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The CpAL Quorum Sensing System Regulates Production of Hemolysins CPA and PFO To Build *Clostridium perfringens* Biofilms

Jorge E. Vidal,[a] Joshua R. Shak,[a] Adrian Canizalez-Roman[b]

Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA[a]; University of Sinaloa School of Medicine, Culiacan, Sinaloa, Mexico[b]

Clostridium perfringens **strains produce severe diseases, including myonecrosis and enteritis necroticans, in humans and animals. Diseases are mediated by the production of potent toxins that often damage the site of infection, e.g., skin epithelium during myonecrosis. In planktonic cultures, the regulation of important toxins, such as CPA, CPB, and PFO, is controlled by the *C. perfringens* Agr-like (CpAL) quorum sensing (QS) system. Strains also encode a functional LuxS/AI-2 system. Although *C. perfringens* strains form biofilm-like structures, the regulation of biofilm formation is poorly understood. Therefore, our studies investigated the role of CpAL and LuxS/AI-2 QS systems and of QS-regulated factors in controlling the formation of biofilms. We first demonstrate that biofilm production by reference strains differs depending on the culture medium. Increased biomass correlated with the presence of extracellular DNA in the supernatant, which was released by lysis of a fraction of the biofilm population and planktonic cells. Whereas Δ*agrB* mutant strains were not able to produce biofilms, a Δ*luxS* mutant produced wild-type levels. The transcript levels of CpAL-regulated *cpa* and *pfoA* genes, but not *cpb*, were upregulated in biofilms compared to planktonic cultures. Accordingly, Δ*cpa* and Δ*pfoA* mutants, in type A (S13) or type C (CN3685) backgrounds, were unable to produce biofilms, whereas CN3685Δ*cpb* made wild-type levels. Biofilm formation was restored in complemented Δ*cpa*/*cpa* and Δ*pfoA*/*pfoA* strains. Confocal microscopy studies further detected CPA partially colocalizing with eDNA on the biofilm structure. Thus, CpAL regulates biofilm formation in *C. perfringens* by increasing levels of certain toxins required to build biofilms.**

The Gram-positive, spore-forming bacterium *Clostridium perfringens* is the most widely distributed pathogen in nature (1–3). This anaerobe affects humans and animals, producing severe diseases, including food poisoning (4), gastrointestinal syndromes and enterotoxemias (3, 5, 6), gas gangrene, and numerous histotoxic infections (7–9). With the exception of food poisoning caused by *C. perfringens* enterotoxin (CPE), a common characteristic in most toxigenic *C. perfringens* vegetative infections is the rapid progression of the disease to a final, often fatal, outcome. The virulence of *C. perfringens* is directly related to its prolific production of more than 17 different potent toxins. Current schemes, however, utilize differential production of four lethal typing toxins (alpha [CPA], beta [CPB], epsilon [Etx], and/or iota [Itx]) to classify *C. perfringens* isolates into five pathogenic types (A to E) (2, 10–13). *C. perfringens* type A, for example, must produce CPA, whereas type C strains produce CPA and CPB. All toxinotypes make other biomedically important toxins such as perfringolysin O (PFO) or CPB2 (10).

C. perfringens type A and type C strains have been isolated from human cases of severe disease (2). *C. perfringens* type A produces nearly ~90% of all gas gangrene cases (3, 9, 13). Gas gangrene, or myonecrosis, is considered one of the most fulminate infections caused by a Gram-positive organism in humans and animals (8, 9). Infection starts from the site of a recent surgical wound or trauma, where *C. perfringens* type A strains must first attach to the disrupted epidermal epithelium and proliferate while at the same time producing toxins that necrotize the tissue (8, 14–16). Tissue destruction associated with *C. perfringens* infection progresses rapidly to involve an entire extremity (8, 14, 17–19). Amputation remains the single best life-saving treatment, although mortality still remains high (8, 9, 15, 16).

The production of *C. perfringens* alpha toxin (CPA) and, to some extent, PFO has largely been implicated in clostridial myonecrosis (3, 9, 13, 20, 21). For example, a *C. perfringens* type A strain (*cpa*$^+$ *pfoA*$^+$) was highly virulent in a mouse model of clostridial myonecrosis, whereas isogenic *cpa* or *pfoA* mutants showed reduced virulence (including reduced tissue destruction) (17, 19, 22, 23). Elimination of both CPA and PFO production (a double *cpa-pfoA* toxin gene mutant) removed most of the histopathological features typical of clostridial myonecrosis (24).

C. perfringens type C is the etiologic agent of human enteritis necroticans (also called pigbel, darmbrand, or "gangrene of the bowel"), which originates in the intestine (2). Enteritis necroticans (EN) is particularly aggressive in diabetics and immunocompromised patients from developed countries (they survive less than 48 h after the first appearance of symptoms) (25–30). EN is characterized by its sudden onset with abdominal cramps, shock, bloody diarrhea, acute inflammation, and pronounced necrosis of intestinal mucosa (27, 31). Necrosis of the intestinal epithelium always coincides with the presence of Gram-positive rods characteristic of *C. perfringens* attached to the surface of the necrotic mucosa that, when visualized by microscopy, appear to form biofilm-like structures (28, 29). CPB, the most important toxin for developing EN (32–34), has been detected on the apical side of

Received 20 February 2015 **Accepted** 23 March 2015

Accepted manuscript posted online 30 March 2015

Citation Vidal JE, Shak JR, Canizalez-Roman A. 2015. The CpAL quorum sensing system regulates production of hemolysins CPA and PFO to build *Clostridium perfringens* biofilms. Infect Immun 83:2430–2442. doi:10.1128/IAI.00240-15.

Editor: S. R. Blanke

Address correspondence to Jorge E. Vidal, jvidalg@emory.edu.

doi:10.1128/IAI.00240-15

intestinal cells underneath those attached bacteria, indicating that *in situ* production of CPB is important for pathogenesis (29). A similar EN disease produced by type A and type C strains affects chickens and other domestic birds. Chicken EN globally costs the poultry industry $2 billion per year (35).

We recently demonstrated that *C. perfringens* strains encodes a functional *agr*-like (accessory gene regulator) quorum sensing (QS) system that controls production of some *C. perfringens* toxins (36, 37). The *C. perfringens* Agr-like (CpAL) system is related to the Agr system from *Staphylococcus aureus* and other Gram positives (36, 38–41). Signaling through CpAL requires a secreted pheromone encoded by the *agrD* gene, which is processed to a functional cyclic peptide by a transmembrane protein encoded by the *agr*B gene (36, 37). The CpAL system regulates *in vitro* production of CPA and PFO in all studied toxin types, including type A and type C strains (36, 42–44). CpAL also regulates production of *C. perfringens* beta toxin (CPB) in type B and type C strains (42, 43). Moreover, a functional CpAL system was required *in vivo* to produce necrotizing enteritis in rabbit ileal loops by regulating intestinal levels of CPB (42).

Another QS system encoded by genome-sequenced *C. perfringens* strains, the LuxS/AI-2 system, was implicated in transcriptional regulation of *pfoA*, but not *cpa* or other toxin genes, in mid-exponential-phase planktonic cultures of type A strain 13 (S13) (45). Although studied *in vivo* and *in vitro*, LuxS-AI-2 regulation has not been identified in *pfoA*-encoded *C. perfringens* type C strain CN385 (42, 46).

As mentioned earlier, *C. perfringens* strains may form biofilm-like structures on host tissues or epithelia. Biofilms are bacterial communities surrounded by a matrix providing the mechanical stability, mediating their adhesion to surfaces, and forming a cohesive, three-dimensional polymer network that protects them from the host response and antibiotics (47). Biofilm production in *C. perfringens* has been poorly studied. Initial attempts to characterize *C. perfringens* biofilms demonstrated that biofilms are produced *in vitro* by some strains (48, 49). The matrix has been recently studied and found to be made of some proteins, β-1,4-linked polysaccharides and extracellular DNA (eDNA) (49, 50). A type IV pilus (TFP) is also required for biofilm formation; these pili were located on the biofilm matrix (48).

More recently, Obana et al. reported in 2014 that biofilms formed at 37°C have an increased biomass and a phenotypically different biofilm matrix in comparison to those formed at 25°C. Furthermore, incubation at 37°C, but not at 25°C, upregulated levels of *pilA2* mRNA, which encodes the main pilin subunit of the matrix-associated *C. perfringens* TFP (50). These data suggest that *C. perfringens* biofilms are differentially regulated. In the present study, we investigated QS regulation of *C. perfringens* biofilms and demonstrated that the CpAL, but not the LuxS/AI-2 system, is required for proper formation of the biofilm structure. Moreover, CpAL-regulated CPA and PFO toxins, but not CPB, were required for biofilm formation, and transcription of the genes *cpa* and *pfoA*, but not *cpb*, was upregulated in biofilms compared to planktonic cultures.

MATERIALS AND METHODS

Strains, culture media, and chemicals. Wild-type strains and mutant derivatives utilized in the present study are listed in Table 1. Media for culturing *C. perfringens* included FTG (fluid thioglycolate medium; Difco Laboratories), TGY (3% tryptic soy broth [Becton Dickinson]; 2% glucose [Fisher Scientific]; 1% yeast extract [Becton Dickinson], 0.1% sodium thioglycolate [Sigma-Aldrich]), brain heart infusion (BHI) agar (Becton Dickinson). Sodium thioglycolate, a reducing agent, present in TGY maintains a low oxygen tension in the medium. Blood agar plates were prepared with BHI and 5% defibrinated sheep blood (Remel, Lenexa, KS); egg yolk agar plates were prepared with BHI containing 4% (vol/vol) egg yolk. For culturing *Escherichia coli*, Luria-Bertani (LB) broth (1% tryptone [Becton Dickinson], 0.5% yeast extract, 1% NaCl) and LB agar (1.5% agar [Becton Dickinson]) medium were used. All antibiotics used in the present study were purchased from the Sigma-Aldrich Chemical Company.

TABLE 1 Strains and plasmids utilized in this study

Strain or plasmid	Description	Source or reference
Strains		
CN3685	Type C strain isolated from peritoneal fluid of a sheep with struck	33, 34
JGS1495	Type C strain isolated from a piglet with necrotic enteritis	60
Strain 13	Type A, gangrene strain	75
JGS1075	Type C strain isolated from porcine enteritis	76
JGS1076	Type C strain isolated from porcine enteritis	76
JGS1659	Type C strain isolated from porcine enteritis	76
CPJV20	CN3685Δ*luxS*	This study
CPJV21	CN3685Δ*agrB*	This study
CPJV22	CN3685Δ*plc*	This study
CPJV23	CN3685Δ*pfoA*	This study
CPJV25	CN3685Δ*cpb*	This study
CPJV26	S13Δ*agrB*	This study
CPJV27	S13Δ*plc*	This study
CPJV28	S13Δ*pfoA*	This study
CPJV30	CPJV27 carrying pCP13plc	This study
CPJV31	CPJV28 carrying pCP13pfoA	This study
CPJV32	CPJV21 carrying pCPJVagrB	This study
Plasmids		
pJIR750	*E. coli-C. perfringens* shuttle vector carrying a chloramphenicol-resistant gene, *catP*	54
pJIR751	*E. coli-C. perfringens* shuttle vector carrying an erythromycin-resistant gene, *ermB*	54
pJIR750ai	pJIR750 with *plc* targeted intron in antisense orientation	52
pCPJVlux	pJIR750ai with *luxS* targeted intron in an antisense orientation	This study
pCPJVagrB	pJIR750ai with *agrB* targeted intron in a sense orientation	This study
pCPJVpfoA	pJIR750ai with *pfoA* targeted intron in an antisense orientation	This study
pCPJVcpb	pJIR750ai with *cpb* targeted intron in an antisense orientation	This study
pCP13plc	pJIR751 carrying the *plc* gene from strain 13	This study
pCP13pfoA	pJIR750 carrying the *pfoA* gene from strain 13	This study

Production of *C. perfringens* biofilms. Strains were inoculated into FTG and grown overnight at 37°C. To produce biofilms, these FTG cultures were inoculated (diluted 1:10) in triplicate into either 8-well glass slides (Lab-Tek), polystyrene 96-well plates (Corning), CellBIND surface polystyrene 24-well plates (Corning), or tissue culture-treated polystyrene six-well plate (Corning) containing FTG or TGY with no antibiotics, followed by incubation at 37°C for the indicated time.

Quantification of biofilm biomass. Biofilm development was evaluated using the classic crystal violet staining method as previously reported (51). Alternatively, biofilms were stained by fluorescence with mem-

TABLE 2 Primers utilized in this study

Primer[a]	Sequence (5′–3′)	Source or reference
cpb-Fwd	GCGAATATGCTGAATCATCTA	77
cpb-Rev	GCAGGAACATTAGTATATCTTC	
JEV135L	TAAAAACCGAAAAGAAGAAAAA	This study
JEV126R	TATGTAGGTTAGAGTCATACA	
JEV165L	TTGAATTCCCCAGTTATTCACGATTAAAG	This study
JEV166R	TTGGATCCTTAATTGTAAGTAATACTAGATCCA	
JEV167L	TTGAATTCAGGTTAAAACCTGTTTTTGATTGA	This study
JEV168R	TTTCTAGATTTTGTAAATACCACCAAAACCAAT	
JEV181L	ACCTCCACTTATGGTTTCAAATGT	This study
JEV182R	TTGTTATGTTCTTGTGCATCTCCT	
JEV193L	CTAGCATGAGTCATAGTTGGGATG	This study
JEV194R	ACATGTAGTCATCTGTTCCAGCAT	
polCJVL	AATATATGATACTGAAGAGAGAGTAA	46
polCJVR	TCTAAATTATCTAAATCTATGTCTACT	
luxS-L	CCTAAAGGAGATATAGTTTCAAAG	46
luxS-R	TCACTAGAGTTAGCTAAGAGTCAT	
KO-IBS*	AAAAAAGCTTATAATTATCCTTAATTGCCATACTTGTGCGCCCAGATAGGGTG	34
KO-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATACTTGCTAACTTACCTTTCTTTGT	
KO-EBS2	TGAACGCAAGTTTCTAATTTCGATTGCAATTCGATAGAGGAAAGTGTCT	
IBS-pfoA**	AAAAAAGCTTATAATTATCCTTAATTGCCATACTTGTGCGCCCAGATAGGGTG	52
EBS1d-pfoA	CAGATTGTACAAATGTGGTGATAACAGATAAGTCATACTTGCTAACTTACCTTTCTTTGT	
EBS2-pfoA	TGAACGCAAGTTTCTAATTTCGATTGCAATTCGATAGAGGAAAGTGTCT	
566/567s-IBS†	AAAAAAGCTTATAATTATCCTTATTCCACACTGAAGTGCGCC-CAGATAGGGTG	53
566/567s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCACTGAATATAACTTACCTTTCTTTGT	
566/567s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTTGGAATCGATAGAGGAAAGTGTCT	
luxS-IBS‡	AAAAAAGCTTATAATTATCCTTATACTCCAAAGCTGTGCGCCCAGATAGGGTG	46
luxS-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAAAGCTTCTAACTTACCTTTCTTTGT	
luxS-EBS2	TGAACGCAAGTTTCTAATTTCGGTTGAGTATCGATAGAGGAAAGTGTCT	

[a] Some primers were used to prepare a *cpb* mutant (*), a *pfoA* mutant (**), an *agrB* mutant (†), or an *lux*S mutant (‡), as indicated.

brane-permeant fluorescent reagents SYTO9 (Molecular Probes) or DAPI (4′,6′-diamidino-2-phenylindole). These molecules stain nucleic acids. Briefly, biofilms were washed three times with sterile phosphate-buffered saline (PBS; pH 7.4) and incubated with 5 mM SYTO9 for 20 min at room temperature or with DAPI (30 nM) and incubated for 5 min. After two washes with PBS, fluorescence (arbitrary units) was quantified using a VICTOR X3 multilabel plate reader (Perkin-Elmer). Biofilms were photographed using an inverted Evos fluorescence microscope (Advanced Microscopy Group). Fluorescence arbitrary units of a chosen experimental condition (e.g., wild-type [wt] biofilm 8 h postinoculation) were set to 100% of biofilm biomass and used to calculate the percentage of biofilm biomass of all other tested conditions.

Quantification of eDNA. Strains were inoculated into 24-well plates and incubated for the indicated time at 37°C. The supernatant (~1 ml) was removed, centrifuged for 5 min at 12,000 × *g* in a refrigerated centrifuged (Eppendorf) and filter sterilized using a 0.45-μm-pore-size syringe filter (Fisher Scientific). This supernatant was mixed with 0.5 volume of ethanol and vortexed for 10 s, and then extracellular DNA (eDNA) was purified using the QIAamp eDNA minikit (Qiagen) according to the manufacturer's instructions. To quantify the amounts of DNA, a quantitative PCR (qPCR) assay targeting the *cpa* gene was utilized. Reactions were performed with IQ SYBR green super mix (Bio-Rad), 300 nM concentrations of each primer (JEV193L and JEV194R [Table 2]) and 2 μl of DNA template. Reactions were run in duplicate using a CFX96 real-time PCR detection system (Bio-Rad) under the following conditions: 1 cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 55°C for 1 min, and 72°C for 1 min. Melting curves were generated by a cycle of 95°C for 1 min and 65°C for 1 min, followed by 80 cycles starting at 65°C with 0.5°C increments. For quantification purposes, standards (1, 10, 100, and 1,000 pg of purified chromosomal DNA per reaction) were run in parallel to generate a standard curve. Amounts of eDNA in pg/ml were calculated by using the software Bio-Rad CFX Manager.

Viability of biofilms and planktonic cells using the Live/Dead assay. *C. perfringens* type A strain 13 or type C strain CN3685 was inoculated in 96-well plates containing TGY, followed by incubation for 4 or 8 h at 37°C. Biofilms made 4 or 8 h postinoculation were washed three times with sterile PBS and immediately stained utilizing a Live/Dead BacLight bacterial viability kit (L7012; Invitrogen, Grand Island, NY). The assay has successfully been used to investigate biofilms made by *Clostridium difficile* and *C. perfringens* (48, 57). Planktonic cells were removed at the indicated time and stained as described above. The concentration of dyes was utilized according to the manufacturer's recommendations. Stained biofilms, or planktonic cells, were washed additionally two times with PBS, and fluorescence measurements (arbitrary units) were obtained with a Victor X3 multilabel plate reader (Perkin-Elmer). Fluorescent dyes included in the kit are incorporated, or not, into bacterial cells as a function of membrane integrity and therefore viability. Preparations were also observed and photographed utilizing an inverted Evos fluorescence microscope (Advanced Microscopy Group).

Construction of CN3685 and strain 13 null mutants using TargeTron technology. The following genes were insertionally inactivated using a *Clostridium* TargeTron gene knockout system (Sigma-Aldrich): *cpb* (34), *pfoA* (34), *cpa* (also known as *plc*) (52), *luxS* (46), and *agrB* (53). The *cpa* mutant was prepared with plasmid pJIR750ai, included in the TargeTron gene knockout system. Derivative plasmids to insertionally inactivate all others were prepared by cloning target-specific sequences as follows: a set of primers (IBS, EBS1d, and EBS2) that had been previously published (Table 2) were utilized to generate a target-specific ~350-bp PCR product; this target-specific fragment replaced the *cpa*-specific fragment on pJIR750ai. An intron plasmid which integrated an ~900-bp intron fragment into the target gene was then electroporated into either strain 13 or CN3685. Except for the mutation into the *agrB* gene, in which the intron was integrated in the sense orientation, the intron fragment was inserted in the antisense orientation in all other target genes. Transformants

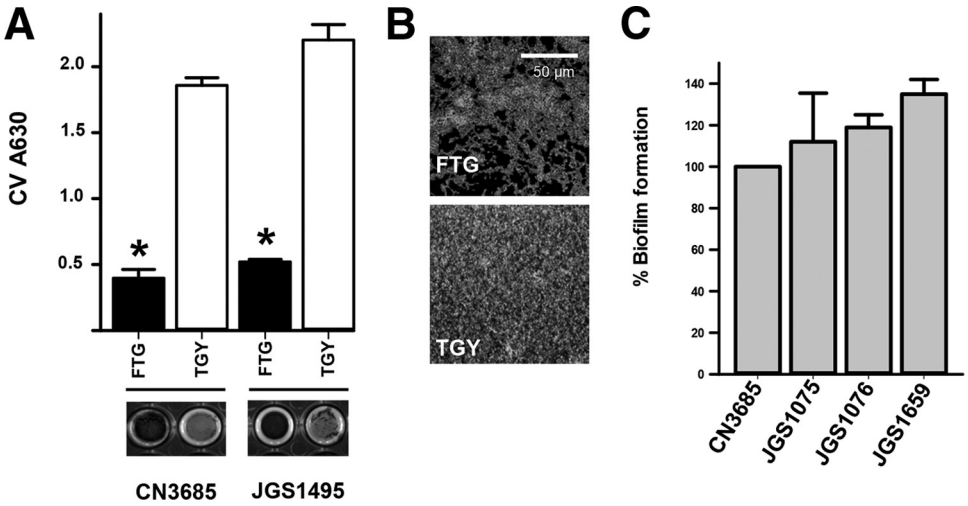

FIG 1 *C. perfringens* biofilms are differentially produced depending on the bacterial culture medium. (A) Type C strain CN3685, or JGS1495, was inoculated into either FTG or TGY, and biofilms were incubated for 24 h. A set of wells containing biofilms were washed and photographed (bottom panel), and then the crystal violet absorbance was obtained. Asterisks indicate statistical significance ($P < 0.05$), as evaluated by a Mann-Whitney U test. (B) Another set of wells was stained with DAPI, and fluorescence micrographs were obtained. Experiments were repeated at least three times. (C) *C. perfringens* strains were inoculated in TGY medium and incubated for 24 h. Biofilms were stained with crystal violet, and the A_{630} was determined. Arbitrary units obtained from CN3685 were set to 100% biofilms, and those made by the other strains were calculated. Error bars represent the standard errors of the mean calculated using data from at least three independent experiments.

were selected on BHI agar plates containing 15 μg of chloramphenicol (Cm)/ml and then PCR screened for an intron-disrupted gene using the following target-specific primers: *cpb* (cpb-Fwd and cpb-Rev), *cpa* (JEV193L and JEV194R), *pfoA* (JEV181L and JEV182R), *agrB* (JEV135L and 126R), and *luxS* (luxS-L and luxS-R). Our PCR screening was designed as to amplify an ~200- to 300-bp product from wild-type genes but amplified a larger approximately 1.1- to 1.2-kbp product from those mutants. Mutants carrying an intron insertion were grown in FTG medium without Cm for ~10 days, with daily subculturing, to cure the intron-carrying donor plasmid. Curing was shown by lack of growth on Cm-containing BHI plates.

Preparation of *cpa* and *pfoA* complemented strains. Plasmids pJIR750 (ATCC 87015) and pJIR751 (ATCC 87016) (54) were purified and doubly digested with restriction enzymes, EcoRI and BamHI or EcoRI and XbaI, respectively. Digested plasmids were again purified using a QIAquick gel extraction kit (Qiagen). Simultaneously, the *pfoA* or *plc* gene, including promoter regions, was amplified by PCR using the primers JEV165L and JEV166R (*pfoA*) or the primers JEV167L and JEV168R (*plc*), purified and doubly digested with enzymes EcoRI and BamHI or EcoRI and XbaI, respectively. Digested fragments were ligated to pJIR750-*pfoA* (pCP13-*pfoA*) or pJIR751-*plc* (pCP13plc) using T4 DNA ligase (Promega) and transformed into competent cells of S13Δ*pfoA* (pJIR750-*pfoA*) or S13Δ*plc* (pJIR751-*plc*) by electroporation as previously described (36). Transformants were plated onto BHI plates containing Cm or erythromycin, respectively, and isolated colonies were screened for the presence of both the intron-disrupted mutant gene and plasmid-encoded wt genes as mentioned above.

Preparation of an *agrB* complemented strain. Taking advantage of the fact that the insertion of the intron within *agrB* created a conditional mutation (i.e., the intron fragment was integrated in the sense strand), complementation of the *agrB*-null mutant CPJV21 was achieved at the mRNA level by removing the intron from disrupted *agrB* mRNA, thus restoring a functional *agrB* transcript (55). Removal of this intron was achieved by reintroducing pCPJVagrB, which encodes the LtrA protein required to splice out the intron insertion, into CPJV21. Transformants were selected on BHI agar containing 15 μg of chloramphenicol/ml. Strain CPJV32 was grown at 30°C to allow maximum expression of the LtrA protein.

RNA extraction and analysis of RNA preparations. Planktonic or biofilm cells were added with 1 volume of RNAprotect Bacteria (Qiagen) and immediately centrifuged for 15 min at 15,000 × *g* in a refrigerated centrifuge (Eppendorf). Total RNA was then extracted from the pellet by using an RNeasy minikit (Qiagen) as outlined by the manufacturer and additionally treated with 2 U of DNase I (Promega) essentially as previously described (46, 51). The integrity of our RNA preparations (i.e., the RNA integrity number [RIN]) and RNA concentration of samples were obtained by using an RNA 6000 Nano kit and a 2100 Bioanalyzer (Agilent Technologies).

RT-PCRs. Reactions were performed with 100 ng of DNase I-treated RNA as the template. Those reaction mixtures contained 500 nM concentrations of each primer (Table 2), 1× PCR master mix (New England BioLabs), molecular-grade water, and 10 U of avian myeloblastosis virus retrotranscriptase (Promega). Control reverse transcription-PCRs (RT-PCRs) were similarly performed, except for the omission of reverse transcriptase (negative) or the addition of 100 ng of genomic DNA (positive). Reactions conditions were as follows: an initial incubation at 42°C for 30 min and denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 15 s, 55°C for 30 s, and 68°C for 1 min and then a final extension at 68°C for 10 min. RT-PCR products were run in 2% agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen).

Quantitative RT-PCR (qRT-PCR). Reactions were performed with an iScript one-step RT-PCR kit with SYBR green (Bio-Rad), 300 nM concentrations of the indicated primers (Table 2), and 10 ng of DNase I-treated RNA template. Reactions were run in duplicate using a CFX96 real-time PCR detection system (Bio-Rad) and the following conditions: 1 cycle at 50°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 and 55°C for 1 min. Melting curves were generated as described above. The relative quantitation of mRNA expression was normalized to the constitutive expression of the housekeeping *polC* gene and calculated by the comparative CT ($2^{-\Delta\Delta CT}$) method (56).

Hb release assay. *C. perfringens* strains were grown in 24-well plates containing TGY for 4 h; maximal expression of PFO, and therefore hemoglobin (Hb) release, has been observed at this time point (36). Culture supernatant was obtained by centrifugation at 20,000 × *g* for 10 min and then filter sterilized using a 0.45-μm-pore-size filter (Fisher Scientific). The Hb release assay was then performed essentially as previously de-

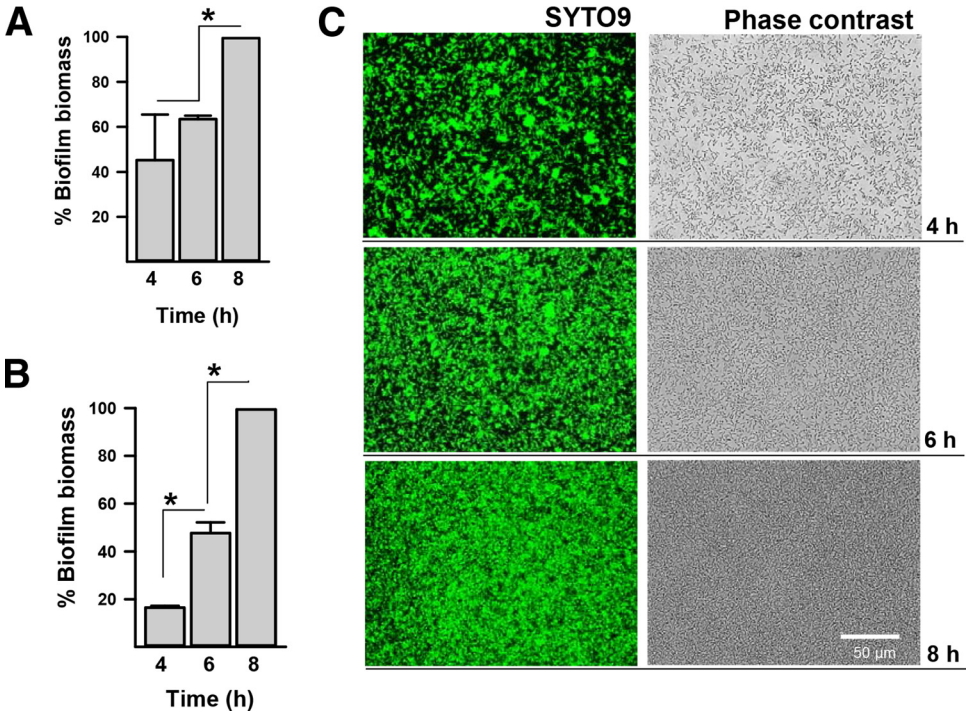

FIG 2 Fluorescence-based method to quantify *C. perfringens* biofilms. Strain CN3685 was inoculated in 96-well plates containing THY and incubated at 37°C for the indicated time. Biofilms were washed with PBS and then stained with crystal violet, after which the absorbance (A_{630}) was obtained (A), or fluorescently stained with SYTO9, and fluorescence arbitrary units were obtained with a fluorometer (B). The biofilm biomass (%) was calculated in panels A and B by setting 100% biomass units obtained for the 8-h time point to calculate the biomass (%) of the other two. *, Statistical significance ($P < 0.05$), as evaluated by the Mann-Whitney U test. Error bars represent the standard errors of the mean calculated using data from three independent experiments. (C) Biofilms stained in panel B were photographed using an inverted microscope and either the EVOS light-cube green fluorescent protein 470/510 (SYTO9) or phase contrast. The scale bar at the bottom right applies to all six panels.

scribed (46). Photographs of erythrocyte pellets in Δ*pfoA* mutants were also obtained.

Detection of CPA by dot blot. Strains were grown in 24-well plates containing TGY for 8 h at 37°C, after which the culture was centrifuged at 20,000 × *g* for 10 min. An aliquot (50 μl) of the supernatant was spotted onto a nitrocellulose membrane, air dried, and then blocked with PBS-Tween 20 (0.05% [vol/vol]) and nonfat dry milk (5% [wt/vol]) for 1 h at room temperature. Membranes were probed with a polyclonal anti-CPA antibody (Bioss Antibodies, Woburn, MA) followed by incubation with horseradish peroxidase-conjugated secondary anti-rabbit antibodies (Jackson ImmunoResearch) and the addition of SuperSignal West Pico chemiluminescent substrate (Pierce).

Confocal microscopy studies. *C. perfringens* strains were inoculated in 8-well glass slides (Lab-Tek) containing TGY and incubated for 24 h at 37°C. Biofilms produced were washed twice with PBS and fixed with 2% paraformaldehyde for 15 min at room temperature. Fixed biofilms were then permeabilized with 0.5% Triton X-100 for 5 min, washed again with PBS, and blocked with 1% bovine serum albumin for 30 min at 37°C. Nucleic acids were stained with SYTO9 as described above. CPA was stained with a rabbit polyclonal anti-CPA antibody, followed by a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 555 (Molecular Probes). Stained biofilms were finally washed three times with PBS, mounted with Vectashield mounting medium (Vector Laboratories), and analyzed with an Olympus FV1000 confocal microscope. Confocal images were analyzed with ImageJ (v1.49k; National Institutes of Health, Bethesda, MD).

Statistical analysis. All statistical analyses presented in the present study were performed using the Mann-Whitney U test and the software SigmaPlot (v12.0; Systat Software, Inc.).

RESULTS

Production of *C. perfringens* biofilms is regulated by environmental signals. Environmental signals, such as those present in bacterial culture media, have been associated with biofilm production in some Gram-positive bacteria (51, 57, 58). We therefore investigated whether the culture medium would impact the formation of *C. perfringens* biofilms. To assess this, we used two classic *C. perfringens* broth media, FTG and TGY (59). As shown in Fig. 1A, we found that the biofilm mass produced in TGY by two different *C. perfringens* type C reference strains, CN3685 (33, 34) and JGS1495 (60), was significantly greater than that produced on FTG. Biofilms produced on TGY were observed in the wells as a thick white pellicle (Fig. 1A, bottom panels). Compared to biofilms made on FTG that covered ~70% of the substrate, biofilms produced on TGY covered the entire surface (Fig. 1B). Similar results were obtained when *C. perfringens* type A strain 13 was inoculated in FTG and TGY (data not shown). We next investigated the biofilm phenotype of other *C. perfringens* type C strains isolated from porcine enteritis (Table 1). All tested strains produced similar biofilm biomass on a microtiter plate at 24 h post-inoculation (Fig. 1C). Together, our results suggest that *C. perfringens* biofilms are regulated by environmental signals.

Development of a fluorescent assay to study *C. perfringens* biofilms. To improve current methods to study *C. perfringens* biofilms, we developed a fluorescence-based method using DAPI or SYTO9. These fluorophores have different excitation and emis-

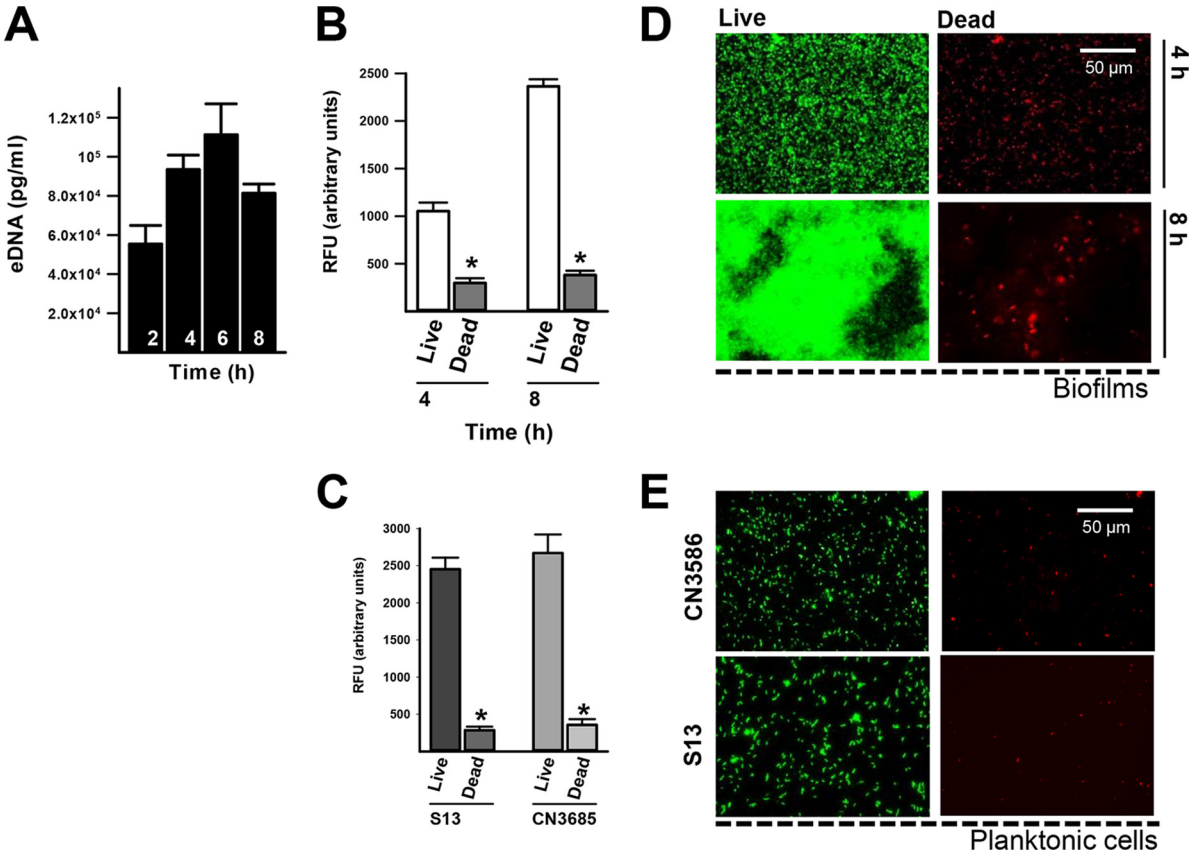

FIG 3 eDNA is released by bacterial lysis into the supernatant of growing *C. perfringens* biofilms. Strain CN3685 was inoculated in 24-well plates containing TGY, followed by incubation at 37°C for the indicated times, after which 1 ml of supernatant was removed. (A) eDNA was purified and quantified utilizing qPCR. In another set of experiments, 4- or 8-h biofilms (B) or planktonic cells (C) were stained using a Live/Dead BacLight kit, and fluorescence arbitrary units were obtained using a fluorometer. *, Statistical significance ($P < 0.05$), evaluated by the Mann-Whitney U test, compared to live bacteria. Biofilms (D) or 8-h planktonic cultures (E) were also photographed with an Evos inverted microscope using the light-cube green fluorescent protein 470/510 (live) or red fluorescent protein 531/593 (dead). The scale bar at the upper right applies to all panels.

sion spectra and are readily available. *C. perfringens* biofilms stained with these fluorescence reporters were compared to the biofilm mass obtained using the classic crystal violet procedure. Fluorescence arbitrary units of biofilms, produced 8 h postinoculation, were set to 100%, and the biomass of all other time points was calculated. The classic protocol, utilizing crystal violet, revealed similar biomass 4 and 6 h postinoculation and a statistically different mass 8 h postinoculation (Fig. 2A). The fluorescence method revealed a statistically different biomass that increased at each time point (Fig. 2B). The latter correlated with microscopic observations of *C. perfringens* biofilms (Fig. 2C). Similar results were obtained when biofilms were stained with DAPI (data not shown).

***C. perfringens* releases eDNA into the culture medium.** The biofilm matrix of other pathogens, such as *Streptococcus pneumoniae* (61, 62) or *Enterococcus faecalis* (63), is partially composed of DNA that has been excreted into the medium (i.e., eDNA). It has been recently reported that eDNA is present in the *C. perfringens* biofilm matrix (49, 50). To assess whether *C. perfringens* cells release DNA to the extracellular milieu, DNA was purified from supernatants of cultures of *C. perfringens* type C strain CN3685. Released eDNA was quantified using qPCRs targeting the chromosomally encoded *cpa* gene (13). The presence of eDNA was detected as early as 2 h postinoculation (5.5×10^4 pg/ml), reaching a maximum peak at 6 h postinoculation (1.1×10^5 pg/ml) with a non-statistically significant decrease in eDNA in the supernatant after 8 h of incubation (Fig. 3A). Increasing presence of eDNA in the supernatant is consistent with the increased biomass formed over time (see Fig. 2). Since the release of eDNA in other species has been linked in part to autolysis, we sought to investigate the viability of *C. perfringens* biofilms and planktonic cells. Our experiments showed that ~25% of the biofilm bacteria appeared to be dead, as scored using the Live/Dead stain, at 4 h after inoculation of strain CN3685 (Fig. 3B) or strain 13 (data not shown). Likewise, ~16% of the biofilm population at 8 h postinoculation consisted of dead bacteria (Fig. 3B and D). Similar populations of dead planktonic cells were observed in 8-h-old cultures of strain 13 or CN3685 (Fig. 3C and E). Our data suggest that eDNA is in part provided by lysis of both planktonic and biofilm bacteria.

The CpAL QS system, but not the LuxS/AI-2 system, regulates *C. perfringens* biofilms. *C. perfringens* strains produce two different quorum sensing (QS) systems, an Agr-like QS system and the LuxS/AI-2 system (36, 37, 45). To investigate whether biofilms are regulated by QS, we prepared CN3685 derivatives CN3685Δ*agrB* and CN3685Δ*luxS* using the TargeTron

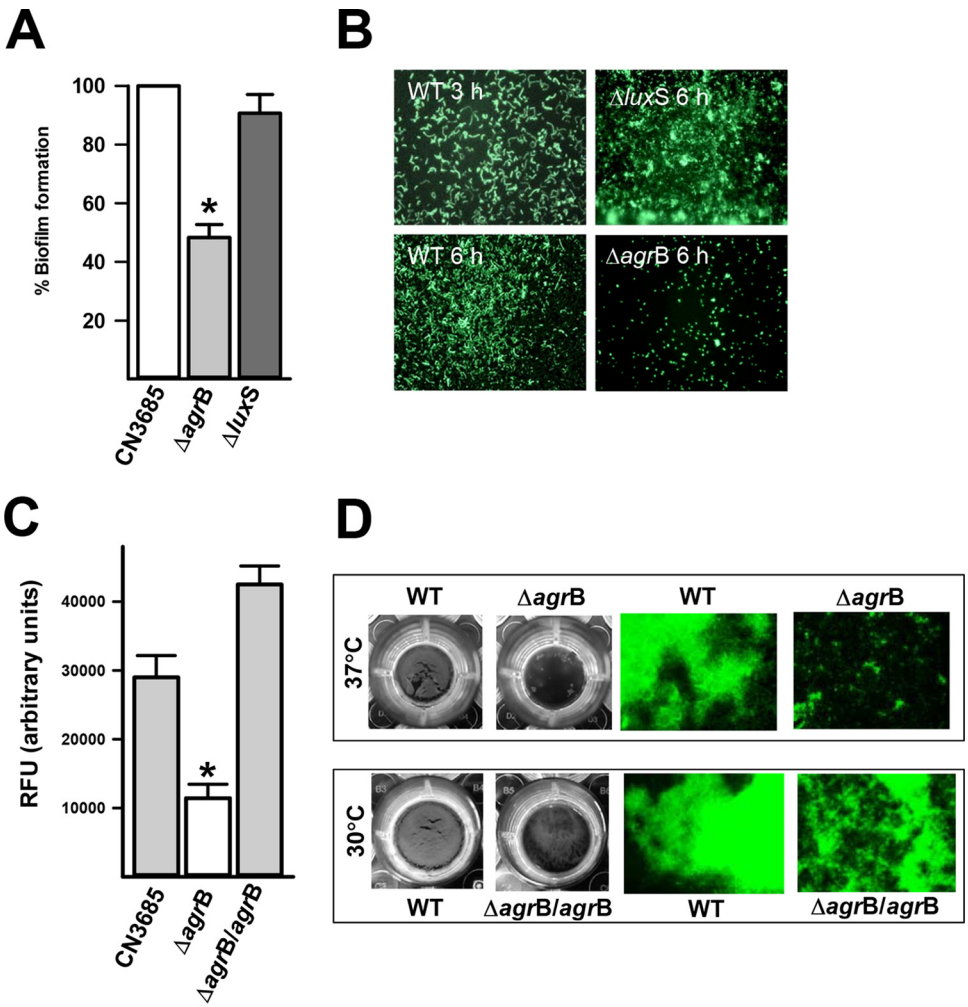

FIG 4 CpAL control of *C. perfringens* biofilms. Strain CN3685 or mutant derivatives were inoculated in 96-well plates containing THY, and biofilms were incubated for 3, 6, or 24 h at 37°C. (A) Biofilms (24 h) were stained with SYTO9, and their biomass was obtained using a fluorometer. (B) Micrographs of fluorescence-stained biofilms at 3 or 6 h postinoculation were obtained using an Evos inverted microscope. In another set of experiments, strain CN3685 or CPJV21 (Δ*agrB*) was incubated at 37°C, whereas strain CN3685 or CPJV32 (Δ*agrB/agrB*) was incubated at 30°C. (C) SYTO9-stained biofilms were quantified by fluorescence. *, Statistical significant ($P < 0.05$), as evaluated by the Mann-Whitney U test. (D) The macroscopic aspect of these strains was photographed, and micrographs were also obtained with a fluorescence microscope. Where shown, error bars indicate the standard errors of the mean calculated using data from three independent experiments.

technology. While the mutant CN3685Δ*luxS* strain produced biofilm levels similar to those of the wt strain, the mutant strain CN3685Δ*agrB* produced significantly less biofilm mass by 24 h (Fig. 4A) or 48 h (data not shown) postinoculation. A time course study revealed that *C. perfringens* wt bacteria had already attached to the substrate at 3 h postinoculation (early biofilms) and covered the entire surface by 6 h later (Fig. 4B). Similarly, the *luxS* mutant produced wt biofilm levels 6 h postinoculation, whereas biofilms of the Δ*agrB* mutant, at the same time point, were not observed (Fig. 4B). Similar results were obtained when the *agrB* was insertionally inactivated in the gangrene-producing strain 13 (data not shown).

To confirm the role of the CpAL system in controlling *C. perfringens* biofilm formation, the mobile group II intron that had been inserted in the sense orientation (i.e., relative to the direction of transcription) was removed by trans-splicing from the intron-disrupted *agrB* gene. To remove the intron, the plasmid pCPJVagrB was reintroduced into CN3685Δ*agrB* to create CPJV32. This strain, CPJV32, was then grown at 30°C to produce maximal expression of the temperature-sensitive LtrA protein which is required for the splicing-induced intron removal (34, 55). Incubation at 30°C allowed strain CPJV32 to produce a significant increase of the biomass, as quantified by fluorescence (Fig. 4C). CPJV32 biofilms were observed as a thick biofilm biomass on the bottom of the wells that covers most of the substrate (Fig. 4D). These experiments confirmed the CpAL system regulates biofilm formation of *C. perfringens* type C strain CN3685.

Upregulated expression of *cpa* and *pfoA* in *C. perfringens* biofilm cells. The CpAL system controls mRNA levels of genes encoding important toxins such as *cpb*, *cpa*, and *pfoA* in planktonic cultures of type A, type B, and type C strains (36, 42, 43). To evaluate biofilm levels of these transcripts, total RNA was extracted from CN3685 biofilms and planktonic cultures and mRNA expression was assessed by qRT-PCR. Of evaluated genes,

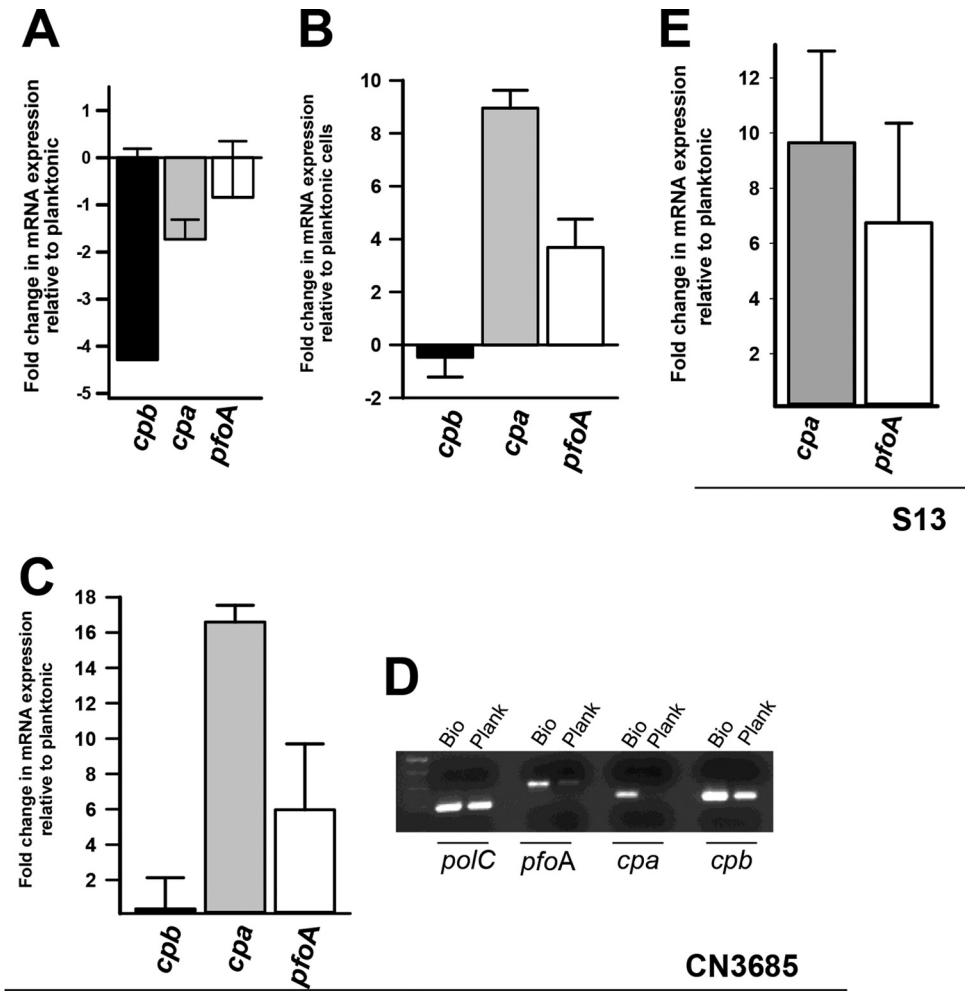

FIG 5 Upregulated transcription of *cpa* and *pfoA*, but not *cpb*, in *C. perfringens* biofilms versus planktonic cells. *C. perfringens* type C CN3685 or type A strain 13, was inoculated in TGY, followed by incubation at 37°C for 4 or 24 h. Planktonic cells and biofilms were harvested, RNA extracted, and used as the template in qRT-PCRs targeting the *cpb*, *cpa*, or *pfoA* gene. Average C_T values were normalized to the *polC* gene, and the fold differences were calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$). Graphs show the fold changes in mRNA expression of 4-h biofilms relative to planktonic cells (A), 24-h biofilms relative to 4-h planktonic cells (B), and 24-h biofilms relative to 24-h planktonic cells (C and E). (D) RNA from 24-h biofilms (Bio) or planktonic (Plank) was utilized in conventional RT-PCRs targeting the indicated gene. Error bars represent the standard errors of the mean calculated using data from at least three independent experiments.

the *cbp* gene, encoding *C. perfringens* beta toxin (61), is maximally expressed in planktonic cultures during the early-mid-log phase of growth (60). In comparison to planktonic cultures, the population of biofilm bacteria 4 h postinoculation had downregulated transcription of the *cbp* gene (~4-fold decrease) but mRNA levels of *cpa* and *pfoA* remained similarly expressed (Fig. 5A). Expression of the *cpa* or *pfoA* transcript was ~9-fold or ~4-fold upregulated in 24-h biofilms, respectively, relative to planktonic cells grown for 4 h (Fig. 5B), whereas *cpb* expression did not change. Moreover, mRNA levels of *cpa* or *pfoA* in biofilm cells increased ~17- or ~6-fold compared to planktonic cells by 24 h postinoculation, as quantified by qRT-PCR (Fig. 5C) or detected by conventional RT-PCR (Fig. 5D).

Relative to 4-h biofilms, however, the levels of *cpa* mRNA gradually increased 8, 16, or 24 h postinoculation, whereas *pfoA* mRNA levels were downregulated in 8-h biofilms, after which its expression remained the same (Fig. 6A). The levels of the *cpb* message did not change in biofilms incubated for 8, 16, or 24 h compared to 4-h biofilms (Fig. 6A). These results indicate that mRNA levels of both *cpa* and *pfoA* are increased in biofilms compared to planktonic cells, at the time points tested here, but that only mRNA levels of *cpa* are increasingly produced as the biofilm structure matures.

To further confirm the upregulated transcription of *cpa* and *pfoA* in biofilm cells, the mRNA levels of those genes were evaluated in total RNA extracted from the gangrene producer, type A, strain 13. As shown in Fig. 5E, mRNA expression for *cpa* and *pfoA* increased ~9- and ~6-fold in 24-h biofilm cells relative to planktonic cells, respectively. Similar expression of *cpa* and *pfoA* transcripts to that presented above for strain CN3685 was observed when expression in 8-, 16-, or 24-h biofilms was compared to the expression in biofilms produced 4 h postinoculation (Fig. 6B).

CPA and PFO are required to produce biofilms. The upregulated production of toxin genes in biofilms and recent findings demonstrating that the CpAL system regulates production of *C. perfringens* alpha toxin (CPA), CPB and perfringolysin O (PFO)

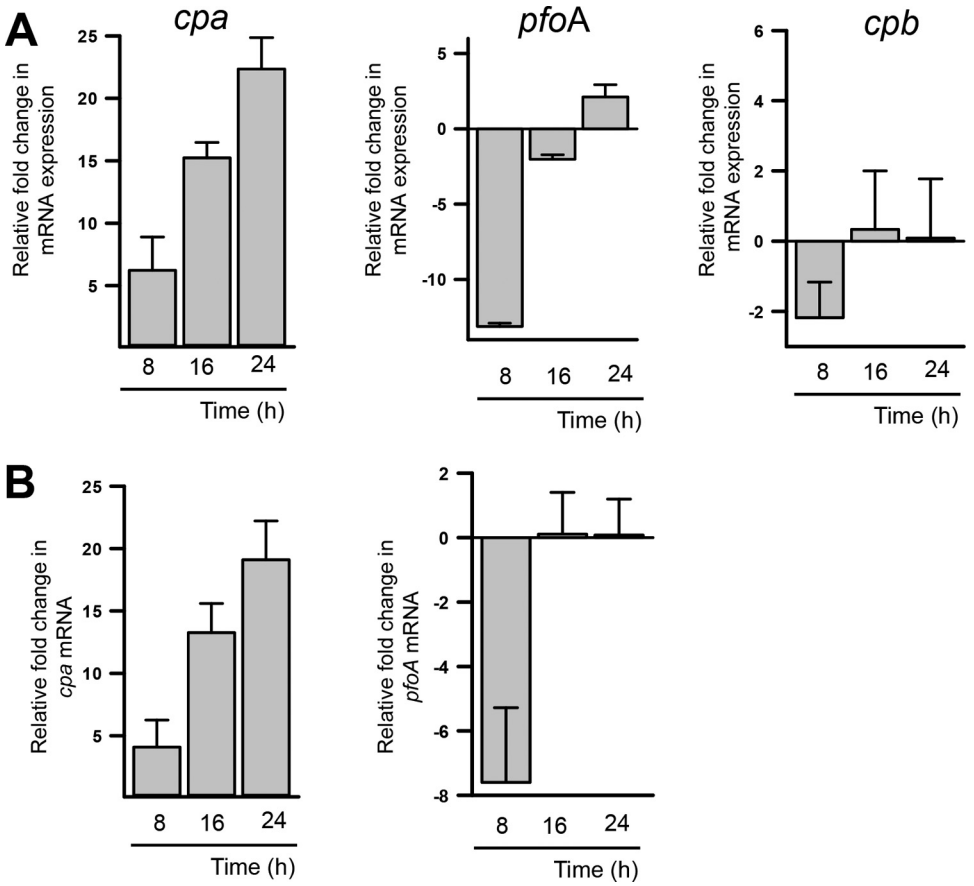

FIG 6 Upregulated transcription of *cpa* in *C. perfringens* biofilms. *C. perfringens* type C CN3685 (A) or type A strain 13 (B) was inoculated into TGY, followed by incubation at 37°C for 4 or 24 h. Biofilms were harvested, and RNA was extracted and used as the template in qRT-PCRs targeting the *cpb*, *cpa*, or *pfoA* gene. Average C_T values were normalized to the *polC* gene, and the fold differences were calculated using the comparative C_T method ($2^{-\Delta\Delta CT}$). The panels show the fold changes in mRNA expression relative to biofilms at 4 h postinoculation compared to 8-, 16-, or 24-h biofilms. Error bars represent the standard errors of the mean calculated using data from at least four independent experiments.

(36, 42, 43) prompted us to directly evaluate the role of these toxins in biofilm formation. To assess this, we inserted an intron separately into *cpa*, *cpb*, or *pfoA*, encoding CPA, CPB, or PFO, respectively (34, 52). The expected phenotypes for the mutation were evaluated in each mutant strain; for example, a strain with an insertionally inactivated *cpa* gene (CN3685Δ*cpa* or S13Δ*cpa*) was not able to hydrolyze phospholipids in egg yolk agar plates (not shown) and did not produce CPA in the culture supernatant (Fig. 7A). An Hb release assay confirmed that strains CN3685Δ*pfoA* and S13Δ*pfoA* did not lyse red blood cells (data not shown), whereby a compact pellet of erythrocytes was observed after 2 h of incubation (Fig. 7A).

Although the gangrene-causing, genome-sequenced, strain 13 produced robust biofilms 24 h postinoculation (Fig. 7B and C), the Δ*plc* and Δ*pfoA* mutants produced significantly less biofilm than did their parent strains. Similar results were obtained for CN3685 when biofilms made by Δ*plc* and Δ*pfoA* mutant derivatives were compared to those produced by the parent strain. In contrast, CN3685Δ*cpb* produced wt biofilm levels at 24 h postinoculation (not shown).

Mutant strains S13Δ*plc* and S13Δ*pfoA* were each complemented with the *cpa* or the *pfoA* wt gene, respectively. As expected, the complemented strains regained the ability to produce CPA and hydrolyze phospholipids or lyse red blood cells (Fig. 7A). Biofilms produced by complemented strains, S13Δ*plc/plc* and S13Δ*pfoA/pfoA*, were restored to wt levels (Fig. 7B and C), confirming the role of these hemolysins in the formation of *C. perfringens* biofilms.

CPA is detected on *C. perfringens* biofilms. Given the increasing upregulated expression of *cpa* in biofilms, the presence of CPA in biofilms was investigated by confocal microscopy. The signal of CPA was detected in optical middle and top sections of biofilms made by S13 and S13Δ*plc/plc*, whereas, at 24 h postinoculation, the CPA signal was absent in the few S13Δ*plc* bacteria that remained attached to the substrate (Fig. 8). In S13 and S13Δ*plc/plc* biofilms, CPA colocalized in specific areas with DNA staining, suggesting that CPA may form a complex with eDNA in the biofilm matrix.

DISCUSSION

Clostridium perfringens infections have been associated with the production of potent and often lethal toxins (11, 64), and yet the microenvironments where strains must produce toxins are different. We have demonstrated here that CPA, a toxin produced by all toxigenic types, and PFO are necessary for *C. perfringens* type A strain 13 and type C strain CN3685 to produce biofilms, since

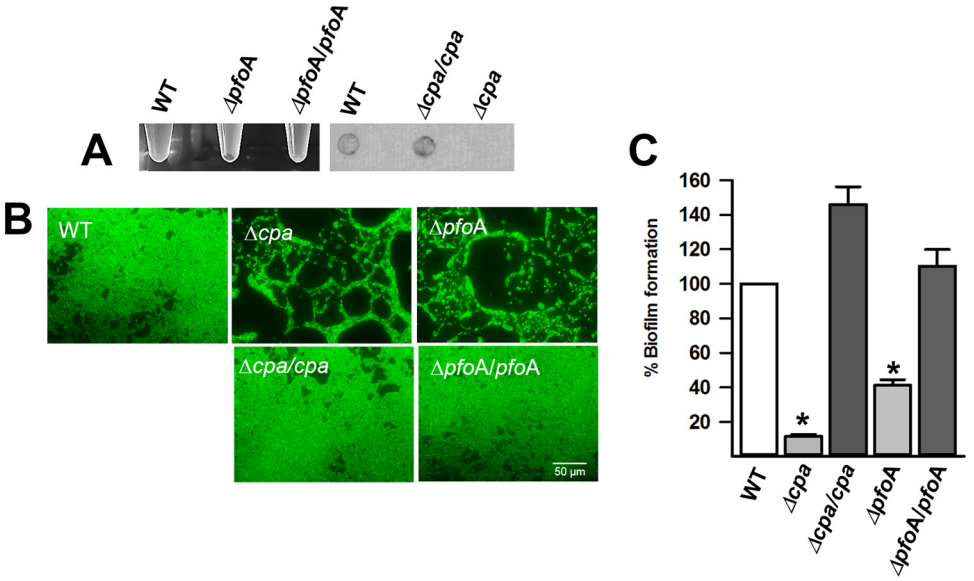

FIG 7 CPA and PFO required for *C. perfringens* biofilms. (A) The phenotypes of wild-type strain 13 (WT) and the S13Δ*cpa* and S13Δ*pfoA* mutants and the corresponding complemented strains were evaluated. The left panel shows the lysis of erythrocytes (note an intact erythrocytes pellet when supernatants from the Δ*pfoA* mutant were used); the right panel shows CPA production as assessed by dot blotting with anti-CPA antibodies. (B and C) Strain 13 (WT), or its derivatives, was inoculated into 96-well plates containing TGY, and biofilms were incubated for 24 h at 37°C. The biofilms were stained with SYTO9, and micrographs of fluorescence-stained biofilms were obtained using an Evos inverted microscope (B), or their biomass (arbitrary units) was obtained using a fluorometer (C). Asterisks indicate statistical significance ($P < 0.05$) compared to the wild type (WT), as evaluated by the Mann-Whitney U test. Error bars represent the standard errors of the mean calculated using data from at least four independent experiments.

their absence rendered strains unable to form wt levels of biofilms. This absence did not have an effect on growing rates of planktonic cells (data not shown). The need of CPA and PFO production for biofilm formation correlated well with the upregulated transcription of *cpa* and *pfoA* toxin genes observed in biofilms compared to planktonic cultures. The specific role that these toxins play in the building of the *C. perfringens* biofilm structure needs, however, further elucidation. Perhaps CPA helps to stabilize the structure by forming complexes with other molecules within the biofilm matrix, e.g., eDNA.

CPA and PFO produce lysis of red blood cells of different species; whereas CPA has phospholipase activity and produces alpha hemolysis on blood agar plates, PFO is a potent pore-forming toxin that induces beta hemolysis of red blood cells (65). Another hemolysin produced by the Gram-positive bacterium *S. pneumoniae*, the pneumolysin Ply, was recently found to be essential for proper assemblage of early pneumococcal biofilms *in vitro* and in a life-like environment (66). PFO and Ply belong to the cholesterol-dependent cytolysin family, sharing~70% homology and ~60% identities. Ply is located on the pneumococcal cell wall (67) and appears to link pneumococcal cells within a growing biofilm structure (66). In contrast, PFO has a signal peptide for its secretion into the supernatant. It is unclear at this point why, or how, secreted toxins such as PFO and CPA may help in the building of *C. perfringens* biofilms. CPA may be incorporated into the matrix, where it was seen partially colocalizing with eDNA in our confocal studies. Although not structurally related to CPA or PFO, recent discoveries showed that a hemolysin produced by *Staphylococcus aureus* forms a nucleoprotein complex with released DNA, leading to a solid biofilm matrix required to build staphylococcal biofilms both *in vitro* and *in vivo* (68). Other exotoxins, such as alpha-toxin (also known as HLA) secreted by *S. aureus* strains, have also been implicated in the formation of staphylococcal biofilms on mucosal tissues and virulence (69).

The composition of the *C. perfringens* biofilm matrix is under active investigation. Two different research groups recently showed that treatment of preformed biofilms with proteinase K, cellulose, or DNase I dispersed *C. perfringens* biofilms, suggesting the presence of proteins, β-1,4-linked polysaccharides, and eDNA (49, 50). Our studies further demonstrated the presence of eDNA in the supernatant of biofilm cultures acting as a source of DNA to be incorporated into the matrix. Although *C. perfringens* strain CN3685, utilized in these studies, carries plasmids ranging in size from ~65 to ~110 kb (70), the eDNA identified corresponded to chromosomal DNA, since our quantitative assays targeted a chromosomally encoded *cpa* gene (13). Chromosomal eDNA was, in part, provided by lysis of a subpopulation of biofilm bacteria and planktonic cells as quantified and visualized by using the Live/Dead cell assay. Since most *C. perfringens* strains carry virulence plasmids (70, 71), the possibility exists that plasmids are also released as a source of eDNA for the matrix.

Regulation of *C. perfringens* biofilms appears to be a complex mechanism which we demonstrated to be mediated by the CpAL QS system but not by the LuxS/AI-2 system. Whereas a mutation in the *luxS* gene did not affect the biomass, our experiments showed that CpAL was required for proper formation of the structure, since the absence of *agrB* (encoding a transmembrane protein required for processing the AgrD pheromone [36]) rendered strain CN3685, or strain 13, unable to produce mature biofilm structures. Instead, Δ*agrB* mutants grew mostly as planktonic bacteria. CpAL regulation of biofilms might involve only a subset of CpAL-regulated factors since the CPB toxin, which is regulated by this system, was not implicated in the formation of biofilms by CN3685. Although not addressed here, the CpAL system could

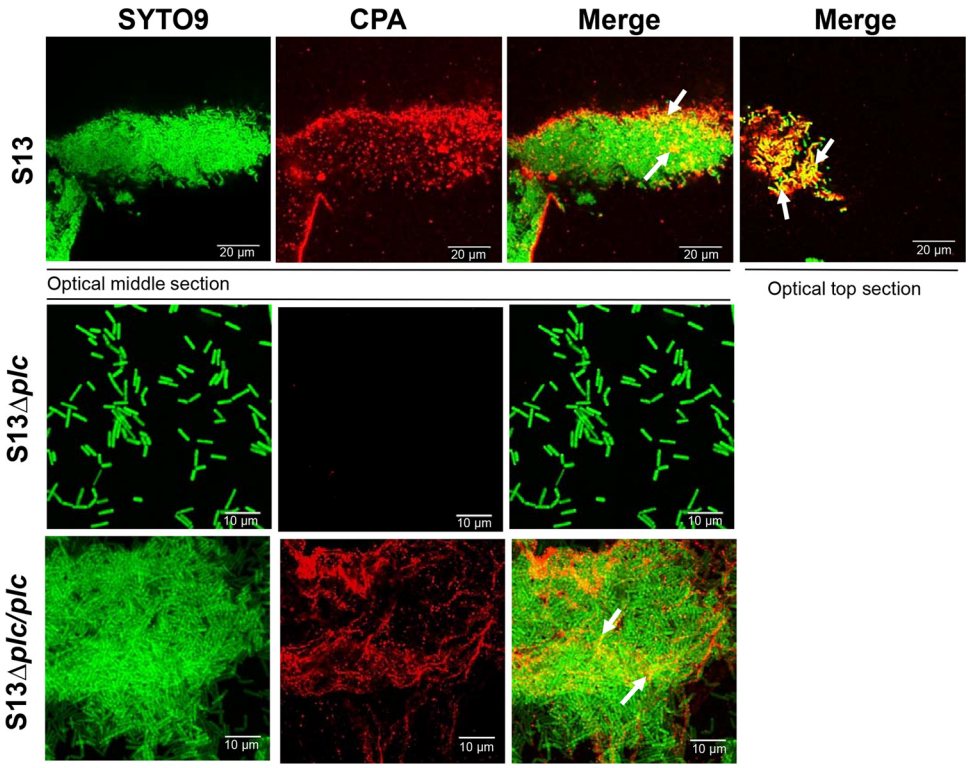

FIG 8 Detection of CPA on *C. perfringens* biofilms. Strain S13Δ*plc* or S13Δ*plc*/*plc* was inoculated into a four-well chamber slide containing TGY, followed by incubation for 24 h at 37°C. Bacteria were stained with SYTO9, and CPA was detected using rabbit polyclonal anti-*Clostridium perfringens* alpha toxin antibodies, followed by goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 555. Optical middle or top sections were obtained with a confocal microscope. Arrows point to areas of colocalization.

activate biofilm-specific downstream regulators. For example, it has been demonstrated that the two-component regulatory system VirR/S transcriptionally regulates toxin levels of CPA, PFO, and other virulence factors in planktonic cultures (72, 73). Whereas the VirR/S system is the logical downstream candidate, since the VirS transmembrane protein appears to be the receptor for the AgrD pheromone (37), another transcriptional regulator called CtrAB has been recently described to indirectly (i.e., via an unknown factor) control the transcript levels of *cpa* and *pfoA* (74). A *ctrAB*-null mutant, however, produced wt biofilm biomass when incubated at 37°C but demonstrated some effects on biofilms formed at 25°C (50). Our data showing upregulated transcription in biofilm cells of CpAL-regulated *cpa* and *pfoA* genes, but not *cbp*, further reinforce our hypothesis that biofilms activate specific downstream signals in response to the CpAL-released AgrD pheromone. Homolog QS systems have also been described in most toxigenic clostridia (41), with most producing biofilms to survive in different hostile environments. Whether these biofilms are controlled by Agr-like systems remains to be investigated.

An additional contribution of the present study was the protocol we developed and validated to fluorescently stain *C. perfringens* biofilms. The fluorescence-based protocol was compared against the classic crystal violet procedure, which, although helpful, was not as sensitive as fluorescence staining of biofilms, which allowed us to detect statistically significant differences of the biofilm biomass at each time point tested. The fluorescence protocol has the additional advantage that biofilms can be stained and quantified within minutes, after which fluorescence micrographs can be obtained from the same preparations. Since there is increasing evidence that other clostridia may produce biofilms in different environments, having a simplified and more sensitive protocol will help to improve our knowledge of biofilms produced by other anaerobes.

In summary, we hypothesize that proliferation of *C. perfringens* strains at the site of the infection may result in activation of the CpAL system, which regulates production of components of the biofilm matrix. The biofilm structure may confer resistance to innate immune molecules and antibiotics while also inducing the degradation of epithelial tissue by the production of toxins. In cases of gangrene produced by type A strains, for example, the border between healthy and necrotic tissue often advances several inches per hour, despite appropriate antibiotic therapy. Therefore, studies of the *C. perfringens* biofilm structure are necessary to better understand the diseases and ultimately help improve the prognosis for these types of infections.

ACKNOWLEDGMENTS

We are grateful to Tohru Shimizu and Kaori Othani from Kanazawa University, Japan, for their gift of strain 13. We also thank Julian I. Rood from Monash University, Victoria, Australia, for providing strain CN3685 and Glenn Songer from Iowa State University for providing *C. perfringens* strains JGS1075, JGS1076, JGS1495, and JGS1659. We acknowledge contributions of Shazaib Jiwani from Oglethorpe University to some laboratory procedures. We also thank June R. Scott, Emory University, for thoughtful suggestions on the manuscript.

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