringens* strains were grown aerobically in GAM broth (Nissui) at 37°C for 4 to 8 h, and DNAs were extracted using the hexadecyltrimethyl ammonium bromide (CTAB) method as described previously (11). Plasmids were isolated by using a QIAprep Spin plasmid kit (QIAGEN), and PCR products were purified with a QIAquick PCR purification kit (QIAGEN). RNAs of *C. perfringens* were extracted from GAM broth culture, using an RNeasy Mini kit (QIAGEN). All other standard molecular techniques were performed as described elsewhere (38). DNA sequencing was performed with a BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems Inc., Tokyo, Japan), and an ABI PRISM model 310 automated sequencer (Applied Biosystems) at the Genetic Center of the University of Miyazaki. Computer analysis of DNA and protein sequences was performed with GENETYX-WIN 5.4 software. RNA secondary structures were analyzed with GENETYX-WIN 5.4 and mfold (47), version 3.2, which is available at http://www.bioinfo.rpi.edu/applications/mfold/old/rna/. Database similarity searches were performed through the National Center for Biotechnology Information by use of the BLASTX and BLASTN algorithms.

PCR techniques. The primers used in this study are listed in Table 1. Primers cpasF1 and cpaR6 were designed from the sequence of *C. perfringens* strain 13, and the other primers for the *cpa* gene were designed based on nucleotide sequences from strain CPBC16ML.

(i) Standard PCR. To detect the *cpa* gene in *C. perfringens*, PCR was performed in a thermocycler (Applied Biosystems), using primer pair cpalphatox1-L/1-R (Table 1 and Fig. 2), as described elsewhere (3). PCR was performed in a final volume of 20 μl, which consisted of 20 pmol (each) of forward and reverse

TABLE 1. Primers used in this study

Primer	Nucleotide sequence (5′–3′)	Binding site[a]
cpasF1	CATCCCTTATATTATGTG	*C. perfringens cpa*
cpaF2	GCATATGATCTATATCAA	*C. perfringens cpa*
cpaF3	CAGAGGGTAATGATCCAT	*C. perfringens cpa*
cpaR6	GTAAATACCACCAAAACC	*C. perfringens cpa*
cpalphatox1-L[b]	AGATTTGTAAGGCGCTT	*C. perfringens cpa*
cpalphatox1-R[b]	ATTTCCTGAAATCCACTC	*C. perfringens cpa*
cpaF8	ACGCTTTCTCTAATACTC	CPF-G2Im within the *cpa* gene of CPBC16ML
cpaF6	GTCAGTGAATATAGAAAATT	CPF-G2Im within the *cpa* gene of CPBC16ML
cpaR8	TAATATTATCTATTACGG	CPF-G2Im within the *cpa* gene of CPBC16ML
cpaR10	AAATATCTTGCCATGGTC	CPF-G2Im within the *cpa* gene of CPBC16ML
Universal F8[c]	AGAGTTTGATCMTGGCTCAG	*C. perfringens* 16S rRNA gene
Universal R15[c]	AAGGAGGTGATCCARCCGCA	*C. perfringens* 16S rRNA gene

[a] Binding sites of the primers are shown in Fig. 2.
[b] From reference 3.
[c] From reference 25.

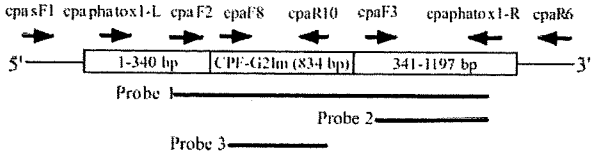

FIG. 2. Schematic structure of the *cpa* gene of *C. perfringens* strain CPBC16ML. Arrows indicate the orientations of the primers for the *cpa* gene. The CPF-G2Im sequence detected in *cpa* is shown as a gray box. Primers cpasF1 and cpaR6 were designed from the sequence of *C. perfringens* strain 13, and all other primers were designed based on the nucleotide sequence of strain CPBC16ML. Probes 1, 2, and 3 are PCR amplicons used as DNA probes for Southern and Northern hybridization.

primers, a 200 μM concentration of each deoxynucleoside triphosphate, 0.5 U of *Taq* DNA polymerase (QIAGEN), 1× PCR buffer, and 50 ng of DNA template. Thermal cycle conditions consisted of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min. After 35 thermal cycles, the reaction was completed with a final extension step at 72°C for 10 min. Following amplification, the resulting amplicons were analyzed by electrophoresis on a 1% agarose gel.

(ii) RT-PCR. Prior to reverse transcription, RNAs purified from *C. perfringens* were treated with 10 U of DNase I (QIAGEN) at 37°C for 30 min. cDNA synthesis was carried out using a cDNA synthesis kit (TaKaRa). Reverse transcriptase was inactivated by heating at 70°C for 10 min prior to RT-PCR. RT-PCR was carried out with primer pair cpaF2/cpalphatox1-R, cpaF8/cpaR10, cpaF2/cpaR10, or cpaF8/cpalphatox1-R (Table 1 and Fig. 2). The reaction conditions and thermal cycles were the same as those for the standard PCR. Nucleotide sequencing of the RT-PCR products was performed as described above.

Cloning and protein expression of the *C. perfringens cpa* gene in *E. coli*. A DNA fragment of the intact *cpa* gene, including its promoter, was generated by PCR using primers cpasF1 and cpaR6 (Table 1 and Fig. 2) from *C. perfringens* strains CPBC16ML, CPBC102, and H4. A six-His-tagged protein expression vector, pQE30-UA (QIAGEN), was used to express the *C. perfringens* alpha toxin in *E. coli* M15 cells. Each *cpa* gene from the three strains was cloned into the pQE30-UA vector, yielding plasmids pTIC119, pTIC120, and pTIC121, respectively, and transformed into *E. coli* DH5α competent cells. Plasmids extracted from colonies were confirmed to include the intact *cpa* gene by PCR. Furthermore, each plasmid was transformed into *E. coli* M15 competent cells in accordance with the manufacturer's recommendations. Transformants were cultured in LB broth with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; TaKaRa), and protein expressed in the cell lysate was purified with a Ni-nitrilotriacetic acid affinity chromatography kit (QIAGEN).

SDS-PAGE and Western blot analysis. To determine the molecular weight and reactivity to antibody of the alpha toxin protein expressed by *E. coli* M15 cells containing the *cpa* gene, the protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% resolving gel (Bio-Rad). After electrophoresis, one polyacrylamide gel was stained with 0.2% Coomassie brilliant blue R260 solution, and the other was electrotransferred onto a nitrocellulose membrane (Bio-Rad) for Western blotting analysis. The membrane was probed with a 1:10,000 dilution of a rabbit anti-alpha toxin polyclonal antibody (kindly provided by J. Sakurai, Tokushima Bunri University, Tokushima, Japan). In addition, supernatants of *C. perfringens* strains CPBC16ML, CPBC102, and H4 were also used for Western blotting analysis.

Hybridization. (i) Southern hybridization. Genomic DNAs from CPBC16ML and strain 13 were digested with EcoRI (TaKaRa), electrophoresed in a 1% agarose gel, and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The PCR products from CPBC16ML amplified with primer pairs cpaF2/cpalphatox1-R (probe 1), cpaF3/cpalphatox1-R (probe 2), and cpaF8/cpaR10 (probe 3) were used as probes (Fig. 2). Each probe was labeled with an AlkPhos-direct kit (Amersham Pharmacia Biotech), and signals were detected by CDPstar chemiluminescence. The sizes of detected bands were estimated using a λ/StyI digestion marker (OneSTEP marker 6; Nippon Gene, Tokyo, Japan).

(ii) Northern hybridization. Northern hybridization was performed as previously described (41), except that the DNA fragments were labeled with an AlkPhos-direct kit (Amersham Pharmacia Biotech) and signals were detected by CDPstar chemiluminescence. The three probes (Fig. 2) examined for Southern hybridization, as described above, were also used in Northern hybridization. To estimate the sizes of the bands detected, 23S rRNA (2.9 kb) and 16S rRNA (1.6 kb) derived from *C. perfringens* strain 13 were used as markers.

Nucleotide sequence accession number. The 834-nucleotide sequence of CPF-G2Im in the *cpa* gene has been deposited in the GenBank data bank under accession number DQ787115.

RESULTS

16S rRNA gene sequence analysis of strain CPBC16ML. Strain CPBC16ML has PLC activity on EY-CW agar plates containing kanamycin, which is a selective medium for the isolation of *C. perfringens*. To confirm whether this isolate is *C. perfringens*, its 16S rRNA gene sequence was determined. A 1,396-bp fragment of the gene showed 100% identity to *C. perfringens* (GenBank accession number Y12669.1) (data not shown). Thus, it was concluded that this isolate is *C. perfringens*.

PLC and hemolytic activities of strain CPBC16ML. To determine if the different size of the *cpa* gene affects the enzymatic activities of the alpha toxin of CPBC16ML, PLC and hemolytic activities were detected on EY-CW and GAM blood agar plates, respectively, and compared with those of reference strains CPBC102 and H4. CPBC16ML showed both activities, as did the reference strains (Fig. 1B and C). This suggested that the larger size of the *cpa* gene did not abolish the enzymatic activities of the alpha toxin produced by CPBC16ML.

Nucleotide sequencing of the *cpa* gene of strain CPBC16ML. Sequencing analysis of the *cpa* gene showed that a segment of 834 bp with no homology to *cpa*, designated CPF-G2Im, was inserted within the *cpa* ORF. The analysis revealed that CPF-G2Im was integrated into the *cpa* gene 340 nucleotides downstream of the initial codon. A BLASTn search revealed that a 661-bp region from the 5′ end (positions 1 to 661) and a 120-bp region from the 3′ end (positions 715 to 834) of CPF-G2Im have 95% and 97% identities, respectively, with two noncoding regions on plasmid pCPF4969 of *C. perfringens* (26) (GenBank accession number AB236336.1). These regions with high similarity to CPF-G2Im were located at positions flanking the ORF encoding a predicted group II intron RT. However, the remaining 53 bp of the intermediate region (positions 662 to 714) of CPF-G2Im had no similarity with any known nucleotide sequence. Furthermore, four short fragments, ranging from 42 to 86 bp, with similarity (90 to 96%) to the Tn*554*-related transposase A (GenBank accession number AE017195) carried on plasmid pBC10987 of *Bacillus cereus* ATCC 10987, were distributed in CPF-G2Im. The amino acid sequence deduced from the nucleotide sequence of CPF-G2Im revealed that an ORF encoding 44 amino acid residues could be predicted for the 3′-end region of CPF-G2Im. However, this short peptide had no similarity with any known proteins and is not likely to be a remnant of a known RT.

Southern hybridization. DNAs extracted from CPBC16ML and strain 13 were digested with EcoRI since the sequencing data showed that there is no restriction site for EcoRI within both the *cpa* gene and the CPF-G2Im fragment. When the DNAs were hybridized with probe 1, which consists of parts of *cpa* and the entire CPF-G2Im sequence (Fig. 2), three bands for strain CPBC16ML and a single band for strain 13 were detected. All of the bands detected for CPBC16ML were larger than that detected for strain 13 (Fig. 3). When probe 2, which does not contain CPF-G2Im, was used, both strains

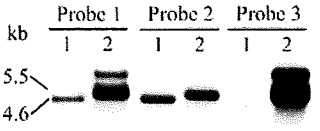

FIG. 3. Southern hybridization analysis of strain CPBC16ML. Genomic DNAs from CPBC16ML and strain 13 were digested with EcoRI, electrophoresed in a 1% agarose gel, and transferred to a nitrocellulose membrane. Lanes 1, *C. perfringens* strain 13; lanes 2, CPBC16ML. Three probes (probe 1, probe 2, and probe 3), as shown in Fig. 2, were used.

yielded single bands, but with different sizes (approximately 4.6 kb for strain 13 and 5.5 kb for CPBC16ML). In contrast, hybridization with probe 3, which contains a partial CPF-G2Im fragment, produced three bands for CPBC16ML, with the same sizes as those seen when probe 1 was used, but no band for strain 13. From these results, we confirmed that CPF-G2Im is present in the *cpa* gene of CPBC16ML. Moreover, it appears that the CPF-G2Im fragment is present not only in the *cpa* gene but also in other locations of the genomic DNA in CPBC16ML.

Cloning and expression of the *cpa* gene in *E. coli* M15. To investigate the expression of the alpha toxin of CPBC16ML, an intact copy of *cpa* was cloned into an expression vector and transformed into *E. coli* M15. The attempt to transform *E. coli* M15 directly with the recombinant plasmid carrying the *cpa* gene initially failed. However, it was easy to transform DH5α cells directly. DH5α cells were then used as an intermediate host. *E. coli* M15 transformants harboring the intact *cpa* genes from strains CPBC16ML, CPBC102, and H4 were successfully isolated. All three transformants showed PLC activity, with the presence of a typical turbidity zone surrounding the colonies on LB agar supplemented with egg yolk (data not shown).

SDS-PAGE and Western blot analysis. The above results demonstrated that the CPBC16ML *cpa* gene was capable of producing functional alpha toxin protein in *E. coli*. Next, the molecular size of the expressed protein was determined. SDS-PAGE analysis revealed a single protein band with the same molecular mass (about 43 kDa) as the proteins of H4 and CPBC102 (Fig. 4A). Western blot analysis showed that single bands of about 43 kDa were detected from the three strains (Fig. 4B). However, the band from CPBC16ML was much weaker than those from the reference strains. Culture supernatants from the three strains of *C. perfringens* were also examined by Western blotting, and the reactivities against antibody were consistent among the strains examined (data not shown). These results confirmed that insertion of CPF-G2Im in CPBC16ML *cpa* neither affected alpha toxin expression nor the molecular weight of the alpha toxin produced.

Northern hybridization. The three probes used for Northern hybridization were the same as those used for Southern hybridization (Fig. 2). Hybridization of probe 1 to the RNAs of strain CPBC16ML yielded three bands (Fig. 5A). The largest band, with a size of about 3.0 kb, was faint (cannot be seen clearly on the photograph). This may be the primary transcript of mRNA for CPBC16ML *cpa*. The second band, with a size of about 1.5 kb, was stronger than the largest one and appeared to be present in *C. perfringens* strain 13 as well. The smallest band, with a size of about 0.9 kb, was detected in CPBC16ML

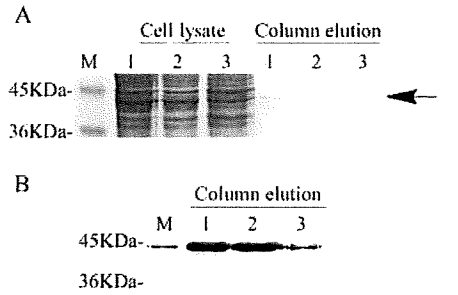

FIG. 4. (A) SDS-PAGE analysis of expressed proteins extracted from the periplasm of *E. coli* M15. Whole-cell lysates and purified His-tagged proteins from *E. coli* M15 carrying the *cpa* gene of the reference strain H4 (lanes 1), the wild-type strain CPBC102 (lanes 2), or the variant strain CPBC16ML (lanes 3) were separated in an SDS-PAGE gel (12%). Lane M contains molecular markers. The arrow indicates the position of the expressed protein purified by Ni-nitrilotriacetic acid affinity chromatography from a cell lysate. The positions (in kilodaltons) of molecular size markers are indicated on the left. (B) Western blot analysis of expressed proteins produced by strains H4 (lane 1), CPBC102 (lane 2), and CPBC16ML (lane 3). The alpha toxin protein was detected by a rabbit anti-alpha toxin polyclonal antibody.

but not in strain 13 (Fig. 5A). When probe 2 was used, CPBC16ML showed a single band of 1.5 kb, as detected in strain 13 (Fig. 5A). In contrast, using probe 3, a single band of approximately 0.9 kb was detected in CPBC16ML but not in strain 13 (Fig. 5A). Although the sizes of the bands detected were not precise because of estimation based on the migration of 23S and 16S rRNAs of *C. perfringens* strain 13 as size markers, these results suggested that the 1.5-kb and 0.9-kb bands corresponded to the *cpa* and CPF-G2Im mRNAs, respectively. From these results, it was assumed that CPF-G2Im may be excised from the flanking *cpa* gene at the mRNA level. The exons then ligate to form an intact *cpa* gene.

RT-PCR analysis. The results of Northern hybridization indicated the possible deletion of CPF-G2Im from *cpa* during transcriptional regulation. To further understand CPF-G2Im

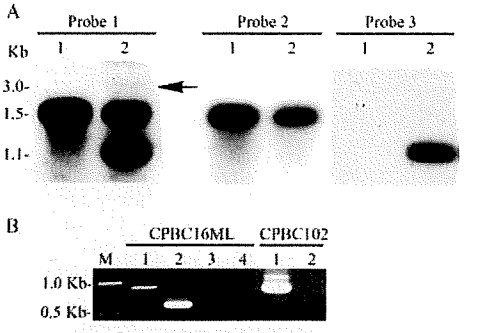

FIG. 5. (A) Northern hybridization analysis of variant CPBC16ML *cpa*. Lanes 1, *C. perfringens* strain 13; lanes 2, strain CPBC16ML. Three bands for strain CPBC16ML, with sizes of 3.0 kb, 1.5 kb, and 0.9 kb, are indicated where probe 1 was used for hybridization. The arrow indicates a predicted primary transcript of CPBC16ML *cpa* mRNA. (B) RT-PCR of CPBC16ML and CPBC102 *cpa* genes. Primer set designations for the lanes are as follows: lanes 1, cpaF2/cpalphatox1-R; lanes 2, cpaF8/cpaR10; lane 3, cpaF2/cpaR10; and lane 4, cpaF8/cpalphatox1-R. Lane M contains molecular size markers. The positions of molecular size markers are indicated to the left of the gel.

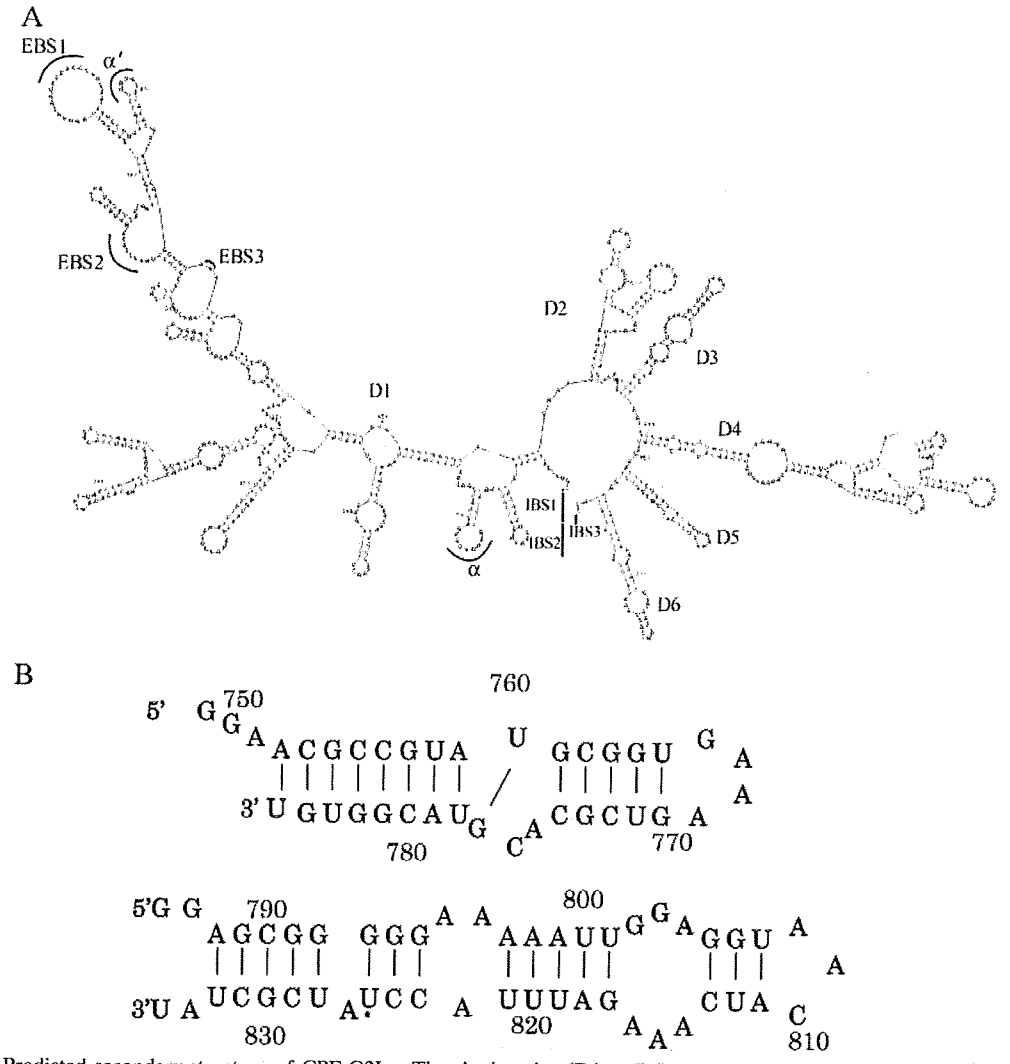

FIG. 6. (A) Predicted secondary structure of CPF-G2Im. The six domains (D1 to D6) emanate from the central wheel. The exon binding sequences (EBS1, EBS2, and EBS3), intron binding sequences (IBS1, IBS2, and IBS3), and tertiary interaction sites (α and α′) are marked. IBS1 and IBS2 are present in the 5′-flanking exon, and IBS3 is present in the 3′-flanking exon (IBS1, 5′-ATAGTT-3′; IBS2, 5′-GGTATT-3′; IBS3, T). (B) Sequence and predicted secondary structure of the 3′ end of CPF-G2Im. The top panel shows the structure of D5, from residues 749 to 785, with a tetraloop (5′-GAAA), and the bottom panel shows D6, from residues 787 to 834, with a bulged A (A_{827}) marked with an asterisk (*). Residues in domains 5 and 6 are numbered according to their positions in the intact CPF-G2Im sequence.

regulation at the mRNA level and the possibility of deletion, RT-PCR amplification was performed. Using a combination of primers designed for CPF-G2Im and for the *cpa* ORF, there was no amplification of the cDNA (Fig. 5B). In contrast, primers designed from the sequences upstream and downstream of CPF-G2Im within *cpa* allowed the amplification of an ~0.9-kb fragment, which was of the expected size, similar to that of the wild-type strain, CPBC102. Using primers specific for the CPF-G2Im fragment, the expected PCR product size of about 0.6 kb was found for CPBC16ML but not for CPBC102 (Fig. 5B). Nucleotide sequencing of the RT-PCR products amplified with primers cpaF2 and cpalphatox1-R indicated that the 834-bp CPF-G2Im fragment was completely deleted from the transcript of its flanking *cpa* gene. Furthermore, using RNAs extracted from an *E. coli* M15 transformant harboring pTIC119 with the complete *cpa* gene of CPBC16ML, RT-PCR gave the same results as those described above (data not shown).

Secondary structure of CPF-G2Im. Although CPF-G2Im lacks a recognizable RT sequence, analysis of the predicted secondary structure of the CPF-G2Im sequence revealed the distinctive characteristics of group II introns, where six helical domains (D1 to D6) emerge around a central wheel (Fig. 6A). There is a consensus sequence of GUGCG at the 5′ end. At the 3′ end, there is a well-defined D5 domain with a 5′ GAAA tetraloop (Fig. 6B.1) and a D6 domain with a short hairpin loop structure with a bulged A residue, the branching point which acts as the nucleophile during the first step in the self-splicing of group II introns, located 8 nucleotides upstream from the 3′ end (Fig. 6B.2). With all of these important char-